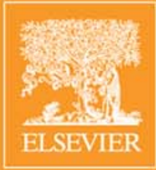

ADVANCES IN PARASITOLOGY

Volume 8

Ben Dawes

Advances in

PARASITOLOGY

VOLUME 8

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Advances in
PARASITOLOGY

Edited by

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VOLUME 8

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PREFACE

The full reviews contained in this volume cover an unusual variety of subjects, the biology of a digenetic trematode which causes "salmon poisoning" disease in mammals, snail problems in African schistosomiasis, the relationship between circulating antibodies and immunity to helminthic infections, electron transport in parasitic helminths and Protozoa, and "river blindness", or onchocerciasis. Four short reviews which supplement contributions of previous volumes are also varied, having the titles: Biological Aspects of Trypanosomiasis Research, 1965—a Retrospect, 1969; Relationships between the Species of *Fasciola* and their Molluscan Hosts; Fascioliasis, the Invasive Stages in Mammals; and Tick Feeding and its Implications.

R. E. Millemann and S. E. Knapp consider the biology of *Nanophyetus salmincola*, a small digenetic trematode which lives in the intestines of dogs, foxes and coyotes in the Pacific region of north-west U.S.A. and serves as the vector of a rickettsia-like microorganism (*Neorickettsia helminthoeca*) that causes "salmon-poisoning" disease. The flukes are transmitted by freshwater snails (*Oxytrema silicula*) throughout western Oregon and nearby parts of the states of Washington in the north and California in the south. Microcercous xiphidiocercariae of this trematode develop in rediae within the snails and finally penetrate into salmonid and other fishes (and also into the Pacific giant salamander), encysting in subcutaneous tissues and many other locations, even in the kidneys, eyes and optic nerves. Following a brief historical account of the disease we are given details of the taxonomy, life cycle and development of the trematode at all stages in named hosts. The American form is now regarded as a subspecies, *N.s. salmincola*, and this is very closely similar to its eastern Siberian relative *N.s. schikhobalowi*, which occurs in human beings as well as other mammals but which does not seem to carry a rickettsia. Some details of the pathology of the trematode in piscine hosts and pathogenesis in mammals is given before the disease is considered in some depth, with information about geographical distribution and etiology, transmission of the rickettsia-like agent, symptoms, diagnosis, treatment and other matters. There are many gaps in our knowledge of this trematode and its rickettsial agent but possible future steps in research are indicated briefly. In a letter to the Editor (18.4.1969) S. E. K. wrote: "we have only equivocal evidence on the isolation of the salmon poisoning agent. We will know by the end of this year whether we will have the agent isolated and this will be, if accomplished, a major breakthrough in the field of comparative rickettsiology". In order to avoid delay in publication, I indicated that such an event can be reported when appropriate in the future in a short review.

A. D. Berrie recognizes the prevalence of schistosomiasis and relates its increased incidence in modern life to agricultural developments before turning to certain difficulties about the study of snail hosts of schistosomes. A dozen species of *Schistosoma* occur in Africa, four of them in Man, and the cercariae cannot be differentiated by simple means. Adult forms indistin-

guishable from human schistosomes occur in a range of wild and domestic animals in Africa and some human forms have been recorded in ungulates, rodents and primates. In some experimental final hosts, *S. bovis* and *S. mattheei* exert a potent immunizing effect against *S. mansoni* and such heterologous immunity, or zooprophylaxis, may have rich epidemiological significance, modifying the severity of human infections, which conversely may be notably severe where bovine schistosomes do not exist, as in Egypt and Brazil.

After dealing with matters of snail taxonomy and distribution, Berrie considers infra-specific variation which may result from isolated populations of snails, species groups of *Biomphalaria* and *Bulinus*, cytological and biochemical investigations and factors which influence the distribution of snails. Snail population dynamics is considered in relation to environmental factors such as temperature, density and food, reproduction and seasonal cycles, the infection of snails in relation to host-finding by miracidia and factors that influence penetration, and development in snails in relation to tissue responses, prepatent period, effects on the snails, and the suppression of infection. The ecology of cercariae is studied in relation to production and emergence, and also survival and infectivity. The dynamic aspects of transmission are treated in the light of both field studies and mathematical models. Finally, the question of control is taken to be attempted by chemical, biological or environmental methods which are discussed briefly; each of these approaches has its difficulties in practice and success is advocated by a combination of such methods. Results during the past decade have advanced knowledge of schistosome larval forms and snail hosts by the development of new drugs for the treatment of schistosomiasis, new molluscicides and biological agents to control snail hosts. Control on a broad scale remains still to be demonstrated but progress is being made and success may come in the near future, although widespread application of promising methods may meet with special difficulties as a result of economic, sociological and political problems.

Ian J. Sinclair points to a distinguishing feature of helminths as their inability to multiply in the definitive host except by reinfection, so that we can usually study the host's response to a single life cycle of parasites in a way that is not possible for microorganisms. Most research bearing on resistance to helminthic infection is concerned with acquired immunity, a phenomenon usually manifested by the appearance of circulating antibodies directed against certain of the parasite's activities and the consequent ability of the host to deal efficaciously with further infections of that parasite. This review deals with the role of circulating antibodies during and after helminthic infection, i.e. their importance in the resistance of the host and the manner in which they help us to understand host–parasite relationships. It begins with a classification of circulating antibodies, notably five main classes of immunoglobulin, and their properties, and then the antibodies known as reagins, some of which belong to the class IgE of immunoglobulins but occur in such low concentrations in the serum that they cannot be titrated by the use of standard serological tests. Reagins may play a part in host resistance to several helminths, having been found in the hosts of *Schistosoma mansoni*, *Litomosoides carinii* and *Trichinella spiralis*. Some consideration is

then given to antibody binding sites, the effect of immune serum on helminths including "self-cure" phenomena and immunological attack on the enzymes of parasites. Antigens then come in for separate treatment, albeit the complexity of the antigenic mosaics of metazoan parasites. Matters of technique are important in this field and valuable information is provided. A section then deals with the interaction of helminth antigens and immune mechanisms of hosts, and although our knowledge does not allow us to formulate definite conclusions from this work some tentative hypotheses can be and are advanced. Various amounts of antigen seem to produce opposite effects to some extent dependent on the age of the host, and parasites may have evolved mechanisms for circumventing the immune responses of the host by decreased antigenic disparity to host's tissue. A section on vaccination then covers such subjects as live and attenuated vaccines, vaccines prepared by *in vitro* culture of helminths and vaccines derived from whole worm macerates. Immunodiagnosis is then considered in relation to tests using whole organisms or else soluble antigens. Finally, a statement is made on the use of antibodies in taxonomy, i.e. as an aid to the evaluation of relationships between different species of helminths, and a section is devoted to conclusions which indicate that although some circulating antibodies have a protective function this has rarely been demonstrated and most antibodies stimulated by helminthic infections may not be involved directly in protecting the host from further infection. Yet, the resistance phenomena demonstrated in the hosts of helminths cannot be explained except on an immunological basis. Circulating antibodies can provide aids to diagnosis and some serological tests give specific results, but the value of such tests is limited because of the more direct approach of obtaining helminthic materials (eggs or larvae) in faeces. Ian Sinclair stresses that the practical uses of serodiagnosis are limited to occasions when parasitological investigations are difficult and perhaps unrewarding, an opinion which many readers will share with him.

Christopher Bryant tackles a difficult biochemical subject about which some information in depth is available for Man and other animals, but not their helminthic parasites. The practical problems posed by parasites include the assembly of sufficient materials for biochemical study, and culturing techniques may be essential in providing the amounts required. Contamination of parasite materials may spoil results and clean parasites may rapidly become moribund. In dealing with the respiratory systems of parasites in the context of their environment, a preliminary account of aerobiosis and anaerobiosis supplies the generally accepted distinction that the former involves haemoproteins known as cytochromes and phosphorylation occurs at substrate or at electron transport level, while the latter does not involve cytochromes and phosphorylation occurs at substrate level only. However, oxygen may not be an absolute requirement and it may be important to dissociate the concept of anaerobiosis in parasites (i.e. the lack of participation by oxygen in the molecular events of respiration) from the absolute requirement by parasites of oxygen for various processes which need this element, notably growth. The concepts aerobic and anaerobic as applied to parasites must be redefined. It is doubtful whether parasitic helminths are

truly aerobic or anaerobic in the accepted sense; in their environments it is likely that there will be some available oxygen which may be used for respiration or other purposes. Parasites may be metabolic opportunists, as far as short term experiments go. Bryant then considers current concepts of electron transport, dealing with haemoproteins and electron transport in classical systems, haemoglobins, catalases and peroxidases, and the tricarboxylic acid cycle and derivative activity. Passing then briefly over the early work on parasites, electron transport is considered in groups of parasitic helminths (cestodes, trematodes, nematodes and acanthocephalans) and then briefly in parasitic Protozoa (mainly trypanosomes and trichomonads). To formulate conclusions is difficult because of great differences of organization between the parasites, as well as shortage of information, but the point is made that electron transport in parasites differs from the classical pattern, but to decide whether or not this is directly related to the parasitic habit would necessitate study of free-living members of various groups to which the parasites belong, and some of these display alternative pathways of electron transport. Bryant cannot say that the adoption of the parasitic mode of life initiated fundamental changes in electron transport, and it may be more probable that the groups of animals which yielded successful parasites already had these modifications because adapted to conditions resembling parasitic environments, which may have imparted to them significant selective advantage.

George S. Nelson notes that onchocerciasis (river blindness) occurs across a wide belt covering a great part of tropical Africa extending from the Atlantic to the Indian Ocean, and also in the Yemen and in some countries of Central and South America. It is a disease with which many medical men are unfamiliar although it affects many millions of human beings, incapacitating large sections of the populace by causing an irritating and unsightly dermatitis and numerous cases of blindness. The causative filarial parasite, *Onchocerca volvulus*, is transmitted by blackflies of the genus *Simulium*, the control of which is the concern of remarkably few scientific experts. In many endemic areas the parasite has escaped detection because medical staffs had too many other pressing problems to deal with. Ironically, there are a few ophthalmologists in urban areas having little onchocerciasis but practically none in rural areas where the disease is prevalent. In this extremely informative review, Nelson has made an all-out effort to cover his subject fully, sections dealing with the geographical distribution of the parasite, geographical variants of *O. volvulus* and possible animal hosts of this species. Pathogenesis of the disease in Man is discussed and illustrated very fully in terms of adult worms and nodules, skin lesions, hanging groin, hernia and elephantiasis, eye lesions, dwarfism, the longevity of the parasite and the persistent duration of the disease. A section on diagnosis deals with skin snips, microfilarial periodicity in the skin and immunological tests, and another of equal practical importance deals with treatment by separate methods of nodulectomy and chemotherapy. Matter dealing with the transmission of *O. volvulus* by simuliid flies excludes purely entomological topics but gives useful references to literature, and deals separately with the main vectors in Africa, vectors in Central and South America, the process of development in blackflies and the

identification of larval forms in these vectors. In the body of this review there is much information and many ideas derived from work in the field and the laboratory, as well as many topics of engrossing interest. The subject of blackfly control has a section to itself. One of the earliest successful control projects was carried out by Dr J. C. C. Buckley on R. Riana in Kenya, using the method of selective bush clearing which was used also against riverine tsetse flies. The insecticide DDT soon came into use and at Jinja it is used in a dosing device built into the dam, making it possible to control all *S. damnosum* breeding for more than 50 miles downstream and eliminating blackflies from an enormous area. Total eradication of *S. naevei* has since been achieved over an even greater area (of 6000 square miles) in Kenya, and Nelson deals with the features facilitating such schemes in some detail. None of the vectors of onchocerciasis has developed resistance to DDT so far, although simuliid resistance is known in Japan. We note that some species of *Onchocerca* are ubiquitous in cattle and horses, but rarely cause ill-health. Bovine onchocerciasis is unobtrusive and may go unnoticed in endemic areas; the worms are very small and they usually occur in the nuchal ligaments, so that it is not surprising that *O. gutterosa* has escaped observation by veterinarians and meat inspectors in this country. Other species in cattle and deer produce onchocercal nodules underneath the skin, which are obvious in carcasses, while *O. armillata* produce striking lesions in the wall of the aorta. Animals may also acquire ocular microfilaria but apparently without the dire consequence seen in many cases of human onchocerciasis.

It is unnecessary to go into details about the subject matter of the short reviews in this volume. In the first one, W. H. R. Lumsden looks at trypanosomiasis research in retrospect and while in the earlier review he considered trypanosomiasis research broadly, he has now channelled his efforts into the narrower experimental and immunological aspects of this subject. Conventions and definitions are laid down, antigens and antibodies considered, methods of study discussed in terms of recent advances on previous practices and with emphasis on stabilation, cloning, nomenclature of antigenic types, purification of trypanosomal suspensions, measurement of infectivity and recognition of antigen–antibody reactions. Much follows about characteristics of infections, immunodiagnosis, recognition of trypanosomal populations and of infected hosts, immunopathology and immunization, before we come to the conclusion confirming his earlier view that the immunological approach is the one most likely to add to our understanding of trypanosomiasis. It is equally certain that this short review will direct effort into profitable explorations and encourage those most in need of help in the difficult subject of trypanosomiasis research.

S. B. Kendall deals with relationships between species of *Fasciola* and their molluscan hosts. Recent Australian studies have shown that *Lymnaea tomentosa* resembles *L. truncatula* in many respects but is rather more aquatic in its habits. Both these hosts of developmental stages of *F. hepatica* are amphibious forms "best adjusted to temporary habitats", whereas the snail hosts of *F. gigantica* are truly aquatic, e.g. *L. natalensis* in Ghana. In this short review there are succinct remarks concerning the range of snail species

parasitized by species of *Fasciola*, host resistance and competition between larval trematodes within a snail, the survival of metacercariae and implications for their survival under field conditions, and the epidemiology of liver fluke infections.

Ben Dawes and Denys L. Hughes have also written briefly about much research on fascioliasis, particularly the invasive stages in mammals. They have been concerned mainly with excystment and metacercarial structure, fascioliasis in cattle, immunity, anaemia and chemotherapy. The work of Ross and colleagues has enriched our knowledge of fascioliasis in cattle. Cattle given a low level infection (200–1300 cysts) did not show increase in the percentage of surviving flukes with increase in infection level, but with 2500 cysts there was reduction in flukes reaching the bile ducts and with 5000–10 000 cysts many immature flukes were trapped in the hepatic parenchyma and fibrosis in the wall of the bile duct was reduced in amount. Much more information from the recent work on pathogenesis and the particular resistance to infection that cattle display is also given, and there is brief discussion of experiments purporting to show acquired immunity to infection in mice, and also work on the problems of anaemia in fascioliasis. This detailed research on anaemia has in the main indicated a need of still further effort, and this is true of chemotherapy and in particular the effect of drugs on the organ systems of the parasite.

Finally, Don. R. Arthur has given a detailed short review on tick feeding and its implications which it would be futile to condense here, but the accounts of fluid flow interchange, the cement and eosinophilic zones, the characteristics of the lesion and associated histopathological change, and feeding and weight changes, will appeal to readers interested in these vectors of parasites.

I am happy once more to express my gratitude to contributors so willing to give generously of their time and energy in order to advance my aim of producing a great fund of up-to-date knowledge of modern biological aspects of parasitology. I am glad to thank members of staff of Academic Press also for their great help and encouragement towards the publication so soon and so carefully of another useful volume.

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November 1969

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Biology of *Nanophyetus salmincola* and "Salmon Poisoning" Disease

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I. Introduction

The digenetic trematode *Nanophyetus salmincola* (Chapin) is the vector for *Neorickettsia helminthoeca*, a rickettsia-like organism that causes "salmon poisoning" disease (SPD). The disease is enzootic in the Pacific Northwest of the U.S.A. and is almost always fatal for dogs, foxes and coyotes. The name of the disease is a misnomer because the salmon are neither poisoned nor do they poison the canid; however, the designation is retained because of long usage.

The first record of SPD was reported from northwestern Oregon and appeared in Henry's Astoria Journal (1814). The writer stated: "Mr. Franchere brought down the Dogs belonging to this place, that had been sent up to the Willamette to pass the Winter there to prevent their death, as living on the Raw Salmon here last summer and Fall caused them to die". Thornton (1849) reported that raw fresh salmon is injurious to dogs and that the poison is in the blood of the fish. He stated that signs of the disease in dogs appear on the

second day after they have eaten salmon and that most dogs die on the tenth day. Cooper and Suckley (1859) stated: "Salmon . . . will make any blooded dog from the States very ill; scarcely one dog out of ten recovers". They were the first to report that dogs that recovered from the disease are immune. Cooper and Suckley concluded that "salmon sickness" was probably distemper. Nash (1882) reported that salmon in Oregon are poisonous to dogs. He described the signs of the disease in dogs, and also observed that dogs that recovered from the disease are immune. Pernot (1911) reported that minute white cysts he observed in the kidneys of salmon and trout caused the disease and concluded that the cysts were amebae. He produced the disease experimentally in dogs by feeding them fish and by subcutaneous injection of blood from a sick dog. He was the first to describe in detail the symptomatology and gross pathology of the disease. Bonebrake (1925) believed that the etiological agent was a bacterium. Donham (1925a) found small trematodes in the intestines of dogs that had died after eating salmon. He concluded that the cysts in the salmon were intermediate stages of the trematode. Subsequently, Donham *et al.* (1926) showed that the small intestinal trematode caused SPD in dogs and that the cyst in fish developed into the adult parasite in dogs.

Simms *et al.* (1931a, b) confirmed Donham's finding that an intestinal trematode was associated with SPD, and reported that the disease could be produced in dogs by intraperitoneal injection of mature trematodes. They stated that the stream snail *Goniobasis plicifera* var. *silicula* was the molluscan intermediate host for the trematode, and that the geographical range of the snail determined the SPD enzootic area. These authors also listed natural and experimental definitive hosts for the trematode.

Simms and Muth (1934) provided further evidence that *N. salmincola* was the disease vector. They postulated that the etiological agent was a rickettsia or a hemosporidian. Cordy and Gorham (1950) were the first to discover the SPD agent and to report detailed descriptions on the pathogenesis of SPD in dogs and foxes. They suggested that the agent belonged in the order RICKETTSIALES, and Philip *et al.* (1953) proposed the name *Neorickettsia helminthoeca* for it.

Coon *et al.* (1938), Cordy and Gorham (1951) and Philip *et al.* (1954b) reported successful treatment of the disease in dogs with sulfanilamides and antibiotics.

The histopathology and neuropathology associated with the disease in dogs was described by Hoeppli (1926) and Hadlow (1957), respectively.

Bennington and Pratt (1960), Gebhardt *et al.* (1966) and Schlegel *et al.* (1968) studied the life cycle of the trematode. They listed the natural and experimental fish, bird and mammal hosts.

The literature has been reviewed by Philip (1955) and more recently by Knapp and Millemann (in press), with emphasis on the mammalian disease aspects.

Nanophyetus schikhobalowi from far eastern Siberian natives was described by Skrjabin and Podjapolskaja in 1931. It has since been relegated to subspecific or strain status (Witenberg, 1932; Gebhardt *et al.*, 1966; Filimonova, 1966, 1968).

II. The Trematode

A. TAXONOMY

The trematodes found by Donham (1925a) were described by Chapin (1926) as a new genus and species *Nanophyes salmincola* of the family Heterophyidae. The generic name was preoccupied and so it was amended to *Nanophyetus* (Chapin, 1928). Ward and Mueller (1926) proposed the name *Distomulum oregonensis* for metacercariae that they believed caused exophthalmia in trout fry. *Distomulum oregonensis* was placed into synonymy with *N. salmincola* by Price (1929a). Witenberg (1932) restudied the original specimens, could not find a genital sucker or seminal receptacle, but observed a large cirrus pouch, concluding that the parasite does not belong to the Heterophyidae. He considered *Nanophyetus* to be a synonym of *Troglotrema* Odhner 1914 of the family Troglotrematidae and so transferred the species to this genus. Wallace (1935) also restudied the trematode and found a seminal receptacle, agreeing with Witenberg that the parasite belongs in the family Troglotrematidae but not concurring about its allocation to the genus *Troglotrema.* A cirrus is present in *T. acutum*, the type species, but absent in *N. salmincola*; also *T. acutum* occurs in the frontal sinuses of the host, whereas *N. salmincola* is an intestinal parasite. For these reasons Wallace considered the genus *Nanophyetus* to be valid and erected the subfamily Nanophyetinae for it and *Sellacotyle mustelae*, a closely related genus described by Wallace (1935) from mink.

Skrjabin and Podjapolskaja (1931) described from natives in far eastern Siberia a new species of *Nanophyetus*, *N. schikhobalowi*, which differs from *N. salmincola* principally in the smaller size of the eggs. However, Witenberg (1932) considered the description to be incomplete and concluded that *N. schikhobalowi* is a synonym of *N. salmincola.* On the basis of morphological and life cycle evidence, Gebhardt *et al.* (1966) and the Russian scientist Filimonova (1966, 1968) relegated *N. schikhobalowi* to subspecific status. The chief differences between the two subspecies are (1) the Siberian form apparently does not carry a rickettsia, and (2) natural human infections with the U.S. subspecies have not been reported, although Philip (1958) successfully infected himself experimentally.

B. LIFE CYCLE AND DEVELOPMENT

1. *General account*

Nanophyetus s. salmincola requires three hosts for completion of its life cycle (Fig. 1). The first intermediate host is the pleurocerid stream snail, *Oxytrema silicula*, found only in Oregon west of the Cascade Mountains, north to the Olympic Peninsula in Washington, and in northern California.* The second intermediate hosts are salmonid and some non-salmonid fishes and the Pacific giant salamander in which the cercariae encyst. The definitive hosts are fish-eating birds and mammals.

* The name of the snail was given by Simms *et al.* (1931a, b) as *Goniobasis plicifera* var. *silicula*, but according to Henderson (1936) and Morrison (1954) the correct name is *Oxytrema silicula*.

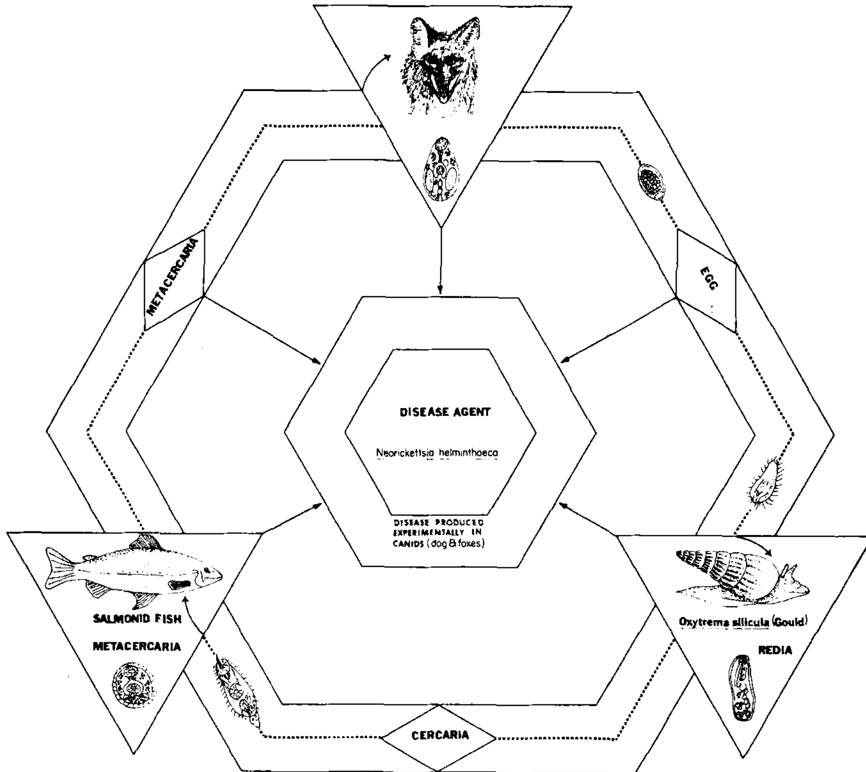


FIG. 1. Life cycle of *Nanophyetus salmincola salmincola* showing the relationship between the trematode and the "salmon poisoning" disease agent. (Knapp, original.)

The first intermediate hosts for *N. s. schikhobalowi*, the Siberian subspecies, are the pleurocerid stream snails *Semisulcospira laevigata* and *S. cancellata*. Second intermediate hosts are salmonid and some other fishes. Definitive hosts are mammals including man. The enzootic area is restricted to northern Sakhalin Island and the mountainous tributaries of the Amur River—the Amgun, Garin, Aneu, Bikin and Khor Rivers in far eastern Siberia (Filimonova, 1966).

2. *Definitive hosts*

There are 32 known natural and experimental definitive host species for the adult parasites (Tables I, II). Adults of *N. s. salmincola* are parasites not only of mammals but also of three species of birds. The Siberian subspecies is a natural parasite of man, whereas the U.S. subspecies has not been reported from man. However, there has not been a systematic survey for *N. s. salmincola* infection in individuals in the enzootic area who might eat poorly cooked or raw fish. Man is susceptible to the U.S. subspecies as shown by Philip (1958), who infected himself by eating raw infected trout obtained from Oregon. Natural definitive hosts common to the two parasite subspecies are the domestic

TABLE I

Definitive hosts for Nanophyetus salmincola salmincola
(*After Schlegel* et al., 1968)

Host	Observed infection*		Reference
	N	E	
Man	–	+	Philip, 1958
Ardea herodias (Great blue heron)	+	–	Schlegel *et al.*, 1968
Lophodytes cucullatus (Hooded merganser)	+	–	Schlegel *et al.*, 1968
Megaceryle alcyon (Belted kingfisher)	+	–	Donham, 1928
Alopex lagopus (Arctic fox)	+	+	Simms *et al.*, 1931a, b
Canis familiaris (Domestic dog)	+	+	Many authors
Canis latrans (Coyote)	+	+	Cram, 1926; Donham and Simms, 1927; Schlegel *et al.*, 1968
Cavia porcellus (Guinea-pig)	–	+	Simms *et al.*, 1931a
Didelphis marsupialis (Opossum)	–	+	Schlegel *et al.*, 1968
Felis domestica (Domestic cat)	+	+	Simms *et al.*, 1931a; Schlegel *et al.*, 1968
Lutra canadensis (River otter)	+	–	Schlegel *et al.*, 1968
Lynx rufus (Bobcat)	+	+	Cram, 1926; Simms *et al.*, 1931a; Schlegel *et al.*, 1968
Meriones unguiculatus (Jird)	–	+	Nyberg and Knapp (unpublished)
Mesocricetus auratus (Golden hamster)	–	+	Philip, 1959; Bennington and Pratt, 1960
Mus musculus (White mouse)	–	+	Philip, 1959
Mustela erminea (Shorttail weasel)	+	–	Schlegel *et al.*, 1968
Mustela vison (Mink)	+	–	Donham *et al.*, 1926; Simms *et al.*, 1931a; Baker, 1950; Schlegel *et al.*, 1968
Neotoma sp. (Woodrat)	–	+	Bennington and Pratt, 1960
Procyon lotor (Raccoon)	+	+	Cram, 1926; Simms *et al.*, 1931a; Bennington and Pratt, 1960; Schlegel *et al.*, 1968
Rattus norvegicus (Norway rat)	+	+	Simms *et al.*, 1931a; Schlegel *et al.*, 1968
Spilogale putorius (Spotted skunk)	+	–	Schlegel *et al.*, 1968

TABLE I (*continued*)

Host	Observed infection* N	E	Reference
Ursus americanus (Black bear)	–	+	Simms *et al.*, 1931a
Vulpes fulva (Red fox)	+	+	Donham *et al.*, 1926; Simms *et al.*, 1931a; Cordy and Gorham, 1950; Schlegel *et al.*, 1968

* N = natural and E = experimental. Negative sign indicates no information available or evidence inconclusive because of small sample size.

TABLE II

Definitive hosts for Nanophyetus salmincola schikhobalowi (*After Schlegel* et al., 1968)

Host	Observed infection* N	E	Reference
Man	+	–	Filimonova, 1963, 1966
Canis familiaris (Domestic dog)	+	+	Filimonova, 1963, 1966
Canis lupus (Gray wolf)	+	–	Filimonova, 1966
Enhydra lutris (Sea otter)	+	–	Filimonova, 1966
Felis domestica (= *F. catus*) (Domestic cat)	+	+	Filimonova, 1963, 1966
Gulo gulo (Wolverine)	+	–	Filimonova, 1966
Martes flavigula (Yellow-throated Asiatic marten)	+	–	Filimonova, 1966
Meles meles (Old World badger)	+	–	Filimonova, 1966
Mustela sibirica (Kolinsky)	+	+	Filimonova, 1965, 1966
Mustela vison (Mink)	+	+	Filimonova, 1965, 1966
Nyctereutes procyonoides (Raccoon dog)	+	–	Filimonova, 1966
Rattus norvegicus (Norway rat)	–	+	Filimonova, 1963, 1965
Selenarctos tibetanus (Asiatic black bear)	+	–	Filimonova, 1966
Ursus arctos (Alaskan brown bear)	+	–	Filimonova, 1966
Vulpes vulpes (Red fox)	+	–	Filimonova, 1966

* See footnote to Table I.

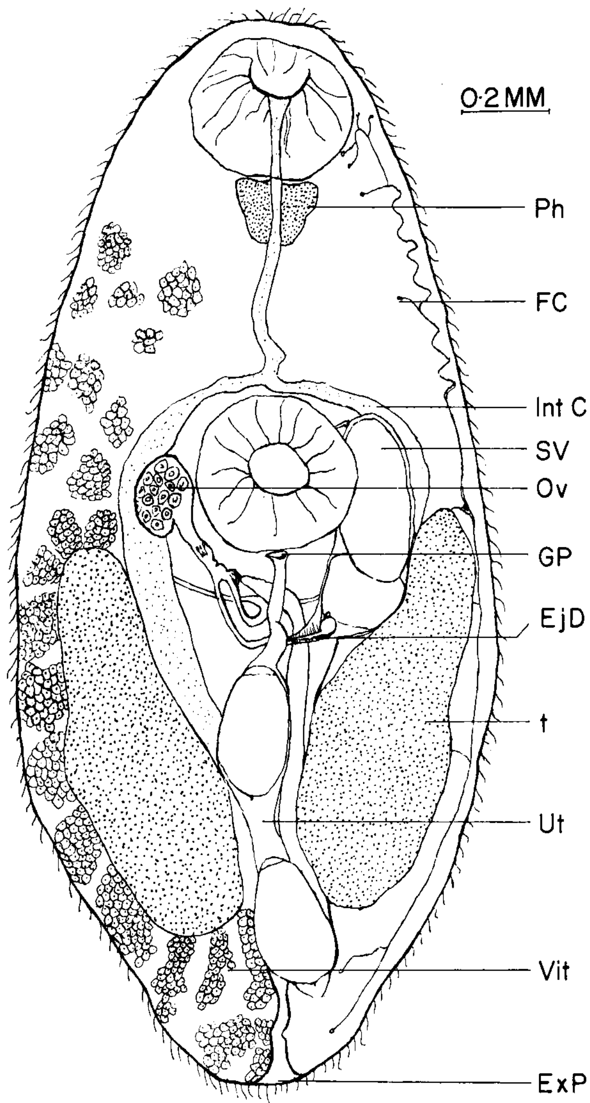


FIG. 2. The adult trematode, *Nanophyetus salmincola salmincola* (Chapin). EjD, ejaculatory duct; ExP, excretory pore; FC, flame cell; GP, genital pore; IntC, intestinal cecum; Ov, ovary; Ph, pharynx; SV, seminal vesicle; T, testis; Ut, uterus; Vit, vitellaria. (After Bennington and Pratt, 1960.)

dog, domestic cat, red fox, and mink, and common experimental hosts are the domestic dog, domestic cat, and white rat.

3. *The adult parasite*

Nanophyetus s. salmincola was first described by Chapin (1926), and later by Witenberg (1932), Wallace (1935) and Bennington and Pratt (1960). The adult worm is 0·8–2·5 mm long and 0·3–0·5 mm wide (Fig. 2). The seminal receptacle first observed by Witenberg (1932) is termed a fertilization chamber by Bennington and Pratt (1960). The uterus contains 5–16 eggs. The cirrus sac contains a large bipartite seminal vesicle but there is no cirrus. Filimonova (1968) studied 165 adult worms obtained from human hosts in the U.S.S.R. and from dogs in the U.S.S.R. and U.S.A.; she found no significant morphological differences between the two subspecies.

Simms *et al.* (1931b) described the early development of *N. s. salmincola* in experimentally infected dogs. Filimonova (1965) studied the development of the Siberian subspecies in experimentally infected rats and dogs from days 3–30 of the infection. On about day 5, the parasites are nearly mature and are producing eggs; on day 10, they reach their maximum size; and on day 30, they begin to deteriorate. Adults of *N. s. salmincola* are eliminated from dogs usually by day 250 of the infection (Simms *et al.*, 1931b), and from hamsters and wood rats by day 51 (Bennington and Pratt, 1960). Adults of *N. s. schikhobalowi* are eliminated from white rats, dogs, cats and mink after 18–51 days, 35–60 days, 30 days and 19 days, respectively (Filimonova, 1965).

The trematodes are buried among the villi or are partially embedded in the small intestine of the animal host. Hoeppli (1926) ascribed the destruction of

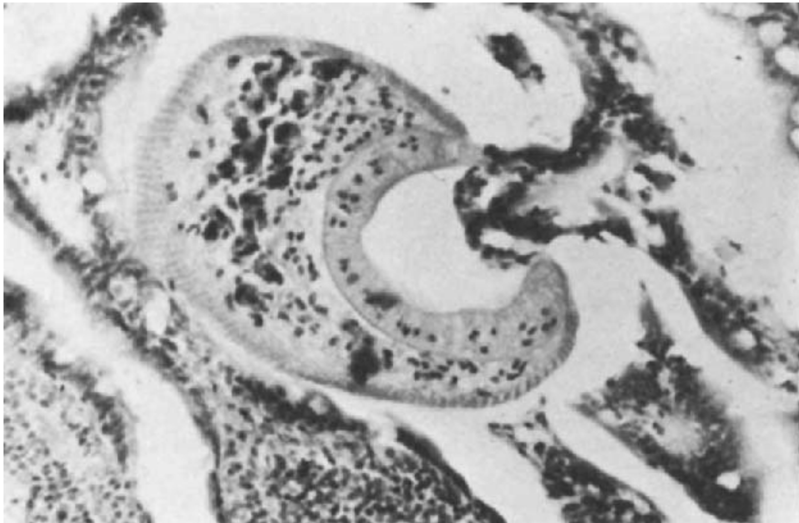


FIG. 3. Histological section of the duodenum of a dog showing the adult trematode, *Nanophyetus salmincola salmincola in situ*. (Knapp, original.) × 300.

the mucosa that he observed to the boring and sucking activity of the parasite. However, Simms *et al.* (1931b) reported that the parasites burrow only into the duodenal tissue and, therefore, Hoeppli's surmise does not account for the observed necrosis of the small intestine below the duodenum. Bennington and Pratt (1960) found no evidence of extensive mechanical tissue damage, although histological sections show intestinal tissue held in the suckers of the parasite (Fig. 3).

Inhabitants of the Siberian villages on the Khor, Olon and Bikin Rivers have high incidences (95 to 98 %) and intensities of infection with *N. s. schikhobalowi* (Sinovich, 1959). Symptoms in human hosts having at least 500 parasites include rumbling and palpation of the sigmoid (32 %) and cecum (43 %), diarrhea (43 %), constipation (16 %), unpleasant sensations in the epigastral region on an empty stomach (14 %), copious nocturnal salivation (16 %) and gastric pains (32 %) (Sinovich, 1959). Trematodes can be removed from human hosts using either fern extract or atebrin and from dogs using arecoline (Sinovich, 1959).

4. *The egg and miracidium*

The eggs of the U.S. and Siberian subspecies measure 64–97 μ × 34–55 μ, and 52–82 μ × 32–56 μ, respectively (Simms *et al.*, 1931a; Witenberg, 1932; Bennington and Pratt, 1960; Filimonova, 1965, 1968). They are light brown in color with an indistinct operculum at one end and a small blunt point at the other, and they are unembryonated when passed in the feces (Fig. 4). Eggs first appear in the feces of experimentally infected animals 5–8 days after they have eaten infected fish (Simms *et al.*, 1931b; Filimonova, 1965).

The time required for eggs of *N. s. salmincola* to hatch when held in standing water at room temperature was reported to be 90 days by Price (1929b), 58

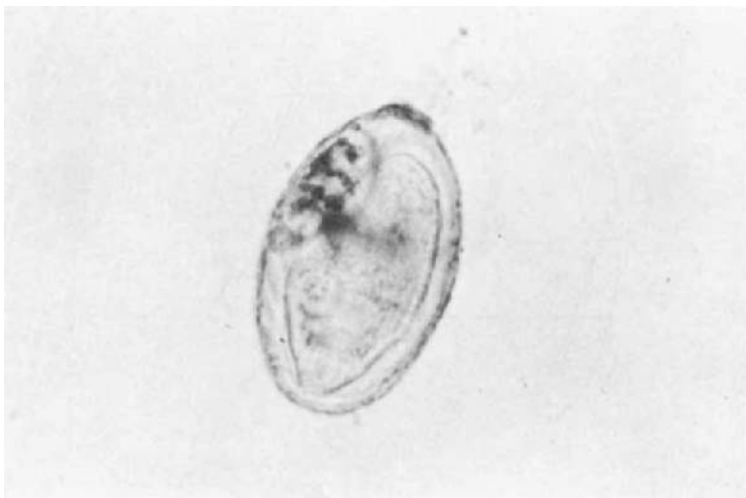


FIG. 4. Embryonated egg of *Nanophyetus salmincola salmincola*. (Knapp, original.) ×480.

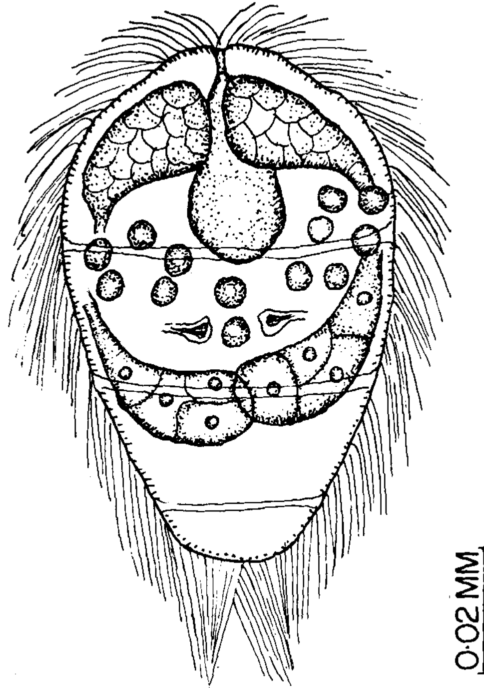


FIG. 5. Miracidium of *Nanophyetus salmincola schikhobalowi* after hatching from the egg. (After Filimonova, 1965.)

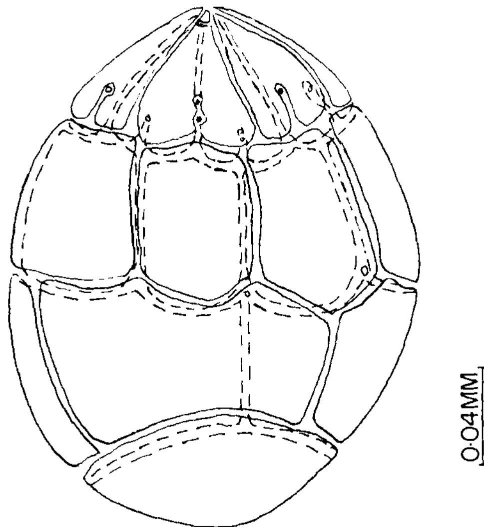


FIG. 6. Diagrammatic illustration of the miracidium of *Nanophyetus salmincola schikhobalowi* showing the distribution of epithelial cells and pores. (After Filimonova, 1965.)

and 75 days by Simms *et al.* (1931b) and 87–200 days by Bennington and Pratt (1960). The last named authors reported that eggs did not hatch in running water and that aeration did not affect the rate of development. We have found (unpublished) that eggs held in test tubes in 60 ml of water at pH 7 and under continuous illumination began hatching about day 31 at the three test temperatures of 21°C, 26°C and 31°C, and by day 156 approximately 75% had hatched. The hatching rate increased with decreasing temperatures, and egg mortality increased with increasing temperatures. Aeration and a sand substrate did not affect the rate of development.

Filimonova (1965) reported that eggs of the Siberian subspecies hatched after 160 days when held in standing water at 16–22°C. The water contained penicillin and was changed three times weekly. If a surface layer of ice was present, then eggs hatched in 35–45 days. Eggs did not develop at 3°C or at 33–37°C. Light did not affect development.

Newly emerged miracidia of the U.S. subspecies measure 0·087–0·105 × 0·037 mm (Bennington and Pratt, 1960). Their anatomy has not been studied. Living miracidia of the Siberian subspecies measure 0·063–0·105 × 0·021–0·042 mm (Filimonova, 1965; Fig. 5). The epithelial cells are arranged in four rows (Fig. 6), with 6, 7, 3 and 1 cells respectively in the first–fourth rows. In the first row, each of four cells has one pore and the remaining two cells each has two pores. Each of two cells in the third row has one pore. There are four pores between the second and third rows and two pores at the apex of the miracidium. Staining the miracidium with neutral red showed one apical gland surrounded by two multicellular glands. Ducts of the latter are directed posteriorly. Between the second and third rows of epithelial cells are two glands with anteriorly directed ducts. Germ cells are situated between the

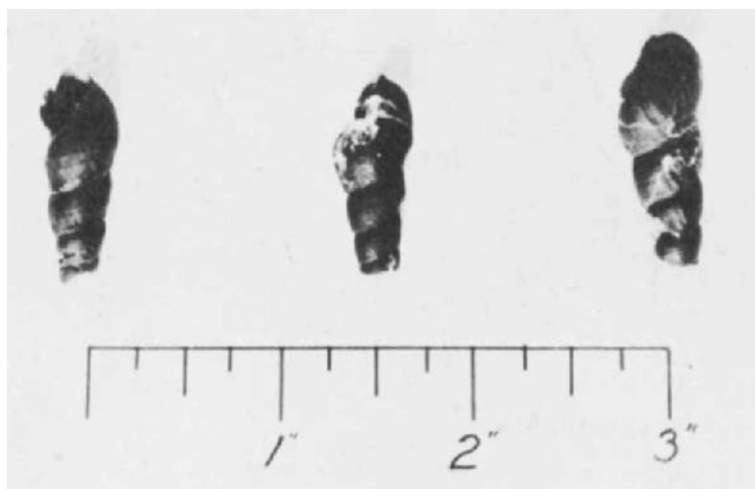


FIG. 7. *Oxytrema silicula* (Gould), the snail intermediate host of *Nanophyetus salmincola salmincola*. (After Simms *et al.*, 1931a.)

anterior and posterior groups of glands. One pair of flame excretory cells is present, and pores lie between the second and third row of epithelial cells.

Miracidia of both subspecies apparently are not attracted to their respective snail hosts (Bennington and Pratt, 1960; Filimonova, 1965). The snail hosts have not been successfully infected experimentally.

5. *The first intermediate host*

The snail host for *N. s. salmincola*, *Oxytrema silicula* (Gould) (Simms *et al.*, 1931a; Fig. 7), is present in large numbers in most of the creeks and streams in the enzootic area (Bennington and Pratt, 1960). Its range extends from the Smith River in northern California, northward through Oregon west of the Cascade Mountains through parts of the Olympic Peninsula as far north as Olympia, Washington (Simms *et al.*, 1931b) (Fig. 8). The geographical range of the snail defines the enzootic area of the trematode (Simms *et al.*, 1931b).

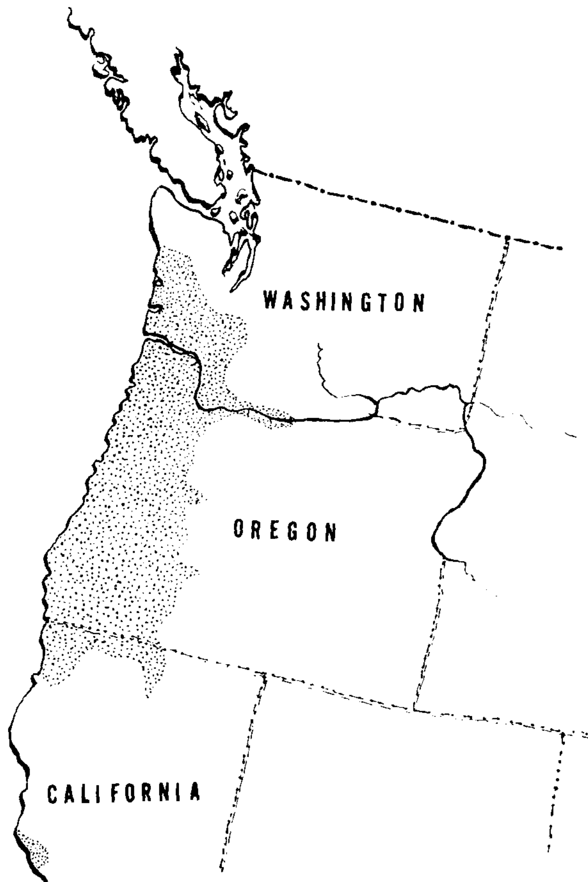


FIG. 8. Distribution of *Oxytrema silicula* (Gould). (Courtesy of Pratt and McCauley, unpublished.)

Snails infected with *N. s. salmincola* also occur in brackish water (0·8–20·5‰ salinity) in western Oregon (Gebhardt, 1966; Baldwin, 1967). Small snails have not been found in saline waters, indicating perhaps that they cannot reproduce in this environment.

The snail is the first intermediate host for at least five species of trematodes other than *N. s. salmincola* (Burns, 1961). Bennington and Pratt (1960) did not find mixed infections involving *N. s. salmincola*, but Gebhardt (1966) did in 15 of 4215 snails he examined, and also found that snails with large numbers of *N. s. salmincola* were not parasitized by other trematode species. We assume that *N. s. salmincola* has a high degree of specificity for *O. silicula*, but experimental proof for this assumption is lacking.

Gebhardt (1966) examined at monthly intervals for 10 months 3225 snails obtained from four western Oregon streams for *N. s. salmincola* infection. The monthly incidences of infection ranged from 9 to 52%. Snails with apertures of 10–13 mm diameter showed the highest incidence of infection. Simms *et al.* (1931b) and Bennington and Pratt (1960) also reported that usually only the larger snails, 2 cm or more in length, are infected. Infection of snails with mature cercariae is seasonal (Gebhardt, 1966). Only immature cercariae occur in snails from early December to early April (Fig. 9). Bennington (1951) also did not find mature cercariae in snails during February and March.

Infected snails collected in the field have been successfully maintained in the laboratory for one year or more (Baldwin *et al.*, 1967). We have (unpublished) successfully reared snails from eggs collected in the field and from eggs laid in the laboratory. These snails, now 2 years old, continue to grow.

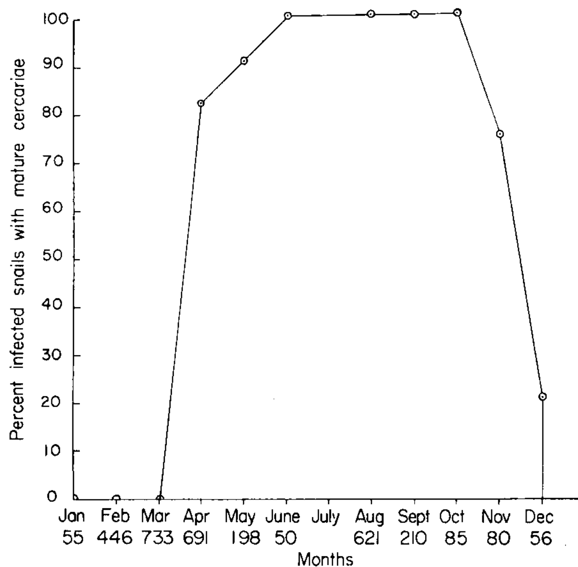
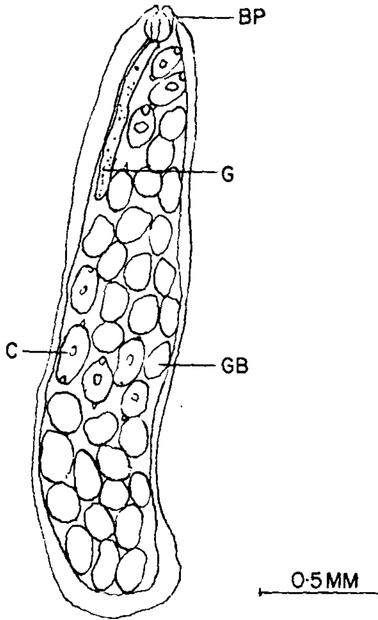


FIG. 9. Monthly incidence of mature *Nanophyetus salmincola salmincola* cercariae in infected snails. The number below each month is the number of snails examined during that month. (After Gebhardt, 1966.)



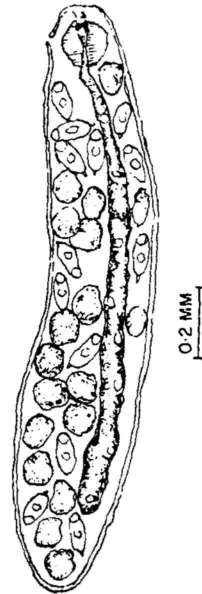


FIG. 10 FIG. 11

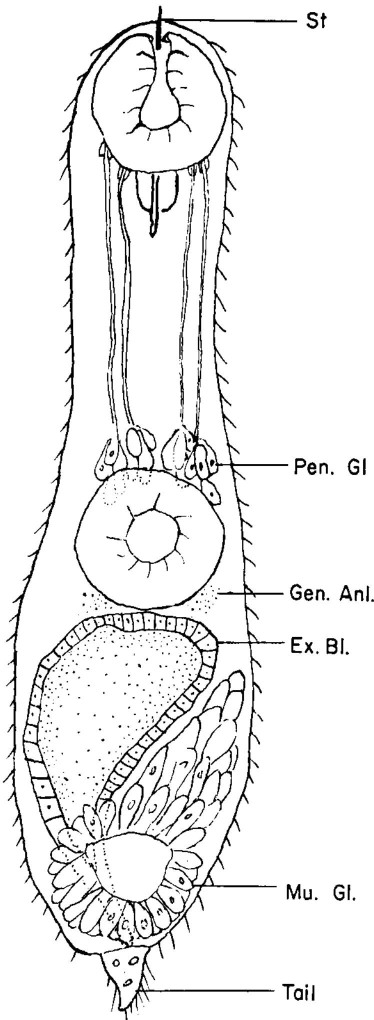
FIG. 10. Mature redia of *Nanophyetus salmincola salmincola* containing numerous cercariae and germ balls. BP, birth pore; C, cercaria; G, gut; GB, germ ball. (After Bennington and Pratt, 1960.)

FIG. 11. Mature redia of *Nanophyetus salmincola schikhobalowi*. (After Filimonova, 1963.)

The snail hosts for *N. s. schikhobalowi* are *Semisulcospira laevigata* and *S. cancellata* (Filimonova, 1965). These molluscs are also parasitized by five other species of trematodes. Mixed infections involving *N. s. schikhobalowi* have been observed (Filimonova, 1965). Infection incidences in snails on the Aneu and Khor Rivers are 9·3 and 16·7%, respectively (Filimonova, 1965). Only the larger snails, 1·6–2·6 cm long, are infected (Filimonova, 1963). Filimonova (1963) reported that the numbers of mature cercariae in snails decreases in August and then increases in September.

6. *The redia*

Sporocysts of both subspecies have not been found (Bennington and Pratt, 1960; Filimonova, 1963). Bennington and Pratt (1960) did not see the daughter rediae of the U.S. subspecies, but Filimonova (1963) observed them for the Siberian subspecies. The rediae of the two subspecies were described by the American and Russian authors respectively. Rediae of these subspecies are very similar (Figs 10 and 11). Measurements in mm (U.S. subspecies given first) are: length, 0·45–3·0, 0·27–3·0; width, not given, 0·08–0·48; pharynx, 0·06, 0·02–0·052 × 0·02–0·048; intestine, to 0·3, 0·05–1·49. Small rediae are very active, and taper to a point posteriorly; large rediae are cylindrical. The birth pore is located near the pharynx. Mature rediae were found in almost all tissues of the snail. The gonads and associated tissues of *O. silicula* are the



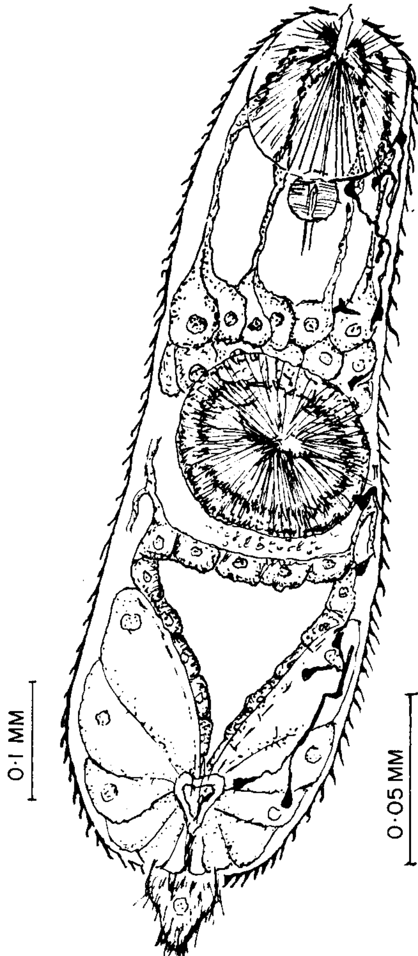


FIG. 12 FIG. 13

FIG. 12. Mature cercaria of *Nanophyetus salmincola salmincola*. Ex Bl, excretory bladder; Gen Anl, genital anlagen; Mu Gl, mucous gland; Pen Gl, penetration gland; St, stylet. (After Bennington and Pratt, 1960.)

FIG. 13. Mature cercaria of *Nanophyetus salmincola schikhobalowi*. (After Filimonova, 1963.)

most frequently parasitized organs, and the digestive gland is next in order of frequence (Law, 1969).

Porter *et al.* (1967) studied the effects of *N. s. salmincola* rediae on the snail host. After the parasites have destroyed the gonads, they invade the hepatopancreas, which is damaged by increased pressure resulting from rapid growth

of the parasites, by active ingestion of the hepatic tubules by the parasites, and by the burden placed upon the snail in disposing of the parasitic wastes. The parasites take up glycogen and lipids from the heptatopancreas either by ingestion of liver cells or by absorption across the body membranes.

7. *The cercaria*

(a) *Morphology and behavior.* The first accurate description of *N. s. salmincola* cercariae was given by Bennington and Pratt (1960). According to them, Donham's (1928) description refers to another trematode, *Acanthatrium oregonense*, and Sinitsin (1931) confused the metacercaria with the cercaria. Cercariae of *N. s. schikhobalowi* were described by Filimonova (1963, 1964). The microcercous xiphidiocercariae of the two subspecies are very similar (Figs 12 and 13). The following descriptions and measurements in mm refer to both subspecies (Siberian subspecies given in parentheses): length, 0·31–0·47 (0·22–0·43); width, 0·03–0·15 (0·03–0·12); cuticle, transparent and armed; tail, 0·03 (0·02–0·04) long with spines; oral sucker, 0·07–0·08 (0·05–0·07); ventral sucker, 0·07 (0·04–0·06); pharynx, 0·02 (0·016–0·023); intestine, absent; stylet, simple $0{\cdot}01 \times 0{\cdot}003$ ($0{\cdot}01$–$0{\cdot}02 \times 0{\cdot}003$); penetration glands, in four clusters of four cells each, the duct of each cell with a dilation near oral sucker; adhesive or mucus gland, near tail with a ventral longitudinal slit-like opening; excretory bladder, dorsal to mucus gland and opening dorsally at base of tail; flame cell formula for U.S. subspecies, not determined for Siberian subspecies, is $2[(3+3+3)+(3+3)]=30$. The non-swimming, creeping cercariae elongate and contract rapidly; the suckers open and close; the stylet is protruded; the aperture of the adhesive organ opens and closes; and the tail contracts and elongates. Cercariae of the Siberian subspecies can live for 1½ months in non-aerated water at 6°C.

(b) *Development.* Gebhardt (1966) held snails, which had immature *N. s. salmincola* infections, at room temperature (20° to 22°C). He observed mature cercariae in these snails after 7 days, and 90% of the snails had mature cercariae on day 15.

(c) *Release from snails and survival.* Law (1969) studied emergence of *N. s. salmincola* cercariae from *O. silicula*. The optimum pH and temperature for cercarial emergence are 7·6–8·0 and 19°–20°C. The numbers emerging increase with increasing temperatures from 3°–20°C and then decrease with decreasing temperatures from 20°–30°C. Approximately five times as many cercariae emerge during the day (8.00 a.m. to 8.00 p.m.) than at night (8.00 p.m. to 8.00 a.m.). Cercariae leave the snail through the ctenidial leaflet (Fig. 14). They then enter the mantle cavity and drift out with the exhalent water current on the right side of the snail's head. Cercariae reach the ctenidial leaflet by one of three routes. The main route is from the gonads or digestive gland to the peri-intestinal sinus, the anterior renal organ and the afferent ctenidial sinus to the leaflet. The other routes used less frequently are from the gonads or digestive gland to the peri-intestinal sinus, the rectal sinus, the afferent ctenidial sinus and the leaflet; or from the gonads or digestive gland to the peri-intestinal sinus, the rectal sinus, the anterior renal organ, the afferent ctenidial sinus and the leaflet.

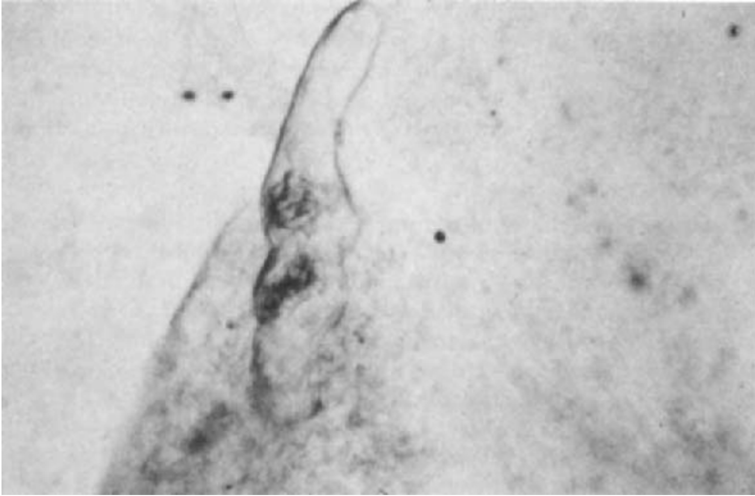


FIG. 14. Mature cercaria of *Nanophyetus salmincola salmincola* emerging from the tip of the ctenidial leaflet of *Oxytrema silicula*. (After Law, 1969.) × 300.

Cercariae of *N. s. salmincola* will emerge from snails held in waters of 4 to 20‰ salinity (Baldwin, 1967). More emerged from snails held at 4‰ salinity than from snails held at the other salinities or from snails in freshwater. Cercariae survived for 75 h in water of 2 or 6‰ salinity compared with 38 h in freshwater. This greater survival of cercariae in waters of low salinity may be explained by the findings of Picken (1937). He reported that the vapor pressure of *Limnaea* [sic] *peregra* blood was equivalent to a solution of sodium chloride at about 4·3‰ salinity. Baldwin (1967) also presented evidence that cercariae from snails collected from brackish water survived longer in waters of 10 to 20‰ salinity than did cercariae from snails collected in freshwater. For example, in water of 12‰ salinity 50% of cercariae from brackish water snails survived 20–38 h, whereas at the same salinity cercariae from freshwater snails survived only 4 h.

Bennington and Pratt (1960) stated that cercariae are released from *O. silicula* in ropy strands of mucus. Baldwin *et al.* (1967) did not observe this, but found that the parasites became entangled in snail mucus if the snails were confined in small vessels.

(d) *Location in stream.* To determine if cercariae of *N. s. salmincola* are concentrated in different parts of a stream, Bender (unpublished) placed uninfected coho salmon 69 mm long in cages that were secured in a riffle, at the surface of a pool, at mid-depth in a pool, or at the bottom of a pool. At the end of 1 week, the average numbers of parasites in fish in the riffle and at the pool bottom were 105 and 102, respectively. The average numbers in fish at mid-depth in a pool and at the pool surface were 73 and 56, respectively. We would expect that the non-swimming cercariae would become concentrated at the pool bottom but not in riffles. However, fish in riffles are exposed to a

greater volume of water in a given period of time than are fish in pools, and therefore they would contact larger numbers of cercariae.

(e) *Control.* Combs (1968) reported that 90% of *N. s. salmincola* cercariae entering hatcheries that use stream water were killed by passing the incoming water through an electrically charged grid. The parallel-plate-type grid was operated at a voltage gradient of 310 V/in. and an exposure time of 0·4 sec.

8. *Second intermediate hosts*

(a) *Species.* The number of natural and experimental second intermediate hosts for the two subspecies is 34 (Tables III and IV). Both subspecies occur naturally in *Oncorhynchus keta*, the chum salmon, and in different species of the families Salmonidae, Cottidae and Cyprinidae. The U.S. subspecies also parasitizes the Pacific giant salamander, *Dicamptodon ensatus.* The following numbers and species of fish were negative for infection with *N. s. schikhobalowi*: 5 *Lampetra reissneri*, 9 *Esox reicherti*, 32 *Leuciscus walecki*, 31 *Phoxinus percnurus*, 45 *P. lagowskii*, 32 *Gobio g. cynocephalus*, 15 *Nemachilus barbatulus*, 15 *Cottus poecilopus*, 20 *Pseudaspius leptocephalus*, 30 *Rhodeus s. sericeus*, 10 *Percottus glehni* and 15 *Pungitius p. sinensis* (Filimonova, 1963, 1964). Filimonova (1963) was unable to infect experimentally the stickleback, *Pungitius p. sinensis*, or the cyprinids *Phoxinus phoxinus* and *P. percnurus*, and Gebhardt *et al.* (1966) were unable to infect experimentally the yellow perch, *Perca flavescens.*

TABLE III

Second intermediate hosts for Nanophyetus salmincola salmincola
(After Knapp and Millemann, in press)

Host	Observed infection*		Reference
	N	E	
Salmonidae			
Oncorhynchus tshawytscha (Chinook salmon)	+	+	Donham *et al.*, 1926; Bennington and Pratt, 1960
O. kisutch (Coho salmon)	+	+	Donham *et al.*, 1926; Baldwin *et al.*, 1967
O. keta (Chum salmon)	+	–	Simms *et al.*, 1931a
O. nerka (Kokanee)	–	+	Baldwin *et al.*, 1967
Salmo gairdneri (Rainbow trout)	+	+	Simms *et al.*, 1931a; Baldwin *et al.*, 1967
S. clarki clarki (Cutthroat trout)	+	+	Donham *et al.*, 1926; Baldwin *et al.*, 1967
S. clarki lewisi (Montana black-spotted cutthroat trout)	–	+	Baldwin *et al.*, 1967
S. clarki henshawi (Lahontan cutthroat trout)	–	+	Baldwin *et al.*, 1967

TABLE III (*continued*)

Host	Observed infection* N	E	Reference
S. salar (Atlantic salmon)	–	+	Gebhardt *et al.*, 1966
S. trutta (Brown trout)	–	+	Gebhardt *et al.*, 1966
Salvelinus fontinalis (Brook trout)	+	+	Simms *et al.*, 1931a; Gebhardt *et al.*, 1966
S. namaycush (Lake trout)	–	+	Gebhardt *et al.*, 1966
Petromyzontidae			
Lampetra richardsoni (Western brook lamprey)	+	+	Gebhardt *et al.*, 1966
L. tridentata (Pacific lamprey)	+	–	Gebhardt *et al.*, 1966
Cottidae			
Cottus perplexus (Reticulate sculpin)	+	+	Bennington and Pratt, 1960; Gebhardt *et al.*, 1966
Cyprinidae			
Carassius auratus (Goldfish)	–	+	Bennington and Pratt, 1960; Gebhardt *et al.*, 1966
Rhinichthys osculus (Speckled dace)	–	+	Bennington and Pratt, 1960
Richardsonius balteatus (Redside shiner)	+	+	Gebhardt *et al.*, 1966
Catostomidae			
Catostomus macrocheilus (Largescale sucker)	–	+	Gebhardt *et al.*, 1966
Centrarchidae			
Lepomis macrochirus (Bluegill)	–	+	Gebhardt *et al.*, 1966
Gasterosteidae			
Gasterosteus a. aculeatus (Threespine stickleback)	–	+	Gebhardt *et al.*, 1966
G. a. microcephalus (Threespine stickleback)	–	+	Gebhardt *et al.*, 1966
Poeciliidae			
Gambusia affinis (Mosquito fish)	–	+	Gebhardt *et al.*, 1966
Percidae			
Perca flavescens (Yellow perch)	–	0	Gebhardt *et al.*, 1966
Ambystomidae			
Dicamptodon ensatus (Pacific giant salamander)	+	–	Gebhardt *et al.*, 1966

* N = natural and E = experimental. Negative sign indicates no information available. 0 = failure to achieve infection.

TABLE IV

Second intermediate hosts for Nanophyetus salmincola schikhobalowi

Host	Observed infection*		Reference
	N	E	
Salmonidae			
Brachymystax lenok (The lenok)	+	+	Filimonova, 1963, 1964, 1965
Coregonus ussuriensis (Amur whitefish)	+	+	Filimonova, 1964, 1965
Hucho taimen (The taimen)	+	+	Filimonova, 1963, 1964, 1965
Oncorhynchus keta (Chum salmon)	+	+	Filimonova, 1963, 1964
O. gorbuscha (Pink salmon)	+	–	Filimonova, 1963
Salvelinus leucomaenis (Kundzha)	–	+	Filimonova, 1965
S. malma (Dolly varden)	–	+	Filimonova, 1965
Thymallus arcticus (Arctic grayling)	+	+	Filimonova, 1963, 1964, 1965
Cottidae			
Mesocottus haitej	+	–	Filimonova, 1963
Cyprinidae			
Phoxinus phoxinus	+	0	Filimonova, 1963
P. percnurus	–	0	Filimonova, 1963
Cyprinus carpio (Carp)	–	+	Filimonova, 1965
Gasterosteidae			
Pungitius s. sinensis (Stickleback)	–	0	Filimonova, 1963

* See footnote to Table III.

Parasites of both subspecies occur in greater numbers in salmonid fishes than in non-salmonid fishes (Filimonova, 1963, 1964; Gebhardt *et al.*, 1966). The former species, therefore, are considered to be the principal second intermediate hosts. Most of the salmonid fish within the enzootic areas are infected with the parasites (Simms *et al.*, 1931a; Filimonova, 1964).

Donham *et al.* (1926) did not find *N. s. salmincola* metacercariae in ocean-caught salmon probably because not enough fish were examined. They assumed, therefore, that salinity had a "cleansing" effect on the infection. Bennington and Pratt (1960) found parasites in several salmon reportedly taken at sea, and Farrell and Lloyd (1962) observed metacercariae in salmon taken near the mouth of the Columbia River. Millemann *et al.* (1964) found 24 of 43 ocean-caught adult coho salmon, *O. kisutch*, and 3 of 4 adult chinook

salmon, *O. tshawytscha*, infected with the parasite. These fish had been in the ocean 2–3 years. The metacercariae in these fish carried the rickettsia because dogs fed some of the fish developed SPD. Farrell *et al.* (1964) reported that coho salmon held captive in salt water retained both the metacercariae and rickettsiae for 33·5 months. Thus, salinity is without any "cleansing" effect either on the trematode or on the SPD agent.

(b) *Cercarial penetration and migration.* Penetration and migration of *N. s. salmincola* cercariae were studied by Bennington and Pratt (1960), Baldwin (1967) and Baldwin *et al.* (1967). Cercariae rarely crawl on the skin of the fish. They become very active when they are within 1 mm of the fish's skin and penetrate it at any point. Cercariae do not enter the fish through the urinary aperture as stated by Sinitsin (1931) or the gills as believed by Simms *et al.* (1931b). Penetration is accomplished within 30 sec to 5 min after contact and can be accomplished in fresh water and also in water of 12 to 20‰ salinity. Cercariae that penetrate the caudal fin enter blood vessels either between or inside rays (Fig. 15). They then migrate to the fin base and reach the internal organs principally by the circulatory system (Fig. 16). Baldwin *et al.* (1967) saw cercariae in the renal blood vessels in histological sections; these probably reached the kidney by the renal portal system, a route first suggested by Bennington and Pratt (1960).

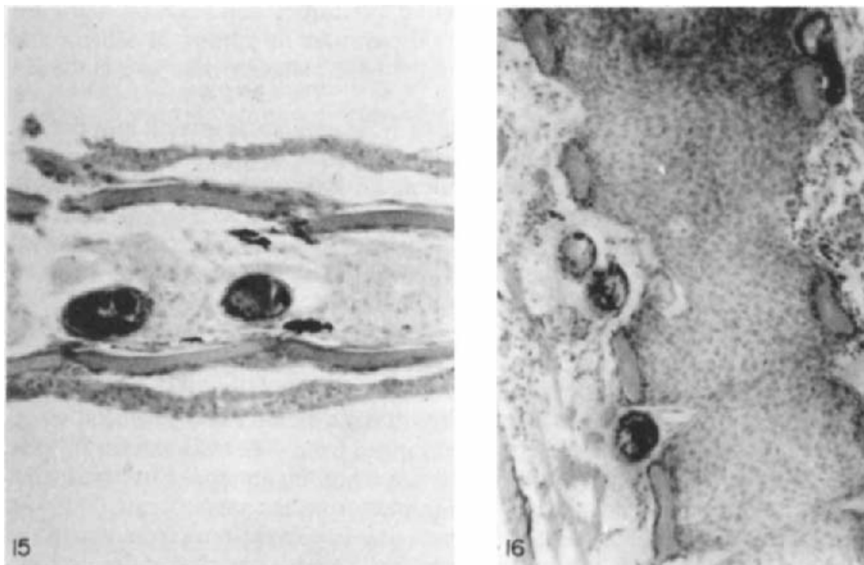


Fig. 15. Cross-section through the caudal fin of a Lahontan cutthroat trout showing two *Nanophyetus salmincola salmincola* cercariae inside a fin ray. (After Baldwin *et al.*, 1967.) ×117·4.

Fig. 16. Cross-section through the base of the caudal fin of a Lahontan cutthroat trout, showing one or two cercariae of *Nanophyetus salmincola salmincola* inside a blood vessel and two other cercariae in the subcutaneous tissue. (After Baldwin *et al.*, 1967.) ×117.

Baldwin *et al.* (1967) infected fish experimentally by introducing cercariae or infected snail tissue into them by stomach tube. The number of metacercariae recovered from these fish was less than the number recovered from fish exposed percutaneously to about the same number of cercariae. Metacercariae from the fish infected through the alimentary tract were infective for the definitive host. Coho salmon in the laboratory will avidly eat freely falling cercariae and can become infected in this manner (Baldwin *et al.*, 1967). Ching (1957) and Baldwin *et al.* (1967) found *O. silicula* snails in the stomachs of fish in nature. Thus, it is possible that fish in nature may become infected orally by eating free cercariae or infected snails.

Cercariae of the Siberian subspecies also penetrate the fish principally through the skin, which is accomplished in a few seconds (Filimonova, 1965).

(c) *Encystment sites.* Encysted metacercariae of both subspecies occur in almost all tissues of salmonid fish, including the eyes and optic nerves, but are most numerous in the kidneys, body muscles, and fins, and less numerous in the gills (Ward and Mueller, 1926; Simms *et al.*, 1931b; Filimonova, 1963, 1964). However, in non-salmonid fish the metacercariae are more numerous in the gills and fins than in the visceral organs (Bennington and Pratt, 1960; Filimonova, 1963; Gebhardt *et al.*, 1966).

(d) *Incidence and intensity of infection.* Most, if not all of the salmonid fish within the two enzootic areas are infected with the parasite (Simms *et al.*, 1931a; Filimonova, 1964). The infection intensities can also be high; for example, Filimonova (1963) found 2000 parasites in salmon in Siberia and Simms *et al.* (1931a, b) in Oregon found 14062 metacercariae in a cutthroat trout 4·6 in long.

A student of ours studied the course of *N. s. salmincola* infection in natural populations of coho salmon and steelhead trout in three Oregon coastal streams (Bender, unpublished). He collected fish twice weekly from March to October 1966 and determined their infection intensities. Few of the coho salmon that emerged from the gravel in March were infected, but all fish collected in mid-April and thereafter were infected. The average numbers of parasites per fish and per gram of fish increased rapidly from mid-April to early July; the infection levels then tended to become stable until early September, when another increase occurred (Fig. 17). Steelhead trout emerged from the gravel in mid-April. All fish collected at this time and subsequently were infected. The numbers of parasites in coho salmon and steelhead trout, 50–60 mm long, collected in September ranged from 400–1565 and from 1735–2002, respectively. Heavily infected fish were not uncommon. Steelhead trout were more heavily infected than coho salmon from the same stream (Fig. 17). For example, the highest numbers of parasites in coho salmon from two of the streams were 415 and 380, whereas the highest numbers in steelhead trout from the same two streams were 1735 and 2002. As mentioned previously, cercariae are more numerous in stream riffles than in pools, and in nature steelhead trout occupy riffles and coho salmon occupy pools (Hartman, 1965). Thus, Bender's finding of the difference in natural infection levels between the two fish species may be explained.

Bender (unpublished) also determined the daily infection levels in coho

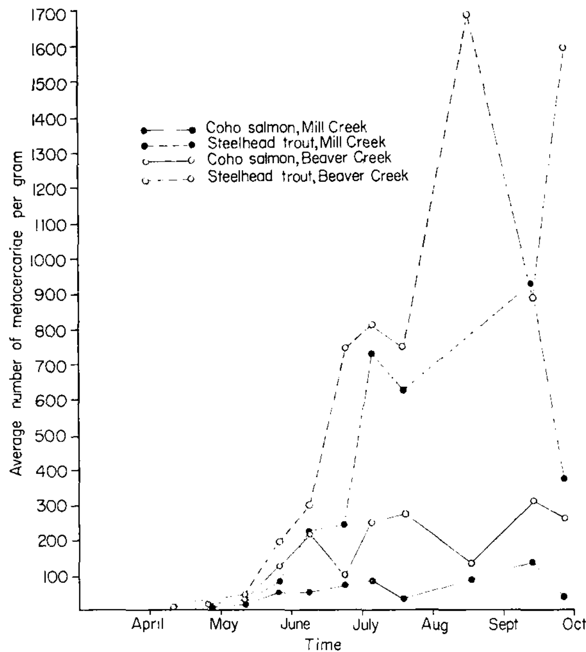


FIG. 17. The average numbers of *Nanophyetus salmincola salmincola* metacercariae in coho salmon and steelhead trout expressed as parasites per gram of fish. The fish were collected from two western Oregon stream from April 1 through September 1966. (After Bender, unpublished.)

salmon under natural conditions. A 300 ft section of a stream was isolated using upstream and downstream blocking seines. Resident fish were removed by electrofishing and replaced with an equal number of uninfected marked salmon. Samples of fish were examined daily for 20 days. During the first 9 days fish acquired 6–143 parasites each day, and thereafter the numbers tended to become stable. After 20 days, the average number of parasites in fish was approximately 400. The largest number was 856.

(e) *Pathogenesis and pathology*. The following discussion pertains only to the U.S. subspecies. There is no information on the effects on fish of infection with *N. s. schikhobalowi*.

(i) Natural infections. Ward and Mueller (1926) believed that the metacercariae caused the deaths of brook trout fry that had died in an Oregon hatchery during a severe epizootic. They found a direct relationship between the degree of exophthalmia and the number of parasites in the optic nerves. However, Simms *et al.* (1931a, b) believed that fish could tolerate large numbers of parasites. Simms (1933) reported heavy infections in young rainbow and brook trout that were dying in an Oregon hatchery, and many of these fish had exophthalmia, but he also found heavy infections in apparently healthy fish from streams and hatcheries. Bennington and Pratt (1960) reported the occurrence of extensive lesions in the fins, gills, tail,

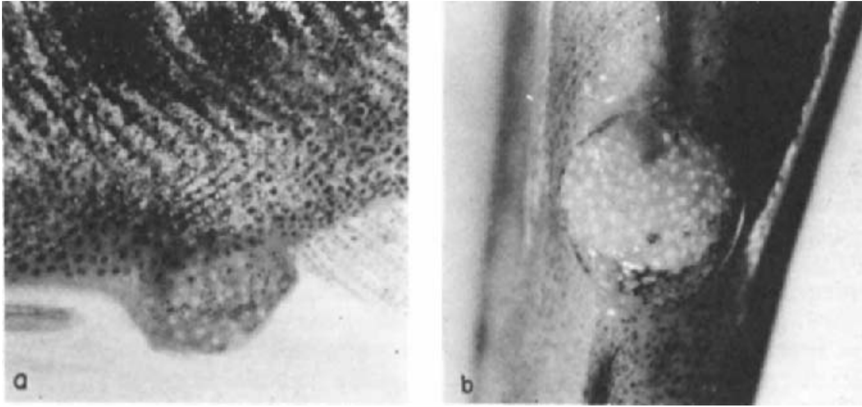


FIG. 18. Coho salmon from Sam Creek, Oregon, with mass of prolapsed intestinal tissue at the anus. The protruding tissue contained large numbers of encysted *N. salmincola salmincola* metacercariae that appear as white spots in the photograph. a—lateral view; b—ventral view. (After Bender, unpublished.)

retina and cornea of wild infected fish and concluded that these effects were produced by migrating parasites. Coho salmon collected by Bender (unpublished) from an Oregon stream had badly eroded fins that contained many parasites. Approximately 60% of the coho salmon he collected in September had a large mass of prolapsed intestinal tissue that almost blocked the anus (Fig. 18). This tissue contained many encysted parasites, and the surrounding tissue was frequently hemorrhagic. It is not likely that these fish could have survived and migrated to sea. Prolapse of the rectum may occur in heavy *Trichuris trichiura* infections in man with toxicity or trauma as the presumed cause. Similar irritation may have caused the condition in Bender's fish.

Wood and Yasutake (1956) studied the histopathology associated with encysted parasites in yearling coho salmon. They found little tissue reaction or inflammation around the parasites, but around some they did see fibrous walls and occasionally hyperplasia of epithelial cells. There was evidence of "marked obstructive and mechanical injury" to ventricle muscle fibers of the heart, the retina, the kidney tubules, the pancreatic tissue and the gall bladder wall. Metacercariae in the aorta were considered large enough to obstruct this vessel. Wood and Yasutake (1956) concluded that practically every organ of an infected fish is weakened physiologically. The evidence strongly suggests that the parasite is pathogenic to fish in nature, at least under some conditions.

(ii) Laboratory infections. Shaw *et al.* (1934) exposed young rainbow and brook trout to infected snails and reported that the fish remained healthy even though some were heavily parasitized. Bennington (1951) and Bennington and Pratt (1960) concluded that the parasite is pathogenic to fish because 12 chinook salmon, 2 dace, 1 sculpin and 2 goldfish died 6–24 h

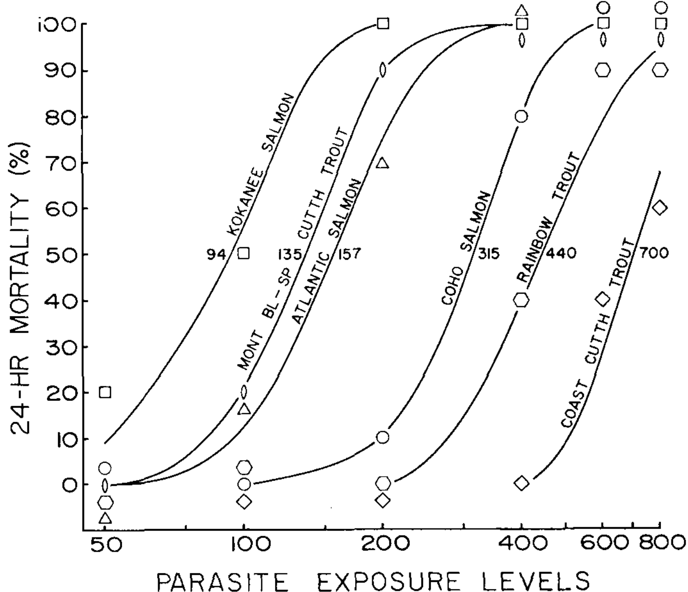


FIG. 19. The 24-h mortalities of salmonid fish exposed to known numbers of *Nanophyetus salmincola salmincola* cercariae. The number beside each curve is the LEL_{50} value for that species of fish. (The value for rainbow trout was given incorrectly as 340 by Baldwin *et al.*, 1967.) Mont Bl-Sp Cutth Trout, Montana Black-Spotted Cutthroat Trout; Coast Cutth Trout, Coastal Cutthroat Trout. (After Baldwin *et al.*, 1967.)

after exposure to infected snails. Farrell and Lloyd (1962) reported that rainbow trout died after exposure to infected snails.

The definitive study on the pathogenicity of the parasite to fish in the laboratory was done by Baldwin *et al.* (1967). They exposed six species of salmonid fish, 29–42 mm in fork length, for 1 h to known numbers of parasites, and then determined the 24-h lethal exposure levels (24-h LEL_{50}—the exposure level, in numbers of cercariae lethal to 50% of the fish in 24 h) and the 24-h lethal dosage levels (24-h LD_{50}—the infection level, in numbers of metacercariae that results in 50% mortality of the fish in 24 h). Most of their fish exposed to large numbers of parasites died within the first 24 h (Fig. 19). No control fish died during this time. After exposure to 200 parasites, 100%, 90% and 70% of the kokanee (*O. nerka*), Montana black-spotted cutthroat trout (*S. clarki lewisi*) and Atlantic salmon (*S. salar*), respectively, died during the first 24 h, but this exposure level proved fatal to only one coho salmon and no rainbow trout (*S. gairdneri*) or coastal cutthroat trout (*S. clarki clarki*) (Fig. 19). All of the coho salmon and 90% of the rainbow trout died within 24 h after exposure to 600 parasites, but only 40% of the coastal cutthroat trout died (Fig. 19). The 24-h LEL_{50}'s for kokanee, Montana black-spotted cutthroat trout, Atlantic salmon, coho salmon, rainbow trout and coastal cutthroat trout are 94, 135, 157, 315, 440 (given incorrectly as 340 by Baldwin *et al.*, 1967) and 700, respectively (Fig. 19).

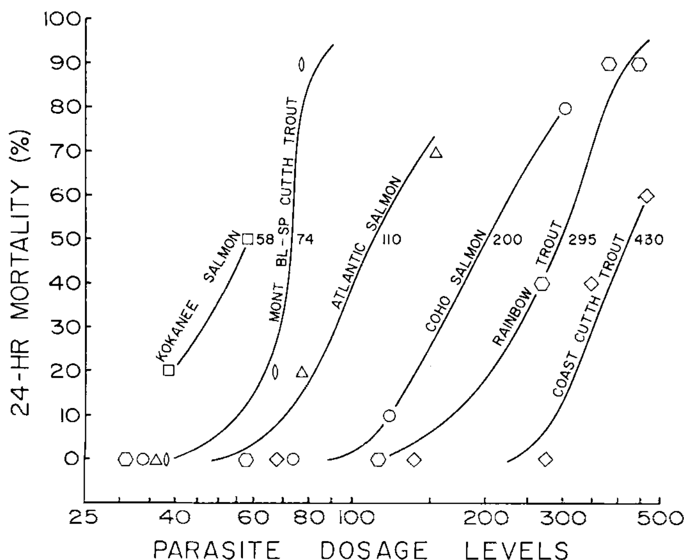


FIG. 20. The 24-h mortalities of salmonid fish infected with *Nanophyetus salmincola salmincola* metacercariae, in relation to estimated numbers of parasites. The numbers (averages) of parasites in these fish were assumed to be the average numbers found in fish that were exposed to the same numbers of cercariae and that survived for the first 24 h of the experiments. The number beside each curve is the LD_{50} value for that species of fish. Abbreviations same as for Fig. 19. (After Baldwin *et al.*, 1967.)

The highest parasite numbers recovered from kokanee, Montana black-spotted cutthroat trout and Atlantic salmon that survived for the first 24 h were 68, 79 and 162, respectively. One coastal cutthroat trout that survived had 531 parasites, and three had 422–531 parasites, whereas coho salmon and rainbow trout that survived had fewer parasites than coastal cutthroat trout but more than the other species (Fig. 20). The 24-h LD_{50}'s for kokanee, Montana black-spotted cutthroat trout, Atlantic salmon, coho salmon, rainbow trout and coastal cutthroat trout are 58, 74, 110, 200, 295 and 430, respectively (Fig. 20).

These results clearly show that the six fish species studied by Baldwin *et al.* (1967) can be divided into three groups having different relative sensitivities to the infection (Figs 19 and 20). The coastal cutthroat trout is the most resistant species, the rainbow trout and coho salmon are intermediate in resistance, and kokanee, Atlantic salmon and Montana black-spotted cutthroat trout are the most sensitive species. The high virulence of the parasite for the last two species may account in part for the unsuccessful efforts by the Oregon State Game Commission to establish these species of fish within the enzootic area.

That the pathogenicity of a parasite usually decreases with increasing evolutionary duration of the host-parasite relationship is a widely accepted principle. The results of the experiments of Baldwin *et al.* (1967) are in accord with this principle, which may therefore explain the differences in sensitivity to *N. salmincola* of various salmonid fishes. Atlantic salmon, Montana black-spotted

FIG. 21. Caudal region of an uninfected Lahontan cutthroat trout 45 mm long. (After Baldwin *et al.*, 1967.) × 7.

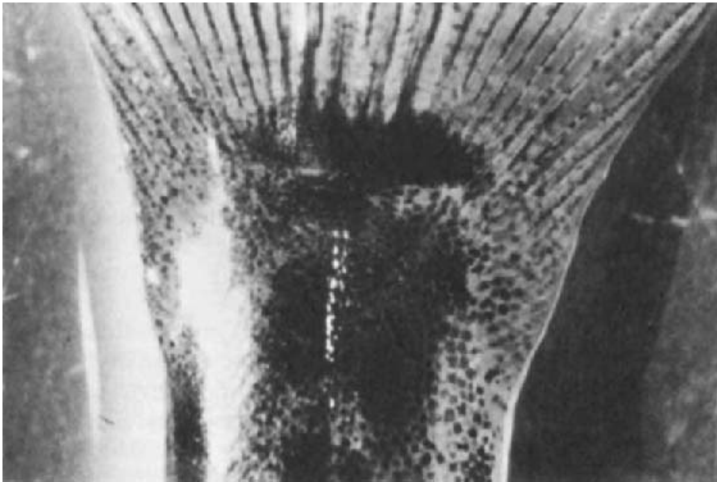


FIG. 22. Caudal region of a Lahontan cutthroat trout exposed to 700 cercariae of *Nanophyetus salmincola salmincola* 24 h before the picture was taken, showing large hemorrhagic area. The entire dark area shown was red in color. The chromatophore pigment was dispersed, but most of the darkness was due to hemorrhage in the dermis (see Fig. 24). (After Baldwin *et al.*, 1967.) × 7.

cutthroat trout, kokanee, and their parent stock, sockeye salmon, except sockeye salmon in the Columbia River that are destined for streams east of the Cascade Mountains, do not occur naturally in the enzootic area of the trematode (Snyder, 1940; Oregon State Game Commission, 1947; Carl *et al.*, 1959);

the other species tested do occur naturally in this area. Thus, kokanee, Montana black-spotted cutthroat trout and Atlantic salmon should be and are the most sensitive of the tested species to the effects of the parasites. The genus *Oncorhynchus* is more recent in origin than *Salmo* (Neave, 1958), and presumably it became established in the enzootic area after *Salmo*. Thus, coho salmon should be and are more sensitive to the parasite than are coastal cutthroat trout. The rainbow trout used by Baldwin *et al.* (1967) came from a stock with an incompletely known history, and therefore we cannot speculate about the relative resistance of this species to the parasite.

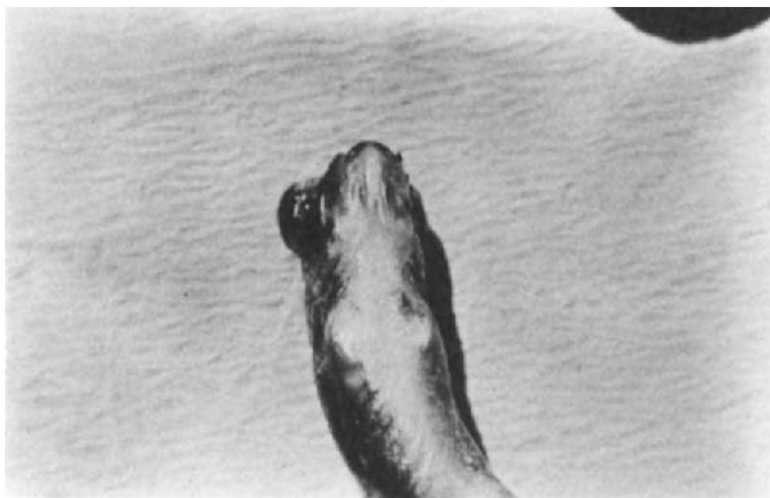


FIG. 23. Exophthalmia in an Atlantic salmon, 76 mm long, exposed to *N. salmincola salmincola* cercariae for 30 min approximately 3 weeks before the picture was taken. (Millemann, unpublished.)

Baldwin *et al.* (1967) described the pathology and symptomatology associated with experimentally infected fish. Diffuse petechiae on the body surface and larger hemorrhagic areas at the bases of the pectoral, pelvic and caudal fins appeared within 2 h after exposure of the fish (cf. Figs 21 and 22). Hemorrhagic areas reached a maximum size 12 h later, and many were visible for 48 h. As first reported by Bennington and Pratt (1960), papules were present on the fish's skin, presumably where the parasites had penetrated. Orbital hemorrhage was observed in many fish.

Exophthalmia was not present in the fish infected experimentally by Bennington and Pratt (1960), Farrell and Lloyd (1962) and Baldwin *et al.* (1967), but we (unpublished) have produced this condition in Atlantic salmon 76–85 mm long by exposing them in the laboratory to parasites once for approximately 30 min (Fig. 23). Control fish held under the same conditions in parasite-free water did not develop exophthalmia. In contrast with the findings of Ward and Mueller (1926), we found few parasites (4–21) in the

eyes and optic nerves of our fish, but we recovered 1200–1400 parasites from the other tissues, including the kidney, and therefore the exophthalmia may be due to edema resulting from kidney damage by the parasites. Exophthalmia occurs in other infectious diseases of fish involving kidney damage such as bacterial kidney disease, viral hemorrhagic septicemia, and Sacramento River chinook disease.

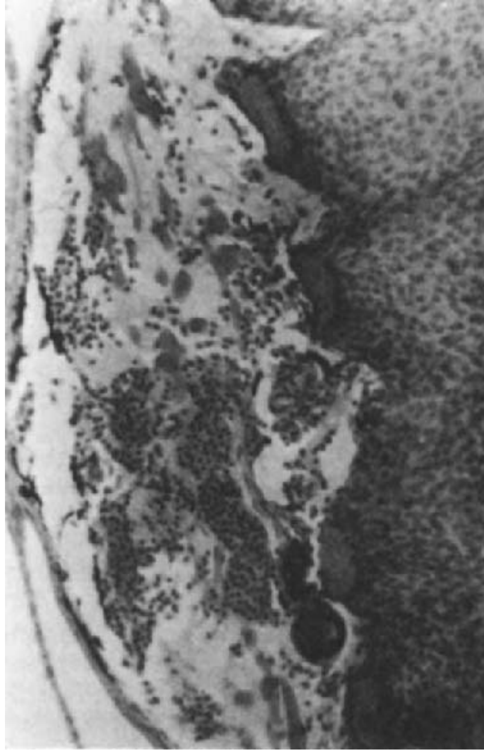


FIG. 24. Cross-section through the base of the caudal fin of a Lahontan cutthroat trout 52 mm long exposed to 500 cercariae of *N. salmincola salmincola*, showing hemorrhagic area in loose connective tissue and a cercaria near a fin ray and blood vessel. (After Baldwin *et al.*, 1967.) × 117·4.

Histopathological changes reported by Baldwin *et al.* (1967) include extravasation of red blood cells in the loose connective tissue between the dermis and muscle bundles in the hemorrhagic area at the caudal fin base (Fig. 24), destruction of muscle tissue in the path of cercarial migration, and partial renal tubule and blood vessel occlusion.

Signs of infection in fish include a decrease in swimming activity, loss of equilibrium, drifting, erratic swimming, an increased respiratory rate and vertical or horizontal tail curvature in some fish (Baldwin *et al.*, 1967).

The pathogenesis of the infection is not understood. The fish used by Baldwin *et al.* (1967) died within the first 24 h of the infection, which is the time

of parasite penetration and migration. Deaths of fish may be due not only to mechanical tissue destruction, but also to toxic substances, e.g. proteolytic enzymes released by the parasites to aid their migration through tissues. We have suggested evidence, from studies in progress, to support this view. We injected salmonid fish intraperitoneally with homogenized cercariae. The fish died 24 to 48 h later and most of them had large hemorrhagic areas in the body musculature that were similar to those produced by living parasites. Our control fish, injected either with distilled water or distilled water in which live cercariae had been held previously, remained healthy.

It is unlikely that fish in nature frequently acquire in a short time the number of parasites that they received in the laboratory studies of Baldwin *et al.* (1967). As noted previously, uninfected fingerling coho salmon held captive in streams acquired 6–143 parasites a day. Farrell and Lloyd (1962) stated, and we (unpublished) have demonstrated in the laboratory, that fish can tolerate daily exposure to small numbers of parasites, and thereby accumulate numbers of parasites that would be lethal if acquired within 24 h. The presence of large numbers of parasites found by Simms *et al.* (1931a, b) and by us in naturally infected fish can thus be explained. However, fish that survive the initial stage of the infection may be weakened physiologically during later stages of the infection, as suggested by Wood and Yasutake (1956), especially if the parasites are encysted in vital organs such as the heart and gills. Studies of subtle effects of *N. s. salmincola* infection on fish in our laboratory (by our student Mr. Jerry Butler) show that the growth of infected coho salmon and steelhead trout is retarded and their swimming performance impaired compared with that of uninfected control fish.

9. *The metacercaria*

(a) *Morphology and development.* The morphology of *N. s. salmincola* metacercariae was studied by Ward and Mueller (1926), Bennington and Pratt (1960) and Gebhardt (1966). Filimonova (1963, 1965) reported on the morphology and development of metacercariae of the Siberian subspecies. The following description applies to metacercariae of both subspecies. Encysted parasites are ovoid in shape (Fig. 25). Newly encysted metacercariae are enclosed by a thin, transparent wall of parasite origin, and older ones also by a fibrous wall of host origin. As first noted by Simms *et al.* (1931a), the parasites increase in size with increasing age, and the excretory bladder becomes filled with granules and increases in size. The granules cause the parasites to appear black by transmitted light and white by reflected light. The stylet gradually disappears with increasing age of the metacercariae.

Gebhardt (1966) studied the development of *N. s. salmincola* metacercariae in experimentally infected fish from days 1–106 of the infection. The diameters (in mm) of encysted parasites and their age in days (in parentheses) were: 0·11–0·13 (1); 0·12–0·15 (5); 0·15–0·19 (15); 0·19–0·21 (30); 0·21–0·25 (50); and 0·23–0·25 (106). The size of parasites was reported as 0·17–0·25 mm by Ward and Mueller (1926), 0·14 mm by Donham *et al.* (1926), and 0·17–0·25 mm by Simms *et al.* (1931a). These discrepancies can be attributed to differences in age of the metacercariae.

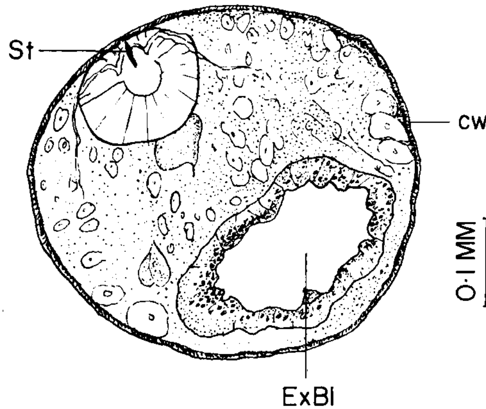


FIG. 25. Infective metacercariae of *Nanophyetus salmincola salmincola* taken from a "wild" fish. CW, cyst wall; ExBl, excretory bladder; St, stylet. (After Bennington and Pratt, 1960.)

Filimonova (1965) also found that *N. s. schikhobalowi* metacercariae from experimentally infected fish increase in size with increasing age. Parasites of 5 h, 5 days, 12 days and 22 days old were 0·09–0·16, 0·13–0·16, 0·16–0·20 and 0·16–0·22 mm in size, respectively. The oral and ventral suckers, the pharynx and cyst wall increased in size with increasing age of the metacercariae, and the stylet gradually disappeared. Granules first appeared in the excretory bladder of 12-day-old parasites.

Metacercariae of the two subspecies have the following measurements (in mm) with those of the Siberian subspecies given in parentheses. Diameter of encysted metacercariae, 0·11–0·33 (0·21–0·35); excysted metacercariae, 0·53 × 0·28 (0·35–0·66 × 0·18–0·34); oral sucker, 0·11 (0·07–0·12); ventral sucker, 0·10 (0·07–0·11); pharynx, 0·03 (0·04–0·05); excretory bladder, 0·25 × 0·18 (0·10–0·24 × 0·07–0·23). The cuticle is covered with spines. Intestinal caeca are present and first appear in 12-day-old metacercariae. In metacercariae of the U.S. subspecies, Ward and Mueller (1926) observed testes and a band of dense nuclei near the acetabulum, which presumably represented the female reproductive system, but Bennington and Pratt (1960) did not see either testes or the nuclei. Filimonova (1963) reported that two testes and an ovary are present in *N. s. schikhobalowi* metacercariae.

(b) *Infectivity*. Metacercariae of the U.S. subspecies are first infective for hamsters when 10 days old (Bennington and Pratt, 1960), and those of the Siberian subspecies are first infective for dogs at 11 days (Filimonova, 1963, 1965).

(c) *Effects of chemicals and temperature*. Donham *et al.* (1926) reported that *N. s. salmincola* metacercariae could be destroyed either by cooking infected fish (temperature not given) or by holding the fish for 6 days in freezing weather during which the temperature ranged from −22° to 1·5°C. Parasites were destroyed when infected fish kidney was spread in a thin layer and dried at room temperature for 5 days (Simms *et al.*, 1931b). Parasites in infected fish held at approximately 1·5°C for 74 days were not destroyed, but were killed

on the 94th day (Simms *et al.*, 1931b). Simms *et al.* (1931a) observed movement of some encysted free metacercariae after they were held at approximately 3°C for 5·5 months but not after 6 months.

Farrell *et al.* (1968) reported that a dog, who according to the owner had not eaten fresh fish but had eaten skin of kippered salmon, became infected with *N. s. salmincola* and also developed SPD. The fish had been soaked previously in a solution of sugar, salt and pepper for 3–4 h and then smoked at a "reported" temperature of 82·2°C for 10–16 h.

Filimonova (1965) reported that *N. s. schikhobalowi* metacercariae in fish held at 20°–22°C were infective after 5 days and viable after 8 days, and in fish held at 4°C the parasites survived 15–16 days.

(d) *As biological tags.* The usefulness of parasites as biological tags to indicate the origin and migration of fish and discreteness of fish populations is well established (Sindermann, 1961; Kabata, 1963; Margolis, 1963; Templemen and Fleming, 1963). A parasite, to be an efficient tag, must meet the following requirements: (1) it should be easily visible; (2) it should not kill the fish; (3) it should live as long as the fish; (4) the infection rates should be stable and high; (5) it should have a restricted geographical distribution; (6) it should have a narrow host specificity. Metacercariae of *N. s. salmincola* satisfy these requirements. *Nanophyetus s. schikhobalowi* has not been reported as a parasite of coho salmon and chinook salmon, which are intermediate hosts for *N. s. salmincola.* Thus, the origin of infected coho and chinook salmon caught on the high seas would be known. With this and other information, we could evaluate the contribution of a part of the Pacific Northwest to the high seas coho and chinook salmon fisheries. Moreover, knowledge of the migration and distribution of the two salmon species would be enhanced.

III. "Salmon Poisoning" Disease

A. Geographical Distribution

The geographical distribution of SPD extends north from the Sacramento River in California to the southern portion of the Olympic Peninsula in Washington and inland to the Pacific slopes of the Cascade Mountains (Fig. 8). As noted previously, the enzootic area is determined by the distribution of the snail *O. silicula,* which is the intermediate host for the trematode vector *N. s. salmincola.* The disease has been reported from dogs in eastern Washington and Oregon (Farrell and Lloyd, 1962; Knapp and Shaw, unpublished). However, they probably became infected by eating infected salmonid fish that had migrated up the Columbia River, or by eating infected trout that had been transplanted from western Oregon or Washington hatcheries into local streams or lakes.

B. Etiology

A rickettsia-like organism, *Neorickettsia helminthoeca,* is the etiological agent of SPD (Fig. 26). The organisms, approximately 0·3 μ in size, are coccoid or coccobacillary in shape, and occur singly, or in multiple colonies, or in morula-like clumps in infected canid cells. Pleomorphic forms that appear as

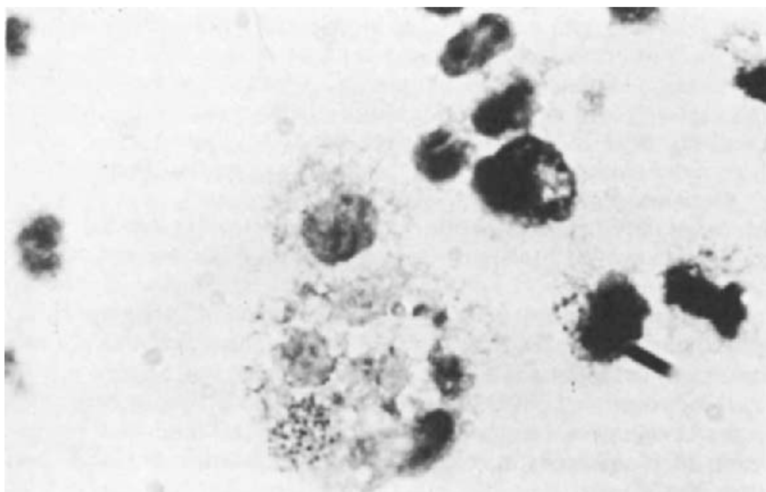


FIG. 26. Lymph node impression smear from a dog that died of "salmon poisoning" disease showing the etiological agent in the cytoplasm. (Knapp, original.) × 2400.

rods of varying lengths up to 2 μ and sometimes bent in crescents or rings, have been observed (Philip, 1955). The organisms stain purple with Giemsa stain, and blue or red with Macchiaviello's stain, and are Gram negative. Attempts to grow the organisms on bacteriological media, in embryonated chicken eggs, and in chick embryo tissue culture have been unsuccessful (Philip, 1955). Simms and Muth (1934) were unable to produce infections in dogs using filtrates of ground infected trematodes or of various dog blood preparations. The organism in lymph node cells resists freezing at −20°C for 31–158 days (Philip *et al.*, 1954a), and in leucocytes it resists temperatures of 4·5°C and 52·5°C for 48 h and 2 min, respectively, but not 60°C for 5 min. (Simms and Muth, 1934). The SPD agent shows some affinities with *Ehrlichia* (=*Rickettsia*) *canis*, the causative agent of a tick-borne canine rickettsiosis (Philip, 1959).

C. TRANSMISSION

Infective SPD agent has been demonstrated in all of the stages of the trematode vector (Fig. 1). Its presence in adult *N. s. salmincola* was shown by Simms *et al.* (1931a, b), Simms and Muth (1934), Philip *et al.* (1954b) and Nyberg *et al.* (1967); in metacercariae by Simms *et al.* (1932) and Philip *et al.* (1954a); in helminth-infected snail livers by Philip *et al.* (1954b); and in helminth eggs by Nyberg *et al.* (1967).

Only canids are susceptible to the disease. It has been transmitted to them experimentally by feeding infected salmonid fish from fresh water (many authors) or ocean-caught salmonid fish (Millemann *et al.*, 1964; Farrell *et al.*, 1964); by injection of infected blood, spleen, and lymph node suspensions

(Simms *et al.*, 1932; Cordy and Gorham, 1950; Philip *et al.*, 1954a); by injection of adult flukes (Simms *et al.*, 1931a; Simms and Muth, 1934; Nyberg *et al.*, 1967); by injection of metacercariae (Simms *et al.*, 1932); by injection of helminth-infected snail livers (Philip *et al.*, 1954b); and by injection of helminth eggs (Nyberg *et al.*, 1967). Philip (1955) reported partial success in transmitting SPD to dogs by allowing the ticks *Haemaphysalis leachi* and *Rhipicephalus sanguineus* to feed on the animals or by injection of suspensions of *R. sanguineus* ticks into dogs.

Raccoons develop a mild temperature rise after eating infected fish or after injection with proved infectious lymph nodes, but otherwise remain symptomless and are refractory to infection with the agent (Philip, 1955). However, trematodes from raccoons carry the agent. Philip (1955) produced disease in a dog by intraperitoneal injection of 500 adult trematodes, but not of lymph nodes, recovered from a symptomless infected raccoon. Simms *et al.* (1931a, 1932) failed to produce SPD in two bobcats and one bear by feeding infected fish, and in one guinea-pig, one white rat and one rabbit by intraperitoneal injection of blood from a sick dog. Cordy and Gorham (1950) could not produce the disease in mink, cats, guinea-pigs, hamsters and white mice. Philip *et al.* (1954a) were unable to adapt the agent to guinea-pigs, hamsters and white mice. Man can be infected with *N. s. salmincola* but apparently is refractory to the disease. Philip (1958) infected himself by eating raw infected trout, samples of which produced a fatal infection when fed to a dog. He suffered no ill effects, but some parasites did attain maturity as evidenced by the appearance of a few eggs in the stools 10 days after ingestion of the fish.

D. SYMPTOMS

Signs of infection in dogs and foxes are similar (Cordy and Gorham, 1950). The incubation period averages 5–7 days, although some dogs have long incubation periods of 19–33 days (Philip, 1955), after which there is a sudden onset of fever and anorexia sometimes accompanied by vomiting and diarrhea or dysentery. The fever, with a temperature peak of 104°–107°F, lasts 4–7 days and is followed by a period of defervescence during which the temperature returns to normal or falls below the normal level. Some dogs that succumb to

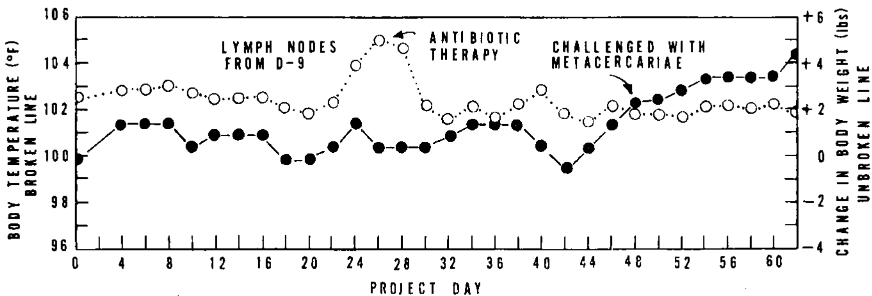


FIG. 27. Body temperature and weight changes for a dog injected intraperitoneally with infected lymph nodes and then subsequently treated and challenged with metacercariae. (After Nyberg *et al.*, 1967.)

the disease may be afebrile, show a slight temperature rise, or show a shortened febrile period (Philip, 1955). Dehydration and loss of weight are marked as a result of the constant inappetence. The animals will occasionally drink excessive amounts of water. A serous nasal discharge may occur early in the febrile period. A conjunctival exudate may be present at the inner canthus. The somatic lymph nodes become enlarged. In the later stages of the disease, the mesenteric lymph nodes are enlarged. Death usually occurs 10 days–2 weeks after onset of signs. The course of a typical infection is shown in Fig. 27.

E. PATHOGENESIS AND PATHOLOGY

SPD is acquired by ingestion of raw infected salmonid fish. It is an almost uniformly fatal disease for dogs. Philip (1955) stated "some 90 percent of naturally infected dogs are reported to die". Simms *et al.* (1931b) reported that only 4 of 102 dogs with SPD recovered naturally. The disease is equally fatal for foxes (Cordy and Gorham, 1950) and coyotes (Donham and Simms, 1927). The way that the etiological agent leaves the trematode vector and gains access to the host tissues is not known. The organisms are present in recticulo-endothelial cells of lymph nodes, tonsils, spleen and intestinal lymph follicles of dogs and foxes, and occasionally in macrophages of the liver, lungs and blood (Cordy and Gorham, 1950).

The principal gross lesions include variable enlargement of the ileocecal, mesenteric, portal and internal iliac lymph nodes (Fig. 28). The thymus may be enlarged and edematous in young animals. The spleen and tonsils may also show enlargement, hemorrhage and hyperplasia. Occasional enlargement of other lymphatic tissues may occur. Necrosis and hyperplasia of intestinal

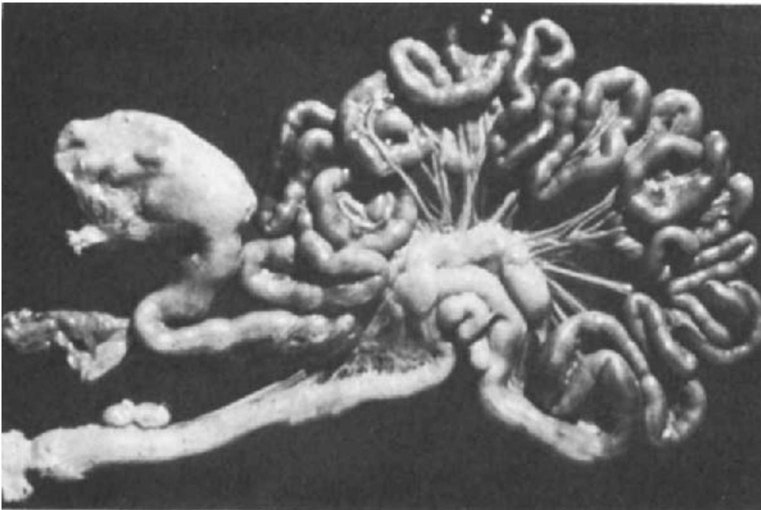


FIG. 28. Intestine from a dog that died of "salmon poisoning" disease. Enlarged visceral lymph nodes are typically characteristic for the disease. (Courtesy of C. B. Philip.)

lymph follicles can lead to ulceration and severe hemorrhage (Cordy and Gorham, 1950).

Microscopic necrotic foci appear in the lymph follicles and may also occur in other tissues. Leucocytic infiltration is a common occurrence. Neuropathological changes are characterized by a non-suppurative meningitis or meningoencephalitis (Hadlow, 1957).

The trematodes embedded in intestinal tissue account for slight tissue damage and are found predominantly in the duodenum, whereas the enteric ulceration and hemorrhage resulting from lymph follicle necrosis are frequently confined to areas of the alimentary tract adjacent to the ileocecal valve.

Cordy and Gorham (1950) compared pathological lesions of SPD in dogs and foxes. Microscopic lesions were more extensive in foxes and apparently the fox is more susceptible to the disease. In most of the dogs examined the livers appeared normal, but in most of the foxes the livers were soft and friable. Hemoperitoneum and hemorrhage of the gall bladder wall, kidney necrosis and heart lesions were observed only in foxes.

The mechanism by which the organisms cause disease in canids is not known.

F. DIAGNOSIS

1. *History and clinical signs*

A history of the animal eating raw fish and appearance of the signs provide a presumptive diagnosis for SPD.

2. *Examination of feces for eggs of* N. s. salmincola

The eggs, which measure 64–97 $\mu \times$ 34–55 μ, first appear in the feces of dogs 5–8 days after the animals have eaten infected fish, and disappear by the 250th day of infection (Simms *et al.*, 1931a). They are light brown with an indistinct operculum at one end and a small blunt point at the other (Fig. 4). Eggs can be detected in the feces in heavy infections by a direct smear. A more reliable method is the technique of Dennis *et al.* (1954), which according to Farrell *et al.* (1955) is applicable to recovery of *N. s. salmincola* eggs. The latter authors described the technique as follows:

> It consists of a washing-sedimentation technique, using a mixture of 5 cc. of a household liquid detergent and 995 cc. of tap water to which a few drops of 1 per cent alum (aluminum potassium sulfate, U.S.P.) have been added. This fluid becomes somewhat gelatinous on standing; therefore, it is prepared at frequent intervals. A 1- to 3-Gm. fecal sample is thoroughly mixed with about 15 cc. of the detergent solution in a large test tube or other container and the mixture is strained into a 50-cc. centrifuge tube. The material on the strainer is washed with more detergent solution until the centrifuge tube is filled to the 50-cc. mark. The suspension is allowed to stand for five to ten minutes and then the supernatant fluid is carefully decanted or siphoned off, allowing 2 or 3 cc. of the liquid and debris to remain in the centrifuge tube. The sediment is again washed by refilling the centrifuge tube to the 50-cc. mark with the detergent solution and allowing it to stand for another five to ten minutes. The supernatant fluid is then separated as before. (The ova will settle quickly in water, but the detergent solution floats off more debris.) A small amount of the sediment is transferred to a slide and examined for fluke ova.

The finding of eggs in the feces does not establish the diagnosis because dogs that have recovered from the disease can be reinfected with the trematode, and the eggs of other canine parasites can be confused with those of *N. s. salmincola.*

3. *Skin test*

A successful skin test has not been developed (Farrell *et al.*, 1955).

4. *Lymph node aspiration and smear*

Farrell *et al.* (1955) described the following technique for diagnosis of SPD. The mandibular lymph nodes medial and ventral to the mandible of the dog are palpated, then one of the nodes is pulled ventrally and a curved intestinal clamp placed behind it. This fixes the node in position. A few drops of fluid can be aspirated from the node using a 20 gauge needle attached to a syringe. The plunger is withdrawn and the node compressed at the same time. The aspirated fluid, which usually remains in the needle, is expressed onto a clean microscope slide. The drop is smeared, air dried, fixed and defatted for 1 min with a mixture of equal parts of ether and alcohol. The smear is stained by either Giemsa's or Macchiavello's technique. In addition to the usual Giemsa technique, which is used if there is any doubt as to the presence of rickettsia, Farrell *et al.* (1955) described a rapid Giemsa method. This involves staining the smears for 2 min with a 1 : 1 dilution of stock Giemsa and buffered water at pH 7·2 followed by washing of the slides. The cells in the smears are examined for the typical intracytoplasmic rickettsial bodies. Extracellular organisms cannot reliably be distinguished from debris.

G. TREATMENT

Various sulfanilamides given orally or parenterally have been successful in treatment (Coon *et al.*, 1938; Cordy and Gorham, 1951; Philip *et al.*, 1954b). Dosage at therapeutic blood levels should be maintained for at least 3 days. Effective results have also been obtained with penicillin, chlortetracycline, chloramphenicol and oxytetracycline. The best results follow administration of large divided doses. If the animal is dehydrated, intravenous fluid therapy is essential to avoid nephrotoxic effects. Treatment in the late stages of the disease may not be successful.

General supportive treatment, aimed at correcting and maintaining fluid and electrolyte balance, providing nutritional requirements and controlling diarrhea, is often essential unless there is a prompt favorable response.

H. IMMUNITY

Canine hosts that recover from the disease either spontaneously or after drug treatment develop a lasting and solid immunity to the disease agent, but not the fluke (Simms *et al.*, 1931b; Shaw and Howarth, 1939; Philip *et al.*, 1953). Simms *et al.* (1931b) reported that dogs from an immune female were susceptible to SPD.

I. CONTROL

There is no vaccine or other prophylatic currently available. Shaw and Howarth (1939) suggested that dog owners feed their animals infected fish and then begin early sulfanilamide treatment to produce solid immunity.

Preventive measures include avoidance of feeding raw salmonid fish to dogs and keeping them away from streams during the period of salmon migration.

Control of the infection by eradication of the snail host, using molluscicides, is impractical.

IV. FUTURE RESEARCH

There is still much to be learned about the biology of *N. salmincola*, the SPD agent, and the disease process. The following are some of the areas that merit further investigation: (1) the chemical and physical factors necessary for development and hatching of the trematode eggs; (2) identification of the parasite stages in the snail immediately after miracidial penetration; (3) the ecological and biological factors involved in restricting *O. silicula* to its present known geographical range; (4) the degree of first intermediate host specificity; (5) pathogenesis of the trematode infection in fish; (6) sensitivity of the metacercariae to physical and chemical agents; (7) confirmation of the assumed conspecificity of the U.S. and Siberian trematode strains; (8) location of the rickettsiae in the various trematode stages, especially in the adult trematode; (9) isolation, *in vitro* cultivation, and characterization of the rickettsiae. Studies relevant to these research needs are either in progress in our laboratories or are planned for the near future.

Farrell (1964) has reported the existence of a second rickettsia-like disease of dogs, which he named Elokomin fluke fever (EFF), that is transmitted by *N. s. salmincola*. He stated that this disease has a high morbidity and low mortality for dogs; that ferrets and bears, which are refractory to SPD, are susceptible to EFF; and that EFF and SPD are serologically distinct. However, experimental proof for the existence of EFF is still lacking because, unfortunately, Farrell has not published any experimental data to support his general statements.

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Snail Problems in African Schistosomiasis

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I. INTRODUCTION

Schistosomiasis is now considered to be the second most important parasitic disease affecting man and it is estimated that there are at least 150 million people infected (World Health Organization, 1965). In a few areas outside the African continent some degree of control has been achieved, but in general the disease is thought to be on the increase, particularly in countries where water resources are being developed. Yet for many years it received scant

attention and there are still a number of countries with a high incidence of infection which regard it as one of their more minor medical problems. However, during the last two decades schistosomiasis has received increasing attention and the latest bibliography lists some 10000 papers up to 1962 (Warren and Newill, 1967).

To study the transmission of schistosomiasis both the parasites and the snail intermediate hosts must be considered together with the complexities of the host-parasite relationship through which each may influence the other in a variety of ways. An additional complication is that several species of parasites and of snails are involved in Africa and both groups show considerable infraspecific variation. As a result many field investigations raise taxonomic problems which cannot be solved readily, and a brief account of these difficulties will be given in the early part of the review.

The study of the transmission of insect-borne diseases has been facilitated by the availability of considerable numbers of trained entomologists. In contrast trained malacologists have been scarce and those with an interest in the ecology of freshwater snails have been rare. Although snails are less mobile than insects other problems cause difficulty in studying them. Growth can occur throughout the life of the individual and the rate of growth may depend on intrinsic and extrinsic factors which vary from time to time. Given good environmental conditions growth, as measured by increase in shell length, tends to be rapid among young snails and less rapid among larger snails. It is seldom possible to equate age with size in the members of natural populations because many factors such as temperature, food supply, parasitism, and the stability of the habitat appear to affect growth, and this problem will be discussed more fully later. Unlike insects, snails do not pass through distinctive stages in their life history and this again adds to the difficulty of defining comparable points in data from different localities.

This review relates mainly to Africa but some of the species of schistosomes from that continent have extended their geographical range into the Near East and America. It would be illogical to exclude relevant information from these other areas and considerable reference is made to studies from them, but it has not been possible to discuss their local ecological problems.

II. The Schistosomes

A. Species of *Schistosoma* in Africa

The species of mammalian schistosomes believed to be present in Africa are shown in Table I. A fuller discussion of these species and different views on their validity are given elsewhere (Amberson and Schwarz, 1953; Schwetz *et al.*, 1953; LeRoux, 1958; Nelson *et al.*, 1962; Pitchford, 1965) and only a few points need be noted here. Reports of other schistosomes such as *S. japonicum* Katsurada, 1904 and *S. spindale* Montgomery, 1906 being found in Africa are unconfirmed and may well derive from observations of abnormal or unfamiliar eggs.

S. haematobium exists in two forms which cannot at present be distinguished morphologically but which are quite distinct in their choice of intermediate

TABLE I

The mammalian schistosomes found in Africa

Species	Intermediate hosts	Usual definitive hosts	Known distribution
S. haematobium (Bilharz, 1852) northern form	*Bulinus:* *truncatus* group	Man	Near East, North and West Africa
S. haematobium southern form	*Bulinus:* *africanus group*	Man	Africa south of the Sahara
S. bovis (Sonsino, 1876)	*Bulinus:* *truncatus* group *africanus* group	Ruminants	Near East, North and East Africa
S. mattheei Veglia and LeRoux, 1929	*Bulinus:* *africanus* group *truncatus* group?	Ruminants and man	East, West and Southern Africa
S. leiperi LeRoux, 1955	*Bulinus:* *africanus* group?	Ruminants	Eastern and Central Africa
S. curassoni Brumpt, 1931	Unknown	Cattle	East Africa
S. intercalatum Fisher, 1934	*Bulinus:* *africanus* group	Man	Congo
S. mansoni Sambon, 1907	*Biomphalaria*	Man, other primates and rodents	Africa south of the Sahara, Egypt and neighbouring countries, Caribbean islands and northern South America
S. rodhaini Brumpt, 1931	*Biomphalaria*	Rodents	Congo and East Africa
S. margrebowiei LeRoux, 1933	Unknown	Wild and domestic animals	Central Africa
S. faradjei Walkiers, 1928	Unknown	Man	Congo
S. hippopotami Thurston, 1963	Unknown	Hippopotamus	Uganda and South Africa
S. edwardiense Thurston, 1964	Unknown	Hippopotamus	Uganda

host (McCullough, 1959). The two forms occur sympatrically in West Africa and it has been proposed that the southern form should be regarded as a distinct species, *S. capense* (Harley, 1864), but this has not yet been accepted (LeRoux, 1958; Pitchford, 1965). *S. bovis* appears to have the same difference in intermediate host to the north and to the south of the Sahara, but in this case the other groups of *Bulinus* have also been implicated (Teesdale, 1962; Kinoti, 1964). In the south and west of the continent *S. mattheei* replaces

S. bovis but the morphological similarity of their spindle-shaped eggs causes considerable problems in identification (Dinnik and Dinnik, 1965; Pitchford, 1965; Blair, 1966). Further problems arise because a number of factors are known to cause variation in the shape of schistosome eggs and it can be difficult to identify the African species, other than *S. mansoni* and *S. rodhaini*, on the basis of egg shape alone (Pitchford and Visser, 1960; Nelson *et al.*, 1962; Pitchford, 1965; Jewsbury, 1968). Also it is claimed that *S. mattheei* and *S. haematobium* can hybridize producing polymorphic eggs, intermediate between the spindle-shaped and oval types, which resemble those described for *S. intercalatum* (Pitchford, 1961).

An important problem in studying naturally infected snails is that there is no reliable method of distinguishing the cercariae of the different mammalian schistosomes. Efforts have been made to find morphological criteria but the methods required are complex and time-consuming (Rowan, 1961; Wagner, 1961) and, in view of the cross reactions obtained when using cercarial antigens to diagnose human infections, there would seem to be little chance of finding an immunological test. In practice it is necessary to expose suitable laboratory hosts to cercariae shed by snails and to recover adult flukes and eggs for identification later. This introduces a series of hazards into the identification and involves the maintenance of animal colonies, while the delay of several months may mean that important information is not available until after the end of an investigation.

B. RESERVOIR HOSTS

Schistosomes apparently identical with the human forms are found in a considerable range of wild and domestic animals in Africa (Nelson, 1960; Nelson *et al.*, 1962; Pitchford and Visser, 1962). Ungulates are the natural hosts of *S. bovis*, which is rare as a parasite of man, and of *S. mattheei*, which may reach a high incidence in man in southern Africa. There are a few records of ungulates being infected with *S. haematobium* or *S. mansoni* but no evidence that they have any significance in the epidemiology of these species.

A number of species of rodents are susceptible to *S. haematobium* and *S. mansoni* (Pitchford, 1959; Gear *et al.*, 1966) and this is also true of *S. mansoni* in South America (Amorim, 1953; Barbosa *et al.*, 1953; Martins *et al.*, 1955). A population of *Mastomys* sp. in South Africa was unable to maintain transmission of *S. mansoni* (Pitchford and Visser, 1962) but other more aquatic rodents may be more efficient in this respect, and it seems possible that rodents could constitute an epidemiologically significant reservoir of infection in some areas, as in the case of *S. japonicum*. Rodents are the natural hosts of *S. rodhaini* and the fact that they can maintain transmission of this parasite suggests that they should be able to do so with other schistosomes in suitable conditions (Stijns, 1952; Berrie and Goodman, 1962; Fripp and Goodman, 1967).

Many primates are susceptible to both *S. haematobium* and *S. mansoni* but only those which regularly visit water are likely to become naturally infected. In this respect baboons are the most obvious choice. High levels of infection

with *S. mansoni* have been recorded in baboons and they seem to tolerate heavy infections over long periods (Nelson *et al.*, 1962). It is not yet clear whether baboon populations can maintain *S. mansoni* infection naturally in the absence of human infections but it has been reported that human infections have been contracted from such a zoonotic situation in Tanzania (Fenwick, 1966).

C. ZOOPROPHYLAXIS

When golden hamsters are exposed to a mixture of cercariae of *S. haematobium* and *S. bovis* the latter parasite becomes dominant and the development of *S. haematobium* is prevented (Teesdale and Nelson, 1958). In Corsica, Sardinia and Sicily *S. bovis* is prevalent in cattle but no *S. haematobium* occurs in man, although the local strains of *B. truncatus* are highly susceptible to both parasites, and this stimulated the suggestion that where man is constantly exposed to cercariae of schistosomes which do not mature in man these may cause some degree of immunity to infection with human schistosomes (LeRoux, 1961). Great numbers of such cercariae occur in certain parts of Africa where *S. haematobium* and *S. mansoni* are rare or absent and this phenomenon could explain the different severity of *S. haematobium* infections observed in different parts of Kenya (Nelson *et al.*, 1962; Nelson, 1966). Although high incidences of infection for both *S. haematobium* in man and *S. bovis* in cattle occur in some areas (Jordan, 1966) it is at present difficult to determine whether the severity of infection is being affected.

S. bovis does not develop into adult worms in rhesus monkeys, and when rhesus monkeys were exposed to several immunizing doses of this parasite and subsequently challenged with *S. haematobium*, they showed a high degree of acquired resistance manifested mainly in a reduction in the number of mature worms which developed from the challenge infection (Hsü *et al.*, 1966). Similarly, *S. bovis* and *S. mattheei* both have a potent immunizing effect against *S. mansoni* in mice and in rhesus monkeys, producing a large decrease in worm loads and in tissue egg densities (Nelson *et al.*, 1968; Amin *et al.*, 1968). In mice, *S. haematobium* had no effect on a subsequent exposure to *S. mansoni* nor had *S. bovis* to *S. mattheei*. If the same is true in man, such natural heterologous immunity, or zooprophylaxis, may have considerable epidemiological significance and could certainly influence the severity of infection in man. This shows considerable variation in different areas and *S. mansoni* is notably severe in Egypt and Brazil where there are no bovine schistosomes.

III. TAXONOMY AND DISTRIBUTION OF THE SNAILS

A. INFRA-SPECIFIC VARIATION

The freshwater environment has two characteristics which seem to be important in the evolutionary history of its snail fauna: it is discontinuous and transient. Most bodies of fresh water persist for only a relatively short time geologically, as a result of which their snail fauna has time to develop considerable infra-specific variation but insufficient time for evolution to proceed to higher levels (Hubendick, 1962). Also, freshwater snails are usually isolated

in small local populations thus creating a favourable situation for the development of divergent forms. Breeding experiments with *Biomphalaria glabrata* (Say) have indicated that allopatric populations develop various degrees of reproductive isolation, which emphasizes the importance of geographic speciation in this situation (Paraense, 1956). The interaction of these factors could explain the observed variability of freshwater snails at the infra-specific level.

In many parts of Africa the alternation of wet and dry seasons gives rise to temporary pools which are often snail habitats. The high reproductive potential of the snails produces large fluctuations in population size, which will be discussed later, and it is obvious that in these circumstances an even more complex and rapidly changing genetic situation may be expected. Some information on variation of radula teeth indicates that such a situation does arise. Specimens of *Lymnaea peregra* (Müller) collected from a relatively stable habitat in Sweden at an interval of nine years showed no significant differences in their teeth (Berrie, 1959), whereas specimens of *Bulinus* (*Bulinus*) sp. collected from temporary pools in South Africa at an interval of only four years showed little similarity in this respect (Schutte, 1965).

B. THE SPECIES GROUPS

Most of the original descriptions of African freshwater snails were based on shell characters only and the type localities are often extremely vague. When morphological characters were examined it became clear that many of the named species were synonymous, and Mandahl-Barth (1957a, b) did a great service by clearing up the large numbers of synonyms and arranging what he considered to be the valid species into the two genera *Biomphalaria* and *Bulinus*. Since then the arrangement has required some modification (Mandahl-Barth, 1960, 1965) and this process will certainly continue, but a solid framework now exists within which current taxonomic problems can be considered.*

The introduction of species groups which include the most closely related species within each genus has proved particularly useful. In addition to the great infra-specific variation in African freshwater snails it has become increasingly clear that it is scarcely possible to recognize conventional species limits particularly within *Bulinus*, as many of the so-called species appear to be able to interbreed and a complete range of intermediate forms can be found in some cases. In this situation the species groups stand as reasonable biological groupings even though their limits are also difficult to define.

1. *Biomphalaria*

Information about the species of *Biomphalaria* mentioned in this review is given in Table II. All members of the genus must be regarded as potential intermediate hosts of *S. mansoni*, but all species and races of *Biomphalaria* are not equally susceptible to all strains of the parasite and in some cases they may be completely refractory (Wright, 1962; Saoud, 1965).

Morphological studies and cross-breeding experiments convinced most

* Throughout this article all references to species of *Biomphalaria* and *Bulinus* follow Mandahl-Barth's nomenclature as far as possible.

TABLE II

Species of Biomphalaria *in Africa arranged in species groups*

Species	Number of subspecies	Main distribution	Schistosomes transmitted
1. *pfeifferi* group			
B. pfeifferi (Krauss, 1848)	Several of dubious validity	Africa south of the Sahara	*S. mansoni* *S. rodhaini*
B. rhodesiensis Mandahl-Barth, 1960	—	Zambia and Malawi	
B. germaini (Ranson, 1953)	—	Sahara and Lake Chad	
2. *choanomphala* group			
B. choanomphala (Martens, 1879)	2	East African lakes	*S. mansoni*
B. smithi Preston, 1910	—	East African lakes	*S. mansoni*
B. stanleyi (Smith, 1888)	—	East African lakes	*S. mansoni*
3. *alexandrina* group			
B. alexandrina (Ehrenberg, 1831)	2	Nile delta	*S. mansoni*
B. angulosa Mandahl-Barth, 1957	—	Eastern and Southern Africa	*S. mansoni*
4. *sudanica* group			
B. sudanica (Martens, 1870)	3	Eastern Africa	*S. mansoni* *S. rodhaini*
B. camerunensis (Boettger, 1941)	2	Western Africa	

malacologists that the snail hosts of *S. mansoni* in Africa and in America are congeneric but raised a considerable controversy over priority and practicality in nomenclature. As a result the International Commission on Zoological Nomenclature (Opinion 735, 1965) have ruled that the generic name *Biomphalaria* is to be given precedence over the generic names *Planorbina*, *Taphius* and *Armigerus* by any zoologist who considers that any or all of these names apply to the same taxonomic genus. The generic names *Australorbis* and *Tropicorbis* which have been used for the snail hosts in America are both of more recent origin and also fall into synonymy. In view of this ruling all snail hosts of *S. mansoni* can now be included in the genus *Biomphalaria* and frequent reference will be made to the extensive studies on the American species *B. glabrata* in this review.

2. *Bulinus*

Information about the species of *Bulinus* mentioned in this review is given in Table III, which shows that the role of the various species in the transmission of schistosomes is more complex than is the case with *Biomphalaria*. The

TABLE III

Species of Bulinus *mentioned in this review arranged in species groups*

Species	Number of subspecies	Main distribution	Schistosomes transmitted
1. *africanus* group (sub-genus *Physopsis*)			
B. africanus (Krauss, 1848)	2	Eastern and Southern Africa	*S. haematobium* southern form
B. nasutus (Martens, 1879)	2	East Africa	*S. bovis*
			S. mattheei
B. globosus (Morelet, 1866)	—	Africa south of the Sahara	*S. intercalatum*
			S. leiperi?
B. ugandae Mandahl-Barth, 1957	—	East Africa	*S. bovis*
2. *truncatus* group			
B. truncatus (Audouin, 1827)	5	Africa north of the equator, Near East, Mediterranean islands	
B. guernei (Dautzenberg, 1890)	—	West Africa	*S. haematobium*
B. natalensis (Küster, 1841)	—	Central and Southern Africa	*S. mattheei*?
3. *tropicus* group			
B. tropicus (Krauss, 1848)	5	Equatorial and Southern Africa	*S. bovis*?
B. obtusispira (Smith, 1886)	—	Madagascar	*S. haematobium*
4. *forskali* group (formerly sub-genus *Pyrgophysa*)			
B. forskali (Ehrenberg, 1831)	—	Africa and Arabia	?
B. senegalensis Müller, 1781	—	West Africa	*S. haematobium*
B. reticulatus Mandahl-Barth, 1954	—	East and Central Africa, Aden	*S. haematobium* in Aden
B. cernicus (Morelet, 1867)	—	Mauritius	*S. haematobium*
B. beccarii (Paladilhe, 1872)	—	Aden	*S. haematobium*

species in the *africanus* group are the main intermediate hosts of *S. haematobium*, *S. bovis* and *S. mattheei* in Africa south of the Sahara and they can be distinguished fairly readily from other species of *Bulinus*. However, within the group, the species are not easy to define and it seems likely that hybrids occur particularly in East Africa (Mandahl-Barth, 1965; Berrie, 1966c).

The species in the *truncatus* and *tropicus* groups are very variable in their morphological characters and many local forms have been recognized. Even

the characters which can be used to separate the two groups are unreliable, particularly in southern Africa (Stiglingh *et al.*, 1962; Mandahl-Barth, 1965; Schutte, 1965, 1966), and a fascinating and frustrating border zone exists which cannot yet be resolved. This problem is of some importance since it seems likely that all members of the *truncatus* group may be potential intermediate hosts of at least the northern form of *S. haematobium* and of *S. bovis*, while there is no adequate proof that members of the *tropicus* group transmit mammalian schistosomes, although occasional reports have cast suspicion on this group at least as regards non-human schistosomes (Teesdale, 1962).

The *forskali* group of snails are typically relatively small with high-spired shells, but the morphological separation of this group from the *truncatus* group is not satisfactory and the affinities of some species, such as *B. reticulatus*, are doubtful. Four species are known to act as intermediate hosts of *S. haematobium* in three restricted areas, two of which are outside continental Africa. Other species, particularly *B. forskali*, have been reported shedding mammalian schistosome cercariae and experimental infections have been successfully achieved, but the number of cercariae produced is generally small and there seems to be no definite proof that this group is seriously involved in the transmission of schistosomes in other parts of Africa.

C. CYTOLOGICAL AND BIOCHEMICAL STUDIES

The chromosomes of the snail hosts have also been studied (Burch, 1960, 1964, 1965, 1967; Natarajan *et al.*, 1965; Brown *et al.*, 1967; Brown and Burch, 1967) and those species of *Biomphalaria* which have been examined all have the haploid chromosome number of 18 which is typical of the Planorbidae. This number, with minor exceptions ranging up to 21, is also found in all species of *Bulinus* except those belonging to the *truncatus* group. In the *truncatus* group the basic number seems to be 36 but populations with 54 and 72 pairs have been recorded. It thus seems that all members of this group are polyploid and this character may prove valuable in distinguishing the *truncatus* and *tropicus* groups whose morphological differentiation is so difficult. However, in southern Africa, *B. natalensis* which is placed in the *truncatus* group on morphological grounds has only 18–21 pairs of chromosomes (Schutte, 1966; Brown *et al.*, 1967).

Trematode miracidia are clearly very discriminating in their selection of intermediate hosts and if we could detect the characters which they recognize this might well advance our knowledge of snail taxonomy. The miracidia probably penetrate in response to some stimulus provided by the body surface of the snails, and the stimulus seems likely to be chemical since miracidia have been shown to respond to water containing snail extract (Davenport *et al.*, 1962). After a miracidium has penetrated a snail its further development depends on its compatability with its new environment. This aspect will be discussed more fully later but again biochemical reactions will be involved which might be useful taxonomic characters. Finally, apart from factors relevant to miracidia, some biochemical variation may be expected between different species, subspecies and even populations.

The miracidia first contact the body-surface mucus of the snails and chromatographic examination of this mucus in a number of species of *Lymnaea* yielded results which fitted well with the taxonomy of that genus (Wright, 1959, 1964), but no such useful information has been obtained with *Biomphalaria* and *Bulinus*. Histochemical studies have been made of the enzyme systems in *Helisoma trivolvis* (Say) (Cheng, 1964) and *B. glabrata* (Muller, 1965), and the alkaline and acid phosphatases of *B. sudanica* and *B. truncatus* have been compared by electrophoresis (Wright *et al.*, 1966). The amino acid composition of the blood and other tissues from several planorbid snails has been examined (Targett, 1962a, b, 1963; Wright and Ross, 1963). The haemolymph of some strains of *B. glabrata* and *B. sudanica* contains a haemagglutinin for vertebrate erythrocytes which is not present in other strains of *B. glabrata* and this may be useful in differentiating populations or as a genetic marker (Gilbertson and Etges, 1967). Immunological studies of the haemolymph antigens of snails have given useful results only at the generic level (Michelson, 1966a, b), while other immunological studies have involved antigens and antisera prepared from various tissues and from egg-proteins of a number of species of *Bulinus* and *Biomphalaria* (Burch and Lindsay, 1966; Wright and Klein, 1967). Perhaps the most hopeful results from the taxonomic point of view have come from electrophoretic studies of planorbid egg-proteins (Wright and Ross, 1963, 1965, 1966). These have detected differences between species and between individual populations just as in the case of morphological characters. In the *africanus* and the *forskali* groups the level of variation in egg-proteins between populations of the same morphological species proved to be greater than the variation between different species in the same group.

The biochemical investigations have yielded much interesting information on variation among the snails and may shed further light on some of the problems of snail-parasite relationship. However, like the cytological studies, the taxonomic importance has so far been restricted largely to the generic and species-group level. It remains to be seen whether further investigations along these lines can distinguish characters which will add discrimination at the species and subspecies level. The integration of these methods with the existing anatomical information may produce absolute criteria for distinguishing the *tropicus* and *truncatus* groups but, when such an approach was adopted in a study of the endemic species of *Bulinus* in Lake Malawi, the affinities of these snails could only be determined by assessing the relative importance of the conflicting evidence obtained (Wright *et al.*, 1967).

D. FACTORS INFLUENCING DISTRIBUTION

Under natural conditions snails are exposed to a range of varying, and often interacting, environmental factors which produce a collective effect on the snails, and it is usually extremely difficult to separate the effect of any one factor and conclude that it is critical. Little clear information has emerged from attempts to correlate the distribution of snails with physical factors, particularly water chemistry.

In East Africa and in Puerto Rico intermediate host snails are not found in waters with low electrical conductivity, indicating very low concentrations of

dissolved salts (Beadle and Visser, unpublished; Harry *et al.*, 1957). The snails tend to thrive better in waters with a reasonably high calcium content, but a study of the egg-laying rate of *B. pfeifferi* showed an optimum at a calcium concentration of 10–36 mg/l and a marked decline at 80–100 mg/l (Williams, N.V. quoted by Harrison and Shiff, 1966). In the Sudan both *Biomphalaria* and *Bulinus* were found where the ratio of sodium to calcium in the water was 1·2 but only *Biomphalaria* was found where it was 0·2 (Abdel-Malek, 1958), and in South Africa the *africanus* group and *B. pfeifferi* were found where the ratio was from 0·5 to 2·0 but when it rose above 2·4 the *africanus* group was present and *B. pfeifferi* was most uncommon (Schutte and Frank, 1964). The scarcity of pulmonate snails in certain Rhodesian streams with a high magnesium content led to laboratory tests which showed that the egg-laying of *B. pfeifferi* was significantly reduced and then inhibited by increasing the magnesium content of the water (Harrison *et al.*, 1966). Water from other Rhodesian streams with a high natural turbidity caused by a mixture of kaolin and illite or sericite, or both, also disturbed the reproductive activity of *B. pfeifferi* and *B. globosus* (Harrison and Farina, 1965).

B. pfeifferi is absent from the low-lying coastal areas in the equatorial region but is present at similar altitudes to the north and south, which suggests that high temperatures may be unsuitable for this species (Sturrock, 1965b). In southern Tanzania the daily maximum temperature in standing water at Mahiwa (altitude 200 m) can rise to over 30°C at most times of the year and at Nachingwea (altitude 350 m) this can happen between October and February, whereas at Songea (altitude 1050 m) such temperatures are unlikely to occur (Berrie, 1966a, 1970a). *B. globosus* flourishes at all three localities but *B. pfeifferi* is found only at Songea and is not known to occur below Tunduru (altitude 600 m) in this region. *B. obtusispira*, the intermediate host of *S. haematobium* in Madagascar, will not reproduce at temperatures fluctuating up to 25°C but reproduces intensively when exposed to maximum temperatures of 35–37°C which cannot be tolerated by *B. pfeifferi* (Brygoo, 1967). High water temperatures occur in the western and southern parts of the island which favour *B. obtusispira*, whereas the forested nature of the central plateau and eastern areas provides shaded conditions in which *B. pfeifferi* can thrive almost down to sea level. The annual isotherms of 28°C and 18°C delineate the main distribution of *S. haematobium* and *S. mansoni* respectively, but local ecological factors must be considered in any attempt to explain the full distribution of the snails and the parasites in Madagascar. A high mortality of *B. pfeifferi* in outdoor aquaria in South Africa was associated with periods of continuous high maximum temperatures of about 25–27°C (Pitchford and Visser, 1965). Adverse effects such as lowered survival and fecundity have been recorded in aquaria at temperatures above 28°C and it is very difficult to maintain this species at temperatures over 30°C (Foster, 1964; Sturrock, R. F., 1966a). The mortality of infected *B. pfeifferi* in the laboratory increases with temperature in the range 22–26°C and is much higher than that of uninfected snails which remains rather constant (Foster, 1964). A similar lowered resistance to thermal stress has been shown in infected specimens of the marine snail *Nassarius obsoleta* Say (see Vernberg and Vernberg, 1963). These data support

the hypothesis that high water temperatures may exclude *B. pfeifferi*, and presumably also the other African species of *Biomphalaria*, from certain areas and also indicate that *B. pfeifferi* may become a rather inefficient intermediate host well before reaching its limiting temperature. The lower figures specified in South Africa may indicate some temperature acclimatization within this species with change in latitude.

In South Africa the *africanus* group, represented by *B. africanus* and *B. globosus*, extends rather farther south and west than *B. pfeifferi*, though all are confined to relatively warm regions (van Eeden *et al.*, 1965; van Eeden and Combrinck, 1966). The extent of distribution of *Biomphalaria*, *Bulinus* and *Lymnaea* increases in the order *B. pfeifferi*, *africanus* group, *L. natalensis* (Krauss), *B. tropicus*, and the distribution of *B. pfeifferi* is also the first to be affected by increasing altitude whereas *B. tropicus* is most abundant at 4000–5000 ft above sea level. Such a relationship between increase in latitude and in altitude is quite well known towards the edge of the range of northern temperate species of freshwater snail. The degree of association between the *africanus* group, *L. natalensis* and *B. tropicus* has been analysed by considering their occurrence singly or in combination in 506 areas (van Eeden and Combrinck, 1966). A high degree of association was found between the *africanus* group and *L. natalensis* and a low degree of association between the *africanus* group and *B. tropicus*, which makes it possible to suggest that the *africanus* group has ecological requirements similar to *L. natalensis* but different from *B. tropicus* even though the factors involved have not been identified. Similar conclusions were reached by studying distribution in the Transvaal (van Eeden, 1965) and temperature seems to determine the distribution of these tropical species in Natal (Brown, 1967).

Although distribution records are still very incomplete there is information to suggest that the ranges of some species may be changing. The recent discovery of *B. africanus africanus* in Tanzania has serious implications because this species is more likely to become established in irrigation channels than the other members of the *africanus* group which already occur in that country (Sturrock, 1964). *B. alexandrina* may be extending southwards in the Sudan, possibly in association with a similar extension of the water hyacinth *Eichornia*, while *B. sudanica* is extending northwards so that the range of *B. alexandrina* now overlaps those of *B. sudanica* and *B. pfeifferi* (Williams and Hunter, 1967). *B. sudanica*, previously thought to be an East African species, has been reported recently from Ghana where it has proved susceptible to infection with the local strain of *S. mansoni* (Onori *et al.*, 1963; Onori, 1965).

IV. Snail Population Dynamics

A. The Effect of Some Environmental Factors

1. *Temperature*

Detailed laboratory studies have been made of the effect of temperature on the biology of *B. globosus* (Shiff, 1964a) and of *B. pfeifferi* (Sturrock, R. F., 1966a; Shiff and Garnett, 1967). The snails were maintained under carefully

controlled conditions so that growth and fecundity could be recorded and life tables constructed for a range of temperatures. The influence of temperature on the growth of *B. pfeifferi* is shown in Fig. 1a. The Rhodesian investigations included the calculation of the intrinsic rate of natural increase for both species

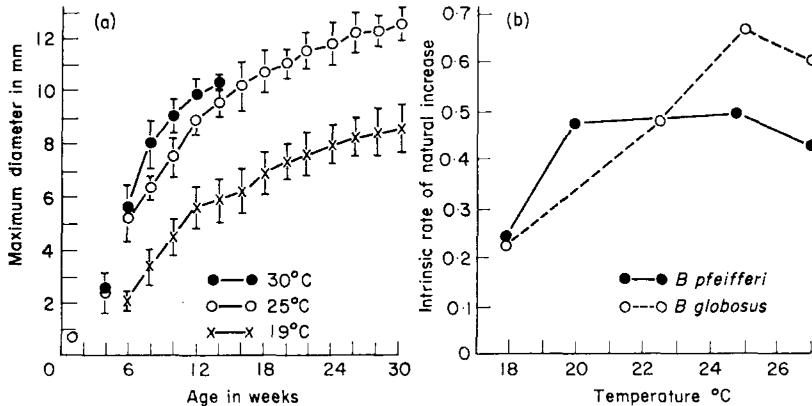


FIG. 1. (a) Growth curves of three groups of *B. pfeifferi* maintained at different temperatures, showing mean values and standard deviation. (After Sturrock, R. F., 1966a.) (b) The intrinsic rate of natural increase of snails maintained at different temperatures. (After Harrison and Shiff, 1966.)

at four temperatures from 18°C to 27°C, as shown in Fig. 1b. In each case the peak value was found at 25°C but *B. globosus* had a much higher peak than *B. pfeifferi*. It has been concluded that the reprodutive rate of *B. pfeifferi* is fairly constant from 20°C to 27°C whereas *B. globosus*, while it will tolerate a similar range of temperature, will respond more dramatically to changes within the range (Harrison and Shiff, 1966). Other studies support the conclusion that the optimum temperature for *B. pfeifferi* and for *B. glabrata* is around 25°C (Michelson, 1961; Foster, 1964; Sturrock, R. F., 1966a).

Selective advantage accrues to a higher intrinsic rate of natural increase only in species that normally live in temporary environments that they never saturate (Slobodkin, 1961). In Rhodesia *B. globosus* lives in habitats subject to catastrophies such as drying up, scouring floods and periods of low temperatures. The high intrinsic rate of natural increase in this species appears to be an adaptation to such conditions, allowing a rapid expansion of the population to exploit favourable conditions when these occur (Harrison and Shiff, 1966). The same could well be true of *B. globosus* and *B. nasutus* in Tanzania, which often inhabit temporary pools in which they multiply rapidly during certain periods of the year (Webbe, 1962b; Berrie, 1970a). Similarly, the ability to survive cold winters and to build up large populations during the warm summer months could account for the ability of *B. africanus* to penetrate further south and west than *B. pfeifferi* does in South Africa (van Eeden *et al.*, 1965; van Eeden and Combrinck, 1966). However, it is often difficult to apply the results of laboratory experiments to field conditions because they are

usually carried out at a constant temperature, and there is scope for experiments involving diurnal and perhaps also seasonal fluctuations of temperature to simulate natural conditions more closely. We also lack sufficient information about the relationship between temperature and the respiratory behaviour of the snails.

2. *Density*

The growth rates and fecundity of intermediate host snails are reduced if they are kept at high densities, and this phenomenon has been studied in laboratory colonies of *B. glabrata* (Chernin and Michelson, 1957a, b), *B. forskali* (Wright, 1960), *B. globosus* (Shiff, 1964b) and *B. angulosa* (Sturrock, 1965a). The data obtained for *B. angulosa* maintained at three densities are shown in Fig. 2. There is evidence that the surface/volume ratio of the water

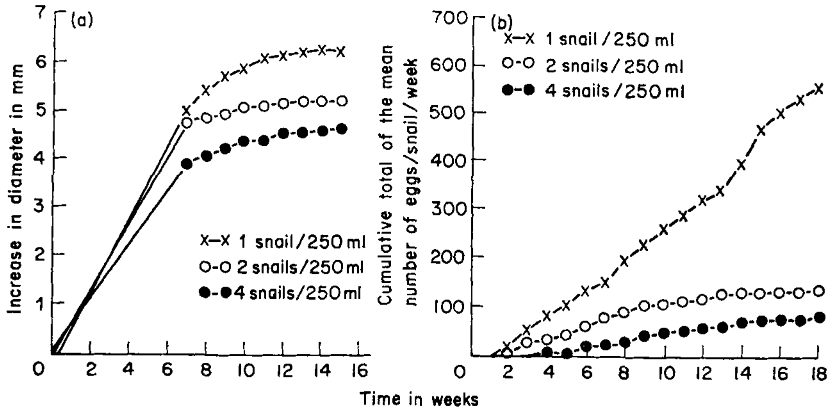


FIG. 2. The effect of crowding on the growth rate (a) and the egg-production (b) of *B. angulosa*. Time is recorded from the start of the experiment when the snails were 4 weeks old and about 3 mm in diameter. (After Sturrock, 1965a.)

used in these investigations may be important with a relatively large surface diminishing the effects of crowding, which could indicate that some unstable substance in the water is involved. The experiments with *B. forskali* showed that if the water was exposed to activated charcoal the growth rate was no longer reduced, which suggested that some substance secreted by the snails was causing the growth inhibition.

Growth inhibition, apparently due to overcrowding, has been reported in a natural population of *B. sudanica tanganyicensis* (Smith) in a small pool in Uganda (Berrie, 1968). Due to drainage operations the volume of the pool was greatly reduced shortly after a new generation of snails had hatched, and these snails experienced growth inhibition for a period of five months while the population density was about 10 snails per litre (Fig. 3). Although some of the snails were large enough to become sexually mature there was no evidence that they were breeding during this period, but when the density of the population was reduced about a hundredfold, growth was resumed and a period of high

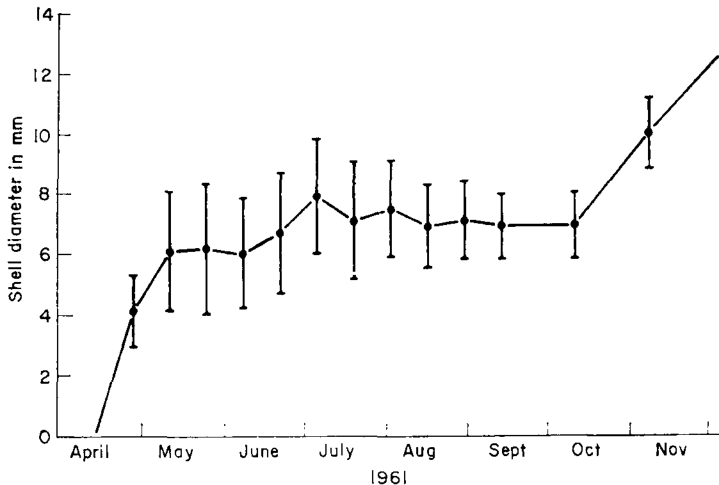


FIG. 3. Growth curve of a generation of *B. sudanica tanganyicensis* during a period of growth inhibition, showing mean values and standard deviation. (Data from Berrie, 1968.)

reproductive activity followed. An organic ester was isolated from the water in the pool and was shown to be capable of causing growth inhibition in the snails and to be destroyed by oxidation (Berrie and Visser, 1963). Now that it is clear that chemical substances are involved in this phenomenon, further investigations may provide information which is useful both for a better understanding of the population dynamics of the snails and the parasites and for exploring new possibilities of snail control.

3. *Food*

Pulmonate snails are basically browsing animals which feed more or less continuously as they move, and there are a number of reports of the food utilized by intermediate host snails (Lietar, 1956; Deschiens, 1957; Abdel-Malek, 1958; Claugher, 1960; Badenhorst quoted by Frank, 1964; McClelland, 1964). Algae seem to be the commonest diet component, with green algae and diatoms being particularly favoured. Blue-green algae are also eaten by the snails and *Oscillatoria formosa* Bory has proved very satisfactory for rearing *Bulinus* in the laboratory (De Lourdes Sampaio Xavier *et al.*, 1968); but some other species may be directly harmful. The snails are not normally found closely associated with filamentous algae but laboratory observations indicate that these cause mechanical obstruction rather than being poisonous to the snails. It has been suggested that in the ecological succession in a pond, *B. globosus* prefer, and in the very young stage require, to feed on the initial growth of micro-organisms which develop on submerged surfaces and that the heavy growths of algae and higher plants which develop later are less favourable (Pringle and Raybould, 1965). Tissues of higher plants may also be eaten particularly if they are in some stage of decomposition, but there is

evidence that snails feeding exclusively on leaves of higher plants grow much more slowly than those feeding on algae.

Field observations around Jadotville, Congo (Kinshasa), indicated a degree of ecological segregation of *L. natalensis*, *B. pfeifferi* and *B. africanus* in respect of the available food and oxygen (Lietar, 1956). *L. natalensis* was restricted to relatively well-oxygenated waters which often had bottom sediments with a high redox potential and provided an adequate diet of fresh plant material in the form of green algae, diatoms and submerged leaves. *B. africanus* occurred in stagnant or semi-stagnant water or in streams with a high content of organic matter where they fed on fine deposits of rapidly decaying organic detritus. The redox potential was always low and the lowest values often corresponded with the highest densities of the snails. *B. pfeifferi* seemed to prefer conditions intermediate between the other two species and was often found together with one or the other of them. Some suspended matter was always present and the sediment contained slowly decomposing vegetable debris on which they fed.

We have no quantitative information about the food requirements of a population of snails or about the factors which may influence the production of suitable food in natural habitats. Until such studies are carried out on the trophic relationships of the snails we remain unable to assess the importance of the food supply in the population dynamics of the snails.

B. REPRODUCTION

The enormous reproductive potential of intermediate host snails is constantly emphasized in the literature and a number of studies have measured their fecundity and determined the influence of certain extrinsic factors such as temperature. Several studies have shown an apparent correlation between the start of a period of rains and increased reproductive activity in populations of intermediate host snails. The stimulus may be provided by an associated drop in water temperature, but other factors such as the addition of nutrients, the dilution of substances in solution, changes in the size of the habitat and possibly associated changes in food supply could be involved, and no adequate evidence is available. The intrinsic factors involved have received little attention as yet although some relevant information has been obtained for other pulmonates. Snail size and time of year are correlated with the development of the reproductive system in temperate freshwater snails (Hunter, 1961; Berrie, 1966b). These snails seem to reproduce when they have achieved an adequate size provided the temperature is high enough and sufficient food is available. In the tropical species egg-laying does not always begin when the snails are large enough to reproduce and the delays must be attributed to unfavourable environmental conditions, possibly including inhibiting substances, which require investigation (Berrie, 1969). The internal control mechanisms are probably chemical and may involve neurosecretion, which has been shown to influence osmotic regulation (Lever *et al.*, 1961; Lever *et al.*, 1965). No investigation has yet reached the stage of considering the interaction of intrinsic and extrinsic factors affecting the reproductive physiology of natural populations of freshwater snails.

C. SEASONAL CYCLES

Many snail populations have been studied for periods of a year or more and it is not possible to consider these observations in detail here (see Smithers, 1956; Lietar, 1956; McCullough, 1957, 1962; Cridland, 1957a, b, 1958; Gaud, 1958; Webbe and Msangi, 1958; Webbe, 1960, 1962a, b; Teesdale, 1962; Crossland, 1963; Shiff, 1964c; Berrie, 1964, 1968, 1970a; Dazo *et al.*, 1966; Hira and Muller, 1966; Odei, 1966). As several species and various types of habitat have been studied the details vary considerably, but it is generally possible to relate seasonal trends in the density and reproduction of the snails to the climate of the region, particularly to the rainfall (Webbe, 1965c). There is usually a fairly clear alternation of wet and dry seasons either once or twice each year but, within this general pattern, there can be great variation in the quantity and distribution of rainfall from year to year. This variability in the major environmental factor complicates the study of snail populations. Most habitats undergo considerable seasonal changes in respect of several related factors such as size, water level, rate of flow, flora, water chemistry, oxygenation and pollution. Optimum conditions for the snails will be produced by certain combinations of factors while other combinations may create very adverse conditions, but we are not yet in a position to define these combinations with certainty.

Rainfall generally provides a stimulus to reproduction and an increase in snail population densities. In some habitats this build-up occurs during the rains but in many streams the snail populations are flushed out by floods and may take several months to recover. *B. forskali* is rather exceptional as it flourishes under flood conditions, and populations of this species seem to undergo rapid increases and declines. Seasonal pools provide suitable habitats for several species of *Bulinus* and are often important schistosome transmission sites. If such pools are not subject to flooding the snail populations start to increase rapidly during the rains and reach a peak early in the dry season (Fig. 4a) but, where flooding does occur, the build-up is delayed and the peak occurs later in the dry season (Fig. 4b). High densities may persist for some time and the decline in the histograms is due to individual habitats drying out rather than to a general decline in density. However, during the last two or three weeks before a pool dries the snail population often undergoes a sharp decline. Permanent pools and dams may become considerably reduced in size during the dry season and, where this results in the disappearance of marginal aquatic vegetation, a rapid decline in snail numbers occurs.

The capacity of the snails to survive periodical drying out of their habitats is well established. Numerous reports exist which show that, while a high mortality occurs, many snails successfully aestivate either under dead vegetation and debris or by actively burying at least the aperture of their shell in the mud. After the start of the rains, when water is again present, the snails emerge from aestivation and laboratory experiments have indicated that after desiccation the intensity of breeding of *B. truncatus* is twice that of normal snails, although their survival time is reduced (Chu *et al.*, 1967).

In a detailed study of *B. truncatus* and *B. alexandrina* in Egypt (Dazo *et al.*,

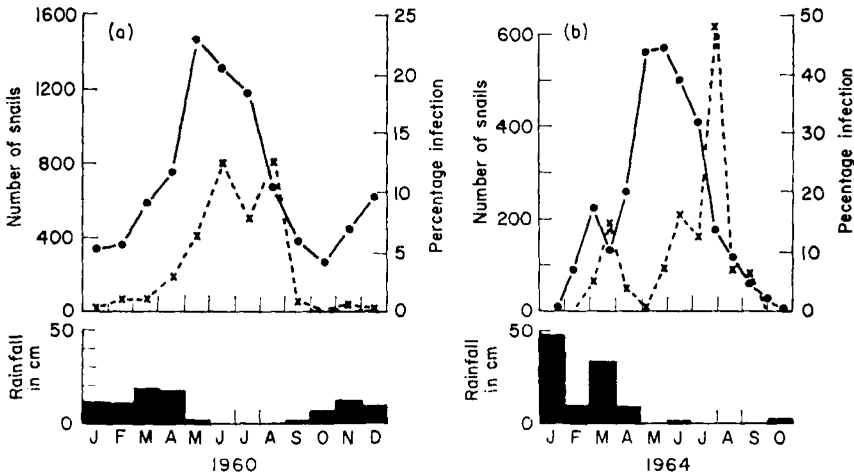
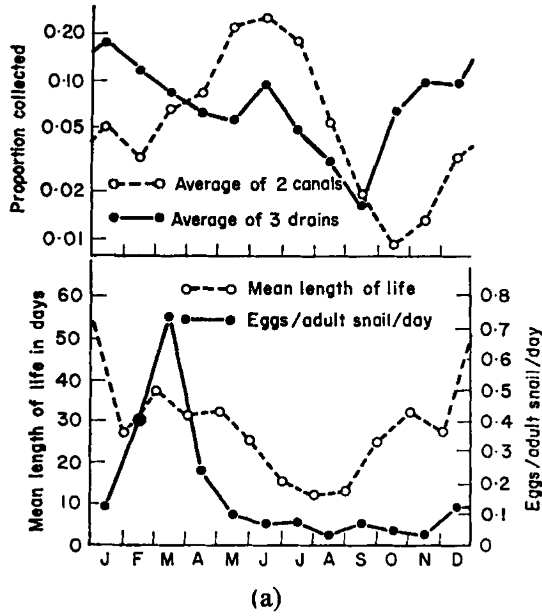


FIG. 4. Seasonal changes in snail numbers and infection rate with *S. haematobium* in small pools in two areas of Tanzania. The continuous line shows the numbers of snails and the broken line the infection rates. (a) *B. nasutus productus* from 19 pools near Mwanza. (After Webbe, 1962b.) (b) *B. nasutus nasutus* from seven pools near Nachingwea. (After Berrie, 1970a.)

1966) growth and seasonal changes of density were recorded and survival and reproduction rates were calculated throughout the year and used to construct ecological life-tables for these species. The density of *B. truncatus* in canals followed the pattern indicated above but populations living in ditches reached their maximum numbers in January and then declined to a minimum in September. This is shown in Fig. 5a together with the changes in average reproduction and survival recorded during the year. The rise and fall of these latter parameters may be taken to indicate improving and deteriorating conditions respectively. The striking fact that the maximum density of snails occurred several months after optimum conditions are indicated, is explained by failure to obtain adequate representation of young snails in the collections, so that the increase in density was not recorded until the snails had grown considerably. This problem is encountered in most studies of snail populations. *B. alexandrina*, like *B. truncatus*, had a peak of oviposition in March but the seasonal pattern of survival in this species was quite different (Fig. 5b). The close relationship between maximum oviposition and high survival resulted in a low, flat peak in population density through the summer months. However, in both species it is noticeable that conditions deteriorated quite sharply from May to July. The infection rates in the snails were highest in June and the infections which matured then would have been contracted the previous month. The possible influence of changes in infection rates which can affect the growth, fecundity and survival of the snails was not considered in this study.

During the study of *B. nasutus productus* which provided the data for Fig. 4a, the reproduction index and the adult mortality of the populations were calculated and these showed some relation to each other but not always



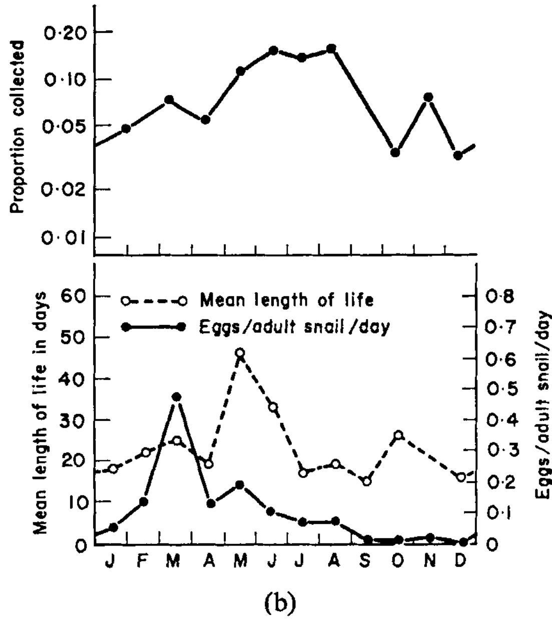


FIG. 5. Seasonal changes in density, survival and fecundity of *B. truncatus* (a) and *B. alexandrina* (b) in an area of the Nile Delta of Egypt. (After Dazo *et al.*, 1966.)

to population density (Webbe, 1962b). Population structure and density varied in different populations with quite large changes occurring from time to time due to the differences in mortality and reproduction which were observed. The data indicate that the length of life of an average adult snail was only 30·3 days.

During cold weather, populations of *B. globosus* in Rhodesia have low rates of fecundity and mortality and the populations contain a relatively large number of adult snails, while in hot weather these parameters are high and the populations are predominantly young (Shiff, 1964c). Thus, *B. globosus* thrives under two sets of conditions which are common in Rhodesia: (1) in large water bodies where optimum egg-laying temperatures are seldom reached but where the temperature is sufficiently high to allow a moderate degree of egg-laying coupled with a high rate of survival and long adult life; (2) in small water bodies where series of adverse conditions alternate with brief periods that are optimum for increase and the survival of the population is ensured by rapid breeding rather than by the longevity of its members. The latter category will include the seasonal pools which are such important transmission sites in many parts of Africa and South America (Smithers, 1956; Barbosa, 1962; Webbe, 1962a; Berrie, 1970a). The ability of the snails to produce rapid responses to changes in environmental conditions enables them to exploit brief periods of optimum conditions which must be an important adaptive factor in colonizing small bodies of water in Africa (Berrie, 1968).

The optimum conditions of food and oxygenation for snails in streams around Jadotville have been discussed above and, because these tend to occur in sequence through the dry season, they are associated with sequential maximum densities of the three species involved (Lietar, 1956). Populations of *L. natalensis* increase very rapidly at the beginning of the dry season when the habitats are well oxygenated and contain growing plants. As the current slows down and plant debris begins to accumulate the populations of *L. natalensis* decline and those of *B. pfeifferi* begin to expand. Finally the breakdown of plant material during the hot months accompanied by growing pollution and deoxygenation creates conditions in which large populations of *B. africanus* build up towards the end of the dry season.

Very few of the above studies have produced the detailed quantitative data which is required to calculate the most important population parameters. The objectives have usually been more limited so that, while we have reasonable indications of season trends, we have little real understanding of the dynamics of these populations. However, during this period suitable techniques for more detailed studies have been developed (Webbe, 1965a) and their application should lead to a much fuller understanding of snail populations.

V. Infection of the Snails

A. Host Location by Miracidia

Schistosome eggs will hatch provided they are shed into fresh water at a reasonable temperature. Light accelerates hatching but there is evidence that this process is largely mechanical and will occur even when the miracidium is

dead (Lengy, 1962). The oxygen requirements of the eggs and of the miracidia are not known. Newly hatched miracidia are very active and the rate of movement of *S. mansoni* miracidia has been measured by two different techniques which each gave a figure of about 7 m/h (Chernin and Dunavan, 1962; Davenport *et al.*, 1962), whereas *S. bovis* miracidia were estimated at just over 1 m/h (Lengy, 1962). The survival and mobility of the miracidia of *S. mansoni* depends on temperature and *p*H (Bénex and Deschiens, 1963). In experiments at 45°C they were highly active and survived for only ½ h; at 25°C they survived about 7 h and at 4°C they were immobile but survived for 24 h. The optimum range of *p*H was 7·5–8·5 but a reasonably good survival occurred within the range of 5·5–10·0. The miracidia appear to be positively phototactic and negatively geotactic but neither of these responses is absolute (Standen, 1949; Chernin and Dunavan, 1962; Lengy, 1962).

Some controversy has arisen as to whether miracidia are attracted towards suitable snails or whether they locate them simply by chance encounters. It is claimed that *S. mansoni* miracidia will attack empty shells and particles of fine gravel and that their movements are random in the presence of *B. alexandrina* (Abdel-Malek, 1950). In experiments with *B. glabrata* a higher concentration of *S. mansoni* miracidia was found near the snail than in other parts of the container (Kloetzel, 1958). When the snail moved the higher concentration could be observed at the former position also, suggesting that remaining mucus might be attractive. Crushed snail was more attractive than a live snail and if snail extract was added to the water this reduced the concentration of miracidia round a live snail. Other experiments using the same species showed that an individual miracidium was very successful in locating and infecting a snail and its ability showed no reduction in containers which allowed an initial separation of 86 cm horizontally and 33 cm vertically (Chernin and Dunavan, 1962). This indicates a scanning capacity adequate to allow infection of snails at normal depths over considerable distances, and no evidence of positive attraction was observed. The miracidia, like the snails, were found most frequently near the outer margins of the containers at the water-air interface; a behavioural characteristic which must greatly increase their chance of meeting. Miracidia of *S. bovis* seem to differ in their response to *B. truncatus*, some being attracted while others are not, and they may also be attracted to faecal matter or mucous secretions of the snail (Lengy, 1962). Miracidia of an Aden strain of *S. haematobium* were attracted to *B. beccarii* and *B. reticulatus* but only after an initial delay of about 15 min (Wajdi, 1964a). A weaker attraction was detected to *B. forskali* which only showed after about 45 min, and little if any attraction existed to *B. truncatus*. This means that complications arising from subspecific variation are involved in experiments of this type. The reactions of miracidia to agar or starch gel pyramids impregnated with various chemicals showed that short-chain fatty acids, some amino acids and a sialic acid would attract miracidia of *S. mansoni* and stimulate attachment and attempts to penetrate the agar (MacInnis, 1965). The attractive components could be removed from snail tissues with various solvents and if butyric or glutamic acid was added to the treated tissues this restored the capacity to attract miracidia and to stimulate attempts at penetration. Biochemical investigations based on this

information might lead to the location and identification of attractive substances in the snails.

Annelid worms of the genus *Chaetogaster* are frequently found commensally on pulmonate snails and are reported to ingest emerging cercariae of *S. mansoni* (Ruiz, 1951). Miracidia of *S. mansoni* were ingested by *C. limnaei* Baer as they tried to attack snails and the miracidia were recovered with undischarged penetration glands in sections of the worms' intestines; but in this case there was no evidence that cercariae were ingested (Wajdi, 1964b). When *B. glabrata* were exposed to 5 miracidia per snail the resulting infection rate among those harbouring *C. limnaei* was 34% compared with 76% in a control group (Michelson, 1964b). Reports exist of *Chaetogaster* ingesting miracidia of other species of trematodes and the commensal relationship seems to include the provision of a defence mechanism for the snails. The adaptive ability of trematodes to utilize host species which have suitable natural relationships is well illustrated by the life cycle of *Trigonodistomum mutabile* (Cort) which uses *C. limnaei* as a second intermediate host (Wallace, 1941). Free larvae of schistosomes and other trematodes are also preyed upon by certain copepods but there is no evidence that this might provide any effective control of transmission (Courmes *et al.*, 1964).

B. FACTORS INFLUENCING PENETRATION

The success of *S. mansoni* miracidia in producing infections in *B. glabrata* (see DeWitt, 1955) and *B. sudanica tanganyicensis* (see Purnell, 1966a) increases with temperature up to the thermal death-point of the snails (Fig. 6a). The increased activity of both organisms with increasing temperature may provide a greater chance of contact between them, but it seems doubtful whether this can be the only factor involved when experimental infections are carried out in very small volumes of water. When *S. mansoni* miracidia were maintained at various temperatures for 6 h it was found that mortality increased linearly with temperature reaching 100% at about 33°C (Purnell, 1966b). In the case of *S. haematobium* the optimum temperature range for infecting *B. truncatus* was 20°C to 30°C but the infection rates fell more rapidly below this range than they did above it, as shown in Fig. 6b (Chu *et al.*, 1966c). However, *B. truncatus* became infected with *S. bovis* equally well at 14°C and 31°C (Lengy, 1962).

Water velocity influenced the ability of *S. mansoni* miracidia to infect *B. sudanica tanganyicensis* in an experimental flowing-water system (Webbe, 1966b). The numbers of miracidia used ranged from less than 1 to 50 per snail and these were tested at water velocities from 0·15 to 1·07 m/sec over an exposure period of 1 h. High infection rates were obtained in all the tests carried out with only a slight reduction at about the 1 miracidium per snail level. There was no evidence that infection rates decreased at distances of up to 12·2 m from the source of miracidia when 25 miracidia per snail were released. It was also shown that the miracidia could penetrate at least 30 cm upstream from their point of entry at a water velocity of 0·15 m/sec. These results indicate that miracidia of *S. mansoni* have a remarkably effective

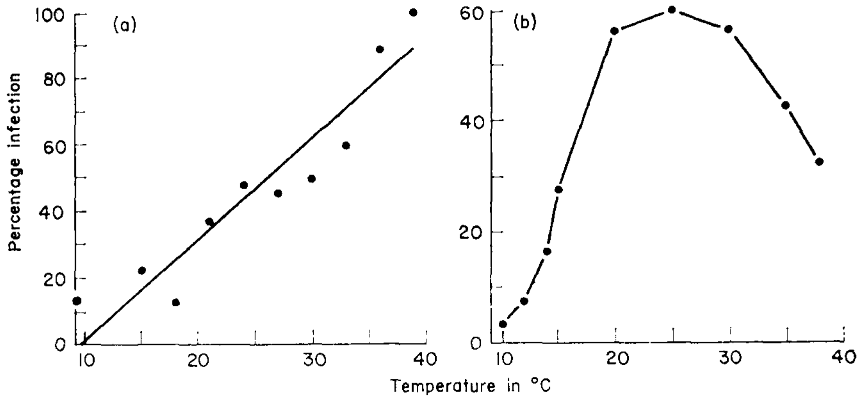


FIG. 6. The effect of water temperature on the ability of miracidia to infect snails. (a) Miracidia of *S. mansoni* in *B. sudanica tanganyicensis*. (After Purnell, 1966a.) (b) Miracidia of *S. haematobium* in *B. truncatus*. (Data from Chu *et al.*, 1966c.)

scanning capacity in flowing water so that snails will become infected under conditions of rapid flow and low miracidial pressure. Since a water velocity exceeding 0·33 m/sec at shell height produces a hydrodynamic drag force sufficient to dislodge *B. glabrata* (see Jobin and Ippen, 1964) it seems likely that snails can be successfully infected at any water velocity which they can withstand.

The relationship between age and infectibility in the snails has given rise to divergent views. It seems likely that highly susceptible strains of snails may show no variation in susceptibility at different ages but there is evidence that such variation does exist in some other cases. One Brazilian strain of *B. glabrata*, which proved highly resistant to infection when over 7 mm shell diameter, showed infection rates of up to 39% if exposed at 1–24 days old (Newton, 1953). Another strain of *B. glabrata* gave higher infection rates if exposed at 2–7 days old than it did at 1½–5 weeks old (Moore *et al.*, 1953). In *B. truncatus* higher infection rates with *S. haematobium* have been recorded in snails 2–5 weeks old than in snails one week old but, as with *B. glabrata*, infections could be obtained even at one day old (Chu *et al.*, 1966a). The age or size of *B. truncatus* did not seem to influence infectibility with *S. bovis* (Lengy, 1962).

The number of miracidia to which a group of snails is exposed influences the proportion of the snails which become infected (Schreiber and Schubert, 1949b). The highest infection rates in *B. glabrata* are obtained when the snails are exposed individually to a number of miracidia closely confined within the vicinity of the snail and, if the snails are exposed in groups, the infection rate varies inversely with the degree of dispersal of the organisms (Standen, 1952). No consistent differences in rate of infection were recorded between *B. truncatus* exposed to 5 miracidia of *S. bovis* each and ones exposed to 20, but a definite increase was noted when 60 miracidia each were used (Lengy, 1962). In natural populations of the marine snail *Velacumantus australis* (Quoy and Gaimard) the incidence of infection with trematodes among the susceptible

juvenile snails is twice as high at densities of less than 20 per sq ft as at higher densities (Ewers, 1964). The incidence among adult snails does not vary with density and this may represent a situation in which the number of miracidia available is not high enough to achieve rapid infection when large numbers of snails are present. If certain strains of *Biomphalaria* or *Bulinus* are exposed to sufficient appropriate miracidia 100% infection will be achieved and failure to achieve such complete success has been attributed to deficient infectivity of the miracidia rather than to innate resistance on the part of the snails (Etges, 1963; Chu *et al.*, 1966b). However, under the same exposure conditions, different strains of *B. glabrata* may show significantly different infection rates and, although the rates can be increased if more miracidia are used, this must indicate that various degrees of innate resistance do exist among the snails (Paraense and Correa, 1963).

VI. Development in the Snails

A. Tissue Responses

When miracidia of a Puerto Rican strain of *S. mansoni* penetrated a Brazilian strain of *B. glabrata* known to be completely resistant to infection, they provoked a marked cellular response and were destroyed within 24 to 48 h (Newton, 1952). No such response occurred to the same miracidia in a highly susceptible Puerto Rican strain of *B. glabrata* and the parasites developed normally in these snails. Cross-breeding the two strains of snails demonstrated that susceptibility to infection with *S. mansoni* is a heritable character probably involving several genetic factors (Newton, 1953) and that hybrid specimens can exhibit the tissue responses of either parent strain or responses of an intermediate character (Newton, 1954). Such tissue responses have since been confirmed in *B. glabrata* by several investigators. A detailed histological study of the course of infection of *S. mansoni* in a highly susceptible strain of *B. glabrata* showed that only a small proportion of the miracidia which penetrate a snail develop into mature mother sporocysts (Pan, 1963, 1965). The mother sporocysts were usually located in the head-foot near the sites of penetration and only rarely provoked a slight tissue reaction. During the third week of infection in snails maintained at 21–24°C, daughter sporocysts began to migrate to the digestive diverticula where they caused a slightly stronger tissue response. Cercariae began to emerge in the fifth week and about 1–2 weeks later a marked tissue reaction was provoked by cercariae trapped and dying in the vascular connective tissue. This reaction coincided with a high mortality of infected snails 6–7 weeks after infection when heavy emergence of cercariae was established. A high mortality has been noted also in *B. truncatus* 12–17 days after exposure to miracidia of *S. bovis* and again 2–3 weeks after the snails started to shed cercariae (Lengy, 1962).

Tissue extracts prepared from infected *B. glabrata* contain substances capable of immobilizing 76–100% of miracidia of *S. mansoni* and also miracidia of *Fasciola hepatica* (Bénex and Lamy, 1959; Michelson, 1963, 1964a). Extracts from uninfected snails immobilize up to 22% of the miracidia and the higher level of activity is found in most snails with an infection more than

nine days old. Substances producing the same effect on *S. mansoni* miracidia can be extracted from *B. truncatus* and *Helisoma caribaeum* d'Orbigny but not from several other species of snails. While further information is required, particularly about the mode of action and the specificity of such substances, they suggest that a developing infection produces some type of immunity to reinfection in the snails. The available evidence on this point from snail infection experiments is not conclusive. When *B. glabrata* were exposed individually to a single miracidium of *S. mansoni*, which resulted in unisexual infections, and then exposed to larger numbers of miracidia, some bisexual infections were obtained (Kagan and Geiger, 1964). In another experiment *B. glabrata* were exposed in groups to *S. mansoni* miracidia on six occasions over a period of 12 days and subsequently shown to contain mother sporocysts which varied greatly in size, indicating repeated successful invasion (Pan, 1965).

None of these results would conflict with the existence of an immunity to reinfection which required about 9 days to develop and was then only partial. However, when *B. truncatus* were exposed to miracidia of *S. haematobium* and exposed for a second time at intervals of 9, 28, 42 or 180 days, histological examination showed that in each case the second exposures had given rise to infections which developed normally and there was no evidence of tissue reaction around the worms or of any other form of immunity (Wajdi, 1966).

B. THE PREPATENT PERIOD

Early experiments in Sierra Leone showed that the prepatent period for *S. mansoni* in *B. pfeifferi* varied from 16 days at 32°C to 34 days at 22°C (Gordon *et al.*, 1934). Studies with *B. glabrata* have indicated slightly longer times but a recent investigation with East African strains of *B. pfeifferi* produced results almost identical with those from Sierra Leone (Foster, 1964). In the laboratory temperatures below 26°C do not allow satisfactory development of *S. mansoni* within the snails and continuous passage below this temperature seems to cause a loss of virulence in the parasite (Standen, 1952; Stirewalt, 1954). In *B. truncatus* the prepatent period of *S. haematobium* increases with age of the snails, from 31 days in snails exposed at one day old to 44 days in snails exposed at 40 days old, but the survival rate also increases from 62 % to 100 % over this range of age (Chu *et al.*, 1966a). The length of the prepatent period also varies with the number of miracidia used, from 46 days with one miracidium to 40 days with 20 (Chu *et al.*, 1966b). A small increase in the prepatent period of *S. mansoni* with increasing age in *B. glabrata* has been noted but is not considered to be significant (Moore *et al.*, 1953).

The data on prepatent periods which have been obtained from experiments conducted in outdoor aquaria at ambient temperatures in South Africa (Pitchford and Visser, 1965) and in Iran (Chu *et al.*, 1966d) give a good indication of the influence of seasonal changes in temperature and confirm the known fact that transmission in natural habitats in these areas is curtailed during the coldest months of the year. The data for *S. haematobium* and *S. mansoni* are shown in Fig. 7. In South Africa the incubation periods for *S. mattheei* are intermediate between the two sets shown and in Iran the periods for *S. bovis*

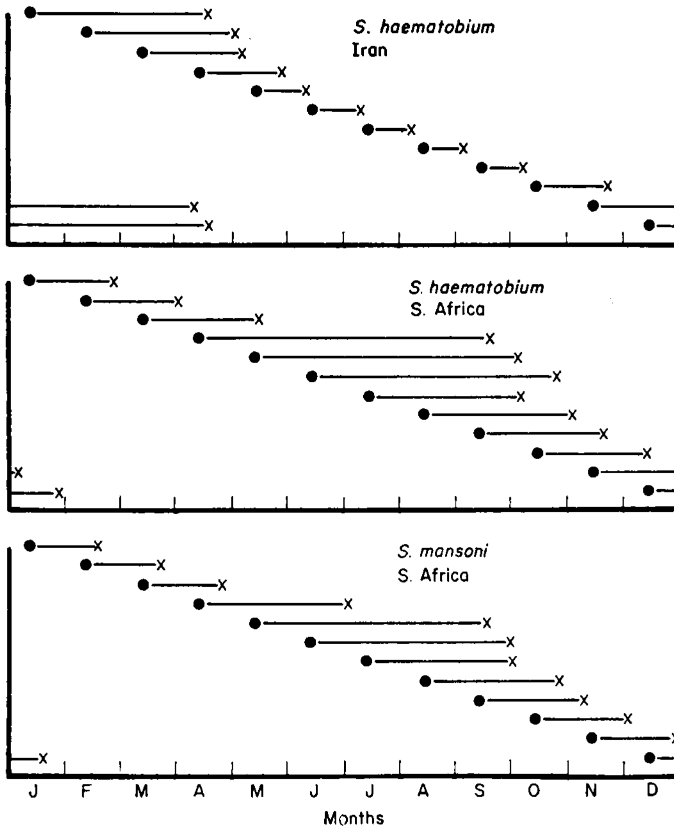


FIG. 7. Seasonal changes in the prepatent period of *S. haematobium* and *S. mansoni* in local intermediate hosts at ambient temperatures in South Africa and in Iran. Circles indicate infection of snails in the middle of each month and crosses show when cercariae are first released. (Data from Pitchford and Visser, 1965, and Chu *et al.*, 1966d.)

are shorter than those for *S. haematobium*. It is clear from Fig. 7 that very few infections mature in the snails during the four coldest months when the mean maximum temperature is about 20°C or less. The South African observations include data which show that cercarial production continues in some snails whose infections have matured before the onset of the cold weather but in other cases production ceases and is resumed after the cold period. In consequence, when the temperatures rise again all the infections which have been contracted over a period of several months mature almost simultaneously and production from previously infected snails increases. If sufficiently large numbers are involved this could create a very dangerous period for the definitive hosts; a situation which is not uncommon in the epidemiology of parasitic diseases, including fascioliasis. Summer temperatures in Iran are much higher than in South Africa, which may account for the minimum incubation period for the northern form of *S. haematobium* in these observations being only

20 days compared with 38 days for the southern form. In tropical Africa temperatures are unlikely to remain low enough at any time of year to prevent maturation of infections in the snails except at high altitudes. Although temperatures may not create a seasonal pattern of transmission in these areas other factors, such as rainfall, can do so.

C. EFFECTS ON THE SNAILS

Some larval trematodes are known to cause gigantism in the infected snail hosts and although this term cannot be applied in the case of schistosomes there is evidence that infection with larval schistosomes does influence the growth of snails. The mean shell diameter of experimentally infected, adolescent *B. glabrata* is consistently larger than that of uninfected controls during the first weeks of infection, but the growth rate then decreases in the infected snails so that by the end of 7 weeks after infection both groups show equal growth and the infected snails are eventually stunted (Pan, 1965). No significant growth responses were noted when large mature *B. glabrata* were infected. Laboratory infections of *B. pfeifferi* of all ages cause a temporary acceleration of growth compared with uninfected controls, as shown in Fig. 8a, and, in this case, the

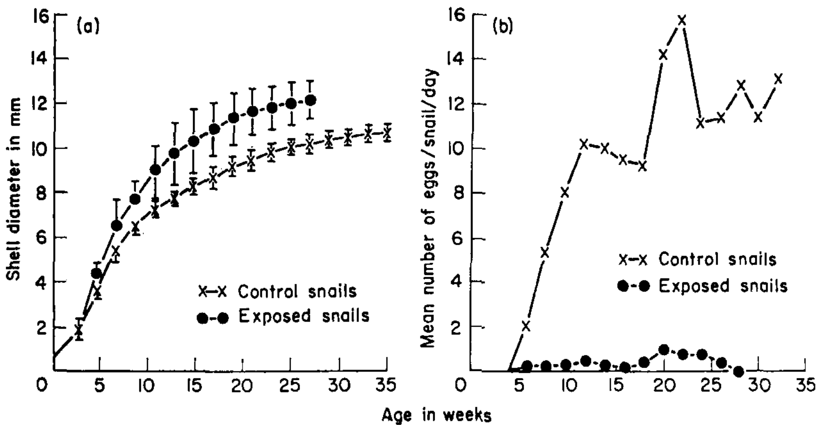


FIG. 8. The effect on the growth rate (a) and fecundity (b) of *B. pfeifferi* of exposure to miracidia of *S. mansoni* at the age of 3 weeks. Snail size is shown as means and standard deviation. (After Sturrock, B. M., 1966.)

controls do not catch up at a later stage (Sturrock, B. M., 1966). The more intense the infection produced in *B. pfeifferi* the greater the increase in growth of the snail. Similarly, *B. nasutus productus* show an increase in growth rate at some stage after infection (Sturrock, B. M., 1967). When infected and uninfected *B. truncatus* were measured at the time of exposure and when they started to shed cercariae 38 days later no difference in growth was apparent for this period (Chu *et al.*, 1966b), but this does not preclude the existence of changes such as those noted in *B. glabrata*. These studies and others agree that the life span of infected snails is generally shorter than that of uninfected snails.

The size distributions of naturally infected *B. sudanica tanganyicensis* and *B. nasutus productus* in northern Tanzania show that most infections are found in the larger size groups (Webbe, 1962a, b). In southern Tanzania the size distributions of naturally infected *B. globosus* and *B. nasutus nasutus* have been compared with the size distribution of all snails examined so that the infection rate for each size group could be determined (Fig. 9) (Berrie, 1966c, 1970b).

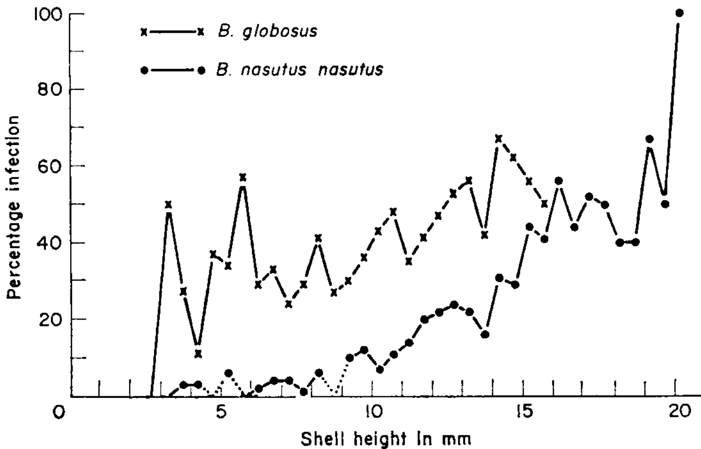


FIG. 9. The relationship between snail size and infection with *S. haematobium* in two populations of snails in southern Tanzania. (After Berrie, 1970b.)

The infection rate rises sharply in the larger size groups and, in populations containing an interbreeding mixture of both species, a correlation exists between the number of snails over 9 mm shell height in each population and the percentage infection recorded from that population. In populations of *B. globosus* a higher level of infection is found in smaller snails but the trend of infection among larger snails is similar. This data must be interpreted with caution since the increased growth of infected snails is a complicating factor.

Infection with larval schistosomes also affects the fecundity of the snails. In South America infection with *S. mansoni* causes a significant reduction in the numbers of egg masses and of eggs laid by *B. glabrata*, and this reduction becomes more marked as infection proceeds until the snails rarely ovulate after they have shed cercariae for a few weeks (Coelho, 1954). Histological evidence shows that the ovotestes remain functional in infected snails and if the infection is lost normal egg-laying can be resumed. Egg-laying declines in *B. glabrata* in the fourth week of infection, before cercariae begin to emerge, and is completely suppressed 5 to 6 weeks after infection in both adolescent and mature snails (Etges, 1963; Etges and Gresso, 1965; Pan, 1965). Egg production may resume on a limited basis about 3 months after infection and may return to a normal rate if the infection is lost. In *B. pfeifferi*, snails infected before reaching sexual maturity continue to lay some eggs throughout the rest of their lives and can resume normal production if the infection is lost, but those infected after maturity become completely sterile (Fig. 8b) (Sturrock,

B. M., 1966). In both *B. glabrata* and *B. pfeifferi* the eggs laid by infected snails have a higher sterility rate and a lower hatching rate than normal, and in *B. glabrata* the offspring of infected snails have a higher mortality rate for up to 10 days after hatching (Coelho, 1954; Sturrock, B. M., 1966). The onset of egg production seems to be delayed in *B. nasutus productus* infected before reaching sexual maturity, and subsequent egg production is greatly depressed (Sturrock, B. M., 1967). The fecundity of *B. truncatus* is also reduced by infection with *S. haematobium* and the reduction of oviposition is proportional to the number of miracidia used to infect the snails which never become completely sterile (Chu *et al.*, 1966b). It has been claimed that the reduction in the number of eggs per mass in *B. truncatus* is more marked than the decrease in the number of egg-masses laid (Najarian, 1961), but the opposite has also been reported (Chu *et al.*, 1966b).

Exhausted daughter sporocysts of *S. mansoni* can undergo regeneration and produce more cercariae so that the infection can persist for long periods in snails (Pan, 1965). Production of cercariae of *S. mansoni* in *B. glabrata* and *B. pfeifferi* and of *S. haematobium* in *B. truncatus* may last for 8 months and these snails may survive their infections and can subsequently be reinfected (Ritchie *et al.*, 1963; Pan, 1965; Pitchford and Visser, 1965; Chu *et al.*, 1966a; Wajdi, 1966). In *B. nasutus productus* the maximum period of production of *S. haematobium* cercariae yet noted is 9 weeks and the infection usually proves fatal to the snails (McClelland, 1965, 1967; Sturrock, B. M., 1967), but other snails of the *africanus* group have remained infected for up to 6 months (Pitchford and Visser, 1965). In Kenya about 50% of naturally infected *B. pfeifferi*, *B. africanus* and *B. globosus* died within 10 days of being brought to the laboratory and 6% of the *B. pfeifferi* were the sole survivors after 2 months (Teesdale, 1962). While this does not indicate the duration of infection it does suggest that the survival of infected snails is much lower than that of uninfected snails. The same conclusion was reached in laboratory and field observations on the survival of infected *B. glabrata* (Barbosa, 1963; Ritchie *et al.*, 1963). Although *B. glabrata* usually appears to be well adapted to its local strains of *S. mansoni* a considerable mortality occurred among infected snails and their average life span after infection in the laboratory was 39 days. *B. centimetralis* (Lutz), which is a poor intermediate host in South America, survived for an average of only 5 days after infection. *B. truncatus* have continued to shed cercariae of *S. bovis* for up to 73 days but 50% of the snails died within 2 months of first shedding (Lengy, 1962). The infection died out in surviving snails but extensive tissue damage remained.

The biochemical requirements of larval trematodes and the effects which these produce on the snails have recently received some attention (Cheng, 1963). Existing information is inadequate to allow their significance to be usefully discussed in relation to problems of disease transmission. Efforts are being made to devise techniques for studying larval trematodes in snail tissues maintained *in vitro* and such methods would greatly facilitate further studies (Chernin, 1964; Bénex, 1966, 1967).

Anything which seriously reduces the fecundity and survival of certain individuals must be a selective disadvantage to them and this will clearly apply

to snails infected with larval schistosomes. Selection pressure might be expected to favour any form of innate resistance to infection among the snails and, since evolutionary processes are presumably tending to maintain the host-parasite complex, some compensatory factors must exist, although there is little factual information as to how these are operating. Recent studies of *Velacumantus australis* showed that this snail is polymorphic in respect of an unbanded or a white-banded shell (Ewers and Rose, 1966; Ewers, 1967). The incidence of infection with larval trematodes was four times greater among unbanded snails than among white-banded ones and infection was shown to cause complete castration or considerable degeneration of the gonads. Banded snails were more frequent among the young snails than among the older snails, so that they were apparently at a selective disadvantage in spite of the advantage gained in respect of parasitism. Predation was investigated and it was found that fish took a higher proportion of banded snails, but it was considered that other kinds of selection were also operating which might be less easy to demonstrate. Such a situation would be an obvious advantage in maintaining the host-parasite complex and it was concluded that polymorphism with respect to resistance to parasite infection might well occur in species where the parasite frequently causes sterility or reduces fecundity and where conditions in nature are such that almost universal exposure to infection occurs. This may well apply to many schistosome transmission sites in Africa.

D. SUPPRESSION OF INFECTION

When *B. glabrata* with mature infections of *S. mansoni* were kept out of water for 30 days degeneration of sporocysts and cercariae occurred after about 16 days and the one-third of the snails which survived the period lost their infections (Barbosa, 1962). In the case of immature infections development stopped when the snails were removed from water and resumed when they were replaced in water, and 5-day-old sporocysts could remain dormant for at least 150 days. In a natural population of *B. nasutus productus*, in a pool which remained dry for 98 days, snails were found shedding cercariae of *S. haematobium* 6 days after the pool refilled (Webbe, 1962b).

Numerous records exist of snails being infected with more than one species of larval trematode and shedding the cercariae simultaneously. Some combinations are quite common and occur at higher frequencies than would be expected from random infections, and this suggests that, in some cases, infection with one species of larval trematode may predispose a snail to superinfection with another species (Ewers, 1960; Bourns, 1963). On the other hand it is many years since it was first noted that echinostomes never featured in double infections in the freshwater snail *Stagnicola emarginata angulata* (Sowerby) in the Douglas Lake region of the United States (Cort *et al.*, 1937). Recent studies in Malaysia have shown that when certain schistosome, strigeid or plagiorchid sporocysts occur in *Lymnaea rubiginosa* (Michelin) together with certain echinostome rediae, an antagonistic reaction takes place which leads to the elimination of the sporocysts (Lie *et al.*, 1965; Basch and Lie, 1966a, b). A similar antagonism has been demonstrated between two species of echinostome

in *L. rubiginosa* (Lie *et al.*, 1966). The active echinostome rediae appear to prey on the sporocysts of the other species and there is also evidence of indirect effects but not of any immunity against the penetration and early development of a second infection. Two species of echinostome larvae from Brazil have shown antagonism towards sporocysts of *S. mansoni* in *B. glabrata* (Lie, 1966, 1967). Mature infections of *S. mansoni* regressed with considerable reduction in production of cercariae when the snails were superinfected with *Paryphostomum segregatum* Dietz. Only a small proportion of snails infected with *P. segregatum* could be successfully superinfected with *S. mansoni* and, in these, production of schistosome cercariae was very low. With *Echinostoma barbosai* Lie and Basch the effect was weak and incomplete, probably involving only indirect antagonism. Rediae of *E. malayanum* Leiper prey on sporocysts and cercariae of *S. spindale* in *Indoplanorbis exustus* (Deshayes) and the intensity of this antagonism is intermediate between the two described with *S. mansoni* (Heyneman and Umathevy, 1968). An unusual feature of this study is that, in 650 naturally infected *I. exustus*, 21·2% were found infected with *S. spindale* and 13·5% with *E. malayanum* while 7·2% were infected with both parasites. This is two and a half times the expected proportion of double infections, which might suggest a positive association between the two parasites. These studies of interspecific antagonism between larval trematodes, which constitute a new approach to biological control, have been reviewed more fully elsewhere (Lie *et al.*, 1968).

In Africa *Biomphalaria* and *Bulinus* act as intermediate hosts of a number of trematodes other than species of *Schistosoma*, and echinostome infections have been recorded in *B. guernei* and *B. senegalensis* from Senegal (Gretillat, 1961). Very little is known about these other trematodes but double infections involving *Schistosoma* occur (Berrie, 1964, 1970b). When *B. truncatus rohlfsi* with an established infection of xiphidiocercariae are exposed to *S. haematobium* miracidia, the number of successful superinfections is very much lower than the number of infections which can be obtained in snails with no existing infection (Paperna, 1967). This case contrasts strikingly with the high degree of association reported from Canada between a xiphidiocercaria and an avian schistosome in *L. stagnalis appressa* Say (Bourns, 1963). To the north of Lake Victoria high incidences of other trematodes have been recorded in snails in areas where very little transmission of human schistosomes occurs (Cridland, 1957a, b, 1958; Berrie, 1964). In south-eastern Tanzania populations of *Bulinus* frequently have a high incidence of *S. haematobium* but infections with other trematodes are relatively scarce (Berrie, 1966a, 1970b). There is no evidence to suggest that the different incidence of infection with other trematodes in these two areas is a significant factor in determining the incidence of infection with *Schistosoma*, but it may be concluded that more attention could usefully be paid to the interrelationship of schistosomes and other trematodes in their intermediate hosts in Africa.

The suppression or eradication of larval schistosomes within the intermediate host has been attempted by exposure to molluscicides at concentrations which are sublethal to the snails; to schistosomicidal drugs used for treatment of the infection in man; or to antibiotics or metabolic inhibitors.

Unfortunately the molluscicides tested have shown no useful effects and such a consolation cannot be expected from unsuccessful mollusciciding operations (Sturrock, R. F., 1966b; Warren, 1967). Other substances have proved effective under laboratory conditions but their practicability under field conditions has yet to be investigated (Warren and Weisberger, 1966; Warren, 1967). Perhaps the most promising results are those obtained with lucanthone hydrochloride which has been used as a therapeutic drug for a number of years. This suppressed the development of *S. mansoni* in *B. glabrata* at a concentration of 0·4 p.p.m. and also prevented the snails from growing or laying eggs.

VII. The Ecology of Cercariae

A. Production and Emergence

The number of cercariae which emerge from infected snails varies considerably between individual snails and from day to day. The initial output of cercariae is low but production increases over a period of days or weeks to reach a peak after about two months and then falls to a relatively constant level which is maintained almost until shedding ends (Faust and Hoffman, 1934; Chu *et al.*, 1966b). It has been reported that *B. nasutus productus* with unisexual infections produced significantly fewer cercariae than those with bisexual infections (McClelland, 1965) and that *B. truncatus* which had been exposed to two or more miracidia shed twice as many cercariae as those exposed to a single miracidium (Chu *et al.*, 1966b). However, with *S. japonicum* in *Oncomelania quadrasi* (Möellendorff) (Pesigan *et al.*, 1958) and with *Fasciola hepatica* in *Lymnaea truncatula* Müller (Kendall, 1949), infection with a single miracidium produced more cercariae per day than multiple infections, and this was attributed to poorer development of sporocysts in multiple infections due to overcrowding. Mature specimens of the two species of *Bulinus* would certainly be larger than the other snails and might provide space for more miracidia to develop fully but an upper limit must still exist, and these relationships could usefully receive further attention.

Numerical data on output are scattered through the literature and a general pattern emerges. *B. glabrata* frequently produces enormous numbers of cercariae. Figures of over 1000 per day and 100 000 during the course of infection seem quite common (Faust and Hoffman, 1934; Schreiber and Schubert, 1949b), while an average of 4598 per day is on record with individual snails producing up to 17 600 in one day (Barbosa *et al.*, 1954). African *Biomphalaria* average about 500 per day and rarely exceed 1500 per day (McClelland, 1965, 1967; Sturrock, R. F., 1965a). The larger species of *Bulinus* are of the same order and only a small proportion exceed 2000 per day (Lengy, 1962; McClelland, 1965, 1967; Chu *et al.*, 1966b; Berrie, 1970b), but in both genera exceptional individuals will yield figures about three times the normal maxima. The *forskali* group appear to produce relatively small numbers of cercariae and the maximum of 200 or more produced by *B. reticulatus* on the first day of shedding (Wright, 1963) seems to be exceptionally high and might be regarded as an additional factor casting doubt on the relationships of this species.

Since the reported output of *B. glabrata* is considerably greater than that of *B. sudanica tanganyicensis*, which in turn is considerably greater than that of *B. angulosa*, and the size attained by these species decreases in the same order, it is reasonable to suppose that snail size is an important factor controlling production of cercariae. The small production from members of the *forskali* group also fits this pattern. In a study of 89 naturally infected *B. globosus* and *B. nasutus nasutus* from southern Tanzania a positive relationship was found between size of snail and output of cercariae (Berrie, 1970b). None of the 15 snails up to 10 mm shell height yielded more than 600 cercariae when exposed to light for 6 h on the day after collection, while the nine which had figures of more than 2000 cercariae were all over 13 mm shell height. However, although the size of a snail may be a factor in limiting the number of cercariae produced, there is still great variation between snails of the same size. When the snails were grouped according to their output of cercariae it was found that 45 gave less than 500 and that progressively fewer tended to occur in each higher class.

The emergence of cercariae from the intermediate hosts does not proceed continuously. The pattern of emergence seems to be partly determined by an innate rhythm and is greatly influenced by external factors. At normal temperatures the most important stimulus is provided by light, while temperature itself has a secondary importance (McClelland, 1965, 1967). The basic pattern for *S. mansoni* has been described in *B. glabrata* (Schreiber and Schubert, 1949a), in *B. angulosa* (Sturrock, R. F., 1965a) and in *B. sudanica tanganyicensis* (McClelland, 1967). The emergence built up rapidly during stimulation with light, reaching a maximum in the second or third hour, and nearly the whole day's production was shed within 5 h. This pattern seemed to be fairly constant at all levels of output except that in light infections the peak occurred earlier because production could not be sustained for so long. The pattern of emergence of *S. haematobium* from *B. nasutus productus* was similar but cercariae were shed more slowly (McClelland, 1967). Maximum output occurred in the fourth hour and, while most cercariae had emerged by the end of the seventh hour, the residual output after that time was higher than in *S. mansoni*.

High temperatures appear to inhibit the production of cercariae and this effect is almost complete for *S. mansoni* in *B. glabrata* at 35°C (Barbosa, 1962). In some areas where schistosomiasis is endemic the maximum water temperatures during the hot season may exceed 35°C but the diurnal fluctuation will usually produce early morning temperatures below this level. It would be interesting to know whether such conditions would alter the emergence pattern of cercariae. Reference has already been made to the effect of low temperatures on the prepatent period in the snails and on the production of cercariae. Data from natural populations in Rhodesia confirm that a break in transmission occurs with both *S. haematobium* and *S. mansoni* during the coldest months (Shattock *et al.*, 1965).

B. SURVIVAL AND INFECTIVITY

It is clear that cercariae may survive for some time after they have ceased to be capable of penetrating a suitable definitive host. They emerge from the snail

with a limited store of energy and once this has been depleted to a certain point their reserves presumably become too small to produce the physical effort required during penetration. Cercariae which have lost their infectivity are of no epidemiological significance and it is of some importance to distinguish them from those cercariae which are still infective. In the static conditions of a laboratory container cercariae will probably expend much less energy in swimming than they would in a natural habitat subject to any form of disturbance, and consequently they may be expected to remain infective for a longer period and to survive still longer. Infectivity may be influenced by environmental conditions and under certain conditions the cercariae may be killed before they lose their infectivity.

Cercariae in domestic water supplies can usually be killed by adequate chlorination but the cercaricidal activity of chlorine probably depends on the concentration of hypochlorous acid which is produced and changes quite considerably according to the pH of the water (Frick and Hillyer, 1966). The availability of calcium and magnesium ions is essential for successful penetration of the definitive host and the concentration of these ions in the water has been shown to affect the infectivity of *S. mansoni* cercariae (Lewert *et al.*, 1966). Variation in the chemical composition of natural waters may explain some of the variability in schistosome infection rates obtained in different laboratories and in the intensity of human infection found in different endemic areas. Mice have been exposed to known numbers of *S. mansoni* cercariae kept in water at 22·7–25·7°C at various intervals after shedding and the resulting worm-burdens have been recorded (Olivier, 1966a). With cercariae up to 3·6 h old, up to 28·5% developed into adult worms. After 12·6 h the rate fell to 6·5% and after 24·6 h to 0·5%.

Studies of the effect of environmental temperature on the success of cercariae in infecting definitive hosts have produced divergent conclusions. When infected *B. pfeifferi* were maintained in tanks at constant temperatures ranging from 18°C to 30°C and mice were exposed to the cercariae released in the tanks it was found that nearly every mouse was infected and the mean worm load did not appear to vary with temperature (Foster, 1964). In another investigation it was found that the success of *S. mansoni* cercariae in penetrating and maturing in mice was strongly influenced by the water temperature (DeWitt, 1965). The optimum temperature was between 30°C and 35°C although infections were achieved at all temperatures tested between 10°C and 40°C. In a third series of experiments *S. mansoni* cercariae were maintained at constant temperatures from 12°C to 33°C and samples used to infect hamsters after 2, 6 and 24 h (Purnell, 1966b). The results suggested that survival of cercariae decreased with age but was independent of maintenance temperature up to 30°C at which increased mortality due to temperature was noted. Infectivity and the mean worm load per 100 original cercariae also declined with age and a further decline in those maintained at higher temperatures was noted among the oldest cercariae.

Significant variation has been reported in the infectivity of cercariae shed by the same snails at different times and this has been attributed to the physiological condition of the snails (Evans and Stirewalt, 1951) and to the progressive

maturation of the intramolluscan larval stages (Wright and Bennett, 1967a, b). When experimental infections were made with cercariae of *S. haematobium*, obtained very soon after the snails had started shedding, a predominance of male worms was obtained, which suggests that male cercariae may mature more rapidly than female ones. As the age of the infection in the snails increased, considerable fluctuations were recorded in the success of cercariae in becoming adult worms. Until this phenomenon is understood more fully it must be given careful consideration in planning any experiment in which the results are represented by variations in the worm burden produced in animals.

Considerable difficulties exist in attempting to study populations of cercariae under natural conditions but several attempts have been made. A pressure filtration chamber was devised in which the cercariae from a given volume of water could be trapped on filter paper and then stained and counted (Rowan, 1957). This worked well in fairly clear water but trials in Africa, where many natural waters are very turbid, resulted in immediate blockage of the filter, and alum flocculation of the suspended matter did not improve the performance because the larvae were trapped in the flocculi (McClelland, 1965). Using this apparatus in Puerto Rico it was possible to show that *S. mansoni* cercariae were present in flowing water for only 6 or 7 h in the middle of the day, but in ponds the concentration of cercariae remained high for a much longer period through the afternoon and evening (Rowan, 1958; Maldonado, 1959). The number of cercariae recovered on cloudy days proved to be as great as on sunny days. Other methods of filtration and of concentrating cercariae which have been suggested are discussed elsewhere (Rowan, 1965) and interest has arisen in the possibility of using a continuous-flow centrifuge to recover cercariae from water (Barrett and Ellison, 1965; Olivier, 1966b). We are still left with the problems of successfully adapting any method to the field conditions encountered in many parts of Africa and of identifying the cercariae after they have been isolated.

Better progress has been made in studying populations of cercariae by the indirect method of exposing laboratory animals in the natural habitats and using the resulting infection rates and worm burdens as an assessment of the transmission potential of the habitat at the time of exposure. This disposes of the problems caused by turbidity and of identifying the cercariae and, epidemiologically, has the advantage of detecting only those cercariae which are infective. However, a delay of several weeks is imposed in obtaining results and, in still water, relatively immobile animals and cercariae may have little chance of making contact. In an extensive study of *S. japonicum* in the Philippines direct counts of cercariae were carried out along with animal exposures in natural waters and both methods produced a similar picture of periodic fluctuations in the density of cercariae (Pesigan *et al.*, 1958).

Rodents have been experimentally immersed in natural waters in the Transvaal and it was found that infection with *S. mansoni* took place between 10.00 and 16.00 h and with *S. mattheei* between 07.00 and 16.00 h (Pitchford and Visser, 1962). This led to a series of experiments in outdoor aquaria (temperature range 20–29°C) containing groups of snails each shedding cercariae of a single species of schistosome (Pitchford and Visser, 1966). At

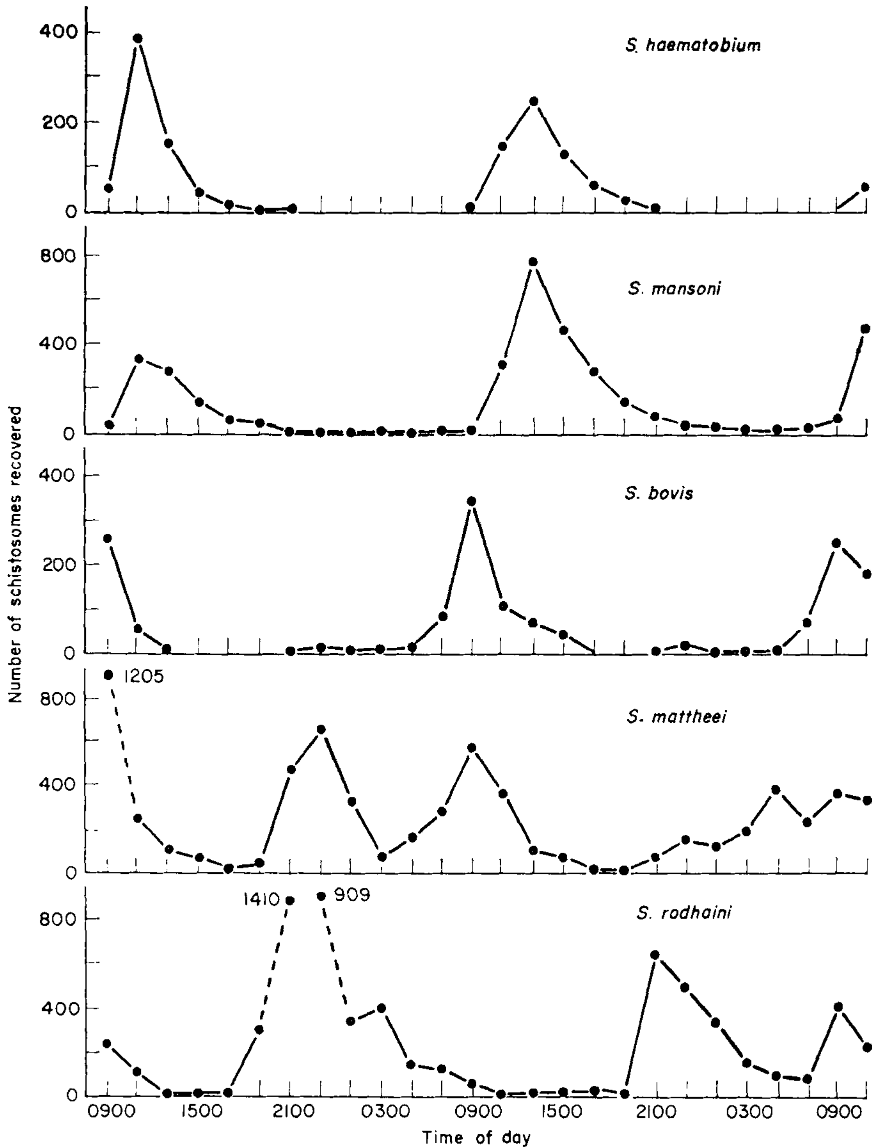


FIG. 10. The numbers of schistosomes recovered from groups of four *Mastomys* exposed for 15 min in aquaria containing appropriate infected snails. A separate group was exposed every 2 h during the observations and adult schistosomes were recovered at autopsy 6 to 10 weeks later. (Data from Pitchford and Visser, 1966.)

intervals of 2 h over a period of 52 h, groups of 4 *Mastomys* were placed in each aquarium for 15 min and allowed to swim around freely. The rodents were later killed and the number of worms which had developed from each

exposure is shown in Fig. 10. Two strains of *S. haematobium*, from South Africa and Iran respectively, were tested and both gave almost identical results with maximum development around noon and no worms developing for 10–12 h overnight. *S. mansoni* had a very similar pattern but the fact that infections continued later in the day at a rather higher level than *S. haematobium* is not in accord with the laboratory data discussed above. *S. bovis* and *S. mattheei* both gave an early morning peak at 09.00 h but *S. mattheei* also had another peak after dark which may represent an adaptation to its wild antelope hosts. *S. rodhaini* showed a sudden rise around sunset which might coincide with the most active period of suitable wild rodents, but this distinctive nocturnal pattern contrasts with laboratory observations which report that most cercariae are released very early in the morning within 1 h of light stimulation occurring (Fripp, 1963, 1968).

In a small seasonal stream flowing into Lake Victoria mice were exposed every 3 weeks and the results showed that a well defined seasonal transmission occurred with *S. mansoni* (Webbe, 1965b). A particularly interesting aspect of the data is that the maximum recovery of adult worms from the mice did not always correspond with the times at which the largest numbers of infected *B. pfeifferi* were found in the stream. This apparent discrepancy was attributed to changes in the state of the stream, particularly the water velocity.

Preliminary experiments with *S. mansoni* cercariae showed that in flowing water with velocities under 0·5 m/sec more worms developed in mice exposed at relatively fast rates of flow than at relatively slow ones (Rowan and Gram, 1959). Further experiments in a Puerto Rican stream showed that the number of worms recovered from immersed mice was reduced when water velocities greater than 1 m/sec were used, but significantly lower velocities were not tested in this situation (Radke *et al.*, 1961). Successful development also decreased quite rapidly with distance from the cercarial source, a tenfold reduction being noted between animals exposed 30 m downstream and those exposed 762 m downstream, but a small proportion of cercariae were able to develop in mice exposed 1524 m downstream. Cercarial densities were found to remain very constant throughout the experimental area and no reduction in number was detected 610 m downstream. This indicates that increased water velocity and distance travelled reduce the infectivity of the cercariae.

Experiments with an East African strain of *S. mansoni* showed that maximum infection rates and worm loads were obtained when mice were exposed in water flowing at about 0·3 m/sec (Webbe, 1966a). The worm load was reduced at both higher and lower velocities. The interpretation of the results of these experiments is complicated by the fact that changes of velocity cause changes in the cercarial density-volume relationship. However, it seems clear that up to a certain point increase in velocity increases the chance of a cercaria making contact with a stationary immersed animal but that above this point some cercariae may either be swept off the animals before they can penetrate or become "fatigued".

Predation by fish may have an important effect on the number of cercariae surviving in natural waters. In the laboratory it has been shown that four *Lebistes* in one l of water are capable of consuming 7000 *S. mansoni* cercariae

in less than 3 h (Rowan, 1958). A study of a heavily polluted stream in Puerto Rico showed that *Lebistes* were not present for 500 m below the source of pollution and the density of cercariae in this stretch was considerably higher than it was further downstream where *Lebistes* were actively feeding (Rowan, 1965). Cover in the form of vegetation or suspended matter may play an important role in the dynamics of natural populations of cercariae and there are some reports which indicate unusually high numbers of cercariae during turbid water conditions.

From these studies of the ecology of cercariae it seems clear that in flowing water the period of high risk for transmission of *S. mansoni* is likely to be between 09.00 h and 16.00 h while that of *S. haematobium* may persist rather later. In the early morning and late evening contact with the water may be much less dangerous. If infected snails are restricted to certain parts of a stream we have some idea of how the risk may decrease with distance downstream. Standing bodies of water are likely to have cercariae present for longer periods unless predation is high or numerous opportunities for host penetration occur, but even here the risk should be considerably lower in the early morning unless *S. mattheei* is involved.

VIII. Dynamic Aspects of Transmission

A. Field Studies

Various aspects of the biology of larval schistosomes and of their snail hosts have been considered in the preceding sections. The snails are affected by a number of features of their physical and biological environment and by the presence or absence of parasites in their tissues. The larval schistosomes must survive two periods of exposure to the hazards of an aquatic environment and on each occasion must find a suitable host before their reserves of energy have been expended. During their development in the snail host they are affected directly by many characteristics of the host and indirectly by the factors which affect the host itself. Field studies of transmission involve the investigation of a dynamic situation in which the interaction of these factors may be constantly changing. It is, therefore, essential that a wide range of data be recorded and that parallel laboratory investigations be carried out using the same strains of snail and parasite, otherwise the results may prove impossible to interpret fully.

The percentage infection rate in snails is frequently recorded but, unless this is accompanied by adequate data on the size distribution of the snail population and of the infected snails, it may prove misleading. The appearance of a cohort of young snails which have not had time to develop mature infections will cause a sudden reduction in the percentage infection, but, unless there has been increased mortality amongst the older snails, the actual number of infected snails in the habitat may be unaltered. A quantitative assessment of the density of the snail population is necessary to determine the number of infected snails present, and a study of the relationship between the size of the infected snails and their output of cercariae is required to determine the rate of production of cercariae in the habitat.

Similarly, to record a reduction in the density of a snail population may not mean that transmission by the population has been reduced unless data on sizes and infection rates are obtained. It is also possible that a reduction in density may change a rather stable situation in which little transmission is taking place into a dynamic situation with a far greater potential for transmission (Berrie, 1968).

Snail populations often live in situations which create great difficulties in applying quantitative sampling methods and few field studies have been able to obtain adequate samples of small snails or of egg masses. While some available studies provide useful comparative figures for important population parameters such as density, recruitment, growth and mortality, few, if any, can claim to have obtained exact figures. At one time of year it may be possible to collect three times as many snails in a given period as at another time of year and the proportion of small snails in collections may change by a factor of ten; but it is seldom possible to express this data as numbers per unit area or volume of habitat. The striking lack of information about the food supply required to maintain the intermediate host-parasite complex has already been stressed. Some of the small habitats containing snail populations represent circumscribed ecosystems in which the flow of energy through the system could be measured to show how the production of snail and parasite tissue is related to the energy resources of the system. The quantitative techniques which have become available for investigating these ecological and physiological problems should be applied to studies of the snail hosts of schistosomiasis so that the population parameters can be determined more accurately. When such data are available it should be possible to achieve a much fuller understanding of the dynamic aspects of transmission.

B. MATHEMATICAL MODELS

A further stage in attempting to understand the dynamics of transmission is to construct a mathematical model of the situation. The model should incorporate all the important factors known to influence transmission in a form which can be manipulated mathematically to show how these factors interact and how changes in them affect the rate of transmission. The construction of a quantitatively accurate model requires a very considerable amount of basic data and has only been attempted in connection with the large internationally financed studies which have been carried out on *S. japonicum* in Leyte and on *S. haematobium* and *S. mansoni* in the Egypt-49 project (Hairston, 1962, 1965a, b). Here demographic techniques were used to construct ecological life-tables for the snails. Constructing similar tables for the parasites was more complicated because reproduction occurred twice in the life cycle; direct observation of reproduction and mortality in the two hosts was not possible; and the survival of the parasite between the periods of reproduction had to be estimated. The parasite population in endemic areas does not appear to change greatly unless the area is disturbed. Even in areas where the transmission rates are quite different the parasite populations are able to come into equilibrium and the net reproductive rate must remain constant at approximately

1·0. There is evidence to support the conclusion that compensatory mechanisms operate over a range of transmission rates to keep the parasite population in equilibrium (Hairston, 1965a).

In the absence of adequate data it is possible to construct a model which does not primarily depend on a quantitative knowledge of the factors affecting transmission, but rather on their nature and relative importance. Computer analysis allows each factor to be considered over a range of possible values and the effect of each variable on transmission to be assessed. This gives a qualitative picture of transmission that shows which factors are most important and, therefore, most merit quantitative measurement. This approach also indicates that the parasite population tends to reach a stable equilibrium if the system is undisturbed (Macdonald, 1965).

An important conclusion from both approaches is that there is a lower limit of transmission below which the compensatory mechanisms fail to operate. This has been termed the break point and, once the transmission rate falls below this level, further diminution is not only progressive but accelerating, so that the infection will die out in the area. This is attributed to the fact that, as the parasite population in the definitive host declines, there will be an increasing probability that single parasites which enter the definitive host will remain unmated and infertile. This gives rise to a critical level of reproduction in the definitive host above which the parasite population will increase until it is stabilized by compensatory mechanisms, and below which it will be unable to maintain itself (Hairston, 1962, 1965a; Macdonald, 1965). These conclusions indicate that before the parasite can become established in a new area it must be introduced on a scale large enough to carry it above the break point. This would involve the immigration of large groups of infected people rather than isolated individuals.

IX. Control

The transmission of schistosomiasis may be reduced by preventing the contamination of water with viable eggs; by keeping people away from water containing cercariae; or by eliminating the intermediate hosts. Each of these objectives may be attacked in several ways. Contamination is reduced if infected people are given medical treatment which either cures the infection or suppresses the egg output of the parasites, and also if adequate latrines are provided and used. However, many available drugs have unpleasant side effects which deter patients and, in endemic areas, persons who have been cured are likely to become reinfected, perhaps more severely. Contact with infective water may be discouraged by siting houses as far as possible from potential snail habitats and by providing a safe piped water supply, and adequate facilities for washing and swimming. It may also be necessary to fence dangerous bodies of water. Such measures kept the incidence of infection with *S. mansoni* below 2% in children on a South African irrigation scheme (Pitchford, 1962), which compares favourably with the results of other control measures which have been employed in South Africa (Pitchford, 1966).

Snail control is now a very complex subject and an entire review in this

series has already been devoted to one aspect (Berg, 1964). It may be attempted by chemical, biological or environmental methods and a recent monograph gives a detailed exposition (World Health Organization, 1965). Most ecologists hope that suitable biological or environmental methods will become available, but the necessary research is time-consuming and expensive. Research on molluscicides has progressed more rapidly due to the interest of the chemical industry and at present there is no generally applicable method of snail control other than by molluscicides. These are relatively expensive and complicated to apply properly on a wide scale and they usually kill other organisms, notably fish which constitute an invaluable source of protein in many African countries where malnutrition is widespread. A single molluscicide treatment can be extremely successful but the effects are temporary and repopulation occurs quite rapidly. Most treatment has, therefore, been applied to restricted areas which were known to be active transmission sites and which were suitable for repeated treatment. The successful treatment of an irrigation system (Crossland, 1963) and a small river (Webbe, 1964) are good examples from East Africa. Both programmes involved strategic timing of treatment based on previous studies of the bionomics of the snail populations, and similar measures could probably be applied widely in this region (Webbe, 1965c). A more extensive snail control programme has been operated in Rhodesia for a number of years. In this case a blanket treatment was given to whole stream systems and local treatment was subsequently applied at any point where the snails reappeared (Clarke *et al.*, 1961; Shiff and Clarke, 1967; Clarke and Shiff, 1968). This approach takes no account of seasonal trends in snail populations but has the practical advantage of spreading work evenly throughout the year.

Each approach has difficulties in practice and it is clear that a combination of methods will have more chance of success. The aim must be to reduce the parasite population to a level below the break point and it is possible to use the qualitative mathematical model to predict the results which may be expected from different approaches to control (Macdonald, 1965). While heavy contamination of the water may facilitate the introduction of the parasite to an area, it appears to have little effect on the ultimate level of endemicity. This seems to be determined almost exclusively by the number of snails present, the frequency of contact with the water and the longevity of the worms. Safe water supplies are, therefore, more important than latrines in combating schistosomiasis. The model indicates that the parasite population could be reduced to the break point in just over four years by the combination of therapeutic treatment with either snail control or reduction of exposure.

A preliminary assessment of observations on young children in the Egypt-49 project has produced evidence that transmission of both *S. haematobium* and *S. mansoni* has been interrupted as a result of molluscicide treatment extending over only 2 years (Farooq *et al.*, 1966). A reduction of new infections would be most noticeable in this group and a greater time would be required to produce a large reduction of incidence in the whole population. Within a period of about a year, over 50% of the children under 5 years old became negative spontaneously in respect of each parasite (Farooq and Hairston, 1966). It appears that the mean life span of adult worms may be much less than the

three years assumed in the mathematical model, in which case control measures should take effect more rapidly than was predicted.

During the past decade our knowledge of larval schistosomes and their intermediate hosts has advanced considerably. Much effort has been devoted also to developing new drugs for treatment of the disease and to finding new molluscicides and biological agents to control the snails. Those who have expected some major discovery, which could lead rapidly to widespread control, have been disappointed. Sceptics who have claimed that schistosomiasis is a sanitation disease, which can only be controlled by a radical improvement in the standard of living in endemic areas, may feel justified. The fact that control measures have been applied successfully to relatively small transmission sites shows little progress since the days when copper sulphate was the only available molluscicide and information about snail populations was rudimentary. We still require a demonstration that control can be applied on a wider scale and that it can produce an effective decrease in the incidence and intensity of infection throughout the area. An isolated endemic area with a static population and a relatively simple surface water system should provide the best opportunity. Recruiting and training suitable personnel is difficult and such an operation is bound to prove costly. However, there are indications that reasonable success should be possible within a few years with existing methods, and some projects, such as Egypt-49, are making progress in this direction.

Even if existing methods are proved to be effective, enormous difficulties can be foreseen in attempting to apply them on a very wide scale. The disease is most prevalent in developing countries which can least afford the cost of such campaigns, and which have many rival demands for any available funds. Since control measures proposed by health authorities will inevitably involve interference with water that is used for many purposes, the effective co-operation of several government departments may be required. While piped water supplies and good sanitation seem highly necessary to those reared in western cultures the same views must not be assumed in other cultures. Among the more obvious problems are the ritual washing prescribed by the Moslem religion, the attractiveness of bathing and the possible unpleasantness of pit latrines in hot climates. The problems of the social acceptability of new developments in hygiene and disease control have received rather little attention and should be more fully considered before large schemes are implemented. We are faced with a situation in which the problems are not merely biological but also political, economic and sociological.

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The Relationship between Circulating Antibodies and Immunity to Helminthic Infections

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I. Introduction

Many animals are able to resist helminth infections, and the response of a host to such infections appears to be similar to that produced to bacteria or viruses. The defence mechanisms employed are similar for all types of invading organism. The distinguishing feature is the inability of helminths to multiply in the definitive host by means other than reinfection. It is possible, therefore, to study the host's response to a single life cycle of the parasite in a way which is not practicable for micro-organisms.

There is some evidence to suggest that natural or innate resistance can play a part in counteracting a helminthic infection, but little detailed knowledge of the degree to which it affects the outcome of the disease. Most investigations in this field are concerned with acquired immunity. This phenomenon is usually manifested by the appearance of circulating antibodies directed against

the tissues or secretions of the parasite and normally results in the ability of the host to deal quickly and effectively with a further infection due to that particular parasite.

The elucidation of the precise role of antibody in resistance to helminths is rendered difficult by the number and complexity of the antigens involved. The worms consist of a whole range of structural, secretion and excretion antigens producing a stimulation of the host which may vary qualitatively and quantitatively as the parasites develop. The antibody response is therefore correspondingly complex not only in the range of antibody produced but in the relative amounts and importance from the protective point of view. No doubt humoral antibodies play a part in resistance to helminth diseases, because all the manifestations of resistance can be transferred passively in some cases.

Serological techniques such as the complement fixation test have been useful in studying the general immune response of the host. This was particularly so in the earlier investigations where it was necessary to demonstrate that an immunological phenomenon was involved. However, these tests cannot be expected to give precise information on individual antibodies or the part played by these antibodies in resistance. Some of the newer serological techniques for demonstrating precipitating antibodies may possibly solve this problem as they allow the study of individual antigen-antibody systems even when they are not isolated from other systems.

The purpose of this review is to consider as far as possible the role of circulating antibodies during and after the course of helminthic infections, their importance in the resistance of the host, and the uses to which they may be put in furthering our understanding of host-parasite relationships.

II. Classification of Circulating Antibodies

Serum antibodies belong to a group of globulins which are noted for their extreme heterogenicity with respect of their electrophoretic mobility and molecular size. There are, at present, five main classes of immunoglobulin (Ig): IgG, IgM, IgA, IgD and IgE. Although their nomenclature has been confused in the past, better understanding of their structure and properties has made it possible for a committee of the World Health Organization (1964) to reach agreement on definitions and names.

Much work has been done on the properties of these immunoglobulin classes in relation to the serological response of the serum to bacteriological and viral diseases, and Pike (1967) has produced a review of this literature. Of the five classes only two, IgG and IgM, have had their serological properties investigated extensively. In the agglutination test smaller amounts of IgM are required for a positive reaction than of IgG. On the other hand IgG is much more effective in producing precipitation and complement fixation than IgM. An interesting practical implication of this is the need to test the sera of infected animals using more than one technique in order to ensure that both IgG and IgM antibodies are titrated. Confirmation of this is given in the work of Soulsby and Stewart (1960). They found that the serological reactions

detected by the complement fixation test and the haemagglutination test were independent of each other, and that it was possible to absorb out the antibodies responsible for the haemagglutination reaction with only a small reduction in the complement fixing antibody titre. The system they used was an infection of *Haemonchus contortus* in sheep. Ross (1961), working on *H. contortus* infections in cattle, came to a similar conclusion. He demonstrated that the complement fixing antibody was confined to the IgG fraction while the haemagglutinating activity was found in the β_2-globulin fraction of sera from infected cattle.

The serological properties of IgA are less well understood but its most outstanding characteristic is a high concentration in body secretions. The *iso*agglutinin found in human saliva and colostrum has been shown to be IgA by Tomasi *et al.* (1965), who also determined that IgA was synthesized in local glands and that the secretions derived antibodies from a source different from that which supplied circulating antibodies to the serum. Of the other two immunoglobulin classes, IgD accounts for less than 1 % normal human serum immunoglobulin and has not yet been shown to have serological activity. IgE, however, has been associated with reaginic activity by Ishizaka *et al.* (1966), and will be dealt with under that heading.

Generally IgM is said to be produced early in a primary antibody response followed by a greater and more persistent IgG response (Pike, 1967). This assumption has been questioned by Robbins *et al.* (1965), and with certain antigens and immunological schedules only IgM antibodies have been produced (Sandberg and Stollar, 1966). Crandall and Crandall (1967) found with *Ascaris suum* infections in mice that the principal precipitating antibody was a macroglobulin, and that although the titre was increased with a further infection of *Ascaris* there was no indication that the IgG type of antibody was produced. In contrast to this, the immunoglobulin in rats infected with *A. suum* was found to be of the IgG type as judged by chromatography on Sephadex G200 and the resistance of their precipitating activity to 2-mercaptoethanol.

In another study on experimental trichinosis in rabbits, Crandall *et al.* (1967) estimated the proportion of cells producing three different classes of immunoglobulin by staining with contrasting fluorochromes in the fluorescent antibody test and then counting the specifically stained cells. The results showed an increase in IgM producing cells in the mucosa early in the infection, followed by an increase in IgG producing cells late in the infection and in hyperinfected animals. The proportion of IgA cells remained uniformly high (80–90 %) in the mucosa while in the spleen they accounted for only 2–10 %, and at neither site were there increases attributable to the infection. When cells from the lymph nodes and spleen were studied, the IgM producing cells increased late in the infection, and after hyperinfection, IgG and IgM antibodies produced against *Trichinella spiralis* were demonstrated in the serum and IgG antibodies were found in the mucosal extracts using the fluorescent antibody test. However, using the more sensitive Ouchterlony double diffusion precipitin test, both IgG and IgM antibodies to *T. spiralis* were demonstrated in the mucosa.

Dobson (1967) found with *Oesophagostomum columbianum* infections in

sheep the presence of antibodies associated with particularly high concentrations of β-globulins after the first infection and with β- and γ-globulins after a further infection. He also showed that this intestinal worm infection induced antibodies at greater titres within the mucosa of the gut than in the serum.

III. Reagins

Prausnitz and Kustner (1921) demonstrated that the wheal and erythema type of skin sensitivity was associated with a serum antibody. They produced passive sensitization of human skin by intradermal injection of serum from a sensitive patient and elicited a typical wheal and erythema by subsequent injection of antigen into the same site. As there was not agreement on the nature of the skin-sensitizing component of the donor serum it was given the neutral label of reagin. Evidence that reagins were indeed antibodies and that their production could be stimulated immunologically, has appeared quite recently (Fine and Abram, 1960), and Johansson *et al.* (1968a) showed that some at least belong to the newly discovered class of immunoglobulins designated IgE. This discovery has alleviated a major difficulty in our understanding of this type of antibody. The other difficulty is that reagins exist only in very low concentrations in the serum so that to titrate them using the standard serological tests is impossible. However, their isolation is imminent and this problem may not be insoluble. Reagins can be demonstrated most easily in living tissues, even when the test is conducted *in vitro*. The use of living tissues was considered unavoidable because the most important property of reagins is their great affinity for skin and some other body cells. The nature of this attachment causes the release of histamine and other pharmacologically active substances when the specific antigen combines with the cell bound antibody, and it is these substances which are titrated in the standard serological techniques.

Recently, determined efforts have devised serological tests which are not dependent on living tissues. By means of an adaptation of the extremely sensitive radio-immunoabsorbent technique of Wide and Porath (1966), IgE can be measured in normal sera at concentrations far lower than was possible previously (Johansson *et al.*, 1968a). A test devised by Coombs *et al.* (1953) uses a red-cell-linked antigen antiglobulin reaction, and this has been adapted to titrate allergens, the antigens involved in the hypersensitivity reaction. Here the allergen is fixed onto the red cells by antibody and the antibodies to the allergen added. As the antibodies are of the incomplete form, no reaction takes place until antiglobulin serum is added. These antiglobulin sera can be prepared to the specific immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, and it is possible to correlate the titres of these classes with the presence of reagins.

In a study of patients with an allergy to castor bean allergen, Coombs *et al.* (1968) have shown a good correlation between IgE and the clinical state of the patient. Raised levels of IgE globulins have also been correlated with the occurrence of *Ascaris lumbricoides* infections in Ethiopian children (Johansson *et al.*, 1968b). The African children had 16–20 times the amount of IgE globulins found in a control group of Swedish children, and when a group of

the African children shown to be infected with *Ascaris* was used, the disparity was even greater. This finding strongly suggests that the worm infection stimulated a reagin-like reaction, although there is no evidence as yet that this is an effective protection against the infection.

In *Nippostrongylus brasiliensis* infections a correlation exists between circulating antibody and resistance to the disease. Mulligan *et al.* (1965), working on the self-cure phenomenon in the rat to *N. brasiliensis*, demonstrated that the humoral antibody, passively transferred, was important in interfering with the establishment of the infection. As the protective antiserum had no parasiticidal effect on the worms *in vitro* and no immediate effect on their oxygen uptake it was considered that the antibody acted through its role in local anaphylaxis.

Ogilvie (1967) and Jones and Ogilvie (1967) also investigated these reagin-like antibodies to *N. brasiliensis*, finding a close association between these antibodies and the immune response in rats. The antibodies were detected by homologous passive cutaneous anaphylaxis and could be shown in all rats one week after they had acquired resistance to the initial infection. Reagin formation was induced by an infection of living worms and was greatest for the adult stage. When injections of worm extract were used, only some rats responded by producing reagins and then only after the primary infection. Further infections produced "blocking" antibodies which had the same antigenic specificity as the reagins, but could not be attached onto the skin of the rats. This is in line with common experience in these matters, as the crude extract of helminths must contain a great deal of material which the immunological mechanisms of the host do not encounter in natural infections. However, Jones and Ogilvie (1967) have shown that a saline extract of adult worms has the power to neutralize, *in vivo*, the passive transfer of immunity with sera from immune rats. Evidently there are substances in the extract of the same antigenicity as those which stimulate protection when administered through a live infection of the worms. It would seem, therefore, that the method of presentation of the antigen is at fault, and further progress in this line of research must now depend to some extent on the isolation of the antigens responsible for the reaginic stimulation. Some work directed to this end by Jones and Ogilvie (1967) has shown that the allergen from *N. brasiliensis* could be partially purified by column chromatography using Sephadex G200. Other tests have shown that it appears to be a protein with a molecular weight of 12000–17000.

Reagins probably play an important part in the resistance of the host to several helminths. Ogilvie *et al.* (1966) reported finding them in rats infected with *Schistosoma mansoni*. Here there was a correlation between their presence and protection, and passive transfer experiments showed that this resistance could be conferred by circulating antibodies. Two other examples of reaginic antibodies in helminth infected hosts are quoted by Ogilvie (1967)—against *Litomosoides carinii* (Worms, unpublished) and against *Trichinella spiralis* (Denham, personal communication).

The close association of reagins with active immunity in infected rats and the involvement of reagins in the neutralization of passive immunity indicates

a protective function for these antibodies. However, it is not possible to suggest that they are the only antibodies to act in this way. Ogilvie and Jones (1967) found in *N. brasiliensis* infections with thymectomized rats that a direct relationship did not exist between the degree of acquired resistance and the level of reagins in the sera of the hosts. Edwards *et al.* (1967) investigated the occurrence and properties of reagins to *S. mansoni* in monkeys, and here too no correlation was found between reagins and resistance to reinfection; nor could resistance be transferred passively by injection of serum. The contrast between these results and those obtained by Ogilvie *et al.* (1966) must refer to the type of hosts used. Smithers and Terry (1965) pointed out that in the rat the host-parasite relationship is weighted in favour of the host and that under these conditions any immunological activity is magnified whereas, in the monkey, a more stable relationship exists and the same immunological activity has little effect on the parasite. It is therefore not possible at the present time to come to any firm conclusion as to the role which reagins play in the production of immunity.

IV. Antibody Binding Sites

With the advent of methods for labelling antibodies with dyes which fluoresce in ultraviolet light or with electron dense substances it has become possible to examine the sites in the worms where the antibodies react with their specific antigens. This has opened the way for investigations which may enable immunologists to study the mode of action of protective antibodies on the worm. These techniques have been applied only on a limited scale, but the results which have been obtained encourage the hope that much more can be learnt in this field. Fernando (1968a) working with heterophile antibodies in rabbits showed that they were increased in titre after an infection of *Toxocara canis*. This antibody combined with its homologous antigen which was present in infective eggs and the intestine of the adult worm. He also found that the cuticle had little antigenicity with this type of antibody.

The argument about the antigenicity of the nematode cuticle has raged for many years. Although cuticular precipitates have been observed by many workers (Oliver-Gonzalez, 1943; Sadun *et al.*, 1962) these have been ascribed to the flow of secretion and excretion products over the cuticle from the oral and excretory pores of the worm. However, Crandall *et al.* (1963) used sections of *A. suum* larvae to demonstrate the attachment of fluorescein-labelled antibody to the cuticle of all stages of larvae, including the second stage which did not form precipitates in immune serum. This result and the uniform distribution of stain round the cuticle of the sectioned material made it appear unlikely that the results were due to the accretion of antigen from the major pores of the larvae. Examination of adult worms by the same technique revealed specific staining on the periphery of muscle cells but not on the cuticle. Taffs and Voller (1963) also used fluorescent antibodies and confirmed that the cuticle of *Ascaris* larvae is antigenic.

It is still possible, however, that the antigens which react on the cuticle are not part of the outer layer, but secreted by the pore canals which are present

in the cuticle (Bird, 1958). The possibility was strengthened by Despommier *et al.* (1967), who found that ferritin conjugated gamma globulins from hyperimmune rabbit serum were attached to the outside of the cuticle of *T. spiralis* larvae and nowhere else. However, this antibody did form a precipitate with secretion and excretion products and possibly the same antigen–antibody system is involved. Unfortunately, absorption studies which might have decided the issue were not employed.

Soulsby (1965) investigated the nature and origin of functional antigens in helminths and found that antibodies produced in guinea-pigs resistant to *A. suum* were localized in areas of the worm where DNA was present. He postulated that the functional antigen might be associated with nuclear protein and that the immune mechanism of the host might produce an inhibition of protein synthesis.

Although work in this field is only in its preliminary stages, there are many lines of research open and it is to be hoped that the extension of our knowledge will be rapid. It would be particularly useful to apply labelling techniques to antibodies produced against chemically defined or fractionated antigens, as this would seem to be a logical development of the work. In this way it would be possible to circumvent the difficulty of using antisera produced in response to a wide spectrum of antigens some of which, although potentially capable of stimulating antibodies, are ignored by the host.

V. Effect of Immune Serum on Helminths

A. *in vitro*

An approach to the study of protective antibodies has been made by observing the effect of hyperimmune serum on parasites, and in a number of cases precipitates have been found at the pores of the helminths. It is not possible to assess the significance of this phenomenon in resistance unless we can show that the interaction of antibody and antigen impairs the vitality of the worm. Oliver-Gonzalez (1940) using immune serum to *T. spiralis* found precipitates at the oral pores of larvae extracted from muscle and at oral, anal and vulval openings of adults. Treated worms had shorter survival times than the controls.

Jackson (1959) confirmed the production of precipitins but found no parasiticidal activity. He used the fluorescent antibody test to demonstrate that the precipitate contained antibody which usually occurred at the oral opening of adults. In sections of the worm specific fluorescence was observed in the digestive tract and occasionally in the reproductive organs. This suggested that the antigens involved in the precipitates might be secretions.

In other cases the exposure of larvae to immune serum reduces their infectivity. This holds for *Ancylostoma caninum* (Otto, 1948) and *N. brasiliensis* (Thorson, 1954). Heyneman and Welsh (1959) described a series of experiments on the life-cycle stages of the dwarf mouse tapeworm, *Hymenolepis nana.* These cestodes were incubated in antisera produced by injecting macerates of adult worms into rabbits, and both eggs and cysticercoids were greatly

reduced in infectivity compared with the controls which were subjected to normal serum. Immune serum also affected adult worms by completely enveloping them in a layer of precipitate and rupturing the cuticle.

It is important, however, to evaluate carefully experiments carried out *in vitro* using humoral antibodies because there is not necessarily a correlation between these results and those obtained when the parasite occurs in the host. Taliaferro and Sarles (1939) found precipitates which appeared to be similar to those observed *in vitro* around worms in the tissues of rats immune to *N. brasiliensis*. However, for helminths which are restricted to the gut, the degree to which they come in contact with the tissue fluids and serum of the host is lessened. *Haemonchus contortus* and *T. spiralis* are known to have some contact but this may not be true for all gastro-intestinal parasites.

The part played by antibodies found in the gut mucosa has received little attention. Soulsby (1960b) using haemagglutinating techniques failed to demonstrate muco-antibodies. However, Douvres (1962) found that precipitates were formed round *Oesophagostomum radiatum* larvae immersed in media containing extracts from the intestines of a resistant calf, and Dobson (1966a, b, c) demonstrated antibodies to *O. columbianum* in mucous exudates and gut extracts from infected sheep using haemagglutination, precipitin and percutaneous anaphylaxis tests.

B. PASSIVELY TRANSFERRED ANTISERUM

One of the classical methods of determining whether circulating antibodies can protect an animal from disease is to transfer serum from an animal which has recovered from the infection into another which has had no experience of the organism, and then compare the results obtained from these animals upon infection with those obtained from suitable controls. In some instances there has been clear evidence that passive immunity can be conferred to a susceptible host by the administration of hyperimmune serum from an animal infected with helminths. Chandler (1938) and Sarles (1939) first demonstrated this for a helminth infection and both used *N. brasiliensis* infections in rats for their experiments. Culbertson (1942) was successful in passively transferring immunity to *T. spiralis* in rats and Sadun (1949) did the same for *Ascaridia galli* infections in chickens.

Numerous investigations have attempted to define the effect of circulating antibody on *Ascaris* infections. Oliver-Gonzalez (1941) found that immune serum had a parasiticidal effect on *Ascaris* larvae and Taffs (1961a) was able to transfer immunity passively from one guinea-pig to another. Crandall (1965) attempted similar transfers between mice but succeeded in reducing the worm burden in only one out of three experiments. However, the results she obtained in experiments with mice which had undergone parabiotic union were more uniform. The level of infection was significantly lower in susceptible mice which had been joined parabiotically to resistant mice and this confirmed that there was an immunological basis for resistance to the worms. Kelley and Nayak (1965) working on *A. suum* were able passively to immunize piglets against the migrating larvae with immune serum and showed that the globulin fraction imparts a resistance comparable to the whole serum.

Wilson (1966) also used immunoglobulins to confer strong immunity to *Dictyocaulus viviparus* in guinea-pigs, although Jarrett *et al.* (1955) achieved only limited success with immune serum injected into calves. These results follow a recognizable pattern because it is well known that helminths in unusual hosts are much more susceptible to all forms of attack. Miller (1967) found that it was possible passively to protect dogs against hookworm infection and Hamilton (1968) obtained similar results for cats infected with the lungworm *Aelurostrongylus abstrusus.* In both cases the amount of serum injected was massive, the pups in Miller's experiment receiving twice the volume of their circulating blood. Thorson (1954) showed that the protective capacity of hyperimmune serum taken from rats infected with *N. brasiliensis* could be removed by absorption with the secretion and excretion products of the larvae but not by absorption with larval tissues.

In all the infections mentioned above the nematodes invade the body of the host during their life cycle and there is little evidence as yet that passive immunity can be conferred in infections which concern only the alimentary canal. One possible exception was reported by Luisenko (1956), who found that some degree of passive protection could be obtained against *H. contortus* infections in sheep. Filmer and McClure (1951) demonstrated that circulating antibodies to *Trichostrongylus* spp. infections in sheep could be detected in the colostrum of ewes and subsequently appeared in the circulation of their lambs, but that protection was not transmitted to the lambs. Turner (1959) also failed to transfer passive immunity to *Strongyloides papillosus* in lambs although the same serum killed the larvae *in vitro*. The implication would appear to be that humoral antibodies can have a protective effect only on those infections which have a parenteral stage in their life cycle, and that helminths which are confined to the gut are acted upon by resistance phenomena which are not mediated, at least in a direct way, by the circulatory system.

C. THE "SELF-CURE" PHENOMENON

This term was first used by Stoll (1929a, b) to designate a phenomenon found in lambs infected with *H. contortus.* When the lambs were subjected to reinfection, the numbers of eggs passed in the faeces fell precipitously and at *post mortem* examination nearly all the worms were found to have been eliminated. Thereafter the lambs became refractory to reinfection.

This phenomenon occurs in a number of host-parasite relationships including *Trichostrongylus* spp. infections in rabbits (Michel, 1952), *D. viviparus* infections in cattle (Wetzel, 1948) and *N. brasiliensis* infections in rats (Mulligan *et al.*, 1965). Stewart (1950) showed that this reaction was associated with changes in the complement fixing antibody titres in sheep infected with *H. contortus* and undergoing "self-cure". He found that as the egg counts fell the antibody titre rose and as the egg counts rose the antibody titre dropped. This was most noticeable when individual animals were studied. Taffs (1964a) also found a similar correlation between antibody titre and faecal egg count in an investigation on *A. suum* infections in pigs. In seven out of ten cases there was an inverse relationship between the level of circulating antibodies as

judged by the conglutinating complement absorption test and the faecal egg count. Adult worms were also eliminated after the administration of a second infection of *Ascaris* eggs.

Further work on the serology of this phenomenon has been carried out by Soulsby and Stewart (1960), who showed that strong precipitating antibodies occur at the time of "self-cure" and considered that the third moult of the *H. contortus* larvae was mainly responsible for this stimulation. The exsheathing fluid produced a strong specific precipitating line in the agar double diffusion test with antisera produced by the animals at this time. This line of research was continued (Soulsby, 1960a) and a correlation was found between the loss of immunity in sheep after undergoing "self-cure" to *H. contortus* infections and the disappearance of precipitins from the antisera. It was shown, however, (Stewart, 1950) that the "self-cure" reaction is not strictly specific. In an experiment involving *Trichostrongylus* spp. infections in lambs it was found that an *H. contortus* infection superimposed on the existing one had the effect of eliminating both populations although the reverse could not be demonstrated.

Several studies have been undertaken to examine the role of circulating antibodies in the mechanism of "self-cure". Stewart (1955) found that the administration of a second dose of infective larvae was not essential, as he was able to induce the phenomenon by injecting exsheathing fluid into the abomasa of infected sheep. It seems that the antibodies in the sheep did not act directly on the worms and were more likely to induce expulsion of the infection by a local anaphylaxis in the mucosa induced by the combination of the antigens of the exsheathing fluid and their specific antibodies. This is in agreement with the results of his earlier work (Stewart, 1953) in which the "self-cure" reaction could be suppressed by the administration of antihistamine drugs.

Mulligan *et al.* (1965) produced evidence in favour of this hypothesis in their studies of *N. brasiliensis* infections in the rat. They could not demonstrate any deleterious effect of immune serum on the adult worms *in vitro* but when adult worms were transferred into previously uninfected rats which were treated with immune serum, there was a significant reduction in the numbers of worms recovered compared with the numbers found in the control rats which received normal serum. It would seem, therefore, that humoral antibodies do not promote "self-cure" directly. However, they are involved in reducing the suitability of the environment of the worms and in this way are responsible for their elimination.

D. IMMUNOLOGICAL ATTACK DIRECTED AGAINST PARASITIC ENZYMES

In considering the relative immunological importance of secretion and excretion products and somatic antigens it is tempting to wonder whether the former may contain enzymes which the parasite uses either in digestion and metabolism or, in the case of invasive larvae, in penetrating the hosts' tissues. In these hosts the antiserum may have a protective function due to the presence of anti-enzyme antibodies, a mechanism first proposed by Chandler (1935)

and elaborated by Chandler (1953) and Thorson (1953; 1956a, b). In his work with *Ancylostoma caninum*, Thorson (1956a) showed that oesophageal extracts of the adult worm displayed proteolytic activity, which could be inhibited by antiserum from immune dogs but not by normal serum. Some protection could also be induced by injecting this oesophageal fraction into uninfected dogs, because fewer and smaller worms were recovered from vaccinated dogs as compared with the controls (Thorson, 1956b). As this worm sucks the host's blood this is a particularly apt system for an investigation into the influence of enzymes in stimulating protective antibodies. Access of circulating antibodies to the worm in this case is relatively straightforward and could directly impair the viability of the parasite.

Trichinella spiralis is another worm which makes good contact with the host's tissues and thus is vulnerable to humoral antibodies. Dusanic (1966) has shown that the enzyme lactic dehydrogenase is secreted by the larvae through the oral pore and stimulates the production of specific antibodies in the rabbit.

Little is known about enzymes which might help the parasite in its invasion of the host tissue and consequently even less is known about the antibodies which these might stimulate. Although an inhibitor to the collagenase type of enzymes found in *S. mansoni* has been demonstrated in the sera of infected individuals and increases in concentration with the development of the infection, Lewert *et al.* (1959) have shown that it is a component of normal human serum but, unable to obtain increases in its concentration in infected laboratory animals, concluded that it was probably a nonspecific substance whose titre increased at the time of tissue damage.

The effect of immune serum on metabolic activity is also virtually unknown. Schwabe (1957) found that immune serum inhibited the respiration of the free-living third stage of *N. brasiliensis* but had little effect on the parasitic third stage. Dobson (1967) also found a direct effect on the oxygen uptake of the third stage larvae of *O. columbianum* in the presence of specific antibodies. Crandall and Arean (1964) studied the effect of host resistance on *A. suum* larvae implanted in diffusion chambers in mice; although no parasiticidal effect was detected, only the larvae which were in chambers in normal mice grew. This inhibition of growth in immune mice was probably due to antibody action rather than non-specific changes produced by the infection, because there was no inhibition in the growth of larvae implanted in diffusion chambers in mice immunized against *T. spiralis*. When they removed the inhibited *Ascaris* larvae from the immune host, growth was resumed in non-immune mice, perhaps an indication that the immunological attack was directed against the nutrition of the parasite and might involve anti-enzymes.

Several workers have been engaged recently on elucidating the enzymic structure of helminths. Bogitsh demonstrated esterases and an alkaline phosphatase in *Posthodiplostomum minimum* (1966a, b) and in *Hymenolepis* cysticercoids (1967). Fripp (1967) made a similar study on three species of *Schistosoma* and showed differences between the mammalian and parasitic enzymes. Thorpe (1968) made a comparative study of the enzyme histochemistry of immature and adult *Fasciola hepatica*.

As more is learnt about the type and location of these enzymes in the parasite and the sequence in which they are formed in the life cycle, their effect on the immunological status of the host will become better understood. The differences found between host and parasitic enzymes suggest that an immunological attack at this point might be rewarding.

VI. Antigens

One of the major difficulties involved in obtaining significant results from the antibodies produced in animals infected with helminths is the complex nature of these parasites which often require intermediate hosts during their life cycle. The development of helminths from the embryonic and larval stages to the sexually mature forms must include many biochemical and physiological changes, all of which makes the antigenic mosaic more and more complex.

At best, our knowledge of the biochemistry of these parasites is fragmentary and the part which the various enzymes and metabolic products of the worms play in stimulating immunity is almost unknown. However, a start has been made on this problem, and in early studies by Melcher and Campbell (1942) and Melcher (1943) two polysaccharides from *T. spiralis* were isolated which gave specific reactions with sera obtained from experimentally infected rabbits. More recently Dusanic (1966) investigated the occurrence of antibodies to the enzyme lactic dehydrogenase in *T. spiralis*; the worm secreted the enzyme during the infection and it stimulated antibody production. Dusanic also demonstrated the presence of lactic dehydrogenase in the oral precipitates found on larvae when immersed in immune serum.

Much of the serological work has been done with crude extracts of adult worms or larvae and this has led to a lack of preciseness in the results. Many workers have shown that numerous antigens can be demonstrated using such macerates and antisera in the double diffusion precipitin test. Chordi and Kagan (1965) found 19 antigenic components in sheep hydatid fluid, Damian (1966) 11 bands in an analysis of *S. mansoni*, and Tanner and Gregory (1961) 11 in a system using partially purified antigens from *T. spiralis* larvae and immune serum. It seems likely that the limiting factor is not the number of antigens available in these crude extracts but the ability of the immunized animal to respond to the spectrum offered and the sensitivity of the precipitin test.

For some years now attempts have been made to fractionate the crude antigenic material in order to reduce its antigenic complexity and if possible to isolate the active portions. This work has been hampered by the unstable nature of many of the antigenic components, but recently it has been greatly stimulated by a number of biochemical techniques which have enabled the antigens to be handled without denaturation and which have produced fractions of the soluble extracts in reasonable quantities.

One of the most useful of these techniques, introduced by Sober *et al.* (1956), is column chromatography with cellulose derivatives or the more easily handled cross-linked dextran materials which are sold under the trade name "Sephadex". These have been used to separate soluble antigens by their molecular size or by their ionic charges. Kent (1960) was among the first to

adopt this technique for use with helminthic antigens, and chromatography yielded six protein groups from an *A. suum* extract. Bénex (1967) obtained four fractions from *F. hepatica* on the basis of molecular size alone and Kronman (1965) recovered nine antigens from *S. mansoni* cercariae, one of which appeared to be completely specific for the antibodies found in infected persons. Baisden and Tromba (1963) working on the swine kidney worm, *Stephanurus dentatus*, also obtained nine fractions using DEAE-cellulose chromatography columns. However, the separation was poor and many of their fractions contained common antigens. Norman and Kagan (1966) also demonstrated the limitations of the technique, being unable to separate host and parasite antigens from sheep hydatid fluid either by molecular size or by ion exchange chromatography. On the other hand Wide and Porath (1966) have increased the scope of the method by coupling antibodies onto sephadex and using the conjugate to inhibit antigen solutions and titrate enzyme concentrations. Gel filtration on Sephadex has also been used (Hogarth-Scott, 1967) to estimate the molecular weight of allergens from a number of nematodes.

Other fractionation procedures have been used. Geyer (1967) subjected *F. hepatica* extracts to electrophoresis in polyacrylamide gel and with differential staining demonstrated 14 protein, 10 lipoprotein and 6 glycoprotein fractions. Although this method cannot fractionate large quantities of material, it seems to effect better separation than column chromatography. Dusanic (1967) has also used polyacrylamide gels to show a difference in mobility between the host and parasitic forms of the enzyme lactic dehydrogenase.

Very little use has yet been made of enzymes to characterize helminthic antigens, although the commoner proteases such as pepsin and trypsin have been employed in the separation of antigens of greater specificity in immunodiagnosis. Tanner (1963), however, studied the enzymatic degradation of a major precipitating antigen of *T. spiralis* larvae and concluded that it was mucoprotein in character.

Baisden and Tromba (1967) used zone and barrier electrophoresis to separate the antigens of the swine kidney worm. Barrier electrophoresis gave poor separation of antigens and none of the fractions was specific for the antibodies produced by the pig in response to infection. Zone electrophoresis in agar gel produced much better results in this respect but could only fractionate small quantities of material.

It is a commonly held view that somatic antigens are not as important in stimulating immunity in the host as are the secretion and excretion products. The latter include the exsheathing fluids liberated by the larvae at the time of moulting. Although this exsheathing fluid has been shown to be a powerful antigen (Soulsby and Stewart, 1960) and may be implicated in stimulating resistance, little is known about the content or structure of its antigens.

Similarly, helminths have been kept in physiological solutions and maintenance media and these have been shown to contain antigenic material derived from the worms. However, they are almost certainly a mixture of breakdown products from moribund worms, secretions and excretions. This situation is unlikely to improve until it is possible to culture the worms throughout their

complete life cycle. Then the antigenic material from the medium can be used with the assumption that it approximates to the products liberated by the helminths in the host.

VII. The Interaction of Helminth Antigens and Immune Mechanisms of the Host

Several workers have shown that different stages of the worms can elicit different types of antibodies in the host which are to a degree stage-specific, but it is also known that for some helminths, at least, one stage is as efficient as another in stimulating resistance. Michel and Sinclair (1963) found no difference in the protection produced in groups of lambs subjected to only the third stage of *Dictyocaulus filaria* larvae, transplanted worms or an infection which was allowed to develop naturally. The precipitins stimulated in these animals were qualitatively similar. The amount of antibody produced by these groups varied but this was probably a reflection of the amount of antigenic stimulation which the lambs received (unpublished results). These results were partly borne out by Ogilvie (1965), who showed that immunity produced by adult *N. brasiliensis* was capable of inhibiting the development of larvae. However, she also found that females were much more effective than either males or immature worms in stimulating resistance. From these results it would appear that it is not just a question of biomass, as the difference in weight between males and females is too slight to produce the results obtained.

Other factors apart from the application of the correct antigen are also important in the stimulation of resistance, e.g. the age at which the animals first become infected can influence the degree to which the host responds. Although it is now realized that the foetus is capable of producing an immune response, the effectiveness of this during the prenatal and neonatal periods is much below that of a mature animal. Silverstein *et al.* (1963) have shown that the immunity produced in foetal lambs varies markedly towards different antigens, indicating a gradual and selective acquisition of immunological competence. It has also been found that the mechanism of immunity is much more easily inactivated by large quantities of antigen during these early stages than later in life. This specific suppression of the immune response to the particular antigen under study is referred to as immunological tolerance (Smith, 1961).

Kassai and Aitken (1967) have produced a form of immunological tolerance to *N. brasiliensis* in young rats. If the rats were infected before four weeks old the infection persisted much longer than a comparable infection in adult rats and the young rats were not resistant to a subsequent infection. An unsuccessful attempt to produce tolerance with this rat-*Nippostrongylus* system was reported by Brambell (1964), who used very few larvae to promote the phenomenon, which may explain his failure.

Dresser and Mitchison (1968) reviewed the literature on immune tolerance and showed that for some antigens there are three levels which, when injected into the host, produce radically different responses. At a very low and very high level the antigen produces immune tolerance, while at intermediate levels the antigen stimulates antibody formation. If these results, obtained for single,

relatively simple proteins, can be used as a guide to interpret the situation for a helminth infection in which the host is subjected to a changing spectrum of complex antigens, then it is just possible to think of Kassai and Aitken's results as being due to tolerance induced by a high level of antigen. Brambell's failure to induce tolerance with a smaller dose would also fit in this scheme.

This field is of great importance in elucidating the relationship between the parasite and its host, and it is to be hoped that much more will be undertaken in this direction. It would be particularly interesting to follow the production of antibodies in these immunologically unresponsive animals, as it may well be that they are stimulated to some helminthic antigens but not others. This would be in line with Dresser's (1962) work on bovine gamma globulins in which he found that although he had induced tolerance to the main component in mice, an impurity administered at the same time stimulated the production of antibodies.

There have not been many investigations into this aspect of parasitism, but Soulsby (1963a) demonstrated some effect when *Cysticercus bovis* and *Mecistocirrus digitatus* were given to young calves. When calves were infected with either species a few days after birth, little or no antibody was detected by the complement fixation or conglutinin absorption tests, the infection persisted and they were unable to resist a challenge infection some months later. Older animals infected in the same manner produced good antibody titres and became resistant to reinfection. Unfortunately, this work did not demonstrate clearly what differences existed between the unresponsive animals and normal, as opposed to immune, animals. Moriarty (1967) produced immunological unresponsiveness to *Echinococcus granulosus* cyst fluid when this antigen was injected into neonatal rats. These animals had antigen elimination rates which were much longer than those found in immune rats or normal rats.

Whether it is possible to induce tolerance by very small infections of worms is not known at present. Dineen and his co-workers in a series of papers (Dineen, 1963; Donald *et al.*, 1963) have developed a theory that there must be a minimum level of parasitism before the host's immune defence mechanism can be mobilized. Above this level the viability of a further infection is impaired. They suggest that during the course of the host-parasite relationship the immune responses of the host may have acted as a selective pressure on the parasite and favoured the genetic variants of the worm which had a reduced antigenic disparity with the host. The result of this would be that as the host-parasite relationship evolves, appreciable parasitic burdens may be tolerated by the host below the threshold level of antigenic information required for stimulating immunological control. They have shown that this theory holds for *Nematodirus spathiger* infections (Dineen *et al.*, 1965a) and *H. contortus* infections (Dineen *et al.*, 1965b).

Dineen and Wagland (1966) found that the presence of small numbers of *H. contortus* interferes with the resistance of sheep to reinfection, compared with sheep in which the primary infection had been terminated with anthelmintics. They cite this as a case of immunological paralysis or tolerance. However, the unresponsive sheep had at least one manifestation of resistance, because a much higher percentage of the challenge infection became inhibited

in these sheep compared with the percentage of inhibited forms found in control animals which received a primary infection from the same culture of larvae. This is the first suggestion in their work that an infection providing an antigenic stimulus below the threshold value for stimulation of resistance, enables the animal to accept a larger infection than a normal animal can.

Thus, there might be a neutral zone in the effects produced by graded amounts of antigen. First a state of tolerance set up by very small amounts of antigen. Then a range below the amount of antigen required to stimulate antibodies where resistance phenomena are not active. Above this threshold the immunological mechanisms are functional and finally a situation where the immunological system is swamped by an excess of antigen and tolerance is once more evident.

Although this theory is mainly speculative, there is some evidence to suggest that helminths are able to adapt the spectrum of antigens which they present to the host in such a way that the antigenic disparity is reduced. This has probably been effected over an evolutionary period by many helminths. A possible example of this is the microfilaria, *Dirofilaria repens*, which does not normally stimulate resistance in dogs even in an infection which lasts many months. Mantovani and Sulzer (1967) found that the antibodies which were produced reacted only with the internal components of the microfilariae and not with the external antigens. However, if large numbers of microfilaria were given in several injections to dogs, circulating antibodies were produced which destroyed the helminths and could also be shown to agglutinate them *in vitro* (Wong, 1964). This serum had also a therapeutic effect on homologous microfilaria infections. From this it seems that only in exceptional circumstances was there enough antigen available for the host to produce protective antibodies.

Smithers and Terry (1967) have investigated the survival of *S. mansoni* adult worms in rhesus monkeys after transfer from mouse, hamster and monkey donors. Worms transferred from monkeys established themselves readily while most of the worms taken from hamsters were killed and those from mice suffered a severe check before adapting themselves to their new host. The hypothesis is put forward that the worms adapted themselves to the host by antigenic mimicry. Thus when the adults were transferred to a new host of a different species, the original host's antigens carried by the parasite were recognized as foreign and the worms were attacked by the immune mechanisms of the new host. Smithers (1968) provided evidence for this by showing that monkeys, immunized against mouse tissue antigens, eliminated worms transferred from mice. It is not known how the host antigens are incorporated into the parasite.

The state of our knowledge is not sufficient to draw definite conclusions from this work but some tentative hypotheses can be advanced on the effect of worm antigens in stimulating antibody production. It seems that various amounts of antigen can produce diametrically opposed effects which are to some extent dependent on the age of the animal. The parasite has also evolved mechanisms for circumventing the immune responses of the host by decreasing its antigenic disparity to the host's tissue.

VIII. Vaccination

Any treatment which affects the natural course of events in the stimulation of resistance to helminths gives an opportunity for studying the effect such a change in conditions has on the production of humoral antibodies. By comparing the results in the natural and the artificial situation it should be possible to increase our knowledge of the mechanism of immunity and the part played by circulating antibodies.

In the field of vaccination parasitologists have been able to follow the lead of the bacteriologists who have worked out the forms which this may take. Vaccines may be classified into the groups termed live; naturally and artificially attenuated; or they may be extracts or metabolites of the infective organism.

A. Live Vaccines

While infective organisms are the least satisfactory type of vaccine in bacteriology because the reproductive potential of bacteria makes it extremely difficult to give a dose which is large enough to promote immunity without causing disease, this factor does not apply to helminths and in some cases good immunity can be stimulated in this way. Administration of small, controlled doses of *A. lumbricoides* larvae by Sprent and Chen (1949) and Oliver-Gonzalez (1956) produced good results. Rubin and Lucker (1956) and Jarrett *et al.* (1959) were also successful in stimulating resistance to *D. viviparus* in this way.

Vaccines employing related but less pathogenic species of worms have also been tried, but the results have been disappointing. Chandler (1932) found that a previous infection of *Longistriata adunca* conferred little resistance to rats challenged with *N. brasiliensis*. There was no significant difference between the controls and the treated group in the number of adult worms recovered although the egg production of those worms in the treated group was only one-tenth of that found in the controls. More encouraging results have been obtained with two species of *Hymenolepis* in rats and mice by Heyneman (1962), who found a high degree of cross-immunity. A measure of success was also obtained by Parfitt and Sinclair (1967) in stimulating resistance to *D. viviparus* in calves by a previous infection of *D. filaria*. It is interesting to note that although the establishment of *D. viviparus* in these calves was markedly reduced by the prior cross-infection, there appeared to be very little cross-reaction between the circulating antibodies stimulated by these two species. A similar effect was observed in guinea-pigs when the same system of infections with *D. filaria* and *D. viviparus* was used (Sinclair, 1967).

B. Attenuated Vaccines

By far the most practicable demonstration of immunological control of a nematode disease is based on the X-irradiated larval vaccine developed by Jarrett *et al.* (1958) to combat parasitic bronchitis in cattle. This vaccine has been produced commercially and is now widely used. Michel and MacKenzie and their collaborators (1965) investigated the occurrence of circulating antibodies in calves treated with this vaccine to *D. viviparus* and compared

them with those occurring in calves infected with normal larvae. Although they could not correlate these with any of the manifestations of resistance which they found in the calves, there were gross differences in the quantity of complement-fixing and precipitating antibodies produced by the two groups. The animals which received the X-irradiated larvae had much less circulating antibody and were also less resistant to a challenge infection than those which received the normal larvae. However, they were capable of producing an anamnestic response which was not markedly different from that which occurred in the animals infected with normal larvae.

Experimental work has also been reported on other X-irradiated vaccines, and good protection has been stimulated to *H. contortus* and *Trichostrongylus colubriformis* infections (Mulligan *et al.*, 1961) and in the dog to hookworm infections (Miller, 1966). Miller found that X-irradiated larvae were superior to normal larvae in stimulating resistance to challenge and suggested that this was due to the pathogenic effect of the infective larvae on the pups.

It is well established that irradiation of infective larvae can disrupt their development without removing their ability to stimulate resistance, but several workers have noted that it is not always possible to obtain reproducible results (Lucker and Vegors, 1960). A better understanding of the immunology of the disease might well throw some light on this problem, and certainly much more must be ascertained about the antibodies which are stimulated under these conditions.

C. VACCINES PREPARED FROM *in vitro* CULTURE OF HELMINTHS

The potentialities of *in vitro* culture of helminths are now well recognized, and although culture of worms through their entire life cycle has been attained for very few of the parasitic helminths, the method has made available collections of antigens elaborated at various stages of their development. Helminths are not known to produce toxins which elicit the formation of antibodies comparable to those encountered in some bacterial diseases, but materials such as exsheathing fluid, described by Sommerville (1957) and Rogers and Sommerville (1957, 1960), may be important as antigens. At least three antigens were shown to be present in the exsheathing fluid of *H. contortus* and they were demonstrated by the double diffusion precipitin test. Soulsby (1960a) showed that the precipitins formed in sheep to the exsheathing fluid of *H. contortus* disappeared after the "self-cure" phenomenon and considered that these precipitins were implicated in the immunological expulsion of the worms.

Horchner (1968) was unable to find in sheep infected with *Chabertia ovina* any precipitins to exsheathing fluid in the sera of these sheep. However, he was able to show that when sheep were injected with worms of different stages and with secretion and excretion products from these worms, the antibodies stimulated by these treatments were to a great extent specific for the stage of worm administered.

Thorson (1953, 1954) working with *N. brasiliensis* in rats found that the secretion and excretion products stimulated some protection and that conversely the antibodies in immune sera could be removed by these products but

not by larval tissues. Similar results using products of *T. spiralis* have been reported by Campbell (1955) and Chute (1956).

Silverman *et al.* (1962) obtained a high degree of protection in laboratory animals to *D. viviparus*, *T. colubriformis* and *S. papillosus* infections using antigens prepared from *in vitro* cultures of infective larvae which had reached the fourth stage of development. Large doses of this *H. contortus* and *T. colubriformis* antigen were effective also in protecting guinea-pigs against infections of *D. viviparus*, although only homologous antigens could confer resistance to *T. colubriformis* in these animals. These observations are encouraging but it is of course hazardous to extrapolate results obtained in small animals to include infections in the natural hosts of these nematodes. Silverman (1965a) extended this work to include *H. contortus* infections in lambs. Using vaccines prepared from fourth and early fifth stage larval cultures he achieved a measure of protection, but with third stage larvae and adult worms he could not demonstrate any effect. This may be a particularly useful observation, because Urquhart *et al.* (1963) found that X-irradiated *H. contortus* larvae failed to protect young lambs, although this vaccine induced good resistance in older animals.

The use of *in vitro* culture to produce antigens which can stimulate immunity appears to be a promising line of research, even if progress has not been as swift as might have been predicted a decade ago, following the original breakthrough in axenic culture (Weinstein, 1958). Several factors have probably influenced this, the most important being the difficulty experienced in culturing the complete life-cycle of the parasite and the use in culture media of undefined biological materials which interfere in analyses of the secretion and excretion products of the parasites.

D. VACCINES EMPLOYING WHOLE WORM MACERATES

Little success has been obtained in vaccinating animals with dead helminthic material. The antigens can be very potent and will stimulate large amounts of circulating antibody, but resistance to infection with live helminths is not usually produced even when these antibodies seem to be identical with those found in a resistant host following natural infection. Thus Crandall and Arean (1965) were unable to demonstrate any protective effect of non-viable *A. suum* larval and egg preparations in mice, although some immunity was stimulated when living material was used. Similarly Smithers and Terry (1967) found that *S. mansoni* adult worms, killed immediately before being introduced into the mesenteric veins of the host, conferred no resistance to the recipients.

To explain these failures it has been postulated that the antigens which induce protective immunity are produced only in small amounts by the living parasite and that when the host is presented with antigens prepared from dead helminths they are either destroyed in the preparation of the extract or are concealed by the large amount of nonfunctional foreign material made available to the host's reticuloendothelial system. However, the method is not always completely unsuccessful and Coleman *et al.* (1968) found that an *H. nana* homogenate, when injected into mice confers a strong immunity; one-half of

the mice which were immunized resisted the challenge infection completely, while the rest harboured only 1 % of the infection found in the non-immunized mice.

Wade *et al.* (1961) halved the number of *D. viviparus* worms established in guinea-pigs by injections of lyophilized worms and third stage larvae, and had even better results when rabbits were used as experimental hosts. In this case, however, the infection was not induced in the normal host and the balance of the host-parasite relationship was very much in favour of the host. Under these circumstances small shifts in the balance will be magnified greatly.

IX. Immunodiagnosis

In many cases helminth diseases can be diagnosed most easily by clinical observation but immunodiagnosis has been used in confirmation. There are, however, instances such as hydatid disease and visceral larva migrans, in which the parasite is difficult to demonstrate or where the parasite has only a short reproductive period in the host, as in most cases of dictyocaulosis in cattle. It is under these circumstances that immunodiagnosis has great potential value. In theory there is little difficulty in the demonstration in the host's serum of specific antibodies to a particular helminth, and much research has examined various techniques which might be used for diagnosis, but not as yet with outstanding success. Many serological tests have been devised in attempts to produce reliable and specific diagnoses, and these can conveniently be divided into those which use intact parasites and those which employ soluble antigens.

A. Tests which employ whole organisms

The number of tests using intact organisms is relatively small compared with the multiplicity of tests using soluble antigens. There are only five of major interest, and all are used in the diagnosis of schistosomiasis. None of the tests seem to give significantly better results than the others and the selection of the test depends on the availability of the stage in the life cycle which is required for the test and the predeliction of the laboratory concerned.

The miracidial immobilization test was introduced by Senterfit (1953), who found that heat-inactivated sera from animals infected with *S. mansoni* immobilized the miracidia while normal sera did not. Kagan (1955) extended this investigation using various laboratory animals as hosts, confirmed the previous results and also found that this test was more sensitive than the cercarienhüllen-reaction, in which the membrane of the cercariae swells when placed in immune serum. In another investigation of the cercarienhüllen reaction, Pifano and Ron Pedrigues (1957) found that 78 % of sera from a collection of 1330 taken from infected animals gave a positive reaction. This is the most extensive study on this test which has been carried out so far and it has demonstrated a discouraging number of false negatives.

Another test using cercariae has been described by Liu and Bang (1950). Here heated sera from infected monkeys was found to agglutinate the cercariae. Several workers (Anderson and Naimark, 1960; Jachowski and Anderson, 1961) have demonstrated this as a sensitive test, giving only a small percentage of

false positives and negatives. The interpretation of the results, as in most agglutination tests, presented some difficulty. Naimark *et al.* (1957) found that this test demonstrated antibodies in the sera of monkeys earlier in the infection than either the complement fixation test or the circumoval precipitin test, which is a method of detecting antibodies by their ability to form precipitates round living *S. mansoni* eggs. Oliver-Gonzalez (1954) showed that no precipitates were formed round the eggs in normal serum and by absorption techniques found that it was a true antibody–antigen reaction. In a further study of this reaction, Oliver-Gonzalez *et al.* (1955a) showed that the test is specific for three species of *Schistosoma*, except for a slight cross-reaction between *S. mansoni* eggs and *S. japonicum* and *S. haematobium* antisera. Although the specificity of this test is good, apart from cross-reactions which occur with the sera of patients with trichinosis, the sensitivity is not as great as that obtained with tests using soluble antigens or the cercarial agglutination test (Jachowski and Anderson, 1961). This lack of sensitivity may be due, in part, to the stage specificity of the antibodies detected by this test. Oliver-Gonzalez (1954) could not absorb them with either adult or cercarial antigens and showed that the titre was greatest in the chronic, egg producing stage of the infection. Oliver-Gonzalez *et al.* (1955b) found that there was a rapid decrease in titre of this antibody when the infection was terminated and considered that the test may well have a use in determining the state of the infection.

The fluorescent antibody test has an advantage over the others in that formalin-fixed miracidia or cercariae may be used as well as fresh material, if the larvae are treated with bovine albumin to block non-specific absorption before they are treated with immune serum (Anderson *et al.*, 1961). The procedure for the test is simple. The organisms are incubated with the immune serum, washed and then exposed to fluorescein-labelled antiglobulin. Fluorescence is observed only on the larvae treated with immune serum and not on those treated with normal serum. The test is about as sensitive and specific as the cercarial agglutination test (Sadun *et al.*, 1961) but it shows the usual cross-reaction with the sera of patients suffering from trichinosis (Sadun *et al.*, 1960). These authors showed that a spot of dried blood yields sufficient antibody to carry out this test, which is the most flexible technique available at present. Selka (1967), using a modification of the fluorescent antibody test, investigated *Schistosome* infections in mice. Two-thirds of these mice produced antibody titres higher than 1/10240, and the titres were also higher in mice with chronic rather than acute infections. There was also a correlation between the titre and the number of eggs retained in the intestines of the mice.

This test has been used successfully for the diagnosis of human trichinosis (Sadun *et al.*, 1962) and a high degree of specificity has been claimed for it. Promising results have also been obtained by Sulzer and Chisholm (1966), who tried this method on pigs artificially infected with *T. spiralis*.

Some exploratory work by Hogarth-Scott (1966) has been done on visceral larva migrans using fluorescent antibodies. Precipitates of a specifically antibody nature were demonstrated at the orifices of the second stage larvae of *T. canis* and *T. cati* when placed in the sera taken from rabbits infected with these nematodes. However, cross-reactions were found between those two

species. Comparable results were obtained from human sera taken from suspect cases of visceral larva migrans.

B. TESTS WHICH EMPLOY SOLUBLE ANTIGENS

There are many tests which use soluble antigens and when account is taken of the different types of antigen which can be prepared in various ways the range is almost unlimited. The proliferation of these tests is partly a measure of the interest in this field and also unfortunately reflects the difficulties encountered in producing reliable results. It is possible here merely to skim over the surface of the problem, and for fuller information the reader is advised to consult the reviews of Soulsby (1963b), Thorson (1963) and Kent (1963a).

1. *The complement fixation test*

This test has had a long and useful life in helminth serology from the time of Ghedini (1906), who used the contents of hydatid cysts as a diagnostic antigen. Stewart (1948) demonstrated antibodies to *H. contortus* and *Trichostrongylus* spp. infections in sheep, using a boiled antigen that became a standard for several years. However, this polysaccharide antigen has limited specificity for nematode diseases (Sinclair, 1964). Fernando (1968a) has shown a strong cross-reaction between the heterophile antibodies found in normal rabbit serum and antibodies produced in the rabbit against *T. canis* infections. Using the complement fixation test he observed that the heterophile antibody titre rose markedly when the rabbits were infected on the first and second occasion. These antibodies resembled those which react with the Forssman antigen and this could account for their lack of specificity, as the antigen is found in many animals. In a further examination of the antibodies produced in rabbits to *T. canis* infections, Fernando (1968b) compared the efficacy of the complement fixing and precipitin tests and although there was an overall similarity in the results, the boiled antigen used in the complement fixation test lacked at least two antigenic components which appeared in the unheated macerate.

Much effort has been expended on improving the antigen used in the test. Witebsky *et al.* (1942) reduced the nonspecificity of *T. spiralis* larval antigen by using a boiled alkaline extract. However, components of this antigen were shown to occur in several members of the *Salmonella* family (Weiner and Price, 1956), and Sleeman (1961) and Sleeman and Muschel (1961) have used a more refined, chemical fractionation procedure to isolate two antigenic components, a glycoprotein and a nucleoprotein, both of which occurred in Witebsky's antigen. The glycoprotein was highly specific for antibodies to *T. spiralis* but they appeared later and disappeared sooner than the antibodies to the nucleoprotein. The nucleoprotein antigen reacted with antibodies to several other diseases including schistosomiasis, syphilis and ancylostomiasis.

An extensive trial by Jezyna *et al.* (1967) on 128 patients suffering from trichinosis gave results found to be dependant on the severity of the infection; 94% of chronic cases gave a positive complement fixation reaction, 93% of severe cases, 76% of moderate cases, and 69% of mild cases. The authors consider the test to be highly sensitive and specific in the later stages of the infection.

The test was also used in the diagnosis of fascioliasis in sheep by Bénex *et al.* (1959), who used a saline extract and showed that whilst a negative result reliably excluded the presence of the disease a positive reaction did not necessarily mean that the parasite was present. Positive reactions were also obtained with sera collected from animals infected with *Dicrocoelium dendriticum.* Clonorchiasis has also been investigated with a view to producing an acceptable form of serodiagnosis. Sadun *et al.* (1959) found that acid soluble protein and lipid free extracts of *C. sinensis* gave positive results with most *Chonorchis* infected persons while only 5% of trematode free individuals reacted. However, cross-reactions with other trematode infections were extensive.

The complement fixation test has not been of much use in the diagnosis of Ascaris infections. Minning and McFadzean (1956) demonstrated the appearance of these antibodies in experimentally infected animals but could not detect them in patients with the disease. Taffs demonstrated antibodies in the sera of infected pigs (1961b) and in laboratory animals (1964c), using a polysaccharide extract derived from the ovaries and uteri. He found, however, that the test was less sensitive than the conglutinating complement absorption test. Tomanek and Prochazka (1967) have indirectly demonstrated the poor degree of specificity of *Ascaris* material by using it in a complement fixation test to demonstrate antibodies to *D. viviparus* in cattle.

Much more success has been claimed for the test in the diagnosis of human cysticercosis. Nieto (1956) used an acetone–alcohol extract of *Cysticercus cellulosae* and cerebrospinal fluid from the patient and produced a specific test which has good sensitivity. This was not so for tests carried out with sera, giving several cross-reactions with antigens prepared from other helminths. The sensitivity was also reduced. The difference in the antibodies found in two sites in the same patient underlines the importance of localized antibodies in certain helminth diseases with the corollary that the serum, although the most accessible course of antibodies, does not always contain the most specific type.

The use of the complement fixation test in the diagnosis of hydatid disease has been investigated for more than 60 years (Ghedini, 1906; Vartic, 1966) but there is still no reliable method. Vartic used an ether extract of the hydatid fluid but could not obtain a positive result for 16% of the proven cases of the disease in cattle, although his antigen showed no sign of the anticomplementary activity which plagued earlier workers, perhaps another illustration of the fact that the most easily obtainable material, in this case the antigen, is not always the best. With increasing expertise in the techniques of *in vitro* culture a greatly increased range of antigens has been made available for diagnostic tests. Little has as yet been done to take advantage of this, but there is no doubt that the secretion and excretion products will increase the specificity of this and other tests. Fife *et al.* (1967) prepared an exoantigen from *S. mansoni* cercariae. Testing with homologous and heterologous antisera they showed this antigen to be suitable for serodiagnosis and apparently detected some antibodies which did not react with the somatic antigens.

Several workers have investigated possible relationships between the complement fixing antibody titre and the helminthic disease which has led to their production. Weber (1958) could find no correlation between the amount

of antibody produced and the infecting dose of *D. viviparus*, the number of larvae eliminated in the faeces of the calves or the clinical severity of the infection. Michel and Cornwell (1959) working with the same system were also unable to show a correlation between antibody titres and the ability of the calves to resist reinfection with *D. viviparus*.

However, other workers have been more successful. Soulsby (1956) found a correlation between the antibody titre and the eggs eliminated in the faeces of sheep infected with gastro-intestinal nematodes, and Ross *et al.* (1960) showed that Zebu cattle having some genetic resistance to gut nematodes had lower egg counts and higher antibody titres than a more susceptible strain of the same breed. Tanner (1968) working with experimental trichinosis in rabbits demonstrated that the antibody titre was proportional to the degree of muscle parasitism, which was the effective amount of antigenic stimulation each animal received, and did not give a positive correlation with the degree of resistance of the animal. The work was characterized by great variation in the response of the host to the parasites, both in the numbers recovered from individual rabbits and in the antibody response.

It seems therefore that the complement fixation test is unlikely to yield more than a general statement on the parasitic condition of animals and that in diagnosis the results must be examined with some reserve. The test cannot be used to determine the degree of resistance of the host to reinfection.

2. *Flocculation and agglutination tests*

There are many variations of these tests employing different carriers for the soluble antigen. Basically, any particle which can adsorb the antigen onto its surface, without altering the combining site and which is of a suitable size to demonstrate the combination of antigen and antibody, may be used. The particles may be naturally occurring minerals such as bentonite, manufactured materials such as latex, cholesterol–lecithin crystals, or erythrocytes.

Hydatid fluid antigens have given satisfactory results in the indirect haemagglutination test, and have been found by several workers (Garabedian *et al.*, 1957, 1959; Arabatzis and Papapanagiotou, 1963) to be better for diagnosis than the complement fixation test or the intradermal test. About 90% of confirmed cases gave a positive reaction, while no "false positive" reactions could be demonstrated in 105 sera from patients with other diseases (Garabedian *et al.*, 1959). Arabatzis and Papapanagiotou (1963) had 2% of "false positive" reactions but these could be distinguished from the experimental group by their lower titres. However, Pinella (1961) found that neither the haemagglutination nor the latex test was specific for sera from cattle and sheep suffering from hydatid disease. This lack of specificity might be due to the crude antigen used. Proctor and Elsdon-Dew (1966), however, did not encounter this difficulty in a study of porcine cysticercosis. They also used a crude saline extract for the indirect haemagglutination test but reported good specificity. Positive reactions were obtained for all the pigs which were condemned on slaughter, and a quarter of a similar number of lightly infected pigs gave positive reactions as did 9 out of 290 pigs thought to be uninfected. Although there are obvious

dangers inherent in results based on slaughterhouse material, the test would appear to be promising as a diagnostic tool for some cestode infections.

Little has been done to investigate the possibilities of these tests for diagnosing fascioliasis. Bénex (1964) reported on a technique using a lipid free antigen prepared from adult flukes which was adsorbed onto latex particles. This conjugate gave good agglutination with sera from infected animals.

Anderson (1960) developed a slide flocculation test for schistosomiasis using a lipid free cercarial antigen adsorbed onto cholesterol–lecithin crystals. This test was less specific than the complement fixation test, but claimed to be simpler than many serological techniques and adaptable for field work. The antigen crystal conjugate has been used in antibody adsorption studies (Anderson, 1962; Anderson *et al.*, 1963) to remove cross-reacting antibodies to *T. spiralis* from the sera of patients infected with *S. mansoni*, using a *T. spiralis* cholesterol lecithin complex, without removing the homologous reaction.

Many types of agglutination have been used in the search for a good diagnostic test for *T. spiralis*. Norman *et al.* (1956) achieved good results using a crude antigen extract adsorbed onto bentonite particles. However, Vogel *et al.* (1957) found that cholesterol lecithin particles were more specific than either the bentonite flocculation or the complement fixation test, and Price and Weiner (1956) using a haemagglutinating technique showed that it was preferable to the precipitation test, particularly as the antibodies were detected earlier in the infection. Secretion and excretion products from *T. spiralis* have been used to increase the sensitivity of the bentonite flocculation test and worked well when used with human but not pig sera (Norman and Sadun, 1959). Kampelmacher and Streefkerk (1964) using the latex slide test reported similar results with pig sera, although they found that the test was specific for the disease when sera from infected rabbits or humans were used.

Studies on *Ascaris* infections using these techniques have met with little success. Here again this is probably a measure of the lack of specificity of the antigens employed. This is all the more frustrating as the large size of worms permits antigens to be prepared from selected tissues instead of the whole worm. Oliver-Gonzalez (1943) showed that these tissues elicited different antibody responses in rabbits and were able to absorb their homologous antibodies selectively. However, Kagan *et al.* (1959) used haemagglutination and bentonite flocculation tests with purified *Ascaris* antigens and found that they were not specific, with the possible exception of the non-embryonated egg antigen. Taffs (1964b) working with *A. suum* in rabbits and guinea-pigs was more successful. Using a conglutinating complement absorption test, he detected antibodies after three infections and found evidence to suggest that the absorption titre was proportional to the degree of resistance of the animal. Herlich and Merkal (1963) have also found a relationship between the resistance of the host and the haemagglutination titre. In this case their system was *Trichostrongylus axei* infections in calves and they showed an inverse correlation between the antibody level and the faecal egg count, although there was no evidence of a protective function associated with the antibody titre as judged by the number or size of the worms recovered at necropsy.

It would seem from these investigations that the agglutination tests are on the whole a sensitive method of estimating the production of antibody in the host and have their place in field surveys such as that of Kagan and Cahill (1968) in Somaliland, where they tested for *Schistosoma haematobeum*, *E. granulosus* and filarial antibodies using a variety of agglutination techniques. However, the specificity of the antigens used in the tests is still suspect and more work is required before much reliance can be placed on these tests. There is also little evidence that the antibodies demonstrated by these techniques are closely connected with any manifestation of resistance.

3. *Precipitin tests*

Although precipitation tests have been used in helminthology to identify antibody–antigen complexes for many years (Fairley, 1923; Coventry, 1929; Rothfeld, 1935), it is only since Ouchterlony (1948) published his method for double diffusion of antibody and antigen in agar gel that the potential of this test has been realized. This form of the test is particularly useful as it enables single antibody specificities to be detected with crude antigen preparations, and it can also be used to search for specific antigens for diagnostic purposes.

Kagan and Norman (1961) and Biguet *et al.* (1962a) have used this test to show that the hydatid fluid and the scoleces of *Echinococcus* have common antigens. Chordi and Kagan (1965) found that the scoleces were antigenically poorer than the hydatid fluid, but they demonstrated that the latter contained host proteins which reduced its specificity. In this work they used the immuno-electrophoretic technique in which the antigen was placed in a well in the agar and then subjected to an electric current before the pattern of precipitin arcs was developed with antiserum.

Norman and Kagan (1966) and Norman *et al.* (1966) confirmed these results and evaluated the suitability of the antigen derived from sheep hydatid fluid in the diagnosis of the human disease. They also attempted to improve the specificity of the antigen through separation of the host and parasitic components of the hydatid fluid by column chromatography, but the precipitin test showed that their efforts were only partially successful.

Abrantes and Avila (1968) compared antigens made from scolices and hydatid fluid of *E. granulosus* using this test; the antigen made from scolices was better for diagnostic purposes than the hydatid fluid, since 78% of the sera taken from surgically proven cases of hydatid disease gave a positive result with antigen from scolices, while only 18% of the same sera were positive with the antigen from the hydatid fluid. Both antigens appeared completely specific and gave no "false positive" reactions with control sera. This encouraging result for the scolex antigen would have been even better but for the inclusion of the results obtained from cases where the cysts were restricted to the lungs. Here only a third of the cases gave a positive result. This was perhaps due to a weaker antigenic stimulus by the cyst which becomes isolated by a fibrous barrier formed by the lung.

Although there is doubt as to whether *F. hepatica* infections stimulate immunity in the host, circulating antibodies have been used in diagnostic

studies. Ichihara *et al.* produced a series of papers (1956a, b, c) in which they were able to detect fluke infections in cattle and goats with an accuracy of 90% using the precipitin test. Teodorovic *et al.* (1963) found a characteristic precipitin reaction in the slow γ-globulin region with immuno-electrophoresis. The serum was obtained from a human patient suffering from a *Fasciola* infection.

Attempts have also been made to fractionate the *F. hepatica* macerate in order to obtain antigens which do not cross-react with other helminths and which give a simplified precipitin pattern. Korach and Bénex (1966a, b) have described the production, by physico-chemical techniques, of a lipoprotein fraction which is antigenic in rabbits and which produced only one precipitin in the gel diffusion test. However, among sera from proven cases of fascioliasis in sheep and man only those displaying high titres in the complement fixation test reacted with the lipoprotein antigen. Bénex (1967) used column chromatography to fractionate a lipid free antigen and two of these fractions gave precipitin lines against rabbit antiserum.

The lack of specificity of various tests for schistosomes has prompted Damian (1967) to investigate the antigenic relationship between *S. mansoni* and the serum of one of its hosts, the albino mouse. Four common antigens were found using the Ouchterlony technique. The rabbit antiserum produced against *S. mansoni* also had an increased concentration of sheep haemolysins and this was taken as an indication of the presence of the Forssman antigen. As this antigen is known to be present in the mouse, it represents another shared antigen. Further work along these lines by Berggren and Weller (1967) demonstrated a circulating antigen in mice given massive doses of *S. mansoni*. In an immuno-electrophoretic study of this antigen they discovered that it was not host related and could be correlated with the worm burden and the time of infection.

The antigenic complexity of *T. spiralis* has been demonstrated convincingly using the precipitin technique (Woodhouse, 1956) and at least ten antigens have been resolved from a larval extract, five of which were heat labile. As many cross-reactions occur when somatic extracts are used, Mills and Kent (1965) have investigated the secretion and excretion products recovered from this worm. Precipitin tests showed that in infections of mice and rabbits the antibodies appeared to the metabolites after 30 days and reached a peak in 50 days. Beck and Anfinsont (1965) obtained similar results for the period of antibody production in infected pigs and rabbits using somatic antigens, and so the metabolic products may provide a useful diagnostic antigen.

Advances have been made in the production of specific antigens from *Ascaris* tissues since the early work of Canning (1929). Although he could differentiate between various tissues obtained from *A. lumbricoides* using rabbit antisera, the corresponding tissues of other ascarids also reacted. Attempts have been made to isolate individual antigens by chemical methods (Kagan *et al.*, 1958) and by column chromatography (Kent, 1960). In the latter investigation, six fractions were produced from a lipid free extract of *A. suum* female worms and the reactions of all of them were specific for *Ascaris* using the precipitin test. No cross-reactions were found from sera from a wide variety of other helminthic infections.

Baisden and Tromba (1967) working with the swine kidney worm *S. dentatus* separated an antigen from worm material by zone and barrier electrophoresis. This reacted specifically with sera from infected pigs while other antigenic fractions were shown to react with normal serum.

4. *Fluorescent antibody test*

Little attention has been paid to the possibilities of this test when applied to soluble antigens. However, Camargo *et al.* (1965) evolved a slide fluorescent antibody technique with adult worm antigen for the serodiagnosis of schistosomiasis. Da Silva and Ferri (1968) used this to investigate the immunity produced by *S. mansoni* homogenates in mice, and found a tendency for the test to give higher titres in the immune mice, but the results were scarcely satisfactory for the diagnosis of disease. Duxbury and Sadun (1967) also used this type of test for the diagnosis of bancroftian filariasis and onchocerciasis and reported that over 80% of confirmed cases gave a positive reaction. Few cross-reactions were observed.

The test does not seem to have much practical importance at present, because the results are dependent upon the availability of specific antigen. Along with all the other diagnostic tests, its usefulness will increase with the production of better defined antigens.

X. The Use of Antibodies in Taxonomy

Various attempts have been made to use serology as an aid to elucidating the relationship between different species of helminths. It is reasonable to suppose that different species of worms contain different antigenic determinants and that as the closeness of the taxonomic relationship decreases so will the number of common antigens, but the risks inherent in the work are manifold. Antibodies are produced to only a few of the antigens out of a wide spectrum which are presented to the animal selected to provide the antiserum, and there is no way of ascertaining whether the antigens which do stimulate antibodies can be considered as representative for that particular species of worm. Even if this were so it is perhaps doubtful whether the relationship between one species and another is directly related to the number of antigens which they have in common.

Another difficulty is the almost complete lack of knowledge about the genetics of helminths. It is widely believed that helminths are genetically stable organisms and this theory is based on the strong host specificity which many exhibit. However, the work of Dineen (1963) and others suggests that the parasite reduces its antigenic disparity with the host as much as possible. Smithers and Terry (1967) have shown that schistosomes can also adapt to a new host. This is done extremely rapidly and although it is almost certainly a phenotypic effect, an underlying genetic basis is probable. From these considerations a certain amount of genetic variation within a helminth species is well within the limits of credibility. If this is so it should follow that there is a

degree of antigenic dissimilarity within a particular species which might confuse an analysis of the antigenic relationship between species.

The difficulties are increased when closely related species or strains of the same species are examined. Taffs and Voller (1963) attempted to differentiate *A. suum* and *A. lumbricoides* by the fluorescent antibody test with antisera prepared in rabbits and monkeys, but were unable to show any serological difference. Unfortunately no absorption tests were carried out so the question remains open. Kagan (1958) using the haemagglutination test found that antisera produced against *Ascaris* cross-reacted with *Toxocara* antigens and vice versa. Inhibition techniques were found to render the antisera specific for genera but not species. Recently Jeska (1967) and Rahman (1967) have confirmed these findings. Rahman used an intragel absorption test and showed that there were several antigens specific for *A. suum* and *T. canis*. Extensive cross-reactions were also found and in one case the heterologous antigen produced more precipitin bands than the homologous antigen, leading to the unlikely conclusion that the relationship between *A. suum* adults and *T. canis* larvae is closer than between *T. canis* adults and *T. canis* larvae.

Trichinella spiralis is known to cross-react serologically with several other helminth species and this has hampered the search for a reliable diagnostic test. Ivey (1965) has shown that some of the difficulties have arisen from the selection of the serological test. He found cross-reactions between *T. spiralis* and *T. canis* using the Schultz-Dale and passive cutaneous anaphylaxis tests, but not when the haemagglutination and precipitin tests were used.

Strong evidence that in some cases antigenic cross-reactions between species are strong enough to cause cross-immunity has been presented by Parfitt and Sinclair (1967). Here an infection of *D. filaria* in cattle produced resistance to a challenge infection of *D. viviparus* and a cross-reacting precipitating antibody. Cox (1952) was able to produce some resistance to *T. spiralis* infections in mice by prior subcutaneous infection with *A. caninum*, and Louch (1962) increased resistance to *T. spiralis* in the rat following an infection with *N. brasiliensis*. In the latter case no precipitins were found when antiserum from *N. brasiliensis* infected rats were tested against *T. spiralis* larval extracts, and the protection might have been due to nonspecific inflammation of the gut.

Biguet *et al.* (1962b) have investigated the relationships between *F. hepatica* and seven other species of helminths using immuno-electrophoresis. They obtained 15 precipitins using a rabbit antiserum to *F. hepatica* but only five of these were specific for *Fasciola*. Cross-reactions were found to the other ten antigens when tested against *S. mansoni*, *D. dentriticum*, *Taenia saginata*, *Onchocerca volvulus* and *T. spiralis*, although only three precipitins were produced in each case. Only one precipitin line was observed for *A. lumbricoides* and *E. granulosus*. In a similar survey, Geyer (1967) obtained 23 precipitins to an *F. hepatica* extract with homologous rabbit antiserum. Extracts of *D. dendriticum* gave four precipitin lines, *S. mansoni* three lines and *A. lumbricoides* one line with this antiserum. Lines were not found when the hydatid fluid of *Echinococcus* and *Cysticercus tenuicollis* were used. These results are in overall agreement and offer some encouragement in the search for specific antigens but they are of little use as a basis for taxonomy.

This is partly due to the difficulties already mentioned and also to the extremely wide range of helminths used in these studies.

Where work has been restricted to related families, the chance of meaningful results is increased. Kravtsov (1967) used serological techniques to ascertain the systemic position of some diphyllobothriid tapeworms, and found antigens specific for the species used and others which were specific for families. Some antigens were common to all the tapeworms. With absorption techniques the antigens of *D. dentriticum* were removed more completely using *D. latum* antisera than were the antigens of *Spirometra erinacei*, giving an indication of the probable relationships between these three species. However, many more careful investigations are required before much reliance can be placed on a system of taxonomy based on serological tests.

XI. Conclusions

Some circulating antibodies have a protective function but this has been demonstrated only occasionally and it would seem probable that most of the antibodies stimulated by helminthic infections are not involved directly in protecting the host from further infection. At the same time it has been shown that most helminths engender resistance phenomena in the host and it is difficult to supply anything but an immunological basis for this.

The protective effect of humoral antibodies has been demonstrated for helminthic infections which have been put into hosts other than the normal species. The results obtained under these conditions are better than those which occur in the normal host-parasite systems, and this suggests that the antibodies have a part to play in producing resistance but that under normal circumstances it is not very extensive.

Apart from their role in protection, circulating antibodies can give a picture of the host's reaction to the parasite over the period of infection. Some correlations have also been found between some of the manifestations of resistance and the antibody titre.

Circulating antibodies can be used as an aid to diagnosis, and some serological tests, notably the Ouchterlony type of precipitin test, give specific results. In general, however, the value of these tests is limited because the examination for helminth material, eggs or larvae, in the faeces is a more direct approach and usually gives satisfactory results. The practical uses of serodiagnosis are therefore limited to the occasions when parasitological investigations are difficult or unrewarding.

Work in this field is hampered by the scarcity of even partially defined antigens and of material from intermediate stages in the worm's life cycle. However, much research effort is now being made in attempts to produce purified, specific antigens from various helminths (Sawada *et al.*, 1965; Kent, 1963b) and to find suitable conditions for the *in vitro* culture of parasitic helminths (Silverman, 1965b). When these difficulties are overcome the way should be clear for great advances in our understanding of immunity to helminthic diseases.

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Electron Transport in Parasitic Helminths and Protozoa

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I. Introduction

A. General Problems

Until recently, comparative biochemistry has held the attention of relatively few biochemists. Biochemical information is available in depth for mammals, plants and microorganisms, but the remaining groups have largely been neglected. The study of the biochemistry of parasitic organisms occupies no-man's land between the known and the unknown. Much work has been attempted and recorded, although a great deal of it is suspect on two counts; techniques employed are often not the best available, and much of the research has been carried out not in biochemical, but in biological departments, where many necessary pieces of equipment are not available.

The comparative biochemist is faced also with the peculiar problems posed by the parasites themselves. Sufficient material for many biochemical studies is usually not available, and elaborate collecting or culturing techniques are

required. It may prove impossible to ensure that the parasite material is completely free of contamination—by intestinal flora, for example, or by components of host tissues which might introduce errors into assay systems. In many cases low activities of enzymes and low concentrations of other substances may present peculiar difficulties in assay procedures. A further complication is that whereas the mammalian biochemist working on liver and the bacterial biochemist working on a given species of organism are reasonably confident that their cell populations are homogeneous, the parasitologist is faced with the necessity of whole-animal work. Thus, it is often difficult to be sure whether in cestodes, for instance, a given enzyme system is associated with proliferating tissue or with egg-producing proglottides. Often the large quantities of lipid material make the isolation of sub-cellular fractions impossible and other biochemical methods very difficult. The amount of protein per gram of body weight is usually low. Thus, the parasitological biochemist has to place reliance on microtechniques, and finally he has to make allowances for the possibility that, as soon as he removes the parasite from the host, he is dealing with a moribund organism. It is therefore important that studies *in vitro*, whether of the whole animal, homogenates or sub-cellular fractions, shall be made to relate to the environment from which the parasite was derived. *In vitro* study at best provides only an approximation of conditions *in vivo*; study of parasites *in vitro* may be more of an approximation still.

In selecting materials for this review, the main aim has been to try to describe the respiratory systems of the parasites in the context of their environment. Thus, one of the topics to be discussed in some detail is the biochemical nature of anaerobiosis. This is followed by a brief treatment of the current views of cytochrome-mediated oxidation and electron transfer in other organisms, and of mechanisms for synthesis of ATP. It is obviously not possible in a review of this length to include every publication in these fields, and some selection has been made. Only work carried out in the last five or so years will receive consideration in any depth. The interested reader is recommended to earlier publications for a more comprehensive survey of the literature (von Brand, 1966; Ryley, 1967; Honigberg, 1967; Baernstein, 1963) and to the excellent bibliographies of Smith (1965, 1968). For a discussion of the properties of parasite haemoglobins, which are not considered here for reasons of space, the review of Lee and Smith (1965) should be consulted.

B. AEROBIOSIS AND ANAEROBIOSIS

It is widely accepted that the early stages of biochemical evolution took place under conditions of reducing atmosphere (Nursall, 1959), and it is probable that during the initial stages there evolved the stepwise degradations of substrates which transferred energy by substrate-linked phosphorylation processes. Later, the storage of chemical energy in molecules such as ATP must have been possible. The pathways of glycolysis and of fermentation are examples of highly organized enzyme systems embodying this principle and both are characterized by the presence of a mechanism which results in the reoxidation of reduced coenzymes by the terminal product of the degradation process.

Pyruvate or acetaldehyde are thus employed as electron acceptors. The products, lactic acid and ethanol respectively, are then discarded as waste. These are by no means the only possibilities, as numerous volatile and non-volatile acids and other metabolites are produced by bacteria and parasites generally (von Brand, 1966).

When abundant oxygen became available, a further function was added to the fermentative or glycolytic ones; oxygen became the ultimate electron acceptor. Thus, the mechanism for the reoxidation of reduced coenzyme depended upon molecular oxygen. The significant point about this system is that it came to involve flavoproteins and iron-containing carrier molecules, which are members of a class of compounds known as haemoproteins (widely implicated in electron transfer in respiratory and non-respiratory systems). As the electrons moved down the electropotential gradient, energy became available for the synthesis of ATP.

The above description provides the generally accepted distinction between aerobiosis and anaerobiosis; the first involves haemoproteins called cytochromes and phosphorylation may occur at substrate or at electron transport level; the latter involves no cytochromes and phosphorylation occurs at substrate level only. However, there is no absolute requirement for oxygen. Any molecule which is transportable to the appropriate site for reaction and has the proper redox potential may be substituted. It is therefore possible to envisage pathways of respiration involving cytochromes, with a phosphorylation efficiency, but in which some molecule other than oxygen acts as terminal electron acceptor. This pathway would be anaerobic in the sense that oxygen is not involved. The formation of an end product capable of undergoing further oxidation, which is nevertheless released into the environment, enables an obvious parallel to be drawn with fermentation or glycolysis. However, such a pathway does not differ in any important respect from the classical concept of the aerobic pathway which has been developed for vertebrates. Differences are of detail only.

It is important to dissociate the concept of anaerobiosis in parasites (which is taken to mean the lack of participation by oxygen in the molecular events of respiration) and the absolute requirement of oxygen by parasites for various other processes, including growth. Many processes require molecular oxygen, and many oxygenases are present in cells. Thus, the direct oxidation of amino acids, egg production (Lejambre and Whitlock, 1967), tanning of egg-shell proteins (Smyth and Clegg, 1959) and even collagen synthesis (Paik and Benoitin, 1963) require oxygen. These are not, however, respiratory processes; they do not result in a net synthesis of ATP, and may even expend it.

The concepts, aerobic or anaerobic, as applied to parasites, need redefinition. It is often not at all clear from the literature whether a parasite is aerobic or anaerobic, as its absolute requirement for oxygen may be irrelevant to overall respiration. Reports of culture experiments in which oxygen is required may similarly be equivocal. Even where oxygen is not required in culture, that is not to say that the cytochrome system necessarily differs fundamentally from the accepted norm for the aerobic organism. It is doubtful, therefore, whether any parasitic helminths are true aerobes or true anaerobes, in the accepted

sense, although the terms may be valid for some protozoa. In the environments that they occupy, it is probable that there will be some oxygen available to parasites, and it is natural that this will be utilized both for respiration and for other processes. It seems likely that they have assumed a duality of function: it is this that complicates *in vitro* work, as over the short terms of most experiments at least, parasites are metabolic opportunists. If a molecule is present it may well be utilized. In this context, studies on cestodes by Cheah (1967a, b) have shown that, largely obscured by a cytochrome system which functions in the absence of oxygen, there is a normal cytochrome system possessing an oxidase. It is most likely that the latter is investigated in *in vitro* measurements of oxygen uptake. *In vivo* it apparently plays a subordinate role.

II. Current Concepts of Electron Transport

A. Haemoproteins and Electron Transport in Classical Systems

Haemoproteins are proteins in which the prosthetic groups are derived from protoporphyrin IX, which is also called protohaem or haem. Haems are square-planar chelates of Fe; the Fe can form complexes with two other binding agents, or ligands. Combination with the first ligand results in the facilitated binding of the second. These complexes are known as haemochromes or haemochromogens, and are important in characterizing haemoproteins. Haemoproteins are very widely distributed in nature, and are involved in at least three important functions: they may be oxygen-carrying entities, such as the haemoglobins, they are concerned in the reduction of peroxides (peroxidases and catalases), and they are involved in electron transfer between dehydrogenases and terminal electron acceptors which are usually cytochromes (Mahler and Cordes, 1966).

A complete discussion of the cytochrome systems hitherto detected in microorganisms, insects, mammals and higher plants, would be out of place here. Excellent accounts of the chemical properties of cytochromes are available (Morton, 1958; Falk *et al.*, 1961). Figure 1 summarizes the current concepts of the array of cytochromes in the electron transport system which is found in mammalian mitochondria. The system falls naturally into four complexes (Green and Goldberger, 1967). Phosphorylation occurs at points along the chain and the number of molecules of ATP synthesized varies, depending on the substrate used. It should be stressed that the inter-relationships of the components of the electron transport system, in particular of the various cytochromes, remain controversial, and the accompanying figure is merely one schematic representation which has been widely accepted.

Current views about the mechanism of phosphorylation are in a state of flux. The orthodox view considers that coupling of electron transfer to phosphorylation involves one or more hypothetical "high-energy" phosphorylated intermediates (Chance and Williams, 1956; Rasmussen *et al.*, 1965; Chance, 1965). However, unorthodoxy, in the form of the chemiosmotic coupling theory proposed by Mitchell (1961, 1966), suggests that phosphorylation is achieved by the establishment of a pH gradient across the mitochondrial

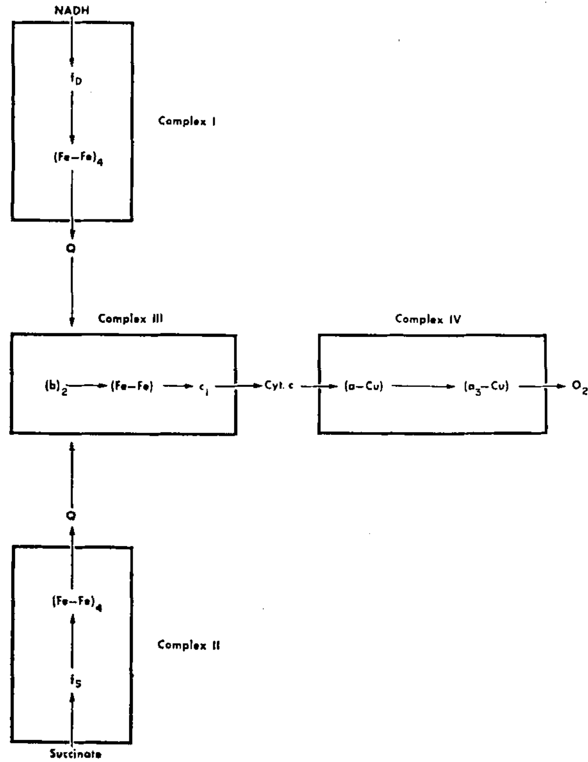


FIG. 1. Distribution of the oxidation-reduction proteins among the complexes of the electron-transfer chain. Fe–Fe represents non-haem iron; f_S, the succinic dehydrogenase; and f_D, the NADH dehydrogenase. (Modified with permission from Green and Goldberger, 1967.)

membrane, and its subsequent discharge by the formation of ATP. The latter view has recently received some promising experimental support, although the evidence for both views is yet incomplete.

B. HAEMOGLOBINS, CATALASES AND PEROXIDASES

Haemoglobins in parasites have been treated very adequately by Lee and Smith (1965) and will not be considered in any detail here. It is sufficient to say that they are concerned with transporting oxygen (haemoglobins) or storing it (myoglobins). The function of such molecules, even in the parasite most frequently studied, *Ascaris lumbricoides*, is far from clear.

Similarly, the function of catalase is not clearly understood. It is apparently able to bring about certain oxidations by hydrogen peroxide. It is extremely widespread in cells. Peroxidase is also widespread and is involved in reactions requiring the participation of oxygen (Morell, 1954; Newton *et al.*, 1965; Hosoya and Morrison, 1965). However, the roles of both enzymes remain enigmatic and, *in vivo*, may be concerned in electron transport, proton transport, respiration or detoxication.

C. THE TRICARBOXYLIC ACID CYCLE AND DERIVATIVE ACTIVITY

Another important function of mitochondria is the oxidation of acetyl CoA, formed in a variety of ways, including the Embden–Meyerhof Pathway, to carbon dioxide and water by means of the tricarboxylic acid cycle. No further explanation is needed of this cycle, except that it appears to be capable of modification in ways that may result in at least part of it functioning in the absence of oxygen, provided that an acceptor for electrons produced during this process is present. Thus, Hoberman and Prosky (1967) show that fumarate is readily reduced to succinate in perfused rat liver under conditions of reduced oxygen tension. That a reduction of fumarate mediated by NAD in mammalian systems was possible was first suggested by Dewan and Green (1937). Slater (1950) later showed that NADP could be reoxidized by fumarate in a heart muscle preparation. Sanadi and Fluharty (1963) showed that in submitochondrial particles from the same source the reaction appears to be a reversal of the energy-linked reduction of NAD by succinate.

In parasites, the conversion of fumarate to succinate, dependent upon NADH, is often found. Kmetec and Bueding (1961) have shown that in *Ascaris* this reaction depends on a flavoprotein dehydrogenase. Cheah and Bryant (1966), working on *Moniezia expansa*, demonstrated that not only was NADH necessary for the reaction to take place, but a cytochrome component was implicated as well. Succinate as an excretory product is common among parasitic helminths and flagellate protozoa. In helminths the route of its formation often involves the fixation of carbon dioxide by pyruvate or phospho-enol pyruvate to yield malate or oxaloacetate, and the subsequent conversion of these products to fumarate and succinate. This is similar to a metabolic pathway first described in bacteria by Wood and Werkman (1936).

Although Sanadi and Fluharty (1963) consider that fumarate reduction in mammals is mediated by the same enzyme that is responsible for succinate oxidation, there is evidence that in Protozoa the forward and back reactions are mediated by quite distinct enzymes. This offers a real possibility for a separate pathway which operates in the absence of oxygen (Baernstein, 1953a, b).

Succinate is only one end product of respiration formed by parasites. A whole variety of substances are produced by Protozoa and helminths, and many of them are equally the end products of particular respiratory pathways. The significance of the variety of end products is that they probably represent "hydrogen sink" mechanisms which permit the reoxidation of coenzymes which have become reduced in the earlier reactions of respiration. Possibly, heavy depositions of fats in many helminths and in cultured forms under imperfect conditions (Zdarska, 1966; Smyth, 1968, personal communication) may also represent a means of removing an end product of metabolism.

III. EARLY WORK ON PARASITES

As von Brand (1966) has pointed out, the tricarboxylic acid cycle does not usually function in the absence of oxygen, but many of its individual reactions may still occur and have importance. Thus, attention paid to the separate steps

of the cycle may be more fruitful than studies of overall cycle activity. Even under conditions of oxygen plenty, the definitive experiments are those which involve direct assays of enzyme activity rather than those which depend on the detection of intermediates. In the latter case, detection may depend upon rate of formation, rate of utilization and the size of the endogenous metabolic pool. However, information can often be gained only by these techniques, owing to the scarcity of material. Negative evidence obtained in this way is often meaningless; positive evidence is acceptable.

Amongst other factors which affect enzyme determination are the methods of assay. Thus, in *Trypanosoma cruzi*, Baernstein and Tobie (1951) and Baernstein and Rees (1952) did not find succinic dehydrogenase, but Seaman (1953) and Agosin and von Brand (1955) detected succinic dehydrogenase activity and attributed the different results to differences of homogenization.

Aconitase, isocitric dehydrogenase, succinic dehydrogenase, fumarase and malic dehydrogenase have been detected in the flagellates *Crithidia fasciculata* and *T. cruzi*. In *T. rhodesiense* only the first four enzymes have been demonstrated, whereas in the ciliate, *Opalina carolinensis*, only aconitase has not been found. Of these enzymes, succinic dehydrogenase appears to be the most widespread as it has been detected in representative flagellates, rhizopods, sporozoa and ciliates (see table in von Brand, 1966). In all probability the distribution reflects the ease of assay of the enzymes.

Similarly, succinic dehydrogenase is probably ubiquitous in parasitic helminths, although the complete tricarboxylic acid cycle has been unequivocally demonstrated in very few. *Echinococcus granulosus* and *Fasciola hepatica* have been subjected to very complete studies which support the operation of the cycle in these parasites (Agosin and Repetto, 1963; Prichard and Schofield, 1968a), and there is substantial evidence suggesting its presence in *A. lumbricoides* muscle (Oya *et al.*, 1965), in *Ditylenchus* (see Krusberg, 1960) and in *M. expansa* (see Davey and Bryant, 1969).

This incomplete survey of the numerous papers on enzymes of the tricarboxylic acid cycle does indicate that a vast amount of routine investigation must still be carried out. Few generalizations can be made, except to say that where a rigorous attempt to detect tricarboxylic acid cycle activity has been made, some activity has usually been detected. However, this is by far the smaller component of respiration in helminths, and the modified Wood–Werkman pathway by which succinate is formed is far more in evidence. Of the few parasites studied, *E. granulosus* is the single exception. It lives buried in the crypts of Lieberkühn, where more oxygen may be available.

IV. Electron Transport in Parasitic Helminths

A. Cestodes

The earliest observations on cytochromes in parasites were made by Keilin (1925), whose original paper on a new class of respiratory pigments in living organisms contained several references to *Ascaris*. The first report that cytochromes were present in cestodes also was by Friedheim and Baer (1933), who found that respiration of *Diphyllobothrium* was not inhibited by carbon

monoxide. They rightly concluded that the respiratory system did not contain a cytochrome system like that previously identified in mammals. Further examination with a hand spectroscope revealed in adult *Diphyllobothrium* and *Triaenophorus* a well defined absorption band between 520 and 530 mμ and characteristic of cytochrome. Combining these observations, Friedheim and Baer justifiably concluded that the respiration of cestodes was different from that of free living organisms. Van Grembergen (1944) repeated much of this work with *Moniezia benedeni*; succinate oxidation was inhibited both by KCN and by carbon monoxide, although the degree of inhibition was much less than in mammalian systems. Thus, 10^{-2} M KCN reduced oxygen uptake by only 50%, whereas in mammals concentrations of 10^{-5} M result in 95% inhibition. Van Grembergen was working on pulped material, in which spectroscopic examination revealed a well defined absorption band at 558 mμ, which became intensified on the addition of dithionite, a reducing agent. A second, less clear absorption band at 530 mμ also appeared. Addition of dithionite and pyridine showed that the pyridine haemochromogen had absorption bands at 558 and 526 mμ. Van Grembergen interpreted these results as meaning that both cytochromes b and c were present, and as only two absorption bands appeared he suggested that the two spectra overlapped. In spite of the absence of absorption bands characteristic of the cytochrome a complex, he concluded that the earlier workers were not justified in attributing a special type of respiration to cestodes.

By a manometric technique Read (1952) showed that cytochrome c reduced by ascorbate stimulates oxygen uptake by homogenates of *Hymenolepis diminuta*, suggesting the presence of cytochrome oxidase. However, in the absence of ascorbate, cytochrome c was not reduced by his preparations.

A more complete study on the electron transport system of a cestode is that of Cheah and Bryant (1966), who worked on a preparation from *M. expansa* which was not homogeneous but contained a high proportion of mitochondria. The oxidation of succinate and NADH involved separate pathways and was accomplished by a different route from that found in mammalian tissue. The major cytochrome component, with its α-absorption band at 557 mμ, corresponded with the band first detected in *M. benedeni* by Van Grembergen (1944). It was this cytochrome that was implicated in the reduction of fumarate to succinate. Only small amounts of cytochrome a were found and there was some evidence for the presence of cytochrome c.

Scheibel *et al.* (1968) disputed the view that cytochromes are involved in the conversion of fumarate to succinate in helminths. They correctly point out that the formation of hydrogen peroxide is more characteristic of flavoprotein than cytochrome, and that in solubilized preparations from *Ascaris* the rate of reduction of cytochromes is at least several hundred times slower than the rate of reduction of fumarate by NADH. However, in a solubilized preparation, the integrity of the particulate fraction containing the cytochromes has been disrupted, and hence the experimental situation is even further from conditions *in vivo*. Cheah and Bryant (1966) considered that hydrogen peroxide accumulating in their particulate fractions under aerobic conditions was probably symptomatic of the participation of flavoproteins in terminal oxidations.

However, the participation of the cytochromes was also indisputable, and they suggested that the cytochromes were the components mainly concerned with terminal oxidation *in vivo*, under conditions of oxygen deficit.

Subsequently, after more complete study, Cheah (1967a) concluded that *M. expansa* has a branched respiratory chain system with two terminal oxidases, one of which is an o-type cytochrome (similar to that found in many bacteria) with absorption maxima at 552 and 556 mμ, and is the major terminal oxidase concerned with fumarate reduction. Hydrogen peroxide was thought to originate from this pathway in the presence of oxygen. The second oxidase appeared to be similar to the classical cytochrome oxidase. These conclusions were based on data obtained with various inhibitors of electron transport. Thus, antimycin A at low concentrations (10^{-6} M) inhibited the transfer of electrons from cytochrome b to cytochrome c; at higher concentrations (10^{-4} M) it inhibited electron transfer between a new cytochrome component, cytochrome 556 (*M. expansa*) and the o-type cytochrome previously mentioned. Similarly, carbon monoxide inhibited cytochrome oxidase and also formed a complex with the o-type cytochrome (Fig. 2). The branching of the cytochrome chain was thought to occur at the level of vitamin k, because of the inhibitory effect of dicumarol on both pathways. This remains uncertain because the actual participation of vitamin k in the electron transfer system has still not been unequivocally demonstrated. In a subsequent note, Cheah (1968) fully established the presence of the orthodox cytochrome system in *M. expansa*, but showed that the concentration of cytochrome oxidase was only one-tenth that of the o-type cytochrome.

The general conclusions to be drawn from this work, that at least two pathways operate, one utilizing oxygen and the other fumarate, are acceptable. Details are not above criticism. One major source of error lies in the purity and the integrity of the preparations employed for the work on cytochromes. The difficulties involved in the preparation of mitochondria from *M. expansa* are great. Christensen, Nicholas and Bryant (1968, unpublished data) have since succeeded in producing very small quantities of a mitochondrial preparation from this cestode, in good condition by electron-microscopic criteria, but bulk preparations cannot be made. However, the techniques employed by Cheah and Bryant (1966) and by Cheah (1967a, 1968) produce mitochondria which are damaged and probably contaminated with some endoplasmic reticulum. Chance *et al.* (1956) published absorption values for mammalian microsomal cytochrome b_5, which are in good agreement with the values for the split α-band (552 and 556 mμ) of the o-type cytochrome of *M. expansa*, except for the approximate shift of 1 mμ to the shorter wavelength. The amount of split is characteristic of a component in the endoplasmic reticulum (Chance, 1965, personal communication). Thus, it is not possible to exclude the view that the branching of the chain described by Cheah (1967a) is artefactual and that there may be two or more systems of different subcellular origin contributing to the overall picture. The same limitations apply to Cheah's (1967b) conclusion that the cytochrome distribution and electron transport system of *Taenia hydatigera* was similar to *M. expansa* (Fig. 3), as to all work involving particulate fractions from parasitic helminths. Nonetheless,

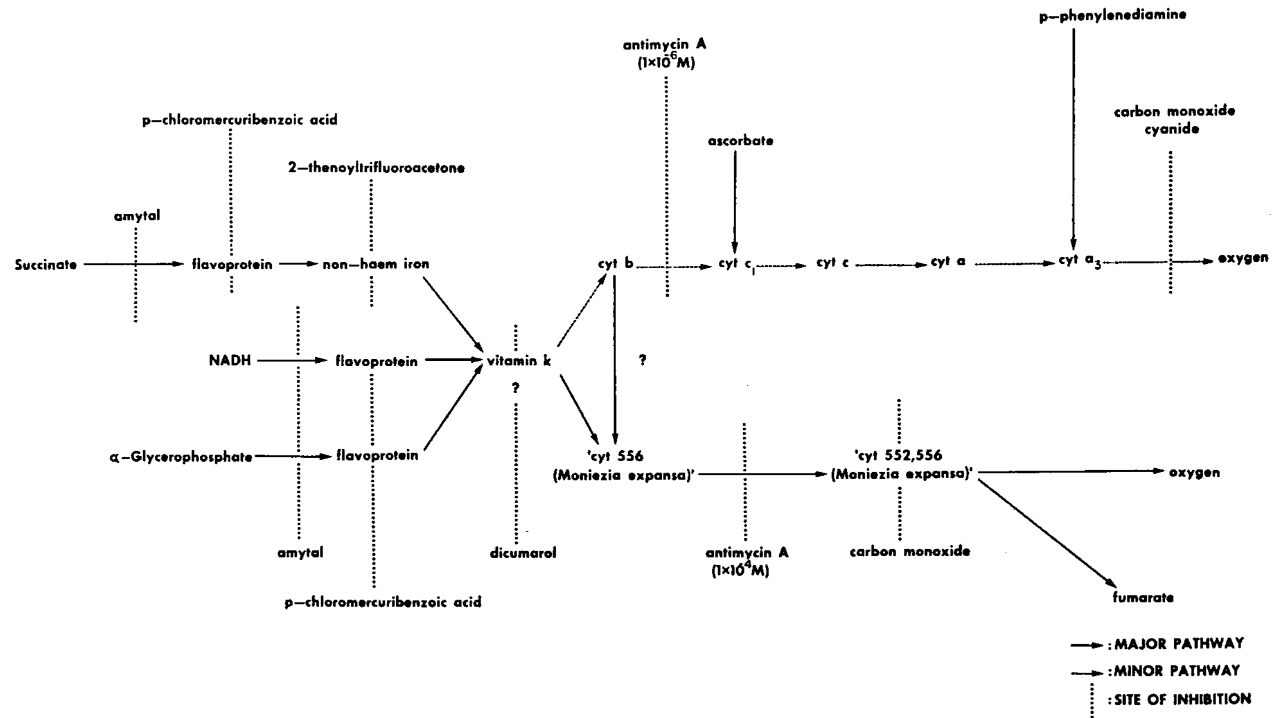


FIG. 2. Proposed pathway for the electron transport system of *M. expansa*. (Reproduced with permission from Cheah, 1967a.)

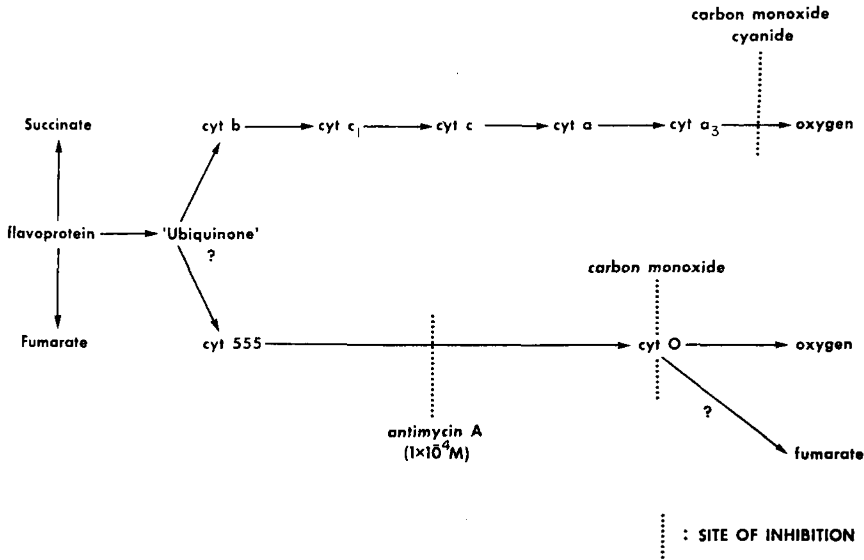


FIG. 3. Pathway for the electron transport system of *T. hydatigena*. (Reproduced with permission from Cheah, 1967b.)

the general mechanisms described in these papers may be applicable to many cestodes. Preliminary examination of *E. granulosus* protoscoleces shows a maximum absorption band at 557 mμ, suggesting that the o-type cytochrome described by Cheah (1967a) is present and mediates the fumarate–succinate transformation (Bryant, 1967, unpublished data). Bryant and Morseth (1968) have demonstrated that in intact living adults of this species, fumarate is readily converted to succinate, so it is not unlikely that the system operates *in vivo* as well as *in vitro*.

Cheah and Bryant (1966) demonstrated that hydrogen peroxide accumulated in the medium when the particulate fraction from *M. expansa* was incubated in air with succinate. Cheah (1967a) showed that it was a product of the activity of the o-type cytochrome. Supernatants obtained after centrifuging whole homogenates of tapeworm at 12 000 g were found to contain a haemoprotein which removed the peroxide as soon as it was formed. Cheah (1967c) carried out a histochemical and spectrophotometric study of this haemoprotein. He identified it as peroxidase by virtue of its ability to form complexes with cyanide, dithionite, carbon monoxide and peroxide.

The histochemical techniques showed that peroxidase activity is concentrated at the base of the cuticle, where the densest populations of mitochondria are found. It was suggested that the function of peroxidase is to destroy hydrogen peroxide, if it be formed, before it can accumulate in sufficiently high concentrations to be toxic. Alternatively, Neufeld *et al.* (1958) suggested that peroxidase occurs with a low level of cytochrome oxidase in tissues which have a high rate of cell renewal. Cestodes may fall into this category, by virtue of an enormous rate of egg production and segmentation.

The presence of peroxidase and/or catalase had previously been demonstrated in *Taenia pisiformis* by Pennoit-de Cooman and Van Grembergen (1942). Recently, Threadgold *et al.* (1968) extended this observation to yet another cestode, *H. diminuta.* A histochemical technique combined with electron microscopy showed that the enzyme is located in the mitochondria of the surface syncytium of the "tegument", and that less intense reaction occurred in the tegumentary cells and parenchyma, while muscle mitochondria proved negative. Rothman (1968) has made similar observations in *H. citelli.* Although it is not yet possible to give a specific function for this enzyme, its presence in mitochondria suggests that it may be related to respiration.

The work of Threadgold *et al.* (1968) illustrates yet another difficulty involved in working with parasite materials. Their determinations of peroxidase activity indicate a very heterogeneous population of mitochondria in *H. diminuta.* This observation is almost certainly true for other cestodes. Hence, preparative procedures may isolate one species of mitochondrion only, from which unjustified generalizations may be drawn. A study of the electron micrographs of *Lacistorhynchus tenuis*, for instance, shows that mitochondria vary considerably in form and size, some having few cristae but others many; similar observations have been made on taeniiform cestodes (Lumsden, 1967; Morseth, 1965).

As oxygen must enter the cestode's body by diffusion through the body-wall, it might be expected that mitochondria with densely packed cristae (a condition usually diagnostic of a highly active cytochrome system) would be present at the base of the tegument. In fact, the mitochondria in the parenchymal cells of *L. tenuis* are significantly larger and contain more cristae membrane surface (Lumsden, 1967). Green and Hatefi (1961) have pointed out that the fewer the number of cristae per unit area of mitochondrion, the lower the oxidative rate, and the greater are the ancillary enzyme activities. Thus, this distribution of mitochondria may represent an adaptation to the peculiar problem of maintaining a permeable tegument under conditions of low oxygen tension, and may be due to either or both of two mechanisms.

As the partial pressure of oxygen, and the concentrations of other metabolites fall along a diffusion gradient between the outside and the interior of the parasite, a greater concentration and multiplicity of electron carriers may be needed internally for efficient oxidation to take place. There is a necessity for parenchymal mitochondria to contain large numbers of cristae. Alternatively, enzyme systems other than those associated with electron transfer may be required at the surface of the parasite for the maintenance of tegumental integrity and active transport mechanisms; hence, the vesicular nature of the mitochondria in the surface cells.

Related to these problems is the problem of ATP synthesis. Scheibel *et al.* (1968) studied the anaerobic incorporation of radiophosphorus into ATP by *H. diminuta.* Low concentrations of uncoupling agents inhibited this incorporation, and the presence of carbon dioxide was essential. No direct relationship between ATP synthesis and fumarate reduction was demonstrated, but these observations suggest that the pathway involving the conversion of fumarate

to succinate has a positive phosphorylation gain, and that similar mechanisms may be of significance in a wide variety of parasites.

B. TREMATODES

From the biochemical point of view, the trematodes are a neglected group. Even the possibility of obtaining kilogram quantities of *F. hepatica* relatively easily has not tempted many workers. Succinic dehydrogenase has been demonstrated in *Schistosoma japonicum*, and succinate utilization has been observed in *Gynaecotyla adunca* and in adults, rediae and cercariae of *Himasthla quissetensis* (see Huang and Chu, 1962; Vernberg and Hunter, 1960, 1963). Metacercariae of *Clinostomum campanulatum* have recently been shown to incorporate radioactivity from uniformly labelled ^{14}C-glucose into glutamic, α-ketoglutaric, citric, fumaric, and succinic acids, which suggests that tricarboxylic acid cycle activity is present (Thomas and Gallicchio, 1967). In *Parorchis acanthus* rediae, utilization of exogenous glucose is strongly dependent on the presence of oxygen in the gas phase of the culture medium, which suggests that there may be an aerobic component of its respiratory metabolism (McDaniel and Dixon, 1967).

Bueding and Charms (1952) showed that cytochrome c and cytochrome oxidase in *S. mansoni* were not present in sufficiently high concentrations to account for more than 10% of the overall oxygen uptake of this trematode. Malic dehydrogenase has been investigated by Conde-del Pino *et al.* (1966) and succinic dehydrogenase by Huang and Chu (1962). A method for the preparation of mitochondria from males of *S. mansoni* has been published, but no respiratory studies were carried out (Smithers *et al.*, 1965).

For *Fasciola* spp. much more information is available. Pennoit-de Cooman and Van Grembergen (1942) and Van Grembergen (1949) demonstrated the presence of succinic and malic dehydrogenases in *F. hepatica.* Bryant and Williams (1962), Bryant and Smith (1963) and Thorsell (1963) found citric, aconitic, α-ketoglutaric, succinic, fumaric, malic and oxaloacetic acids present in the adult fluke. Bryant and Williams (1962) could not, however, detect acids of the tricarboxylic acid cycle in miracidia. Although these results suggested strongly that the tricarboxylic acid cycle was present in adult *F. hepatica*, the appearance of large quantities of succinate indicated that the modified Wood–Werkman pathway was also followed in trematodes. Additional evidence that the cycle was possible was provided by Krvavica and Martincic (1964), who found acetyl coenzyme A in *F. hepatica*, but in much lower concentration than in sheep and cattle livers. Finally, Prichard and Schofield (1968a) demonstrated that all the enzymes of the tricarboxylic acid cycle were present in the liver fluke, although the aconitase and fumarase activities were not associated with the mitochondrial fraction, and the only isocitric dehydrogenase detected was NADP specific.

Prichard and Schofield (1968a) also showed that the oxidation of NADH by fumarate occurred under aerobic conditions, and concluded that the low activities of aconitase and isocitric dehydrogenase indicated a physiological role of the complete cycle of relatively minor importance, the principal function of the enzymes being the formation of succinate by partial reversal of the cycle,

and in anabolic processes. They also demonstrated that the initial carbon dioxide fixation step of the sequence resulting in the accumulation of succinate in *F. hepatica* was mediated by phosphoenol pyruvate carboxykinase. In *S. mansoni* pyruvic kinase is the enzyme responsible for carbon dioxide fixation (Bueding and Saz, 1968).

Prichard (1968) investigated electron transport in a particulate preparation derived from *F. hepatica*. The criticisms of particulate preparations from helminths apply in this case also but the study produced some useful results. As in *M. expansa*, electron transport under aerobic conditions was relatively insensitive to inhibitors, especially cyanide, antimycin A and azide. These observations suggest that the terminal components of the major electron transfer system differ from those found in mammalian systems, and that cytochromes c_1, c and $(a+a_3)$, if present, are in very low concentrations. Hydrogen peroxide was shown to be a product of respiration, suggesting the participation of flavoproteins. A peroxidase was found in the supernatant fraction of homogenates of the whole worm.

Difference absorption spectra, obtained with the particulate fraction, had absorption peaks at 557, 526 and 426 mμ suggesting the presence of a b-type cytochrome. A small absorption band at 444 mμ pointed to the presence of a more orthodox cytochrome b. As in *M. expansa*, a carbon monoxide binding pigment with characteristics similar to that of cytochrome o was detected. Fumarate was readily converted to succinate by the reduced cytochrome of the b type (Fig. 4).

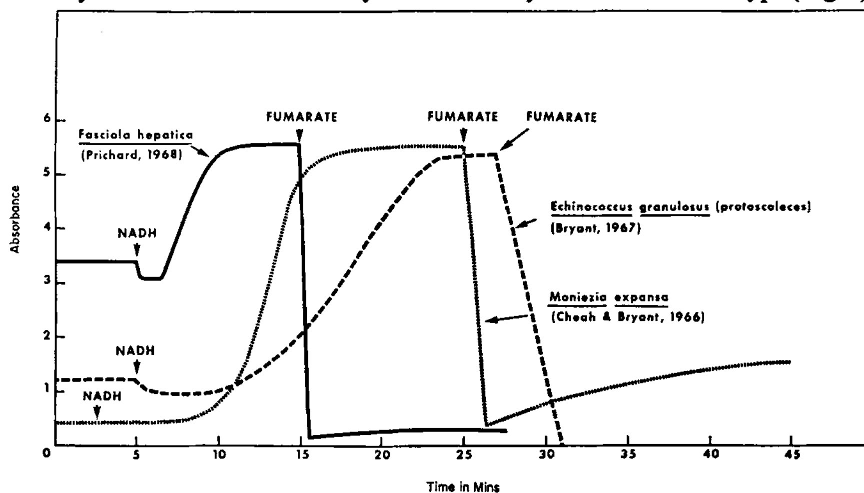


FIG. 4. The reduction of "cytochrome 557" from three helminths by NADH, and its subsequent reoxidation by fumarate. Absorbance is plotted against time at a wavelength of 557 mμ (the α-absorption peak of the cytochrome). Maximum absorbance occurs when the cytochrome is fully reduced. The ordinate is divided into arbitrary absorbance units. Continuous readings of absorbance were taken for 3–5 min, at which time NADH was added. After an initial lag, absorbance increased as the cytochrome was progressively reduced and eventually stabilized at a new, high level. Addition of fumarate resulted in reoxidation of the cytochrome and conversion of fumarate to succinate. Particulate fractions from *F. hepatica* and *M. expansa* were used; the observations on *E. granulosus* protoscoleces were made with whole homogenates.

In summary, Prichard (1968) provides evidence for two pathways of electron transport in *F. hepatica.*

1. NADH → dehydrogenase → flavoprotein → b-type cytochrome →
   → o-type cytochrome ↗ fumarate ↘ oxygen (depending on conditions)
2. Succinate → dehydrogenase → flavoprotein → non-haem iron →
   → b-type cytochrome → o-type cytochrome → oxygen

Traces of the mammalian type of system are present, but do not appear to be contributing significantly to respiration. In important respects, therefore, the respiratory and electron transport processes of *F. hepatica* are similar to those of *M. expansa*. The mitochondrial architecture is also similar, as electron micrographs show very few cristae (Björkman and Thorsell, 1964). In another trematode, *Haematoloechus medioplexus*, peroxidase activity has been observed in mitochondria (Rothman, 1968).

C. NEMATODES

Of all the parasitic helminths, the Nematoda are by far the best known biochemically. The medical importance of pathogenic forms stimulated interest even in the early studies of respiratory metabolism when Keilin (1925) first observed cytochrome in *Ascaris*. Nematodes form a heterogeneous group which excretes an enormous range of substances (see von Brand, 1966), and this suggests a range of metabolic activities adapted to diverse parasitic habits.

Like many other parasites, *Ascaris* can "fix" carbon dioxide. Radioactively labelled succinate was detected in muscle strips when $^{14}CO_2$ was available in the incubation medium. The route involved fixation into pyruvate via a malic enzyme, and the subsequent formation of succinate (Saz and Hubbard, 1957; Saz and Vidrine, 1959). This seems to be the pathway adopted by many other nematodes, and, importantly, it involves two steps at which reoxidation of reduced pyridine nucleotides may occur—the malic enzyme step, and the NADH-linked conversion of fumarate to succinate. Similar pathways have been demonstrated in the canine whip worm *Trichuris vulpis* and in *Haemonchus contortus* larvae (Kmetec and Bueding, 1965; Ward *et al.*, 1968).

In *Ascaris*, the importance of such reductive steps is stressed even further by the occurrence of various highly reduced excretory products. Saz and co-workers showed that propionic acid, which can be derived from succinic acid by decarboxylation, condenses with acetic acid to form methylacetoacetic acid, which is in turn converted to α-methyl butyric acid by a pathway involving two reductive steps (Saz and Weil, 1960; Saz *et al.*, 1958). Figure 5 summarizes these observations. Similarly, reductive processes are responsible for the production of α-methyl valerate (Saz and Weil, 1962).

It is not surprising, because of the importance of the reductive processes, that there is some dissent over the possible occurrence of the tricarboxylic acid cycle in *Ascaris*. The picture is complicated by the varying degrees of success of authors to identify the same enzymes or intermediates of the cycle, and by

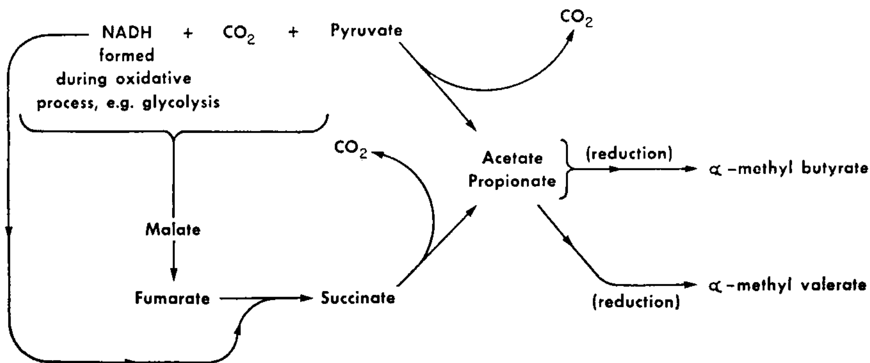


FIG. 5. Formation of end products by *Ascaris lumbricoides*, after Saz and Weil (1962).

the various stages of development which have been studied. Karpiak *et al.* (1965) found that in larval *Trichinella spiralis* citrate, α-ketoglutarate, succinate, fumarate, and malate had no effect on the respiratory rate. They concluded that the tricarboxylic acid cycle was of minor importance in these helminths. Similarly, Mikhailova (1962) found that citrate strongly inhibited the rhythmic contraction of *Ascaris* muscle, that fumarate and malate were also inhibitory, and that the tricarboxylic acid cycle was of little significance. Seidman and Entner (1961) stated that isocitrate and α-ketoglutarate dehydrogenases are absent from *Ascaris*, and the former enzyme could not be detected in developing larvae (Bloom and Entner, 1965). However, Oya *et al.* (1963b) detected isocitrate dehydrogenase in unembryonated eggs, and Oya *et al.* (1965) found direct or indirect evidence for all the enzyme reactions of the tricarboxylic acid cycle in muscle preparations from *A. lumbricoides*, but considered that the relatively low activity of the terminal oxidase system, and the even lower activities of condensing enzyme and aconitase, relegated the tricarboxylic acid cycle to a minor role. Finally, in eggs of *A. lumbricoides* an increasing body of evidence indicates that the tricarboxylic acid cycle occurs (Costello and Brown, 1962; Oya *et al.*, 1963b; Smith, 1967).

A similar confusion exists with respect to electron transport. Kmetec and Bueding (1961) showed that, in a particulate fraction isolated from *Ascaris* muscle, succinate and NADH oxidation are dependent on oxygen tension, Mn^{++}, ethanol and catalase for maximal activity. This is consistent with the view that oxygen uptake is mediated by one or more flavoproteins. In addition, fumarate reduction coupled to NADH oxidation was observed under anaerobic conditions. The enzyme concerned in this reaction was presumed to be identical with the succinic dehydrogenase (Fig. 6).

Previously, however, Kikuchi *et al.* (1959) had presented evidence to show that cytochrome oxidase, NADH oxidase, NADPH oxidase, cytochrome c reductase and succinic dehydrogenase activities were present in muscle pulp from *A. lumbricoides*, suggesting that the classical cytochrome system was implicated in the respiration of *Ascaris* muscle. They also provided evidence of an alternative pathway dependent on cytochrome b, which could be reduced

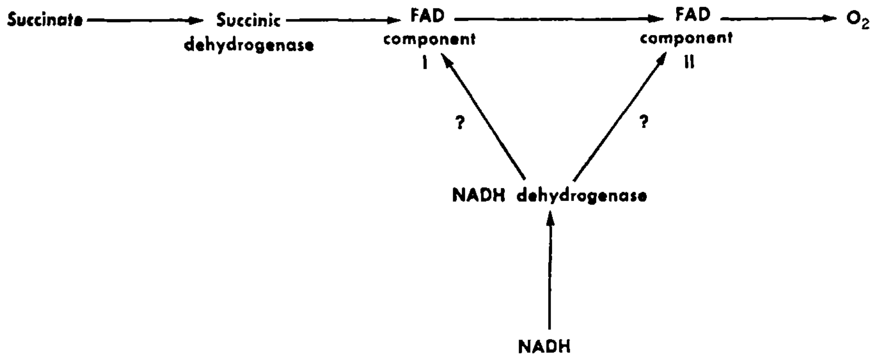


FIG. 6. Electron transport in *Ascaris lumbricoides*, after Kmetec and Bueding (1961).

either by NADH or by succinate. The cytochrome was autoxidizable, and the pathway was relatively insensitive to antimycin A and cyanide (Fig. 7). This is reminiscent of the system subsequently described for *M. expansa* (Cheah and Bryant, 1966; Cheah, 1967a). Kmetec and Bueding (1961) objected to the interpretation by Kikuchi *et al.* (1959) on several grounds. In their own preparations of *Ascaris* muscle, the oxidation of ascorbate had not been stimulated by the addition of cytochrome c; neither was it inhibited by cyanide. Also, the endogenous respiration rate in their preparations was not increased by the addition of cytochrome c. Finally, although Kmetec and Bueding (1961) detected the reduction of cytochrome c by their preparations, the rate of reduction was not commensurate with the rate at which NADH and succinate were oxidized.

The final point is the most significant and is difficult to explain. The remaining ones may be ascribed to different experimental procedures adopted by the two groups, and to the fact that Kmetec and Bueding (1961) were adjusting their incubation media in favour of oxidation by flavoproteins. It is important to note this, for it may mean that under conditions of high oxygen tension the classical oxidase and the b-type oxidase function, and also the flavoprotein system favoured by Kmetec and Bueding (1961). The muscle preparation used by these workers is a conformer—i.e. the rate at which it oxidizes substrates is proportional to oxygen tension—and it has been pointed out elsewhere that preparations from *Hymenolepis* also conform (Scheibel *et al.*, 1968); this does not necessarily mean, however, that flavoproteins form the terminal oxidases *in vivo*. We may reasonably expect that oxygen tensions in the gut will be low, and under these conditions the flavoprotein system may not function efficiently.

Additional evidence supporting the views of Kikuchi *et al.* (1959) was provided by Kikuchi and Ban (1961), who reported the presence of two cytochromes in a particulate fraction from *Ascaris* muscle; one was of the b type, with absorption maxima at 560 and 527 mμ, and the other was of the c type, with maxima at 549 and 520 mμ. In addition, they detected a haemoprotein with absorption maxima at 558, 528 and 424 mμ, which is similar to the o-type

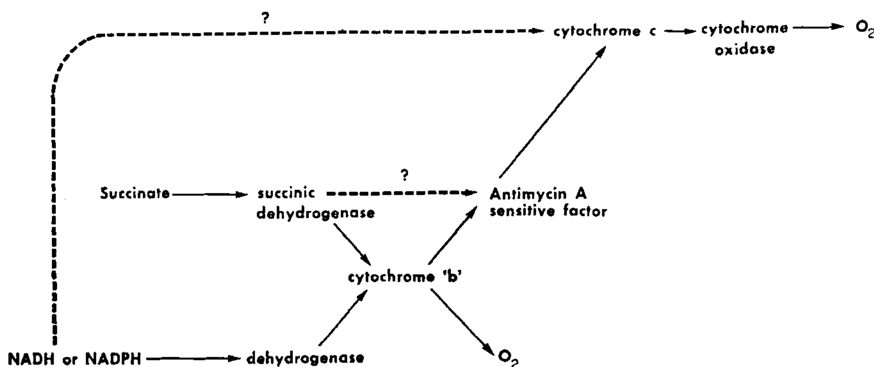


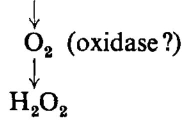
FIG. 7. Electron transport in *Ascaris lumbricoides*, after Kikuchi *et al.* (1959).

cytochrome described by Cheah (1967a, b). The c-type cytochrome was readily reduced by ascorbic acid. The b cytochrome was not, was highly autoxidizable at neutral pH, and was not sensitive to either cyanide or carbon monoxide, although the cytochrome c bound carbon monoxide to a small extent. Reduced cytochrome c from bovine heart muscle was readily oxidized by the particulate fraction, suggesting the presence of cytochrome oxidase activity. Kikuchi and Ban (1961) regard these observations as consistent with the previous conclusion of a dual oxidase system in *Ascaris* (Kikuchi *et al.*, 1959). Further support for their view came from a spectrum of the cytochromes in *Ascaris* muscle (Chance and Parsons, 1963), showing clearly the presence of cytochromes b and c, with absorption maxima at 557 and 549 mμ respectively, although the component described by Kikuchi and Ban (1961) with a maximum at 560 mμ is lacking.

The interpretation of the data for *Ascaris* is confused because this is a genus of intestinal parasites which must be investigated out of the normal environment. It is reasonable to assume, because of the low oxygen tensions the adult might normally encounter, that it possesses an oxygen independent terminal electron acceptor system, mediated by flavoprotein or cytochrome. Because the limitations of the intestinal environment do not apply, study of the electron transport systems of unembryonated and embryonated *Ascaris* eggs provides a better chance of determining what is happening under *in vivo* conditions. However, there is a strong possibility that changes of oxidative capabilities occur as the parasite matures and becomes fully parasitic.

Costello *et al.* (1963) noted an interesting difference between the eggs of *A. lumbricoides*. The unembryonated eggs were capable of oxidizing succinate and malate with a concomitant reduction of exogenous cytochrome c, but this was not followed by its reoxidation. In embryonated eggs, however, reduction of cytochrome c was enhanced by the addition of cyanide, suggesting the presence of an oxidase capable of reoxidizing the cytochrome c. Costello *et al.* therefore postulated two schemes, that for unembryonated eggs based on the work of Kmetec and Bueding (1961).

1. Unembryonated eggs:
   Substrate → NAD → FAD → cytochrome c



2. Embryonated eggs:
   Substrate → NAD → FAD → cytochrome c → O_2 → H_2O
   (cytochrome oxidase)

The appearance of cytochrome oxidase in embryonated eggs was later confirmed by assays of its activity during development (Oya *et al.*, 1963a), and subsequently the participation of FAD in the electron transport system of unembryonated eggs was confirmed (Costello, 1964). These observations were supported by Kmetec *et al.* (1963), who concluded that cytochrome oxidase activity and the cytochrome system generally was constitutive rather than an adaptive response to a specific environmental situation, and was expressed in the free living phase of the animal's life cycle. Further corroboration for the general scheme proposed by Costello *et al.* (1963) was provided by Yanagisawa (1965), who found that the sensitivity of *Ascaris* eggs to cyanide increased as development proceeded, and by Costello *et al.* (1966) who used a more sensitive polarographic technique for measurement of oxygen uptake.

As far as the immature stages of *A. lumbricoides* are concerned, there seems to be a measure of agreement. Unembryonated eggs do not possess cytochrome oxidase, their terminal respiration being mediated by another oxidase, which may be a flavoprotein. As embryonation proceeds, cytochrome oxidase activity appears. Oxygen has been said to induce respiratory enzyme activity and the biogenesis of mitochondria in micro-organisms (Somlo, 1965; Somlo and Fukuhara, 1965). Costello *et al.* (1966) speculate that in the intestines of the host, where the *Ascaris* eggs will not embryonate, oxygen tension is not sufficiently high to permit the onset of development. Once outside the host, the high oxygen tension activates the anaerobic oxidase, possibly flavoprotein in character, allowing cleavage to take place. At the same time other components of the cytochrome system are induced, permitting the subsequent switch to cytochrome oxidase. This attractive hypothesis is well supported by the data.

In the case of the adult, however, two concepts of terminal oxidation exist; Kmetec and Bueding (1961) consider that it is mediated by a flavoprotein, but Kikuchi *et al.* (1959) and Kikuchi and Ban (1961) maintain that two cytochrome systems exist, the first similar to the classical one, and the second involving a b-type cytochrome oxidase. The former view fits better with the observations made on eggs, but information is not yet complete and it is important to determine whether under *in vivo* conditions a cytochrome component is involved in the reduction of fumarate by *Ascaris*.

Interest in electron transport in nematodes other than *Ascaris* is sporadic, and a full account of many isolated experiments would occupy too much space here, even if a meaningful synthesis of the results could be achieved. Important

observations by Krusberg (1960) on respiratory metabolism in the plant pathogens *Ditylenchus triformis* and *dipsaci* reveal several enzymes of the tricarboxylic acid cycle as well as malate decarboxylase (malic enzyme). In *D. triformis*, NADH-cytochrome c reductase and cytochrome oxidase systems were present. Extracts of this nematode showed the presence of cytochromes with absorption bands at 550 and 605 mμ, suggesting the presence of cytochromes c and $a+a_3$ respectively.

Warren (1965) showed that in *Ancylostoma caninum* mitochondria cyanide inhibited succinate oxidation, which suggests the presence of cytochrome oxidase. This agrees with observations on the larval stage of *Necator americanus* by Fernando (1963). Ozawa and Fukushima (1963) demonstrated succinic dehydrogenase, NADH oxidase, cytochrome c reductase and cytochrome oxidase in *Metastrongylus apri*, together with cytochrome b, and concluded that electron transport was accomplished through a cytochrome system. The succinoxidase system of *M. apri* was inhibited only partially by high concentrations of cyanide or antimycin A, which suggests that an alternative oxidase to cytochrome oxidase is also present. Moon and Schofield (1968) found cytochrome c reductase, and perhaps oxidase, which were insensitive to cyanide and antimycin A, in third-stage larvae of *H. contortus*. The larvae also possessed tricarboxylic acid cycle activity (Ward and Schofield, 1967). Finally, Roberts and Fairbairn (1965) found abundant cytochrome oxidase in *Nippostrongylus brasiliensis*, and considered that the flavin enzymes were not important in respiration.

There is little information about the phosphorylating efficiencies of electron transport in nematodes. Oxidative phosphorylation is a notoriously labile process, which may explain the reluctance of biochemical parasitologists to attempt its study. Accurate work is also dependent on pure and tightly coupled mitochondrial preparations, and, as already mentioned, there are few satisfactory techniques available for the extraction of mitochondria from helminths.

Chin and Bueding (1954) studied phosphorylation in particulate fractions and homogenates of *Ascaris* muscle; phosphorylation was dependent on NAD, on a dialysable component of perienteric fluid, and on oxygen uptake. It was uncoupled by 2 : 4-dinitrophenol. Seidman and Entner (1961) used a particulate fraction from *Ascaris* which lacked cytochromes and the enzymes of the tricarboxylic acid cycle; the metabolism of malate was associated with the esterification of ADP to ATP and the process was not oxygen-dependent. Chance and Hagihara (1960) have shown that in mammals succinate-linked NAD reduction requires ATP. In helminths, where fumarate is converted to succinate in the presence of NADH, it is possible that ADP may be converted to ATP. This view is supported by the work of Katsume and Obo (1963), who demonstrated anaerobic phosphorylation in the presence of NADH and fumarate in *Ascaris* muscle. In developing *Ascaris* eggs, Costello and Smith (1964a) have demonstrated an adenosine triphosphatase similar to that of mammalian mitochondria, indicative of the partial reactions of oxidative phosphorylation in mammalian systems (Lehninger, 1964), which may further reflect the differences between the adult and the developing eggs.

Brezna and Lestan (1964) and Zmoray and Lestan (1966) indentified a catalase in the narrow subcuticular zone near the muscle layer in *A. suum*. This is similar to the distribution of peroxidase in the cestode *M. expansa* (Cheah, 1967c). Costello and Smith (1964b) pointed out that if the flavoprotein oxidase described by Kmetec and Bueding (1961) is operative in unembryonated eggs, the subsequent accumulation of hydrogen peroxide would be toxic. They detected catalase activity in unembryonated and embryonated eggs in excess of that necessary for the decomposition of peroxide formed, and suggested that it might form a protective mechanism against peroxide toxicity. In support of this idea we know that in the initial stages of development, catalase activity remains high as the rate of respiration increases, and falls as respiration declines during the third stage of development. Unfortunately, in stage four both cytochrome oxidase and catalase activities rise sharply which suggests that catalase activity is not linked to the flavoprotein oxidase. The reason for the rise is unknown, but Costello and Smith (1964b) suggest it may be related to the future infectivity of the larvae.

D. ACANTHOCEPHALA

The Acanthocephala is a rather neglected group of parasites, perhaps because it is relatively unimportant economically and medically. The worms merit more attention because they damage their hosts very little and are well adapted parasites. The account of the Acanthocephala which has already appeared in this series considers electron transport in some detail (Nicholas, 1967). In summary, lactic, acetic and formic acids are excreted by *Moniliformis dubius* under anaerobic conditions (Laurie, 1957). Succinic acid has been shown to accumulate and lactic acid also is an excretory product in *Polymorphus minutus* (Graff, 1965; Bryant and Nicholas, 1965; Crompton and Ward, 1967a). Crompton and Ward (1967b) demonstrated that ethanol is the major excretory product when *M. dubius* is maintained under anaerobic conditions. Studies on *M. dubius* by Graff (1965) and Bryant and Nicholas (1965), using radioactively labelled substrates, indicated that most of its metabolism is mediated as in other helminths by the Wood–Werkman pathway. Their conclusion that the tricarboxylic acid cycle had little relevance to intermediary metabolism in *M. dubius* was later confirmed in *Macracanthorhynchus hirudinaceus* by Dunagan and Scheifinger (1966), who were not able to detect aconitase and isocitric dehydrogenase activities. However, Crompton (1965) demonstrated the presence of isocitric dehydrogenase in *P. minutus* by histochemical techniques.

Bryant and Nicholas (1966) made a preliminary study of electron transport in *M. dubius*; cytochrome c was readily reduced by the worm, but the rate of reoxidation was slow. Their final conclusion was that a branched electron transfer pathway exists, the major component involving cytochrome b, and a minor component involving a terminal oxidase similar to cytochrome oxidase. This is similar to the pathway subsequently worked out for *M. expansa* (Cheah and Bryant, 1966; Cheah, 1967a) but the limitations discussed previously regarding the integrity of the preparations apply here also. Spectrophoto-

metric examination of the cytochromes of both *M. dubius* and *M. hirudinaceus* revealed the characteristic absorption band at 557 mμ (Bryant and Nicholas, 1967, unpublished data), and hence we may expect that electron transfer in Acanthocephala will involve pathways as indicated for cestodes.

V. Electron Transport in Parasitic Protozoa

A. INTRODUCTION

The great volume of work which has been carried out on many parasitic Protozoa makes it sensible to consider them here. However, the treatment will be brief because of recent reviews by Baernstein (1963), Ryley (1967), Honigberg (1967) and Danforth (1967). The Protozoa are discussed only in relation to a greater understanding of changes in the electron transport systems of parasitic organisms and only forms studied in some detail are included. Even in these cases information may be incomplete and conclusions somewhat contentious.

B. TRYPANOSOMATIDAE

Hoare (1957) divided the trypanosomes into several taxonomic groups, and the various modifications in respiratory metabolism within the Trypanosomidae correlate reasonably well with these divisions.

In Group I are placed the trypanosomes of the *lewisi* group, which differ from the others in various ways, notably marked respiratory sensitivity to cyanide and azide (Baernstein, 1953a, b; Ryley, 1956; Fulton and Spooner, 1959; Zeledon, 1960d). Much work on *T. cruzi* provisionally identified spectral absorption bands diagnostic of cytochromes a and b, but cytochrome c was not detected. Some confusion exists about the presence of cytochrome oxidase in these organisms, because Baernstein (1953a) reported no inhibition of respiration with carbon monoxide, whereas Fulton and Spooner (1959) showed that oxygen consumption in the presence of this inhibitor was reduced by 30%. Baernstein (1953a) and Seaman (1956) detected little or no cytochrome oxidase activity in homogenates. Although succinic and NAD-linked dehydrogenases reduced cytochrome c *in vitro*, these observations may not have any significance *in vivo*, as the reduction of added cytochrome c depends largely on the redox potentials of the components of the electron transport system under consideration, which are obviously non-specific.

The path of electron transport in *T. cruzi* is still far from clear. The absence of a component is far more difficult to establish than its presence and it is not possible to affirm that cytochrome c and cytochrome oxidase are absent. However, the data obtained with the inhibitors suggest that some cytochrome mediated electron transfer occurs. The presence of a fumaric hydrogenase, as distinct from a succinic dehydrogenase, detected by Baernstein (1953a, b), may point to a mechanism similar to those described in Section IV for helminths. *Schizotrypanum vespertilionis* seems to be identical with *T. cruzi* in all aspects of its respiratory metabolism so far examined (Zeledon, 1960a, b, c, d). *T. lewisi*, on the other hand, apparently contains all the components of an orthodox cytochrome system, although the low activity of cytochrome oxidase remains a puzzling feature (Ryley, 1951; Fulton and Spooner, 1959; Grant *et al.*, 1961).

The remaining groups of trypanosomes are considered by Hoare (1957) to be more advanced. In Group II, *T. vivax* is the species for which most information about cytochromes is available. Desowitz (1956) reported partial inhibition of respiration by cyanide, but subsequent workers have not been able to confirm this observation. Carbon monoxide is also not an effective inhibitor and spectrophotometric evidence for cytochromes or evidence of cytochrome oxidase activity has not been obtained (Ryley, 1956; Fulton and Spooner, 1959).

In the bloodstream form of *T. congolense*, a similar condition exists (Fulton and Spooner, 1959). In contrast to previous reports (Ryley, 1956; von Brand, 1951, 1956, 1960) these workers could not detect any effect of cyanide on respiration, carbon monoxide was not effective, and azide only slightly inhibitory. There was no evidence of cytochromes or cytochrome oxidase and it seems unlikely that electron transport is mediated by an orthodox cytochrome system in the bloodstream forms of this trypanosome; nevertheless, a report of increased cyanide sensitivity indicates that cultured forms may contain cytochromes, if on only tenuous evidence (von Brand and Tobie, 1959).

In Group IV (the *brucei* group), *T. rhodesiense* has been most extensively studied. Once again, the bloodstream and culture forms differ in their mechanisms of electron transport. The culture form is cyanide sensitive, and cytochrome a (but not cytochrome b) has been detected in it. Homogenates are unable to reduce added cytochrome, an observation which, with some reservation, suggests that a system other than the classical electron transport system may be operative, and there is some evidence for the presence of a cytochrome system insensitive to both cyanide and antimycin A (Grant *et al.*, 1961; Ryley, 1962). In contrast, the bloodstream form of *T. rhodesiense* is insensitive to cyanide and there is no clear evidence for the presence of an electron transport system dependent on cytochromes (Fulton and Spooner, 1959; Grant *et al.*, 1961; Ryley, 1956, 1962). Transport of electrons is mediated by the coupling of an Lα-glycerophosphate oxidase with an NAD-dependent dehydrogenase.

Other members of the Trypanosomidae are much less well known. Like the trypanosomes, the respiration of the intracellular form of *Leishmania donovani* was found to be less sensitive to cyanide and azide than the culture form (Fulton and Joyner, 1949; Chatterjee and Ghosh, 1959). In *Strigomonas oncopelti*, a parasite of the milkweed plant, spectrophotometric studies show the presence of cytochromes a, b and c. Respiration is sensitive to cyanide, azide and carbon monoxide, and although cytochrome oxidase activity is low there is reasonable evidence to suppose that electron transport is achieved by an orthodox cytochrome system. The oxidation of NADH, however, may take place by an alternative route (Fulton and Spooner, 1959; Ryley, 1962).

C. TRICHOMONADIDAE

The trichomonads are parasitic chiefly in the digestive and reproductive tracts of animals, and their respiratory metabolism is characterized by incomplete oxidations even when oxygen is readily available, as in the trypanosomes. They appear to be adapted to oxygen-deficient environments (Danforth,

1967). In *Trichomonas vaginalis*, cyanide and azide have little effect on oxygen consumption (Ninomiya and Suzuoki, 1952; Read and Rothman, 1955). Increasing the concentration of oxygen inhibits oxygen uptake by these organisms, and peroxide is an end product of respiration (Baernstein, 1955). These observations implicate flavoproteins as oxidases in electron transport. Ryley (1955) found spectrophotometric evidence for cytochromes in *T. foetus*, which is also insensitive to cyanide and azide, but detected no cytochrome oxidase activity. Catalase was present in *T. foetus*, although peroxide still impaired its motility.

Insufficient evidence is related to other trichomonads, but from the few data obtained, their metabolic organization is similar to that of *T. vaginalis* and *T. foetus*, i.e. electron transport does not involve an orthodox cytochrome system; it is doubtful if any cytochromes are involved. The presence of catalase and the possible production of peroxide provide an interesting parallel with the metabolism of several parasitic helminths under conditions of oxygen plenty.

D. Sarcodina and Sporozoa

Entamoeba histolytica alone of the parasitic amoebae has received much attention. Cytochrome pigments have not been detected in this amoeba (Hilker and White, 1959) and cyanide and azide inhibit growth only at high concentrations (Yang, 1959).

In the Sporozoa, the medical importance of *Plasmodium* has resulted in much work but most studies have been restricted to the erythrocytic stages. The problems of obtaining pure preparations of parasites are therefore great and even slight contamination with host haemoglobins can make the spectral identification of cytochromes very difficult. The environment in which the parasites live is rich in oxygen and on *a priori* grounds we might expect to find an array of classical cytochromes, but such information is not available.

The respiration of *Plasmodium gallinaceum*, *P. berghei* and *P. knowlesi* was found to be sensitive to cyanide, and *P. knowlesi* is sensitive also to carbon monoxide (Wendel, 1943; McGee *et al.*, 1946; Marshall, 1948; Fulton and Spooner, 1956), which suggests that cytochrome oxidase may be present. Another blood parasite, *Toxoplasma gondii*, is also cyanide sensitive and has been shown by Fulton and Spooner (1960) to possess cytochromes a, b and c.

VI. Conclusions

In trying to draw any conclusions about the ways in which parasites effect the processes of electron transport and terminal respiration, several limitations must be borne in mind. First, it is unwise to generalize on the basis of work done on vastly different groups of organisms such as the parasitic Protozoa, Platyhelminthes, Nematoda and Acanthocephala. Although they often occupy the same class of environment, a comparison may be only as valid as one between insects and birds. The environment provides a challenge but this has been met by ways which are analogous rather than homologous. Similarly, even within a group such as the Platyhelminthes, a comparison of

Cestoda and Trematoda may be equally inept, although these classes may be more closely related to one another than either is to the Nematoda. The second limitation is that most of the work has been done with *in vitro* systems. Until there are adequate techniques for investigating physiological and biochemical parameters both in the environment and in the parasite without effectively disturbing the host–parasite inter-relationship, such work is, at best, a rough approximation to reality. Add to this the problem that the investigator has often to study simultaneously enzymes from all differentiated tissues within the parasite—ludicrously akin to the mammalian biochemist working on homogenates of whole rats—and it is rather surprising that any reliable conclusions emerge.

The work on helminths has not proceeded to a point where a sufficient number of organisms have been studied to permit confident conclusions, but at least one representative of the four major groups has been examined in some detail. Thus, this final section will consider what has emerged from studies of *M. expansa* and *E. granulosus* (Cestoda), *Ascaris* spp. (Nematoda), *F. hepatica* (Trematoda) and *M. dubius* (Acanthocephala). The position of the Protozoa will be discussed with the object of comparing their processes of electron transport with those of the helminths. The relevance of the heterodox systems found in these groups of organisms to the general problem of parasitism will be considered.

With the single exception of *F. hepatica*, the helminths mentioned in the previous paragraph are parasitic in the alimentary canal of mammals, and the liver fluke belongs to a group with this characteristic. All the parasites are exploiting environments in which one could expect to find an adequate supply of highly reduced molecules, but an inadequate supply of oxygen for their oxidation. Rogers (1949) determined oxygen tensions as high as 30·2 mm Hg in the sheep's gut, but the mean values were generally lower. More accurate data are needed. For a cytochrome system to function efficiently with a terminal oxidase, minimum oxygen tensions of the order of 5 mm Hg are required (Hill, 1936). It is possible for an orthodox cytochrome system to function in such an environment as the gut, although the oxygen tension could drop to levels at which it was rate-limiting, so that an organism occupying this environment should have an alternative system instead of, or as well as, the orthodox system. Only then could the respiratory processes be liberated from the control of the environment. *Echinococcus granulosus*, which may have access to significantly higher concentrations of oxygen, is noteworthy in this context, because its tricarboxylic cycle activity is greater than that of other helminths.

Moniezia expansa, *F. hepatica*, *Ascaris* spp. and *M. dubius* react uniformly to their environments in that carbon dioxide fixation, followed by the conversion of the fumarate so formed to succinate and other highly reduced excretory products, is a very important metabolic pathway. In all these cases fumarate is substituting for oxygen in terminal respiration. The source of the fumarate is effectively carbon dioxide and carbohydrate breakdown products, of which there is an ample supply either in the intestines of the host or generated within the parasite. Even in *E. granulosus* succinate accumulation is significantly high.

The mechanisms by which fumarate is converted to succinate need further clarification. In *M. expansa*, *F. hepatica* and *M. dubius* there is strong evidence to suppose that cytochrome components are involved. In *Ascaris* spp. it may be accomplished either by a flavoprotein or by cytochrome, depending on which school is upheld. If the system is flavoprotein in nature, an explanation for the presence of cytochromes similar to those found in other helminths must be sought.

Although these pathways have an obvious function of permitting the reoxidation of reduced cofactors which have accumulated during metabolic processes, there is little advantage unless a concomitant regeneration of ATP occurs. Information in this area is sketchy, but it is probable that in nematodes and in *H. diminuta* ATP generation does, in fact, accompany the conversion of fumarate to succinate. Prichard (1968) has preliminary data suggesting that it is also true for *F. hepatica*.

When we try to include the parasitic Protozoa in this scheme, shortage of information allows only approximate conclusions, susceptible to reinterpretation. First, the apparent distinction between electron transport mechanisms in cultured trypanosomes and the blood-stream forms is probably real, because the morphology of the cultured forms resembles the stage found in insects. Second, the electron transport systems of trypanosomes, whether bloodstream or cultured forms, differ from the classical model based on observations in mammals and micro-organisms. There is considerable doubt in some cases whether or not it is mediated by cytochromes.

The second generalization similarly applies to the remaining Protozoa considered in this review. There are indications that the main source of difference involves the suppression of an oxygen-dependent terminal oxidase, in favour of an alternative pathway utilizing a different electron acceptor. To this extent, the parasitic Protozoa resemble the parasitic helminths.

The main point—that electron transport in parasites departs from the classical pattern—leads to the important question of whether this difference is directly related to the parasitic habit. In order to provide an adequate answer, it is necessary to examine free-living members of the various groups. This poses a number of problems; they may not exist, or they may be highly specialized and hence not representative of the group to which they belong, or the appropriate experiments may not have been carried out. In the free-living Protozoa which have been studied (*Tetrahymena*, *Paramecium* and *Amoeba*) alternative pathways of electron transport are present (see the detailed account by Danforth, 1967). The presence of alternative pathways, however, does not exclude the possibility that the classical cytochrome system may also be present. Little is known about the free-living helminths, although spectra obtained from homogenates of a fresh-water turbellarian, *Cura pinguis*, and a soil nematode, *Caenorhabditis briggsae*, are similar to spectra obtained from *M. expansa*, and indicate that the branched system may be widely distributed in nature (Bryant and Fletcher, 1966, unpublished data; Bryant *et al.*, 1967).

It seems likely, therefore, that the relationship between the branched respiratory chain and parasitism is not direct. It cannot be said that the adoption of the parasitic mode of life initiated fundamental changes in electron

transport mechanisms, although some changes must have occurred. It is more probable that the groups of organisms which became successful parasites already possessed these modifications because they were adapted to conditions with certain resemblances to parasitic environments. The intestines and many other parasitic locations are special cases of a whole class of environments in which oxygen tensions or the amount of *available* oxygen are low. Many other invertebrates show similar modifications (Simpson and Awapara, 1966). The success of parasites must be due partly to their derivation from non-parasitic groups adapted to such special environments as imparted to them enormous selective advantage.

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Onchocerciasis

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I. INTRODUCTION

Onchocerciasis, or "river blindness", is caused by the filarial worm *Onchocerca volvulus*, which occurs throughout the greater part of tropical Africa, especially in the rain forest regions and the savannah belt that stretches for more than 4000 miles from the Atlantic coast of Senegal to the Indian Ocean in Tanzania. This is the primitive home of the disease, the great endemic area where more than 30 million people are infected. Other foci occur in the Yemen, in Guatemala, Mexico, Venezuela and Colombia.

Onchocerciasis ranks as one of the world's most formidable infectious diseases yet the very name is unfamiliar to most medical men. It is a disease of

underdeveloped and underdoctored rural areas. Although onchocerciasis is rarely mentioned in official morbidity statistics entire communities are afflicted with the unsightly and irritating onchocercal dermatitis, and blindness rates due to this infection often exceed 10%.

It is more than 40 years since Blacklock discovered that *O. volvulus* is transmitted by *Simulium* flies, and although there have been some successful control projects, the greater part of the endemic region has been unaffected by the "advances" in knowledge. With the improvement of medical services in the rural areas many "new" foci of the disease are being discovered and onchocerciasis is now receiving more attention not only from the local public health authorities but also from international bodies, particularly the World Health Organization. Throughout the world, however, no more than a dozen scientists are devoted to the study and control of this disease. The present review is not designed for this small group of experts; it aims to bring onchocerciasis to the attention of biologists, clinicians and public health workers in order to stimulate interest in a fascinating but formidable problem.

Onchocerciasis is an economically important disease, but not a killing disease. It incapacitates large sections of the community and makes them an economic burden. Control of the parasites and their vectors can do nothing but good; it will not increase the birth rate and produce more mouths to feed, but it will release whole communities from an intolerable burden of misery. Also, by ridding the countryside of biting simuliids, vast fertile areas will be freed for agricultural development. Total eradication is still remote but even with the application of existing knowledge and methods of control the intensity of transmission can be so restricted that many areas can be completely freed of this scourge.

II. Geographical Distribution of *O. volvulus*

With the gradual and increasingly successful elimination of many of the more lethal infectious diseases from the tropics, more attention is being given to the less dramatic but often more prevalent helminth infections such as onchocerciasis. In the past many extensive endemic areas escaped detection because the local medical staff, with a doctor-patient ratio of less than one to 50000, had to deal with many other pressing problems. Also the very few trained ophthalmologists in the tropics are almost all confined to urban areas where there is no onchocerciasis. In the rural areas many thousands of people with onchocercal eye disease remain undiagnosed because they never have the opportunity of being examined by anyone competent to make a specific diagnosis. Most of the so called "new" foci have been discovered fortuitously.

The extensive focus in Kenya, which includes the famous "Valley of the Blind" at Kodera, was discovered quite by chance when a pathologist found *O. volvulus* adult worms in a "tumour" sent for examination to the central pathology laboratory in Nairobi (Preston, 1935). Similarly the disease was discovered in the Yemen because Fawdry (1957), who was working in Aden, had noted that a peculiar skin condition known as "Soda" or "Sowda" which was seen in Yemeni patients was in fact onchocerciasis. It was also by chance

that an extensive new focus of the disease was discovered in Uganda because a District Medical Officer working with a very limited budget wanted to know why the local dispensaries were using excessive quantities of sulphur ointment and benzyl benzoate for treating scabies; the "scabies" turned out to be onchocerciasis and it was subsequently shown that some thousands of people were infected within a few miles of the central hospital (Nelson, 1958a). The "cryptic" nature of the disease was emphasized by the fact that for more than 20 years the whole population in this part of Uganda had been inspected annually by medical personnel as part of the sleeping sickness control programme yet onchocerciasis had never been recognized.

Other "new" foci have been discovered when ophthalmologists have seen microfilariae in the eyes of patients attending routine eye clinics, often hundreds of miles from the endemic areas. In Colombia recently the disease was recorded for the first time when a patient from the northern region was examined by chance by an ophthalmologist in Bogotá and microfilariae were seen in the eye. Subsequent investigations revealed that the infection must have been established for many years in Colombia (Assis-Masri and Little, 1965).

Since the discovery of onchocercal eye disease by Pacheco-Luna (1918) and Robles (1919) in Guatemala it was often ophthalmologists working under difficult conditions who demonstrated that onchocerciasis is a serious public health problem. Hisette (1932) in the Congo, Bryant (1935) in the Sudan, Ridley (1945), Budden (1957) and Rodger (1959) in the former British West African territories, Puyuelo and Holstein (1950), D'Haussy *et al.* (1958), Quéré *et al.* (1963), Toussaint and Danis (1965) and Lagraulet *et al.* (1967) in the former French territories; these are only a few of the ophthalmologists who have helped to map the extent of the disease in Africa.

However, the most important contributions to our knowledge of the distribution of this infection have been made by non-medical personnel, by entomologists and biologists: e.g. Gibbins (1939), Barnley (1949), Barnley and Prentice (1958), McCrae and Prentice (1963) and McCrae (1965) in Uganda; Buckley (1949) and McMahon *et al.* (1958) in Kenya; De Meillon (1934, 1957) in South and Central Africa; Raybould (1957), Wegesa (1968) and Hausermann (1966) in Tanzania; Lebied (1950) in the Congo; Lewis (1948, 1953a, 1960a) in the Sudan and many other parts of Africa; Crosskey (1957a), Crosskey and Crosskey (1959) and Davies (1963, 1965) in Nigeria; Crisp (1956), Marr and Lewis (1964) and Noamesi (1964) in Ghana; Garms and Post (1966) in Guinea; Ovazza (1953), Grenier *et al.* (1955) and Le Berre (1966) in many parts of French West Africa; Hoffman (1930) in Mexico; Gibson and Dalmat (1952), Dalmat (1955) and De Leon (1957, 1963) in Guatemala; Lewis and Ibáñez de Aldecoa (1962) in Venezuela. By studying the vectors and the transmission of the disease these workers have greatly extended our knowledge of the distribution of the infection in man.

The cryptic nature of the disease was also dramatically demonstrated in 1958 when Dr. Giaquinto, who was on an official visit from the World Health Organization, discovered a focus of transmission almost within the compound of the "Vector-Borne Diseases Institute" at Amani in Tanzania. At that time the nearest known focus was several hundred miles away but it is now known

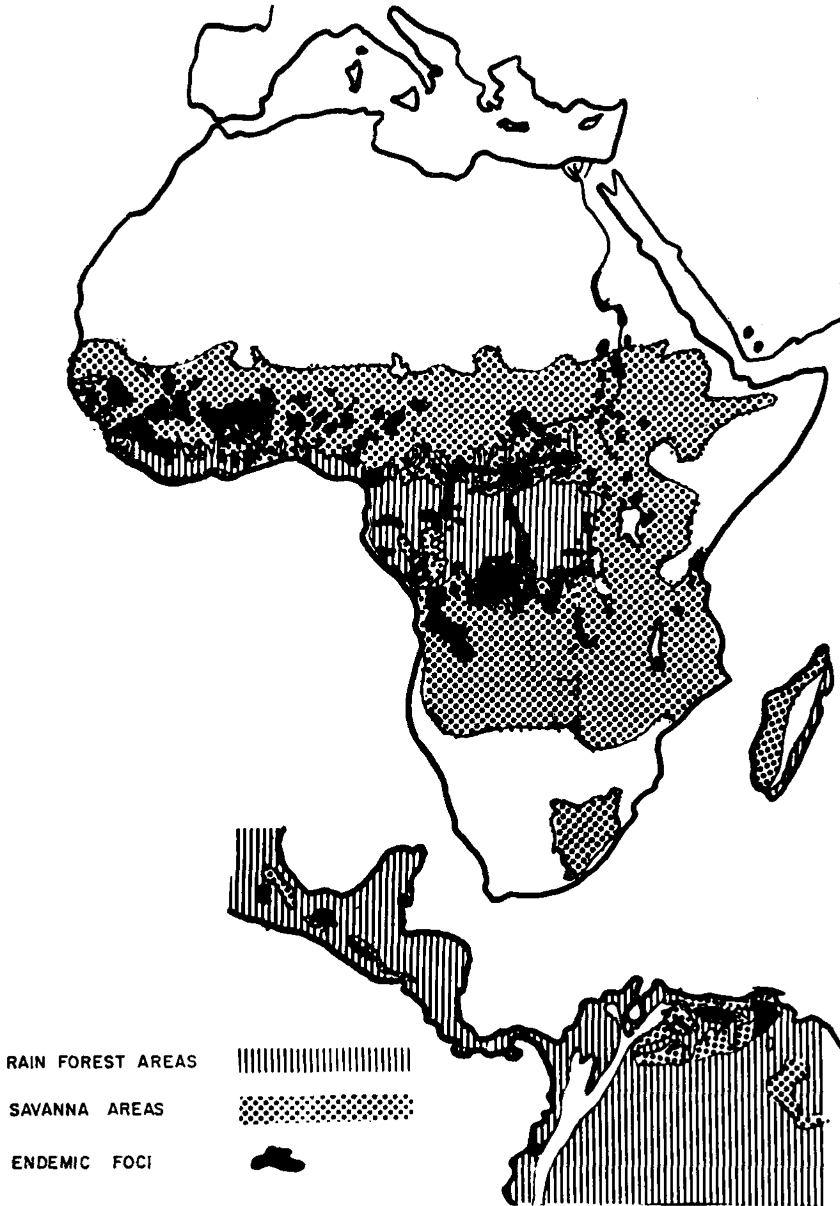


FIG. 1. The geographical distribution of onchocerciasis in man.

that the disease is widespread in Tanzania (Raybould, 1968). Amani is situated among thickly wooded hills with swift flowing streams and plenty of shade; anyone interested in landscape epidemiology would have suspected that there might have been transmission of onchocerciasis in this area. The discovery by

Morgan (1958) of onchocerciasis in the villages along the Nile near the Egypt–Sudan border was, however, quite unexpected; no landscape epidemiologist in his right mind would have suspected that transmission could have occurred in this dry, arid desert region of the Sahara.

Further studies will no doubt show that onchocerciasis is far more widespread than is indicated in Fig. 1. It should be looked for in all parts of the world where simuliids bite man. The disease may not occur in Arctic regions but there is a possibility that it will be found in other areas, especially in Asia and South America where the rivers flowing from the great mountain chains are often ideal breeding places for simuliids.

III. *O. volvulus*, Geographical Variants

Onchocerca volvulus is not a uniform species; there are distinct strains or biological variants of the parasite each with its own inherent properties which determine the pattern of transmission and the subsequent clinical picture in man. As long ago as 1919 Brumpt noted striking differences in the epidemiology of onchocerciasis in Central America and Africa and suggested that the American form should be given specific status under the appropriate name of *O. caecutiens*. It was then believed that the Central American form affected mainly the eyes and that the African form of onchocerciasis was mainly a disease of the skin, but it is now realized that there is far more onchocercal blindness in many of the individual countries in Africa than in the whole of the Central American focus, and Brumpt's specific designation has not been accepted. However, more recent studies by De Leon and Duke (1966) suggest that the Central American and African parasites are at least distinct strains or races of *O. volvulus*. This is also emphasized by the detailed comparative clinical studies undertaken by Woodruff *et al.* (1966a, b) in Tanzania and Guatemala.

Recent observations suggest that the widely held belief that helminths are uniform species is untrue, and with helminths of medical importance strain variants are in fact the rule rather than the exception (Nelson *et al.*, 1966a; Nelson and Saoud, 1968; Nelson, 1969). Variations in the biological properties of geographical variants are known to determine the epidemiology of many helminthic diseases and this is especially true of other filarial infections, e.g. *Wuchereria bancrofti* and *Brugia malayi* with periodic and subperiodic forms each adapted to different vectors, and *Loa loa* with human and simian strains which are quite distinct (Duke and Wijers, 1958). *O. volvulus* is no exception. Apart from the differences between the African and American forms of *O. volvulus* there is also a good deal of clinical and pathological evidence suggesting that there are several variants of *O. volvulus* in Africa itself: the savannah and forest forms in West Africa differ quite markedly in their vector infectivity, their infectivity to chimpanzees and in their general epidemiology and clinical manifestations (Duke *et al.*, 1966, 1967b; Lewis and Duke, 1966; Duke, 1966a, 1967a), and there are also marked differences in the clinical manifestations of onchocerciasis in West and Eastern Africa.

The differences between the Central American and African strains of *O. volvulus* are so great that De Leon and Duke (1966) have suggested that the commonly accepted view that the parasite was introduced into Central America with the slave trade is probably incorrect. They argue that the parasite is indigenous and that the clear differences in the clinical picture and differences in vector susceptibility are unlikely to have evolved in the relatively short period since the days of the slave trade. In support of this argument is the observation that at the present time the disease is much more prevalent in the indigenous Amerindians in Central America than it is amongst the exotic negroes. However, this argument is open to question. At one time negro slaves were far more prevalent in some of the Central American areas than they are today and large numbers were wiped out or dispersed at the time of their emancipation. However, Diaz (1957), Figuerao (1963), Rivas *et al.* (1965) and Fragosa Uribe (1966) have all produced further arguments suggesting that the parasite has been long established in the Americas. It is claimed that apart from the distinct clinical picture in present-day man there is evidence of infection in Pre-Colombian skeletal relics; circular erosions in the skulls are thought to resemble erosions produced by *O. volvulus* nodules on the head. It is also claimed that the roughened skin seen in some of the Maya sculptures represent onchodermatitis. But none of these arguments is very convincing. Circular erosions of the skull are commonly seen in people in the onchocerciasis areas in Kenya but here this is due to the common witch-doctor practice of trephining (Grounds, 1958). Also it is difficult to see how the parasite could have become established in this region except by a mass movement of population from endemic areas such as occurred with the slave trade, or by the transfer of the infection to man from a primary animal maintenance host. (The zoonotic aspects of this infection are dealt with in the next section.)

It is not generally realized how quickly helminths can change their biological properties. There has certainly been ample time for the development of the characteristic features of the Central American *O. volvulus* during the period since the African slaves were first settled in the Americas more than 400 years ago. Almost all the helminths affecting man are actively evolving and producing new variants which are adapted to changing ecological conditions. This type of adaptation is particularly true of filarial worms which are rapidly modified when they are given opportunities of meeting new vectors. This has been demonstrated in the laboratory by Laurence and Pester (1967), who have shown that the infectivity of *Brugia patei* for *Aedes togoi* can be increased from about 40% to nearly 90% after only four cycles of transmission through domestic cats.

IV. Possible Animal Hosts of *O. volvulus*

Onchocerciasis is not normally classified as a zoonosis, i.e. an infection naturally transmitted between man and animals, and *O. volvulus* is generally included in the rather restricted group of parasites that are maintained in nature entirely by interhuman transmission (Nelson, 1965). However, natural infections with *O. volvulus* have been recorded in a spider monkey, *Ateles geoffroyi*, by Caballero and Barrera (1958) and in a gorilla, *Gorilla gorilla*, in

the Congo by Van den Berghe *et al.* (1964). Also Duke (1962a) has shown that the chimpanzee *Pan satyrus* is a good laboratory host for *O. volvulus* although this animal has not been found naturally infected.

Attempts have been made to infect other species of primates and a variety of laboratory animals with *O. volvulus*, but so far without success. It may be that the wrong animals have been used. In our experiments in Kenya we used a variety of primates but *O. volvulus* is the only species in the genus which is found in primates; all other species of *Onchocerca* are parasites of herbivores and it is not easy to distinguish the microfilariae of the adult worms of the different species found in cattle and wild antelopes from *O. volvulus*. There is still a possibility that some of these animals may be reservoir hosts as suggested by both Cameron (1928) and Strong (1937). These suggestions have been neither confirmed nor refuted. The present work on bovine onchocerciasis at the London School of Hygiene and Tropical Medicine Field Station at Winches Farm is designed in part to study this problem.

There have been instances of *Onchocerca* infection in persons who have never left Western Europe. Osborn (1935) reported a case in Liverpool, Siegenthaler and Gubler (1965) a case in Germany and there is one record of human infection in the Southern U.S.S.R. (Azarova *et al.*, 1965). In every case only adult worms have been recovered and it seems likely that the infection was transmitted to man from domestic animals; but the Liverpool patient was a dock-worker handling cotton and it was suggested that he might have been bitten by an infected simuliid that had been imported with the cotton! Since the vectors of the species of *Onchocerca* which infected domestic animals in Europe are markedly zoophilic and always very lightly infected, there is very little opportunity in these situations for the infection to become established in man. But in other areas, e.g. in parts of Africa, the same species of simuliid may be transmitting both the bovine and human *Onchocerca* and it is in these situations that onchocerciasis may be a true zoonosis.

V. Pathogenesis of Onchocerciasis in Man

A. The Adult Worms and Nodules

Irrespective of the strain of parasite the most severe clinical manifestations of onchocerciasis are due to reactions to the presence of the microfilariae. The adult worms are usually of secondary importance. This is in contrast to *Wuchereria* and *Brugia* infections where the main pathology is caused by developing or adult worms. In the early stages of onchocerciasis and in children and in lightly infected individuals the adults of *O. volvulus* produce no detectable reaction and they cannot be palpated. Autopsy studies by Nnochiri (1964) indicate that at this stage of the infection the worms are free in the subcutaneous tissue or merely enclosed in loose fatty or areolar tissue. They may be encountered by chance in operations for hernias (Becker, 1950) and it is not unusual to see worms extruded from abscesses in different parts of the body where they had never been suspected (Oomen, 1967a).

In long standing infections a proportion of the adult worms are found in the characteristic nodules which can be felt and sometimes seen especially over bony prominences. Many of these nodules contain dead or dying worms (Israel, 1959). They occasionally suppurate after treatment or when the patient has concomitant septicaemia (Barlovatz, 1940; Rodger, 1962).

In the African forms of the disease the nodules are mostly distributed around the pelvis especially over the iliac crest, the greater trochanter of the femur, the coccyx and sacrum; a few nodules are found around the knees and other common sites are over the lateral chest wall and spine. Very few nodules are found on the head or upper limbs. In Central America the nodules tend to be more in the upper part of the body, but in Venezuela the distribution more closely resembles the pattern of distribution seen in Africa.

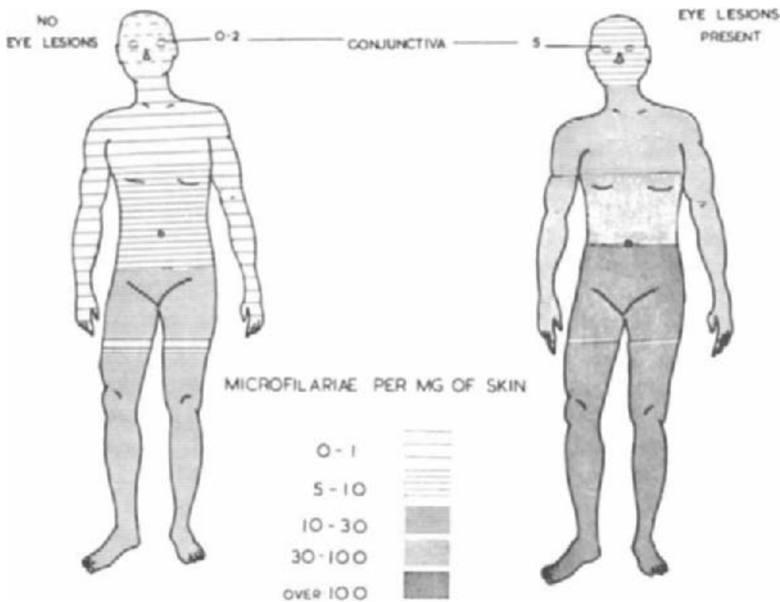
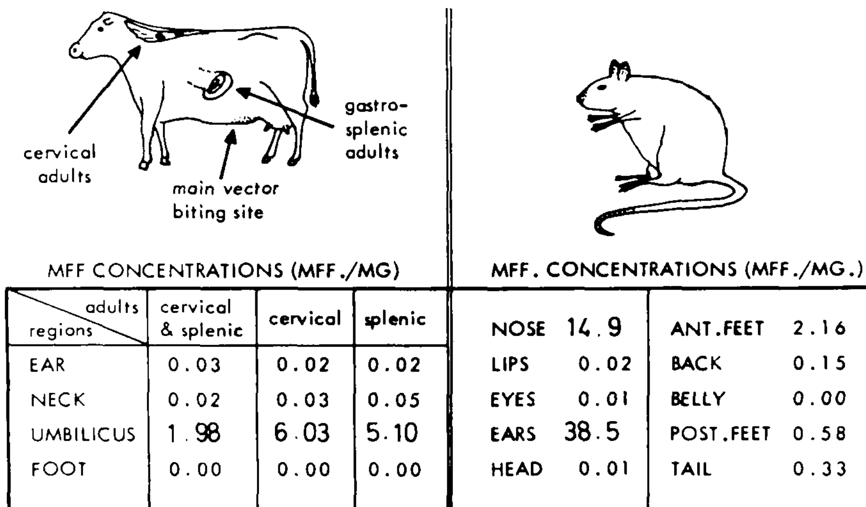


FIG. 2. The distribution and density of microfilariae in the skin in West African onchocerciasis. (Modified with permission from Kershaw *et al.*, 1954.)

The anatomical distribution of adult worms is believed to determine the distribution of the microfilariae and Kershaw *et al.* (1954) have shown that in Africa there is a correlation between the position of the nodule and the density of microfilariae in the skin. In West Africa where the nodules are mainly in the lower part of the body the density of microfilariae in the skin is still highest in the lower extremities even when there are eye lesions. The eye lesions develop in those patients where there are high density infections and where the microfilariae "spill over" into the skin of the upper part of the body (Fig. 2). In East Africa the distribution pattern is different and the main concentration of microfilariae is around the buttocks and upper thigh; the skin below the knees is less affected (Nelson, 1958a; Woodruff *et al.*, 1966a). In Mexico,

Mazzotti (1951a) examined a total of 3310 skin biopsies from 16 patients with onchocerciasis and found that the microfilariae were most easily detected in the skin around the shoulders. This was not seen in a recent study in Guatemala where Woodruff *et al.* (1966b) and De Leon and Duke (1966) found that the highest densities were around the torso, but many of their patients had been subjected to nodulectomy and the removal of worms from the upper part of the body may have modified the distribution of microfilariae.

It has been suggested that the adult worms are in the lower part of the body in the African form of the disease because the African vectors are low biters, the adult worms developing in the anatomical region where the infective larvae are deposited. The reverse is thought to be the case in Central America where the vectors prefer the upper part of the body. If this were true the site of inoculation would also be of considerable importance in determining the pathogenicity in onchocerciasis, in the same way as in filariasis due to *W. bancrofti* and *B. malayi*. In these infections where the mosquito vectors such as *Culex fatigans* and *Mansonia* bite preferentially the lower limbs it has been suggested that it is the low-biting which is responsible for the relatively high rates of elephantiasis of the leg as compared with elephantiasis of the arm (Wharton, 1960; De Meillon and Sebastian, 1967). However, the site of inoculation of the infective larvae may not be the main factor determining the eventual site of the adult worms. According to Duke (1968a) the adult worms of *O. volvulus* always develop around the hip joint in chimpanzees irrespective of the site of inoculation. The parasite seems to have its own directional mechanism. This is also true for *O. gutturosa* of the cow although the infective larvae of this species are deposited in the region of the umbilicus and the adult worms tend to develop around the cervical ligament in the neck. Our studies on *O. gutturosa* indicate that the microfilariae also have a well developed directional mechanism



MFF CONCENTRATIONS (MFF./MG)

regions \ adults	cervical & splenic	cervical	splenic
EAR	0.03	0.02	0.02
NECK	0.02	0.03	0.05
UMBILICUS	1.98	6.03	5.10
FOOT	0.00	0.00	0.00

MFF. CONCENTRATIONS (MFF./MG.)

NOSE	14.9	ANT.FEET	2.16
LIPS	0.02	BACK	0.15
EYES	0.01	BELLY	0.00
EARS	38.5	POST.FEET	0.58
HEAD	0.01	TAIL	0.33

FIG. 3. The distribution of microfilariae of *Onchocerca gutturosa* in the skin of naturally infected cattle and experimentally infected rats.

FIG. 4. Onchodermatitis. The first stage complicated by intense pruritis, a papular eruption and secondary infection.

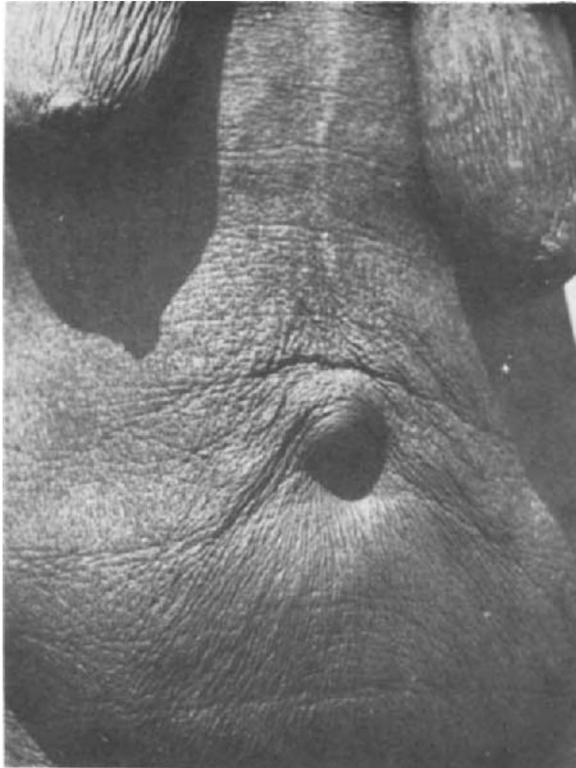
which determines their final position in the skin. They do not merely "sit around" in the skin close to their parent worms but they migrate through the tissues to the skin where there are the best opportunities of being picked up by the vectors. In naturally infected cattle irrespective of the position of the adult worms the microfilariae migrate preferentially to the umbilicus, where they

accumulate in large numbers (Fig. 3). This is a beautiful adaptation to transmission because more than 90% of the vectors bite around the umbilicus. Further studies are in progress to examine the factors responsible for the distribution pattern and it has been shown that when the microfilariae are inoculated into laboratory animals they accumulate in the skin of the ears irrespective of the original site of inoculation whether this be subcutaneous, intraperitoneal or intracardiac. (Nelson *et al.*, 1966b; Eichler and Nelson, 1968.)

B. SKIN LESIONS

Onchodermatitis usually begins with a pruritis which may be accompanied by a papular rash. There is often intense itching and scratching which may result in secondary infection (Fig. 4). In many patients the pruritis persists throughout the whole course of the disease, especially in Africa where there is no difficulty in recognizing an affected community because the whole population seems to be busy scratching. Oomen (1967a) has suggested that the condition of "macular dyspigmentation", consisting of hyperpigmented spots with depigmented centres, is a complication of this early stage of the infection. The relatively mild lesions may persist for weeks or years but some patients may have neither signs nor symptoms of the disease although microfilariae are readily seen in skin snips. If these patients are given a small dose (50 mg) of diethylcarbamazine they will usually develop an acute erythematous and pruritic reaction due to the killing of microfilariae; this is the basis of the diagnostic test introduced by Mazzotti (1951b) which dramatically illustrates the allergic nature of onchodermatitis. The changes in the skin due to diethylcarbamazine have been described by Hawking (1952). It is generally believed that the main clinical features in onchocerciasis are the result of an immunopathological response and the lesions are probably proportional in their severity to the number of microfilariae that die in the skin or eyes. However, there are many instances where the parasites produce no reaction, the patients exhibit no allergic response and high densities of microfilariae are found in the skin and in the anterior chamber of the eye with no other evidence of infection. Rodger (1962) is not convinced by the allergic hypothesis and he has suggested that the pathogenesis may be due to a direct toxic effect caused by the death of the parasites.

The second stage of onchodermatitis is characterized by thickening of the skin due to intradermal oedema, there is often a "peau d'orange" effect especially on the abdominal wall, and the lymph glands in the groin are often enlarged (Fig. 5). In Central America the patients may develop "Erisipela de la Costa" affecting usually the face or upper trunk, and there may be attacks of "Mal morado" with a purplish eruption resembling lichen planus affecting the upper part of the body. These conditions have not been reported in the African form of the disease but there have been occasional records of unilateral skin changes similar to the type of disease reported by Fawdry (1957) and Gasparini (1964) in the Yemen. The Yemeni cases are most unusual in that very few microfilariae are found in the skin. It may be that this is not *O. volvulus* but an abortive infection with some other species of *Onchocerca*.



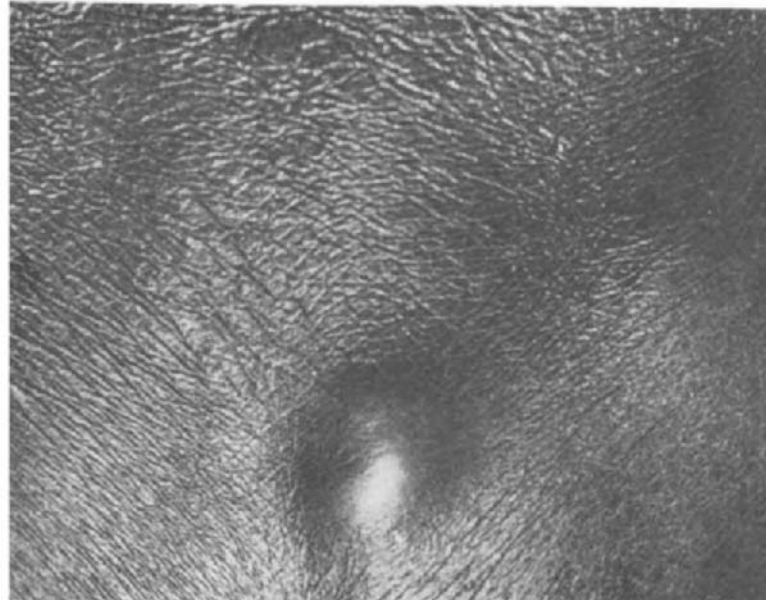


FIG. 5 FIG. 6

FIG. 5. Onchodermatitis. The second stage with intradermal oedema producing a "peau d'orange" effect.

FIG. 6. Onchodermatitis. The third stage with characteristic atrophy resulting in presbydermia, the patients often appearing prematurely aged. A prominent nodule can be seen in the lumbar region.

FIG. 7. Hanging groin. Both the femoral and inguinal glands are often involved. This complication is associated with atrophic skin and may predispose to hernia. Note the characteristic onchocercal depigmentation of the skin of the leg in one of the patients.

The concentration of microfilariae may be much higher on one side of the body than the other, suggesting that the female worms may be predominently unilaterial especially in early cases (De Leon and Duke, 1966). Eventually most

patients develop some degree of lichenification or thickening of the skin especially over the buttocks and thighs. This crocodile skin or pachydermia may be associated with the last stage of the infection, namely the presbydermic or atrophic stage (Hughes and Daly, 1951). Often it is impossible to distinguish the skin changes from those seen in Vitamin A deficiency, and Rodger (1962) has suggested that either the parasite may compete for Vitamin A in the skin or that a Vitamin A deficiency may predispose to heavier infections.

The last stage is characterized by atrophy of the skin with loss of elasticity, which gives the patient a prematurely aged appearance (Figs 6 and 7). This is one of the most distressing features of onchocerciasis, even relatively young members of the community appearing to be old and decrepit. The histological features of this stage of the infection have been illustrated by Van den Berghe (1941a), Wanson (1950), Jamison *et al.* (1955), Jamison and Kershaw (1956) and Hoeppli and Gunders (1962). Depigmentation is often a feature of chronic onchodermatitis (Fig. 7). It is usually most pronounced over the legs and it may be associated with skin papillomata (Kirk, 1947; Browne, 1959a, b). At one time this condition was frequently mistaken for leprosy and the author has seen several patients with onchocercal depigmentation in leprosaria. In these "burnt out" cases the microfilariae are often found in the depths of the dermis separated from the surface by fibrous tissue. They may therefore be inaccessible to the normal "humane" skin-snip-biopsy and to the bite of the *Simulium* (Kershaw, 1966).

C. HANGING GROIN, HERNIA AND ELEPHANTIASIS

The loss of elasticity in the skin is responsible for a relatively rare but a very striking clinical manifestation of the disease, the pseudoadenolymphocele of Rodhain (1952) or the "hanging groin" described by Nelson (1958b) (see Fig. 7). These pendulous sacs are most commonly seen in adult males; they often contain inguinal or femoral lymph glands and they may hang down as far as the knee. Cherry (1959) has described a similar complication in women which resembles the "hottentot apron", a genital distortion which was at one time fashionable in females in South West Africa. A "hanging" perineum has been illustrated as a complication of onchocerciasis in Ethiopia by Oomen (1967a). These complications have not been reported from Central America but the same type of pathological process with loss of elasticity may explain the condition of leonine faces, which is seen in onchocerciasis patients in Guatemala.

In Africa the loss of elasticity of the skin of the groin results in exceptionally high prevalence of hernias (Fig. 8) (Nelson, 1958b; Williams and Williams, 1966). Onchocerciasis is undoubtedly one of the most important causes of hernia and especially femoral hernia in man, yet very few surgeons have recognized the association. Studies on geographical pathology are currently receiving considerable attention in Africa and Burkitt (1966a, b), who has been studying the distribution of surgical conditions, has remarked on the unusually high prevalence of femoral hernia in parts of Uganda, apparently unaware of the aetiological significance of onchocerciasis.

In the past there has been some doubt as to the role of onchocerciasis in the aetiology of elephantiasis, a complication first reported by Ouzilleau (1913) in Equatorial Africa, but it is now generally agreed that in some areas of Africa onchocerciasis does cause gross thickening of the scrotal skin and in some cases there may be some degree of elephantiasis. There should be no

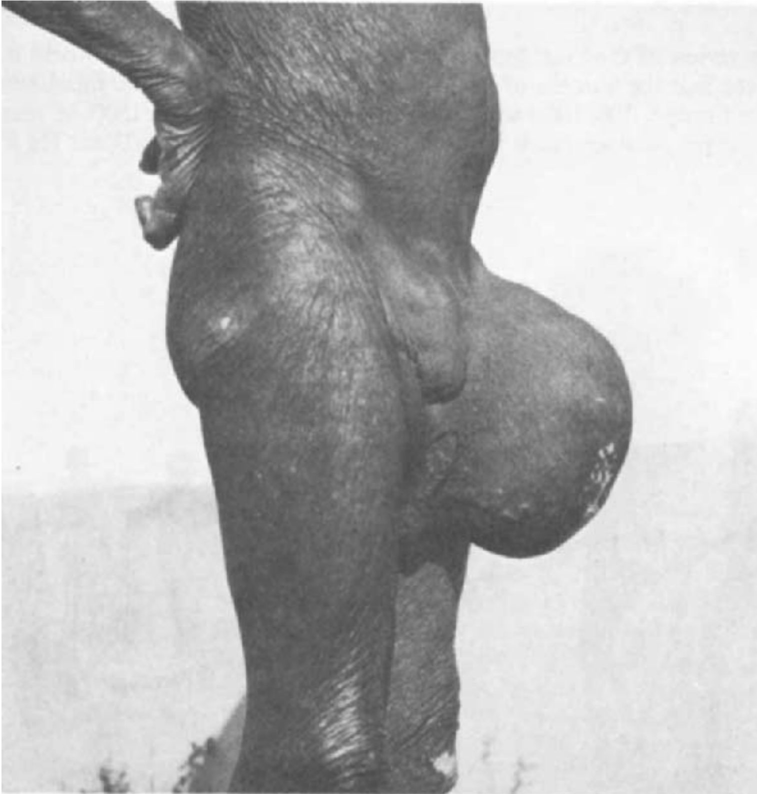


FIG. 8. Femoral hernia in a patient with advanced third stage onchodermatitis. Both inguinal and femoral hernias are common complications of onchocerciasis in Africa.

confusion with the hanging groin where the skin is markedly atrophied and the scrotum is rarely affected but there may be confusion with bancroftian filariasis. A feature which usually distinguishes the two conditions is the absence of associated hydrocoele or elephantiasis of the leg in the onchocercal cases. Gratama (1966) has, however, seen microfilariae of *O. volvulus* in hydrocoeles in Liberia, but Oomen (1967a) found no hydrocoeles in 25 resections of onchocercal elephantiasis of the scrotum in Ethiopia. Elephantiasis of the face has been illustrated as a rare complication of onchocerciasis in the Congo (Piers and Fasal, 1953). This dreadful malformation has also been seen in a woman patient with onchocerciasis in Liberia (Kingston, personal communication).

In practice there should be no real difficulty in determining the aetiological role of *O. volvulus* and *W. bancrofti* because in most regions these are mutually exclusive parasites. Usually this is due to ecological factors affecting the intermediate insect vector, but it is also possible that there are heterospecific immunological factors which prevent mixed infections.

D. EYE LESIONS

In a review of the "Incidence and Causes of Blindness" in the world it was reported that the number of persons with blindness per 100 000 inhabitants is 250 in Europe, 500–1000 where trachoma is endemic and 1500 or more in areas where onchocerciasis is prevalent (Greenslade, 1956). There are many

FIG. 9. Onchocercal eye disease. The blind leading the blind in the savannah region of West Africa. (Courtesy WHO.)

areas in the savannah regions of Africa where 30 % of the population may have impaired vision as a result of onchocerciasis and the blindness rates in the adult population may be over 10 %. In these areas it is not unusual to see chains of blind people being led to the local market by a child with good vision (Fig. 9). In some areas there is a tribal name for onchocercal eye disease which may be associated with a particular river. Long before Europeans arrived in Africa the local people suspected that insects were concerned with transmission of the disease. For example, the people inhabiting "the valley of the blind" in Kenya recognized the skin disease and the eye lesions and when asked by an agriculturist how they contracted the disease they took him to a river and showed

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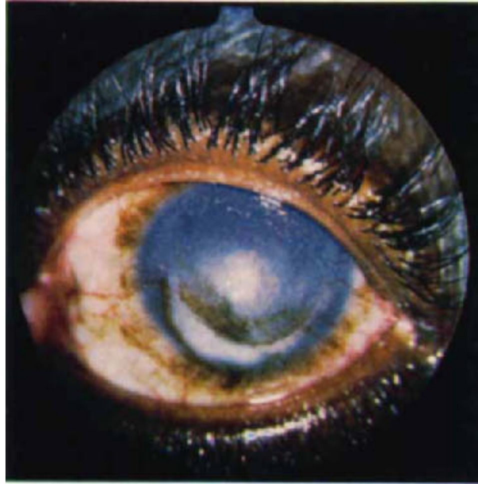


FIG. 10. Onchocercal eye disease. Anterior lesions producing blindness. The cornea shows sclerosing keratitis with vascularization in the lower segment. The remainder of the cornea is oedematous. The underlying pupil is bound down to a complicated cataract. (Courtesy Dr J. Anderson.)

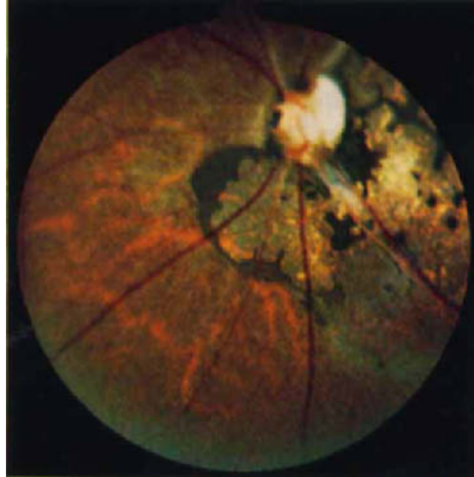


FIG. 11. Onchocercal eye disease. Posterior lesion producing blindness. This fundus photograph shows optic atrophy, narrowing and sheathing of the retinal vessels, retinal atrophy with clumping of pigment, and choroidal sclerosis. (Courtesy Dr J. Anderson.)

him a biting fly which was subsequently identified as *S. neavei* (Dry, 1921). (This was several years before Blacklock (1926) discovered that *S. damnosum* was a vector in West Africa.)

The most severe eye lesions are seen in the drier regions of Africa, and also in Central America. Blindness is less common in the rain forest regions even though infection rates are often very high and there is intensive transmission. In one of the rain forest areas of the Cameroons Duke (1968b) has obtained data on the biting and infection rates of *S. damnosum*, and he has calculated that a single person exposed all day and every day for a year would be bitten by 14 000 infective *S. damnosum* capable of inoculating into him more than 92 000 infective larvae, yet the blindness rate is less than 1 % compared with more than 10 % in the savannah regions where the biting rate of *S. damnosum* is often less intense.

The eye lesions usually take many years to develop and the worst affected section of the community is the adult male population over the age of 40 years, but occasionally with the Central American form of the disease there may be an acute onset in young adults especially when the adult worms are located around the head.

The most prevalent and most characteristic lesions are found in the anterior part of the eye and their severity is related to the number of microfilariae reaching the cornea from the skin of the face through the conjunctiva (Fig. 10). The earliest lesions consist of a punctate keratitis, and this is followed by fibrous tissue invasion which eventually produces a pannus which obliterates the lower part of the cornea. Choyce (1966) has noted that the microfilariae are often attached to Descemet's membrane and he has suggested that they may cause physical damage which results in limbitis. There is frequently an associated iridocyclitis, with the production of a characteristic "pummice stone" appearance of the iris. This may be pear-shaped with a collection of debris forming an hypopyon in the lower anterior chamber. Posterior synechiae develop, the pupil becomes fixed and this together with the post inflammatory fibrosis and exudate overlying the lens may lead to complete blindness. Secondary glaucoma or cataract may also complicate the picture.

In almost all of these cases microfilariae can either be demonstrated in conjunctival snips or they can be seen in the anterior chamber using a high power ophthalmoscope or a slit lamp. The diagnostic features of the anterior lesions are well illustrated by Choyce (1966).

In some cases there may be posterior lesions with choroido-retinitis (Fig. 11). This was first clearly demonstrated by Hisette (1932) in the Congo, Bryant (1935) in the Sudan and Ridley (1945) in West Africa. The retinitis may progress to atrophy with involvement of the optic nerve. The aetiological association with onchocerciasis was at one time questioned by Choyce (1958), but there is now convincing evidence indicating that onchocerciasis is a significant cause of this condition in Africa and Central America (Rodger, 1957; 1959; Budden, 1957; Lagraulet *et al.*, 1957; D'Haussy and Uemura, 1960; Choyce and Woodruff, 1965; Monjusiau *et al.*, 1965). There are, however, many other causes of choroidoretinitis which present problems in differential diagnosis in areas where facilities for ophthalmological work are usually inadequate, and

the onchocercal choroidoretinitis is often difficult to diagnose because there are frequently associated anterior lesions obliterating the pupil. One of the main reasons for controversy is lack of pathological material. The problem of the pathogenesis of ocular lesions in onchocerciasis has been extensively reviewed by Rodger (1960, 1962). His observations and those of Neumann *et al.* (1964) based on the inoculation of microfilariae into the eyes of laboratory animals and chimpanzees have reproduced some of the features of onchocercal eye disease, but this is an unnatural procedure and there is an urgent need to study the whole course of the infection in man and experimental animals.

It is still not known why there are such striking differences in the incidence of onchocercal blindness in different endemic areas. Associated nutritional deficiency, the presence of concomitant infections with other organisms, variations in the strain of the parasite and differences in the intensity of transmission, are all factors which must play some part in determining the severity of the infection, but this problem still remains one of the great enigmas of tropical medicine.

E. DWARFISM

Onchocerca volvulus has been incriminated as a cause of severe dwarfism in Uganda. The so-called "Nakalanga" pigmies seen from time to time in the Mabira forest in Uganda were thought by Johnston (1902) and Pitman (1934) to be a relict group but Raper and Ladkin (1950), who carried out a detailed epidemiological, clinical and pathological study of these dwarfs, have shown that they are in fact offsprings of normal parents. Apart from the characteristic infantilism with associated signs of pituitary deficiency there is one feature that distinguishes them from children of a similar age: they are all severely infected with onchocerciasis. The condition is well known to the local Baganda, who attribute the dwarfism to the children being bitten by the "mbwa" fly (*S. damnosum*). Similar dwarfs have been seen in other parts of Uganda but so far they have only been reported from onchocerciasis foci (Nelson, 1958a; Jelliffe *et al.*, 1962; Stanfield, 1963; Bagenda *et al.*, 1964; Barnley, personal communication). Marshall and Cherry (1961) carried out a detailed study of the endocrine system of a Nakalanga dwarf who died following treatment with Suramin and diethylcarbamazine. The pituitary gland was affected and there was evidence of thyroid and testicular dysfunction. No microfilariae were found in the tissues but this was probably due to previous treatment. It is not known how the parasite causes this syndrome, but microfilariae of *O. volvulus* have been found by Mazzotti (1959) in the cerebrospinal fluid of patients with onchocerciasis in Mexico, and Giaquinto Mira (1934) and Rodger (1960) have seen microfilariae in the optic nerve and retina. These findings suggest that the microfilariae may invade the pituitary from the eye. Until more detailed pathological and endocrinological studies have been carried out on the "Nakalanga" it is unlikely that much progress will be made in understanding the pathogenesis of this condition.

F. LONGEVITY OF *O. volvulus*

All the serious complications of onchocerciasis are due to heavy infections persisting for long periods, and there is evidence suggesting that the disease is

progressive even in the absence of continued transmission. This has been seen in Europeans and North Americans returning to live in non-endemic areas and also by follow-up studies in areas where the vectors have been eliminated. The most striking evidence was obtained in Kenya, where it was found that 11 years after eradication of the vector from the "valley of the blind" the infection rate in the older people was still more than 60%. There were no new infections but many of the people who had had mild eye lesions at the time of the eradication programme were completely blind 11 years later (Nelson and Grounds, 1958). More recent surveys in the same area by Roberts *et al.* (1967) have shown that the infection has now died out and that the maximum life span of the worm is probably 16 years. Observations by Duke (1968c) suggest that the microfilariae will persist in the skin for as long as 30 months after the adult worms have been killed with Mel W.

VI. Diagnosis

A. Skin Snips

The demonstration of microfilariae in bloodless skin snips remains the best method of diagnosis. The technique is simple. A needle or mounted entomological pin is used to raise a small cone of skin which is then cut off under the needle point with a sharp razor blade or fine pair of scissors. There is some discomfort but it is not necessary to use a local anaesthetic. The "skin snip", which is usually about 3 mm in diameter, is placed in a drop of physiological saline or water and allowed to stand for about half an hour before being examined. If the snip is rather thick it should be teased (Duke, 1962b). In the tropical rain forest area of Africa care should be taken to avoid confusion with the much smaller skin microfilariae of *Dipetalonema streptocerca*. If the skin snip is contaminated with blood there can also be confusion with *Loa loa* and *D. perstans*. In cases of doubt the smear should be preserved and fixed with methyl alcohol for subsequent staining with Giemsa or haemalum.

In light infections it may be necessary to take several skin snips before finding microfilariae, and the site for taking the snip will depend on the geographical strain of parasite involved. When only a single biopsy is possible the skin should be taken from the buttock region. Standardization of skin snip size is not practicable but for comparative purposes it is possible to weigh the snips with a torsion balance and express the result as the mean number of microfilariae per mg of skin. This method has proved invaluable for epidemiological and comparative clinical studies such as reported by Kershaw *et al.* (1954) and Woodruff *et al.* (1966a, b). The latter writers claim that variations in the depth of the snips are of no significance and that teasing is unnecessary, but since their data were based only on the numbers that emerged from the snips and no attempt was made to find how many were left behind, their conclusions may not be valid especially in older patients where the microfilariae lie deep in the connective tissue. Our observations with microfilariae in the skin of animals suggest that many of the microfilariae fail to emerge into the saline unless the skin snips are teased (Nelson *et al.*, 1966b). The statistical studies by Rodger and Brown (1957) suggested that weighing of skin snips is

not always necessary and that reliable epidemiological indices can be calculated provided that multiple skin snips are taken. It should be emphasized that histological examination of biopsies is unnecessary, and because of the difficulty in recognizing microfilariae in sections histology is of little value in diagnosis.

Various scarification procedures have been used both for diagnosis and in epidemiological studies (Wanson, 1950; Hughes and Daly, 1951; Nelson, 1955; Basset and Lacan, 1967). They have the advantage of simplicity and speed of operation and avoid duplication of effort in the laboratory. It is claimed by Onori (1963) that scarification and subsequent staining of the impression smears allows a clear differential diagnosis of different species of filarial and malarial parasites in the one procedure. It has even been claimed that scarification is a more accurate technique for diagnosing malaria than blood smears (Van den Berghe and Chardome, 1951), but this claim has not been confirmed (Bruce-Chwatt, personal communication).

B. MICROFILARIAL PERIODICITY IN THE SKIN

Various workers have suggested that the microfilariae of *O. volvulus* are not constantly present in the skin and that their densities in the skin snips will be affected by the time of day when the snips are taken and the local climatic conditions. In surveys in East Africa it has been common practice to apply hot-water bottles to the skin to encourage the microfilariae to migrate to the superficial layers, but there are no data to substantiate the claims for this technique. If marked variations do occur then it is obvious that the factors affecting this fluctuation must be determined if standard methods are to be adopted in surveys. Lartique (1967), in a preliminary study in West Africa, noted a marked periodicity of microfilariae with much higher densities in the skin at 4 p.m. in the afternoon than at 10 a.m. in the morning. In East Africa Wegesa (1966a) has shown that the microfilarial densities are highest between 8 and 10 a.m. in the morning and around 6 p.m. in the evening, coinciding with the peak biting activity of the local vector *S. woodi*. The problem has been investigated in more detail by Duke *et al.* (1967a) in the Cameroons, and they have shown that although there are daily fluctuations in microfilarial concentrations coinciding with the peak biting density of the local vectors, the fluctuations are too small seriously to affect normal diagnostic or epidemiological studies. The periodicity appears to be an adaptation to the biting cycle of the local vector in much the same way as there is an adaptation by the various strains of *Wuchereria*, *Brugia* and *Loa loa* to their local vectors. So far nothing is known about the trigger mechanisms which activate the microfilariae of *O. volvulus* or how far they travel in the skin.

Although there are no records of diurnal periodicity with other species of *Onchocerca*, Sasaki *et al.* (1954) have shown that there is a seasonal periodicity in the number of microfilariae in the skin of horses infected with *O. cervicalis*, and Hawking (1967) has produced evidence of a similar seasonal periodicity in the microfilariae of *Dirofilaria immitis* coinciding with the peak biting activity of the mosquito vectors.

C. IMMUNOLOGICAL TESTS

Various immunological procedures such as intradermal tests, complement fixation reactions, haemagglutination reactions, fluorescent antibody and intradermal tests using antigens prepared from a variety of filarial worms, have been used for the diagnosis of onchocerciasis and other filarial infections (see review by Kagan, 1963). Many of the serological tests are positive in patients with onchocerciasis, but they are all non-specific and give cross reactions with other filarial infections such as *Loa loa*, which is frequently present in the same patient (Biguet *et al.*, 1964). There is, however, some indication from the studies of Ciferri *et al.* (1965) that the intradermal test with *Onchocerca* antigen is more specific than with *Dirofilaria* antigen. On the other hand the haemagglutination reaction using *Onchocerca* antigen is positive not only in other filarial infections but also in persons with guinea worm (Rosé *et al.*, 1966). There has also been a conflict of opinion as to the value of the fluorescent antibody test. Woodruff and Wiseman (1968) have been unable to confirm the promising results reported by Lucasse (1962) and Lucasse and Hoeppli (1963).

Some of these immunological tests may be of value in epidemiological studies and as a rapid check on control methods, but in preliminary surveys and for diagnosis of the infection in individual patients the only completely reliable method is to demonstrate microfilariae, either in the skin or eyes or in the material aspirated from nodules. If these procedures are negative then the Mazzotti test with a provocative dose of 50 mg of diethylcarbamazine is more reliable than the non-specific immunological test. But even the Mazzotti test is not always positive. Occasionally patients with quite high densities of microfilariae in the skin show no obvious reaction and Oomen (1967b) has found that the test is particularly unreliable in Ethiopia. Infections with *D. streptocerca* may mimic the early stage of onchocercal infection and the microfilaria which also occur in the skin can cause some confusion, but Duke (1968d) maintains that the Mazzotti test is negative in cases of streptocerciasis except with doses of more than 200 mg. The test is negative in cases of *D. perstans* infection (Rives and Serie, 1967).

VII. TREATMENT

A. NODULECTOMY

A traditional treatment, especially in Central America, is to remove all palpable nodules especially those around the head. Denodulization is a feature of mass control schemes in Guatemala where medical auxillaries travel throughout the endemic areas excising nodules, and it is claimed that this procedure has a marked effect on the number of microfilariae in the skin and eyes (Diaz, 1957). Similar results have not been seen in Africa where, as has been mentioned, the worms are often impalpable especially in children and young adults and the nodules are mainly around the pelvis. Also many of the larger nodules are merely fibrous reactions around dead and dying worms and the removal of these nodules will have little effect on the production of microfilariae. But with the Central American form of the disease it is obviously a

wise precaution to remove nodules from around the head and upper part of the body as they may contain worms which are producing the microfilariae that invade the eyes. The enthusiasm of Dr. Diaz and his colleagues in encouraging the nodulectomy campaign has undoubtedly reduced the severity of the disease in Central America.

B. CHEMOTHERAPY

Various compounds have been produced which will either kill the microfilariae or the adult worms or sterilize the female worms and prevent them from producing microfilariae, but the treatment of onchocerciasis with drugs is still far from satisfactory. Diethylcarbamazine (Banocide, Hetrazan, Notezine) will kill the microfilariae of *O. volvulus* but not the adult worms (Duke, 1957, 1968e). The severe reactions resulting from treatment with this drug are unpleasant and mass treatment campaigns based on the use of diethylcarbamazine are unpopular. They are also ineffective because of subsequent reinvasion of the skin by microfilariae. On the other hand suramin (Moranyl, Antrypol, Bayer 205, Naganol) will kill both the adult worms and the microfilariae but it is much more toxic than diethylcarbamazine and there have been several fatalities due to exfoliative dermatitis and renal complications (Van Hoof *et al.*, 1947; Massequin *et al.*, 1954; Nelson, 1955). There has been much controversy about the seriousness of these reactions and advocates of mass chemotherapy have suggested that the wide variation in the incidence of toxic manifestations may be due to variation in the quality of the drug prepared by different manufacturers. However, Budden (personal communication) and Duke (1968f) have carried out control trials with different preparations of suramin and they have noted a similar high incidence of complications with all the various preparations. With a total dose in excess of 6 g of suramin reactions similar to that seen in the patient in Fig. 12 are very common and they can progress to an exfoliative dermatitis and death unless corticosteroids are given. It is therefore essential with both suramin and diethylcarbamazine to have close medical supervision of treatment and patients with severe reactions have to be admitted to hospital. This prohibits their use for mass treatment campaigns aimed at eradication of the infection in the underdeveloped rural areas. However, for the treatment of the individual patient excellent results can be obtained if suramin is given following a course of treatment with diethylcarbamazine.

Melarsen W (pentylthiarsaphenyl-melamine), which was introduced by Friedheim and de Jongh (1959, 1960), kills the adult worms but has no effect on the microfilariae (Duke, 1968g). In low dosage it will sterilize the female worms without necessarily killing them. It is effective after only a single injection and at one time there were hopes that it would be of great value for mass treatment. Unfortunately there have been several cases of arsenical encephalopathy following the use of Mel W in the treatment of onchocerciasis (Duke, 1966b; Lagraulet *et al.*, 1966). According to Downie (1966) encephalopathy may also occur in patients with mixed infections with *Loa loa* and *O. volvulus*, and in these cases complication was thought to be due to the action of the drug

FIG. 12. Papular eruption and extreme prostration following six weeks' treatment of onchocerciasis with suramin to a total dose of 5·5 g. The drug is both macro- and microfilariacidal and treatment with corticosteroids may be essential to prevent the development of fatal exfoliative dermatitis.

on the *Loa loa*. These complications are unpredictable, they are not dose-dependent as with the reaction to suramin, and since they may be fatal it is doubtful whether this form of treatment is justifiable for a disease such as onchocerciasis which is disabling but never fatal. Many other drugs have been used including niridazole, thiabendazole and antimony compounds, but they are either ineffective or too toxic (Duke and Moore, 1967; Duke and Hawking, 1967; Duke, 1968h). Studies on human volunteers and on chimpanzees by Duke (1968g) indicate that diethylcarbamazine is not an effective chemoprophylactic against *O. volvulus*, although it is effective against *Loa loa* (Duke, 1963). And although suramin and Mel W have shown some promise in killing off infective larvae they are both too toxic and cannot be used for prophylaxis.

As with studies on pathology of onchocerciasis, research on chemotherapy is handicapped by lack of a suitable laboratory model for screening. At present the filarial parasite which is normally used in the pharmaceutical industry is *Litomosoides carinii*, which lives in the pleural cavity and produces very delicate sheathed blood-microfilariae which are quite different from the robust skin microfilariae of *O. volvulus*. But so far no satisfactory alternative screen has been developed. Vargas (1952) and Chévez *et al.* (1962) have experimented with *O. vvlvulus* microfilariae taken from man and our studies on *O. gutturosa*

microfilariae have shown that they can be maintained alive *in vitro* both in culture media and in deep freeze for long periods. They will also survive in the skin of "proxy" hosts such as mice and rats (Nelson *et al.*, 1966b). This bovine parasite is readily available in abattoirs in many parts of the world and it could provide material for a more detailed study on the activity of compounds against *Onchocerca* microfilariae, but this is far from ideal; what is required is the cyclical maintenance of *O. gutturosa* in the laboratory. However, this is a remote possibility because no species of *Simulium* has been successfully reared throughout more than one generation (Raybould, 1967), and even when cyclical maintenance is achieved the long prepatent period of the parasite and the expense of rearing cattle will preclude the use of this model for screening. It is essential to try and adapt this or other species of *Onchocerca* to laboratory animals.

VIII. The Transmission of *O. volvulus* by Simuliids

A. Introduction

This section is concerned only with the factors affecting the transmission and behaviour of *O. volvulus* in simuliids. It is not proposed to deal with the purely entomological aspects concerning the bionomics and taxonomy of the vectors. These aspects have been extensively reviewed by Dalmat (1955) for Central American vectors, by Lewis (1960a, b; 1961a, b) and Lewis and Hanney (1965) for the *S. neavi* complex and by Le Berre (1966) for *S. damnosum*. Many of the more recent advances in this field have been discussed and published as proceedings of recent symposia (see Lewis, 1968; also *Abstracted Reviews Eighth International Congress of Tropical Medicine*, Teheran, 1968. Pp. 136–138). There have also been many advances in the knowledge of other groups of simuliids, especially in North America where studies on "black flies" have been particularly stimulated by the informal meeting organized by Drs B. V. and D. G. Peterson of the Canadian Department of Agriculture. Their *Simuliidologists' Newsletter* has produced a wide interest in the study of simuliids which has been exceptionally fruitful.

The study by Anderson (1956) on the transmission of *Ornithofilaria fallisensis* and of *Leucocytozoon simondi* by *S. venusutum* and other simuliids in North America has provided ideas which have been immediately relevant to the problem of *O. volvulus* transmission. So also have been the observations by Davies (1957), Doby *et al.* (1959, 1964), Wenk (1965a, b) and McMahon (1968) on the vectors of bovine *Onchocerca* and other European simuliids. One of the most important advances in the knowledge of the vectors of *O. volvulus* has been the recognition of sibling species or other variants in the *S. damnosum* and *S. neavei* complex, as a result of the studies on morphology and behaviour by Lewis (1960c, 1965), McCrae (1968), Lewis and Duke (1966) and Raybould (1967) and on larval chromosomes of *S. damnosum* by Dunbar (1966).

B. The Vectors of *O. volvulus* in Africa

The main vector of *O. volvulus* in Africa is *S. damnosum*. This species breeds on vegetation and on rocks in a great variety of river systems, ranging from the

great perennial rivers such as the Congo and the Nile with their numerous tributaries flowing through thick forest or open grassland, to the seasonal streams on the edge of the desert in the northern savannah regions. This is by far the most important vector.

There are, however, several forms of *S. damnosum* and some are almost entirely zoophilic so that the distribution of this group of simuliids is far more extensive than that of the disease.

Members of the *S. neavei* complex are vectors of onchocerciasis in limited areas in the eastern Congo, in Uganda, Kenya, Tanzania and Ethiopia. The vector in the Yemen is unknown. The *S. neavei* complex is a distinct group which breeds mainly in small streams in highland areas and nearly all species have an obligatory association with fresh water crabs of the genus *Potamonautes*. This remarkable example of phoretic association was first demonstrated with *S. neavei* by Van Someren and McMahon (1950) in Kenya, but the same association is now found with a variety of other species in this group: *S. nyasalandicum*, *S. woodi*, *S. goinyi*, *S. hightoni* and *S. ovazza* (Lewis and Hanney, 1965); *S. neavei* is the main vector throughout East Africa but in Amani in Tanzania this is replaced by the closely related *S. woodi* (Raybould, 1967). It is not known where the females of *S. neavei* and other members of this complex deposit their eggs but the larvae and pupae are found only on crabs (Fig. 13). In the past the identification of the host crab created many problems for the entomologists (Barnley and Prentice, 1958), but much of the confusion has been resolved as a result of the recent taxonomic studies by Williams (1968).

The species of simuliids found naturally infected in Africa are *S. damnosum*, *S. neavei* and *S. woodi*, but Wegesa (1967a) has shown that *S. vorax* is a good laboratory host and that *O. volvulus* will develop to the infective stage in *S. nyasalandicum* and *S. adersi*. Duke (1962c) has also described the development of *O. volvulus* in what was first thought to be *S. aureosimile* but is now recognized as a remarkable new species associated with prawns (Disney, 1969; Lewis *et al.*, 1969, in press). These species rarely bite man so it is unlikely that they will be involved in transmission unless there are major ecological changes which alter their feeding behaviour. Crosskey (1957b) has reported natural infections with filarial larvae in *S. bovis* and Crosskey and Crosskey (1958) have seen infective larvae in *S. griseicolle*, but it is doubtful if these were the larvae of *O. volvulus*. (The problem of identification of filarial larvae in simuliids is dealt with in Section VIII E.)

Lewis (1956, 1957, 1958a, b, 1960a, b, 1965) has studied the physiological age of *S. damnosum* and the differences between nullipars (which do not transmit *O. volvulus*) and parous flies. The latter often tend to bite at particular times of day, therefore the time at which flies are collected can influence the results of the infection-rate surveys. It was expected that in the forest of West Cameroon *S. damnosum* would be relatively long-lived, and that therefore the parous rate would be high, but in fact it proved to be rather low. Le Berre (1966) has studied this phenomenon in various parts of West Africa and found high parous rates in some savannah areas. The causes of this, and its effect on onchocerciasis, require further study.

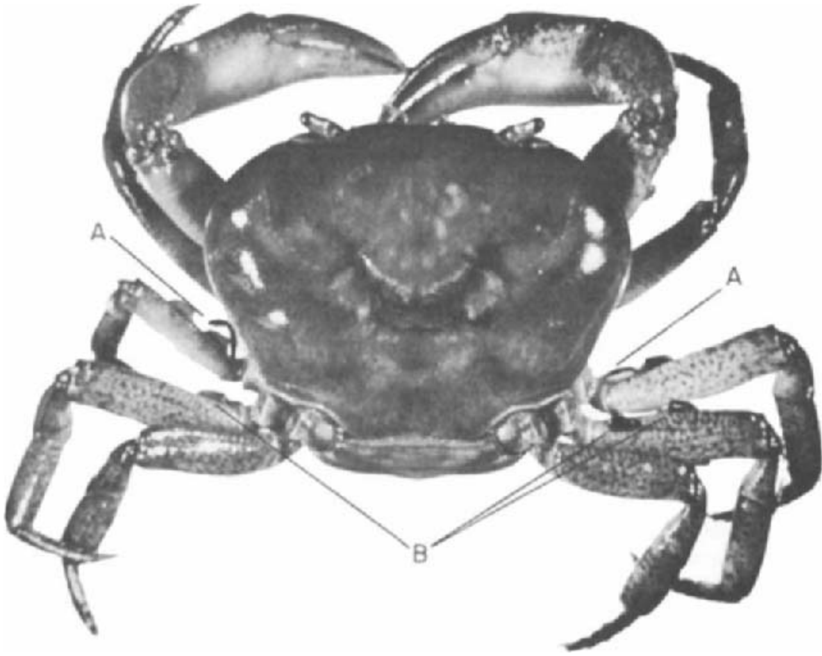


FIG. 13. The phoretic association of the larvae and pupae of *Simulium* on a crab of the genus *Potamonautes*. **A**, larvae, **B**, pupae. (Courtesy Dr J. N. Raybould.)

C. THE VECTORS OF *O. volvulus* IN CENTRAL AND SOUTH AMERICA

In Guatemala and Mexico three species of *Simulium* have been incriminated as vectors, namely *S. callidum*, *S. metallicum* and *S. ochraceum* (Dalmat, 1955). The studies by De Leon and Duke (1966) suggest that in Guatemala *S. ochraceum* is by far the most important vector. It is markedly anthropophilic and the highest biting densities are found between 3000 and 5000 ft above sea level in areas where the disease is most prevalent. It breeds in the numerous small streams and rivers flowing through the thickly wooded volcanic slopes and it is often found biting along the streams flowing through the villages in these regions; even the most minute trickles form breeding places and this makes control extremely difficult (Crosskey, 1968). Both *S. metallicum* and *S. callidum* are strongly zoophilic and it is doubtful whether they are of much importance as vectors in this area. This is also true of *S. gonzales*, *S. veracruzanum* and *S. haematopotum* that have been reported as possible secondary vectors by Vargas (1962) and Cháves Nuñez (1963). However, in Venezuela onchocerciasis has now been recorded in nine states and here *S. metallicum* is thought to be the main vector (Lewis and Ibáñez de Aldecoa, 1962; Rivas *et al.*, 1965; Arends, 1966). *S. metallicum* is a low biter producing a clinical picture similar to that seen in areas of relatively low endemicity in Africa. It is widespread, breeding in small streams throughout the northern region of Venezuela. The recently discovered focus in Colombia is not thought to be continuous

with the focus in Venezuela, and the vector is unknown, although Lewis and Lee-Potter (1964) have recorded several man-biting species in this country.

D. THE DEVELOPMENT OF *O. volvulus* IN *Simulium*

Simuliids were first incriminated as the vectors of *O. volvulus* by Blacklock (1926), who showed that *Simulium damnosum* was the vector in Sierra Leone. The short scarifying proboscis (Fig. 14) is ideally designed for the ingestion of the microfilariae from the skin. It penetrates just deeply enough to pick up the microfilariae from beneath the epidermis. In each geographical region the

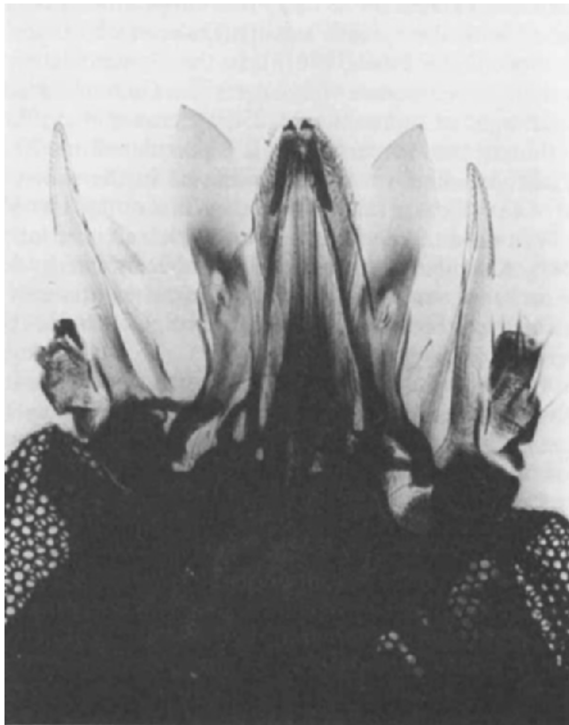


FIG. 14. Proboscis of *Simulium neavei*. The short scarifying proboscis is beautifully adapted for ingesting microfilariae from the superficial layers of the dermis. (Courtesy Mr M. A. Prentice.)

local vectors are adapted to the local strain of *O. volvulus* so that *S. damnosum*, *S. neavei* and *S. woodi*, which are predominantly low biters, are well adapted for picking up microfilariae of the African form of *O. volvulus*. Van den Berghe (1941b) and Duke and Beesley (1958) have studied this aspect of transmission by *S. damnosum*, Raybould (1967) has studied *S. woodi* and Barnley (personal communication) has noted that the same is true for *S. neavei* both in Western Uganda and on Mt Elgon on the Kenya border. In the same way *S. ochraceum* is a high biter and the observations by Strong *et al.* (1934), Dalmat (1955),

Lagraulet *et al.* (1964) and De Leon and Duke (1966) have shown that it is well adapted to transmit the Central American form of *O. volvulus* which has microfilariae in the upper part of the human body.

Not only are the simuliid vectors in each geographical region well adapted to biting the appropriate area of skin of the local human host, but they are also much more efficient intermediate hosts of the local geographical strain of the parasite. This has been well demonstrated by Duke and his colleagues; they have carried out a remarkable series of comparative experiments which has involved the transport of African patients with onchocerciasis to Guatemala and the transport of vast numbers of infected *S. ochraceum* from Guatemala to Africa, and it has been necessary to carry out long-term experiments using chimpanzees infected with the various strains (Duke *et al.*, 1966, 1967a, b; Lewis and Duke, 1966; Duke 1966b, 1967a). In the Guatemala experiment it was shown that when *S. ochraceum* was fed on the Guatemala and African patients the simuliid ingested more than 20–25 times more microfilariae of the Guatemala strain than of the African strain. It is postulated that *S. ochraceum* has a selective attractant effect on the microfilariae in the skin of the local people. This effect of simuliids on microfilariae was first noticed by Strong *et al.* (1934) and it has been used in xenodiagnosis of onchocerciasis in East Africa by Wilkinson (1949). A similar attractant effect has been noted by Moorhouse (1969) in ticks fed on lizards infected with filarial parasites. It seems likely that if the attractant substances could be isolated they might be used to provide a more sensitive method for diagnosis.

The time taken by a particular *Simulium* in feeding is of some importance in determining the number of microfilariae ingested. Crosskey (1962) has shown that *S. damnosum* takes much longer to feed on persons with onchodermatitis than on those with normal skin, and Wegesa (1966b) has shown that the number of microfilariae ingested is proportional to the time taken to feed and not to the size of the blood meal.

If all the microfilariae that were ingested developed to maturity this would undoubtedly cause a heavy mortality in the flies, but a mechanism exists which prevents too many of the microfilariae reaching the thoracic muscles. Lewis (1950, 1953b) has shown that many of the microfilariae fail to leave the midgut and that they are apparently trapped by the peritrophic membrane which forms around the blood meal. The membrane provides an impenetrable barrier and the microfilariae that fail to migrate die and disintegrate in the midgut. Duke and Lewis (1964) have studied this in detail and they have shown that with *S. damnosum* where the mean intake per fly is about 17, less than 50% of the microfilariae escape from the peritrophic membrane, but most of those that do escape eventually develop to the infective stage. Laurence (1966) has observed the time sequence of these events by studying *S. damnosum* killed at intervals after feeding and he has shown that early migration from the midgut is essential if the microfilariae are to survive. Once the microfilariae reach the thorax the majority develop to the fully infective stage and it is very rare to find "chitinized" "encapsulated" larvae in simuliids such as were seen in mosquitoes infected with filarial parasites by Brug (1932), Kartman (1957) and Esslinger (1962).

The morphological characters of the various stages of the development of *O. volvulus* in *S. damnosum* have been studied by Nelson and Pester (1962), Bain (1968) and Duke (1968i). Death of the flies occurs mainly during the maturation of the infection in the thorax. Nelson and Pester (1962) found as many as 72 infective larvae in a single specimen of *S. neavei* from Mt Elgon, and although 42 of the larvae were crowding into the head and proboscis these relatively large larvae measuring 600 μ in length caused surprisingly little damage.

The microfilariae penetrate the thoracic muscle cells and feed on the tissues, which may undergo some degree of liquefaction. Lebied (1950) has shown that the preferred sites of development are the flight muscles of the vector, and in his provocative essay entitled "Une nouvelle théorie endémiologique" he suggests that the consequent limitation in the flight range of infected flies is responsible for the relatively high infection rates in people living near the breeding sites. However, this theory was not supported by observations in Uganda, where Barnley (1958) showed that although *S. damnosum* breeding was confined to the River Nile the flies were widely dispersed through the Mabira forest and transmission occurred at least 30 miles from the River Nile.

In this respect *S. damnosum* is an excellent vector and Duke (1962d) has shown that provided relatively few microfilariae are ingested there is no decrease in the survival rate. This is in contrast with *S. woodi*, which according to Wegesa (1967b) shows a relatively high mortality beginning on the third day after feeding.

Very few critical observations have been made on the optimal environmental conditions necessary for the development of *O. volvulus* in simuliids. In a study in Uganda, Nelson and Pester (1962) noted that the larvae reached the infective stage between days 6 and 7 in *S. neavei* at a temperature of about 21°C with a relative humidity above 75%, but at this relatively low temperature the larvae failed to develop in *S. damnosum*. Wegesa (1966b) found that the optimum temperature for *O. volvulus* in *S. woodi* is 24°C and that no development occurred at temperatures below 18°C. The ambient temperature is of obvious importance in determining the distribution and seasonal transmission of onchocerciasis in much the same way as it affects the distribution of other filarial parasites of man; most of these require much higher mean temperatures for development.

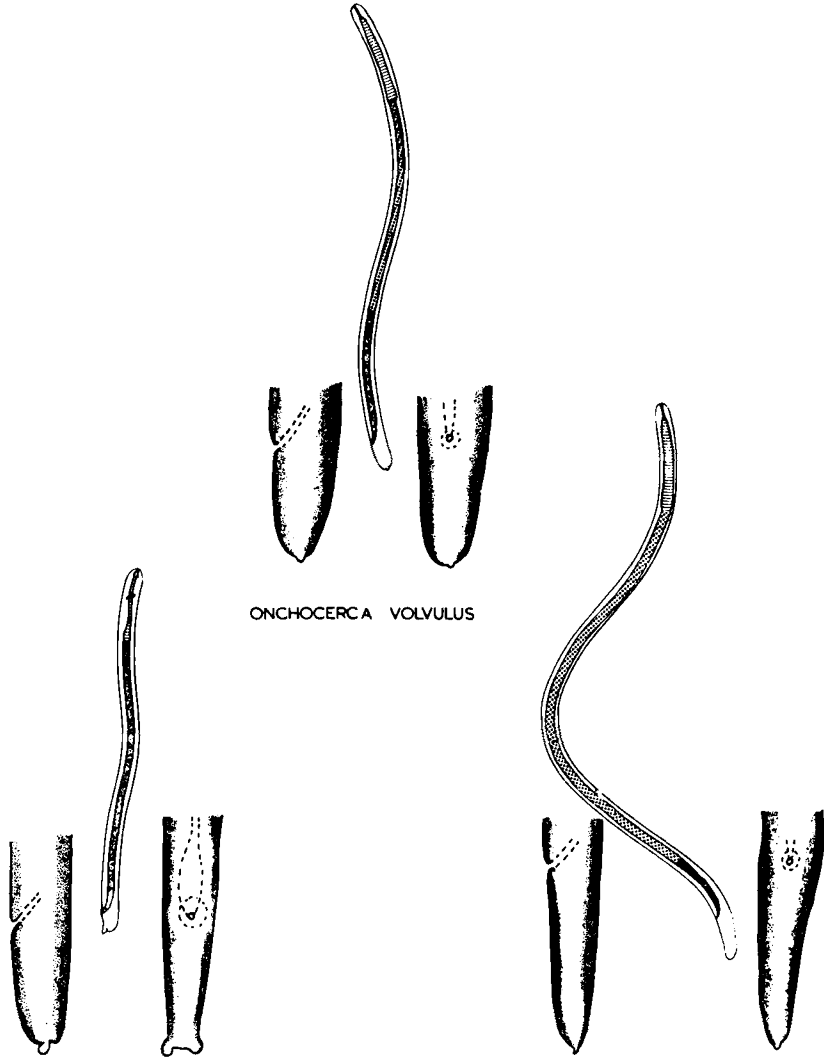
The various factors affecting the development and behaviour of a wide variety of filarial nematodes in arthropodan hosts have been reviewed elsewhere (Nelson, 1964). The epidemiological significance of these factors in relation to the transmission of onchocerciasis has been studied in great detail by Duke and his colleagues in the Cameroons (Duke, 1962d, e, 1968, b, i, j; Duke and Lewis, 1964).

E. THE IDENTIFICATION OF *O. volvulus* LARVAE IN *Simulium*

Wild caught simuliids are often heavily infected with filarial larvae and all too often it is assumed that these are of human origin. Similar assumptions have been made with regard to larvae seen in mosquitoes and this has often

led to the misidentification of the vectors of *W. bancrofti* and *B. malayi*. For example, on the East African coast the author found at least six species of filarial larvae in common man-biting mosquitoes and to identify the larvae it was necessary to carry out numerous feeding experiments on wild and domestic animals so that a reference collection was available (Nelson, 1959). No such reference collection was available for identifying larvae in simuliids, and in studies on the transmission of onchocerciasis in Uganda we found that there were no clear criteria for distinguishing the infective forms of *O. volvulus* from other species. Eventually, it was shown that a high proportion of the infective larvae in the so-called "anthropophilic" *S. neavei* on Mt Elgon were not *O. volvulus* (see Nelson and Pester, 1962). Duke (1967b) has shown that this is also true in the savannah areas of the Cameroons; at Mayo Boki 40% of the larvae in the "anthropophilic" *S. damnosum* were obviously not *O. volvulus*. These observations suggest that the so-called man-biting simuliids are not restricted in their host range and that many of them must feed on animals or birds. This problem is being investigated in West Africa by Disney and Boreham (1969), who found the resting sites of engorged females of several species and identified the blood meals. It is unwise to calculate transmission indices from infection rates in simuliids unless there is confidence in the identification of the infective forms.

Unfortunately the infective larvae of *O. volvulus* have no strikingly characteristic morphological features. Apart from their size it is impossible to distinguish them from other species of *Onchocerca* which are transmitted by simuliids, and unless the larvae are carefully preserved and mounted for examination it is not easy to distinguish them from the infective larvae of other genera of filarial worms. Fig. 15 shows some of the features which distinguish *O. volvulus* from two other types of infective larvae that may be found in simuliids. The "Type A" larvae of unknown origin is commonly seen in *S. neavei* on Mt Elgon (Nelson and Pester, 1962) and *Ornithofilaria fallisensis* is transmitted by simuliids in Canada (Anderson, 1956). The main criteria for differentiation are the total length of the larvae, the caudal morphology and the relative length of the oesophagus and intestine. The site of development in the fly can also give important information because most species are tissue specific and all species of *Onchocerca* develop in the thoracic muscles of their vectors. This will help to exclude other genera of filarial parasites that develop in the fat body or malpighian tubules. The "*Onchocerca*" larvae recorded by De Leon (1957) in the malpighian tubules of man-biting simuliids in Guatemala are now thought to belong to a distinct group of nematodes (De Leon and Duke, 1966). Although *O. fallisensis* has not been reported from any of the endemic onchocerciasis regions it is possible that other species of *Ornithofilaria* and other bird parasites may be transmitted by the vectors of *O. volvulus*; indeed, one of the larvae found by Duke (1967b) in *S. damnosum* closely resembles *O. fallisensis*. The Type A larvae (Fig. 15) which were seen in *S. neavei* are similar to the Type D larvae illustrated by Duke (1967b) and belong to some as yet unrecognized genus. In South America there is a possibility of confusion with *Mansonella ozzardi*, because Cerqueira (1959) demonstrated that in Brazil *S. amazonicum* is a vector of *M. ozzardi* or some closely related species.



	Onchocerca volvulus	*Ornithofilaria fallisensis*	Type "A"
Mean length (microns)	566·0	432·0	803·0
Maximum breadth (microns)	18·7	20·0	21·5
Tail length (microns)	31·2	33·0	48·2
Tail breadth (microns)	13·3	10·8	12·5
Anal ratio	2·3	3·1	3·8

FIG. 15. Infective filarial larvae from simuliids: the characters used to distinguish the species include the length, the caudal anatomy including the anal ratio (which is the mean length of the tail divided by the mean breadth), length of the anterior and posterior oesophagus, and the length of the intestine.

The larvae of *O. gutturosa* are morphologically similar to *O. volvulus* and have a mean length of 493 μ but the range is 427–572 μ, so there is an overlap in length with the larvae of *O. volvulus* which are 440–700 μ long (Eichler, personal communication). Steward (1937) worked out the life cycle of *O. gutturosa* in England more than 30 years ago but nothing is known about the vector of this or any of the other bovine *Onchocerca* in any of the areas where *O. volvulus* is endemic.

There is an obvious need to establish a reference collection of filarial larvae from simuliids and other vectors. This need has been recognized by the WHO Expert Committees on Onchocerciasis and Filariasis (see Technical Report Series No. 335 of 1966 and No. 359 of 1967, which include details of the techniques used for preserving and examining filarial and onchocercal larvae).

One of the main obstacles to studying the life cycles of *Simulium* transmitted parasites has been the difficulty of getting laboratory reared simuliids to feed on

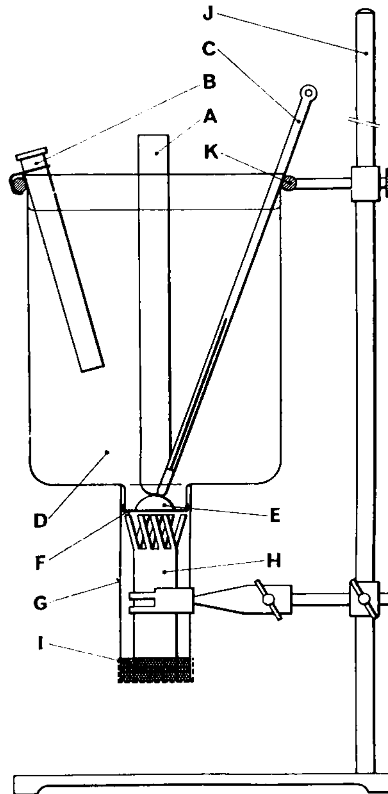


FIG. 16. Apparatus for feeding simuliids on blood through membranes. The membrane **F** consists of the skin of a day-old chick. The apparatus has been successfully used for feeding the vectors of *Onchocerca volvulus* and *O. gutturosa*. **A**, immersion heater. **B**, thermostat. **C**, thermometer. **D**, 1000-ml beaker. **E**, cavity for blood. **F**, membrane. **G**, feeding-tube. **H**, filter-paper. **I**, sandfly netting. **J**, retort stand. **K**, supporting ring (in section). (Courtesy Mr J. P. McMahon.)

man and animals. The techniques developed by McMahon (1968) may help to overcome some of these problems. The simple membrane feeding apparatus illustrated in Fig. 16 has been used successfully with a variety of species of simuliids. By filling the well with blood containing different species of microfilariae it is hoped to use the apparatus to obtain "unknown" infective larvae in much the same way as the "unknown" infective larvae of *Setaria labiatopapillosa* were obtained in mosquitoes which were fed on infected bovine blood through membranes (Nelson, 1962).

IX. *Simulium* CONTROL

The vectors of onchocerciasis can be controlled either by modifying the habitat or by destroying some stage in the life cycle of the fly. One of the earliest successful control projects was carried out by Buckley (1951) on the Riana River in Kenya. This project, which was completed in 1947, demonstrated that the method of selective bush clearing which had proved so successful against riverine tsetse flies was also effective against *S. neavei*. However, the method was never adopted on a wide scale. It was an expensive technique and it was soon superseded by DDT, which was first shown to be effective against African simuliids in this same geographical region by Garnham and McMahon (1947).

In some situations modification of the habitat can still, however, be an effective control measure. For example part of the intensive breeding of *S. damnosum* at the Ripon Falls at the source of the Nile in Uganda was eliminated not by insecticides but by the inundation of the rapids when the Owen Falls dam was built a short distance downstream. By creating lakes where there were formerly cascades and waterfalls large dams such as this can eliminate breeding places for many miles upstream. Downstream there can be the opposite effect and spillways of the dam and fords and other minor engineering work which obstructs river flow can provide extra breeding places for simuliids (Burton and McRae, 1965; Garms and Post, 1966). This is usually of concern only in minor projects, because wherever there are major engineering works such as on the Nile, Niger and Volta there is always strict control of the water flow at the outlets of the dams and this provides an easy method of applying larvicides.

The best example of this type of control is at Jinja in Uganda where a dosing device is incorporated into the dam, and by the regular release of DDT all *S. damnosum* breeding for a distance of more than 50 miles downstream can be controlled from a single dosing point. In this way the fly has been practically eliminated from an area of at least 1600 square miles (Barnley, 1953, 1958). Before control measures were introduced on the Nile the onchocerciasis rate among riverside dwellers was around 99%. The results have been quite spectacular; transmission has been interrupted and a large area of fertile land, which was empty because of the disease and the unbearable biting nuisance of the fly, has been opened up for development. Between 1948 and 1959, i.e. after the fly was controlled, the population in this area increased by 164% compared with only 19% in a neighbouring unaffected area (Prentice and McCrae, 1966).

The Jinja focus, however, is exceptional because here *S. damnosum* is confined to the Nile and there is little danger of reinfestation from tributaries. Elsewhere in Uganda and in other parts of Africa control has been less effective because breeding occurs not only in the large rivers but in innumerable small streams. For control to be effective it is necessary to apply larvicides at many dosing points and at much more frequent intervals. But even under these conditions there has been some success in controlling *S. damnosum*, notably at Mayo Kebbi in Chad (Taufflieb, 1956), at Abuya and Kainji in Nigeria (Davies *et al.*, 1962; Davies 1963, 1965; McMahon, 1967) and also in Northern Ghana and Upper Volta (see Brown (1962) and McMahon (1967) for reviews of the techniques used in these campaigns). At Abuja the *S. damnosum* density was reduced by 90% and the number of infective bites was about one-thirtieth of the pre-control level. This was insufficient to affect the prevalence rate as determined by qualitative skin snip results, suggesting that onchocerciasis transmission can continue in the presence of low densities of the vectors, but the control measures seem to have decreased the severity of the disease in this area (Davies, 1968).

In all these schemes control has been achieved by dosing the rivers with DDT as a larvicide, but in an earlier scheme *S. damnosum* was controlled on the Congo river around Kinshasha by spraying with DDT from the air, using helicopters and other aircraft (Wanson *et al.*, 1949; Lebrun, 1954). It was thought that control resulted from the direct effect of the insecticides on the adult flies and not on the larvae; but it has since been suggested that even here the success may have been due to a larvicidal effect because it is now known that the larvae of *S. damnosum* are killed by DDT in concentrations as low as 0·03 p.p.m. Spraying of larvicides from aeroplanes has become a routine procedure for controlling "nuisance" simuliids in North America, but spraying from aircraft has so far proved either too expensive or impracticable in the main onchocerciasis areas of Africa and Central America.

Total eradication of a vector simuliid has been achieved in only one country, i.e. in Kenya where McMahon *et al.* (1958) eliminated *S. neavei* from an area of 6000 square miles. The success of this scheme has been confirmed through resurveys carried out by Nelson and Grounds (1958) and Roberts *et al.* (1967), who have shown that although there has been a persistence of the infection in the older people due to the longevity of the worms there has not been a single new case throughout the whole region. The successful results can be attributed to the extraordinary determination and organizational ability of the team leader, Mr J. P. McMahon. He insisted on a meticulous preliminary survey of every river and stream throughout an area of 15000 square miles, and during the whole of the ten-year period of the campaign he personally supervized the measurement of river flow, the application of the larvicide and the assessment of the effect of the larvicide. There were several features which facilitated this scheme. (1) The local vector, *S. neavei*, had a very limited flight range and would not traverse open country between watersheds. (2) The affected area was geographically isolated and there was little chance of reinfection from neighbouring territories. (3) Although there were innumerable small streams and rivers which required dosing the *S. neavei* breeding was mainly limited to

the areas between 3500 and 7000 ft, and since this was a relatively prosperous tea-growing region it was often possible to transport the insecticide to dosing points by road. (4) The streams were all perennial so that the campaign could continue throughout most of the year. (5) The discovery by Van Someren and McMahon (1950) that *S. neavei* had an obligatory association with crabs facilitated the eradication scheme, because in assessing the effect of the larvicides the examination of crabs for larvae and pupae proved to be a more sensitive index of the degree of control than searching for adult flies or searching for larvae and pupae on roots and vegetation, as has to be done in *S. damnosum* areas.

The problems of eradication are much more formidable in areas where the vector is *S. damnosum*. This species is found throughout an area of several million square miles, it has a flight range of at least 100 km, and in the savannah region a proportion of the flies may survive throughout the dry season even though most of the rivers dry out completely (Ovazza *et al.*, 1965; Noamesi, 1966). In the Central and South American regions there are also equally formidable problems. It was in Mexico and Guatemala that Fairchild and Barreda (1945) first used DDT against *Simulium*, but here the vectors are much more widespread than the disease and it is now thought that it would be economically unrealistic to attempt control by eradicating the simuliids. In Venezuela the authorities were at one time more optimistic and Convit *et al.* (1961) produced promising results in the control of *S. metallicum*.

In all the endemic areas total eradication of the vector may be impossible but much can be done to limit the intensity of transmission. The severity of the disease varies markedly from village to village. In many places the disease is no more than a nuisance but in every region there are some foci of intense transmission where the blindness rates are high. Priority should be given to control measures in these severely affected areas, for although control may not be totally effective it can bring immediate benefit to the younger generations by reducing the worm load.

None of the vectors of onchocerciasis has so far developed resistance to DDT but there is some evidence from Japan that other species of simuliids can develop some degree of resistance (Suzuki *et al.*, 1963). At the concentration necessary to eliminate *Simulium* larvae DDT has a negligible effect on fish and the treated water is non-toxic to man and animals. There are, however, obvious dangers from using residual insecticides in streams and rivers and many of the unforeseen hazards have been given wide publicity by Rachel Carson (1962) in her book entitled *Silent Spring*. So far there have been no reports of disastrous ecological changes resulting from the use of larvicides in any of the onchocerciasis areas. In fact there have been surprisingly few permanent changes and one of the most remarkable results of the Kenya scheme has been the selective elimination of only one species of *Simulium*; all other species of simuliids have become re-established, including simuliids such as *S. nyasalandicum* which are in phoretic association with crabs. A hazard which was not foreseen in Uganda was a marked increase in molluscs in the Nile after the DDT was applied. Some of the snails which increased in numbers were known to be intermediate hosts of schistosomes but

fortunately they rapidly disappeared within a few months of the larvicidal applications (Cridland, personal communication). There is an obvious need for caution in the widespread use of insecticides, but it is hoped that the fear of hypothetical long-term ecological changes will not prevent their use for the immediate amelioration of one of mankind's most dreadful afflictions.

X. Onchocerciasis in Domestic Animals

Although various species of *Onchocerca* are ubiquitous in cattle and horses they are rarely recognized as causes of ill-health in these animals. Bovine onchocerciasis is so unobtrusive that it goes unnoticed even in areas of high endemicity. In a recent survey of cattle in abattoirs in England *O. gutturosa* adults were found in 746 out of 1591 cervical ligaments and microfilariae were abundant in the skin of infected animals, yet in no case had a diagnosis been made by a veterinarian or meat inspector (Nelson *et al.*, 1966b). A similarly high rate of cryptic infections with *O. gutturosa* has been reported in cattle in many parts of the world (Gnedina, 1950; Supperer, 1952; Webber *et al.*, 1957; Clarkson, 1964), and in Europe both *O. cervicalis* and *O. reticulata* may be prevalent in horses without attracting attention (Supperer, 1952; Nemeséri, 1956). But it is not surprising that these parasites are rarely seen by meat inspectors. The adult worms are very small and they occur most commonly in the ligaments of the neck at the point of insertion into the upper thoracic vertebrae, a site which is not visible in normal abattoir procedures. On the other hand some species of *Onchocerca* such as *O. gibsoni* in cattle and *O. flexuosa* in the deer are much more obtrusive in that they produce nodular swellings under the skin so that they are readily seen when the carcasses are inspected. Perhaps the most obvious of all the species is *O. armillata* which occurs in the aorta of cattle. But again since it is not a normal abattoir procedure to open up the aorta this parasite may go unrecorded even in areas when 100% of the cattle are infected (Abdel Malek, 1958; Patnaik, 1962).

The clinical manifestations of bovine and equine onchocerciasis have received very little attention. As with the human infections the pathological lesions are caused either by the microfilariae in the skin or they result from reactions around the adult worms. Skin lesions such as seasonal dermatitis reported by Underwood (1934), Datta (1939), Dikmans (1948) and Ishihara and Ueno (1958) may be caused by onchocercal infections but the lesions may be due to reactions to biting flies (Riek, 1954). The same is true of ulceration of the udder which may be seen in cattle infected with *O. gutturosa*. However, the fact that some of these lesions respond to antifilarial treatment suggests that the worms have an aetiological role. For example Thienpoint and Bichwe (1957) claim to have cured two cows with dermal onchocerciasis by treating them with diethylcarbamazine, and Thomas (1958) has had some success with the treatment in a case of erysipeloid reaction in a horse with onchocerciasis. There are difficulties in interpreting these observations since cattle and horses are often infected with several species of *Onchocerca* or other parasites producing dermal lesions, e.g. *Parafilaria.* At one time it was generally believed that fistulous withers in horses was due to focal inflammatory processes

around the adults of *O. cervicalis* mainly as a result of secondary infection with *Brucella* and other organisms (Steward, 1934). But this must be a rare complication since relatively high infection rates with *O. cervicalis* are still recorded in England in areas where fistulous withers is very rarely seen (Mellor, personal communication). The adults of both *O. gutturosa* and *O. cervicalis* do occasionally produce quite severe local reactions, and in dissecting out the worms we have frequently discovered chronic inflammatory masses in the region of the cervical ligament.

The adult worms of *O. armillata* produce striking lesions in the intima and media of the thoracic aorta in cattle; the pathology has been described in detail by Chodnik (1958), but apart from slight aneurysmal change there has been no clear account of cardio-vascular disturbances resulting from the presence of these worms in the aorta. *O. armillata* is an unusual species in that the microfilariae are found in the blood and not the skin, and although it is widespread in Africa and Asia the life cycle is unknown. Patnaik (1962) noted that 99% of adult cattle were infected with *O. armillata* in Bhubaneshwar in India and he claimed that the parasite caused epileptiform fits and ophthalmic lesions similar to those reported in human onchocerciasis. It is also possible that *O. armillata* was responsible for the atheromatous lesions seen by Ramanujachari and Alwar (1953) in the aorta of pigs in India.

There have been several observations on the presence of *Onchocerca* microfilariae in the eyes of horses (Iyer, 1938; Böhm and Supperer, 1952, 1954; Nemeséri, 1955, 1956), but there is no clear evidence of their role in the aetiology of equine ophthalmia. The most convincing account of onchocercal eye disease in animals has been produced by Lagraulet (1962), who examined the eyes of 46 horses infected with *O. cervicalis* and noted corneal, uveal and choroidal lesions similar to the lesions which he had seen previously in human cases of ocular onchocerciasis. But as with the human infections there were many animals with numerous microfilariae in the eye but with no obvious lesion. It is this lack of correlation which has made other observers such as Dimic *et al.* (1959) sceptical of the aetiological association. This problem has been reviewed by Roberts (1963) and he suggests that *O. cervicalis* can cause periodic ophthalmia in horses and that riboflavine deficiency may be a possible predisposing factor in much the same way as Vitamin A deficiency is thought by Rodger (1962) to accentuate ocular onchocerciasis in man.

There have been surprisingly few studies on the transmission of bovine and equine onchocerciasis. The life cycle of *O. cervicalis* in *Culicoides nubeculosus* and *O. gutturosa* in *S. ornatum* was worked out by Steward (1933, 1937) in England, but in spite of their wide geographical distribution and the confirmatory observations by Supperer (1952) in Austria and Moignoux (1952) in France no detailed observations have been made on transmission. The same is true of *O. gibsoni*. Buckley first described the development of *O. gibsoni* in *Culicoides pungens* in Malaya more than 30 years ago in one of the most outstanding publications in parasitology (Buckley, 1938), but there have been no further developments in our knowledge of the life cycle of this parasite. Possible reasons for the neglect of these parasites may be their relative unimportance as causes of ill-health in livestock and the difficulties encountered

in maintaining the vector in the laboratory. There is also the problem of a long prepatent period and the possible host specificity of the parasites which may require the use of cattle and horses as maintenance hosts. Recent studies by Nelson *et al.* (1968) indicate that *O. gutturosa* can provide invaluable material for observations on the parasitology and transmission which are particularly relevant to onchocerciasis in man, and Eichler (personal communication) has now used this system to test the efficacy of microfilariacidal compounds. The observations by McMahon (1968) on the vector of *O. gutturosa* have been particularly encouraging and his observations on the feeding of *S. ornatum* have already been extended to studies on *S. damnosum* and *S. neavei*. It is hoped that this type of investigation will eventually lead to the cyclical maintenance of *Onchocerca* species and their vectors under laboratory conditions.

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SHORT REVIEWS

Supplementing Contributions of Previous Volumes

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Biological Aspects of Trypanosomiasis Research, 1965; a Retrospect, 1969

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I. Introduction

A reviewer covering a large subject should not aim to emphasize his own particular interest and experience unduly at the expense of other aspects of the subject perhaps equally important. However, in a more restricted treatment which aims to bring up to date a previous review, one may be perhaps more selective. At the time of the previous review in Vol. 3 of *Advances in Parasitology* the author had ranged widely over many aspects of trypanosomiasis research, but in the last five years has been more restricted to its experimental and immunological aspects, with which the present review will be most concerned.

The ultimate aspiration in this field is the search for methods for the control of trypanosomiasis in man and animals, with its goal of opening huge areas of tropical Africa to man and cattle. Possible methods of control can be considered under four different headings—control of host, control of vector,

chemotherapy, and immunization. Man having so often shown himself intransigent or unreasonable as regards changing his or his cattle's habits for the prevention of trypanosomiasis, and the force of public opinion being so vehement against the destruction of wild animals, there seems little promise in systems based on host control. New vector control methods, such as those based on genetic damage to *Glossina* populations, are under study but so far without significant advance. No anti-trypanosomal chemotherapeutic agents of radically new efficacy have been developed since 1965. The decision to confine the present review to the remaining aspect, the immunology of trypanosomiasis, can therefore be made quite unashamedly. It is still, perhaps even more, the case that (quoting from Lumsden, 1965) "immunology is probably the field of research most likely to influence fundamentally the present trypanosomiasis situation in Africa".

The impact of trypanosomiasis retarding the development of Africa increases rather than diminishes. As an example, Dr. J. A. M. E. Burke (personal communication) estimates the number of new cases of trypanosomiasis in 1968 in the Democratic Republic of the Congo at over 40 000 in a population of about 12 million. An appropriate parallel would be if there were some 30 000 cases of a lethal disease in London which the health service could do little to prevent or cure. American trypanosomiasis has a similar impact in the New World. The present review will deal only with the African disease, but a useful document comparing the African and American situations is now available (WHO, 1969).

II. Conventions and Definitions

Terms proposed by Lumsden (1965) and Lumsden and Hardy (1965) for trypanosome materials seem generally to be accepted:

Isolate. A section of a wild population separated off by transference into artificial conditions of maintenance, usually by inoculation into cultures or into laboratory animals.
Strain. A population, derived from an isolate, maintained in captivity by inducing it to reproduce continuously by serial passage, either mechanical or cyclical, in culture or in laboratory animals.
Stabilate. A population whose reproduction has been arrested by viable preservation on a unique occasion.

In a strain, selection of the continuously reproducing population takes place according to the conditions of passage. In a stabilate, this selection is avoided and selection of the population is restricted to that exerted by the processes of preservation, storage and retrieval from preservation; which seems, with regard to most trypanosome populations in experimental use, to be of little effect.

Clone. Stabilate material, if set up from a strain passaged without particular precaution, may, of course, still be of diverse genetic and antigenic constitution. More uniform populations may be obtained by setting up clones, populations

derived from single organisms, but some special precautions require to be observed (see below).
Infectivity. The capability of a given trypanosome of initiating a population in a new environment—a culture or a host.
Virulence. The capacity of a trypanosome population to produce pathogenic effect in the host infected. This accords with the usage recommended by Wilson and Miles (1964).

Immunological terminology and definitions will follow closely those given by Humphrey and White (1964). For trypanosomes the nomenclature of Hoare (1966) will be followed with the modification that Hoare's three species of *brucei*, *gambiense* and *rhodesiense* will be regarded as subspecies of *Trypanosoma brucei*. Designation will be to subspecies only when definitely intended. Ormerod (1967) has suggested that all these "species" should be submerged in *brucei* but there remains a certain convenience in retaining the three names in a subspecific way to designate populations of particular biological characteristics.

III. Antigens

A. Classification

In a working hypothesis in the previous review (Lumsden, 1965) antigens were classified as internal or external, the latter also evidencing themselves separately from the trypanosome body in the ambient plasma or culture medium as released antigens. These conceptions are still broadly true but it now seems better to classify antigens first of all on the basis of their liability to vary (WHO, 1969). Stable antigens, mainly the internal antigens of the previous classification, probably comprise many of the "run of the mill" constituents of the cells—enzymes, structural proteins, etc., which are common to all the different populations of a given "species" and may also be common to different trypanosome species which can be separated on morphological grounds. It is on the recognition of these antigens that tests for the recognition of trypanosome infection in general are likely to depend. The variant antigens mark the identity of different populations of trypanosomes of the same species which develop in succession in the same individual host. Although it is clear that a considerable number of populations of different antigenic type may succeed one another in a laboratory infection, it has still not been established what is the total potential of a clone. It is known that a "strain" may produce at least some 20 different antigenic types (examples in Brown, 1963, and Watkins, 1964) but whether a clone may continue to produce new types indefinitely in a long-term infection is not yet known. However, Gray (1965b) records the recognition of some 15 distinct types in a clone-initiated, 52-day infection in a rabbit and considered the variation limited only by the life of the host. It does seem important, however, to carry this kind of study further as providing an item of information likely to indicate what mechanism controls antigenic variation. Gray (1965b; and see below) has shown that antigens in succession, though different, are not equivalently antigenic, as judged by the titres of agglutinating antibody which they elicit. Also, McNeillage and

Herbert (1968) have shown differences in virulence between populations of different antigenic type.

Most work on the antigenic variation of trypanosomes has been on *Trypanosoma* (*Trypanozoon*) *brucei*. However, similar variation has been shown to occur in *T.* (*Nannomonas*) *congolense* (Wilson, 1967).

B. LOCATION

As regards the location of these various antigens in the trypanosome body, it seems that the stable antigens are in general internal and so are not necessarily manifested except on disruption of the organism. The variant antigens are certainly manifested on the surface, in which position, presumably, they mediate the direct agglutination reaction. Vickerman (1968) has described the ultrastructure of the trypanosome pellicle and has drawn attention to the existence outside the typical trilaminate membrane, which is the cell boundary, of a "cell coat". This cell coat is uniform over the surface of the body, but occurs only in the blood stream forms and in the metacyclic forms in *Glossina*. It is absent from earlier developmental forms in *Glossina*. From this correlation of possession of a cell coat with existence in the blood stream environment or with preparation for that existence, Vickerman suggests that the cell coat is the logical location of the surface antigens responsible for the differentiation of the various antigenic types of a trypanosome species in its mammal host, and that its loss in the developmental forms in the vector is responsible for the reversion to a "basic" antigenic type at the metacyclic stage.

Wright and Lumsden (1969) and Wright *et al.* (in press) have reported that the living blood-stream forms of *T.* (*T.*) *brucei* observed *in vitro* possess long "filaments" arising both from the posterior end of the organism and from the tip of the flagellum. The filaments were up to 70 μ long and were most easily seen by phase contrast examination of organisms in suspensions containing methylcellulose to retard activity. The filaments may be discarded. These workers provisionally named the filaments "filopodia" and they argue that these are not artefacts such as occur in the stromalytic forms of erythrocytes (Baker, 1967), for various reasons: they can be seen on actively motile organisms suspended in serum or in physiological solutions, immediately after isolation from the host; their length is excessive; stromalytic forms are absent on erythrocytes in the same preparations. Corresponding material was examined by electron microscopy using negative staining and thin section techniques. Tubular extensions of the trypanosome body about 50–60 mμ diam. were observed; and their walls were similar to that of the trypanosome body—a typical trilaminate cell membrane about 8·5 mμ thick covered externally with a cell coat 10–15 mμ thick. The structures seen by phase contrast microscopy appear to be homologous with the tubular extensions seen by electron microscopy. Such extensions of the trypanosome body are likely to be important. If the cell coat is indeed the location of the variant antigens, it is possible that the production and discarding of such extensions may provide the mechanism for the release of the antigen into the environment—the metabolic antigen of Thillet and Chandler (1957) and the exoantigen of Weitz (1960).

IV. Antibodies

Changes in the immunoglobulin levels in hosts infected with a variety of trypanosome species have been the subject of much study. Most attention has been devoted to immunoglobulin M (IgM) because of the very marked changes which take place both in the plasma and in the cerebrospinal fluid in trypanosome infections, and which have useful diagnostic applications (e.g. Mattern, 1964; Lumsden, 1966). Other immunoglobulins have been less studied but Rees (1969) studied both IgM and IgG production in sheep in response to infections with a rat-adapted strain of *T.* (*Duttonella*) *vivax*. IgM levels increased more than two-fold over the first 20–30 days, during the period of maximum parasitaemia, and then decreased slowly. IgG levels remained unchanged for this period but then increased to peaks 2–4 times their original values by 60–100 days. Houba *et al.* (1969), studying *T.* (*T.*) *brucei* spp. infections in *Macaca mulatta* (rhesus monkey), found large increases in serum IgM but no significant rises in IgG or in IgA.

Houba and Allison (1966) and Houba *et al.* (1969) have drawn attention to the fact that antibodies to other than trypanosome antigens are elicited by trypanosome infections. Heterophile antibodies, as evidenced by increases in agglutination titres to normal sheep erythrocytes, reached very high levels in their experimental monkeys and persisted for periods of 6–7 months in animals whose infections were controlled, but not cured, by suramin. Even in an animal judged cured, a moderate titre persisted for at least seven months after cure. They considered that the development of these antibodies is related to high parasitaemic level and attributed to that cause their previous failure to find them in *T. b. gambiense* sleeping sickness. The antibodies were shown to be IgM and by absorption with erythrocytes of various species they were shown to differ from classical Forssman antibodies. They differ, too, from the agglutinins in sera of patients with toxoplasmosis and rheumatoid arthritis; they can be absorbed from infected monkey sera with *T. b. rhodesiense* antigen, although this does not absorb them from the sera of patients with these diseases. Although the rise of normal sheep cell agglutinin titres paralleled the rise in IgM, high IgM levels tended to persist longer than high agglutinin titres. However, these workers showed also that the IgM is probably only to a small degree composed of antibodies to either heterophile antigens or to the parasites, as absorption with parasite antigen would nearly abolish both these activities without producing any detectable reduction in either IgG or IgM levels. The phenomenon of heterophile antibody production in trypanosomiasis is of obvious interest for diagnostic purposes.

V. Methods of Study

A. General Considerations

The reasons for slower progress in the immunology of protozoal infections, as compared with that in other parasite groups such as the bacteria and viruses, seem to be mainly as follows. The more complicated life histories of Protozoa in their great wealth of shape and form tended to attract morphological and

observational studies, seductive but often disappointing, rather than experimental or inferential ones. This tendency was reinforced by the difficulty, or laboriousness, of setting up and maintaining protozoal parasites in the laboratory as type and invariant populations in serial passage either in animals or in culture. The setting up of clones is easy for most bacteria and is practically the first act of a bacteriologist investigating a bacterial population; and the subsequent maintenance of the clone population is generally easy. These operations are much more difficult with the protozoa. However, recent improvements in technique have gone far to remedy this situation and these are summarized below. Attention has continued to be devoted mostly to *T.* (*T.*) *brucei* and we are still in need of convenient techniques for some other species, particularly *T.* (*D.*) *vivax*.

Although the methods described are useful advances on previous practices, much remains still to be done in developing and inventing new techniques and in adopting as routine the improved techniques we already possess. Examples of practices to the detriment of the reproducibility and comparability of experimentation are (i) the assumption that high passage laboratory material is representative of the behaviour of the various named trypanosome species, (ii) the use of numbers of organisms derived directly from a donor animal and suspended in unstipulated diluents or even at low pH, as a quantitative measure of the inocula administered, without recognition of the extreme variation in the number/infectivity ratio which is known to take place with such factors as time in the course of an infection or variation in the pH of the medium (Cunningham *et al.*, 1963b; Lumsden *et al.*, 1965).

B. STABILATION

Viable preservation of trypanosome populations at low temperatures (stabilates—Lumsden and Hardy, 1965) has been increasingly employed to provide standard materials available over long time periods. This method has great advantages over serial passage in avoiding changes in the characteristics of trypanosome materials and it has facilitated the attainment of reproducibility in experimentation. Trends have been away from storage of stabilates in mechanical refrigerators with their inherent irregularity of temperature control and liability to break down, towards storage in contact with solid carbon dioxide or liquid nitrogen. By the latter method, so long as the container is properly designed and the system is kept well filled with refrigerant, it can be assumed that temperature does not deviate from the temperature of vaporization of the refrigerants used (respectively −79°C and −196°C, at sea-level). Storage space in such systems is of course more restricted than in mechanical refrigerators, but this is immaterial since trypanosome materials are typically of high infective potency and only small quantities need be stored. Volumes of about 20 μl in sealed glass capillary tubes are usually convenient. Techniques have usually involved the addition of a freezing protectant such as glycerol or dimethyl sulphoxide and slow cooling, particularly through what appears to be a critical temperature about −30°C (Cunningham *et al.*, 1963a; Cunningham *et al.*, 1965a). The setting up of

large numbers of samples of a stabilate, to act as standards over long periods, by methods of slow cooling, presents some problems in obtaining a suitable slow cooling rate in all the individual samples which can be reproduced on different occasions. Probably the method of Cunningham *et al.* (1963a) is simplest; others demand laborious procedures (Polge and Soltys, 1957) or special apparatus (Walker and Wilen, 1967; Goodwin and Thiel, 1967). However, good results have been obtained by a less demanding technique of rapid cooling, simply by plunging the glass capillaries, containing the material to be preserved, into liquid nitrogen (Herbert *et al.*, 1968). In this case the best results were obtained in suspensions without freezing protectants; as compared with the suspension before cooling, organisms diminished by about antilog 1·1 and infectivity by about antilog 1·8 ID_{63}, i.e. about one organism in 11 survived and about one infective organism in 60.

C. CLONING

For a clone population to be accepted as representative of the single organism from which it is derived, certain precautions require to be observed and stated (Lumsden *et al.*, 1967). First, it must be beyond reasonable doubt that only one organism is present in the initial inoculum. This may be ensured, as far as is humanly possible, by the use of separate drops of suspension small enough to be included completely in the field of the microscope, and requiring the singularity of the organism seen in it to be vouched for by three separate observers. Less stringent arrangements than this may fail, as is shown by negative controls (P. D. Marsden, personal communication). Second, the population derived from that individual must be grown up in an environment as free as possible from any factors likely to select arising mutants. For instance, if the population is grown up in a mouse, calculation will show, even assuming no organisms are destroyed, that a parasitaemia recognizable by microscope (about antilog 6 organisms per ml) cannot be expected before about 17 divisions, i.e., on a six-hourly interdivision interval, before about 100 h. By this time, and even more if the infection is allowed to proceed to levels suitable to use in agglutination tests (see below), antibody response on the part of the host will have begun to select a new antigenic type and the population may well be of mixed antigenic constitution. It is necessary, therefore, to grow up the population in such a way as to avoid this. On present knowledge it appears that passage at intervals of three days or less will fulfil that proviso; blind passage (i.e. passage without evidence of parasitaemia) of a massive inoculum will be necessary for at least the first passage of the series.

D. NOMENCLATURE OF ANTIGENIC TYPES

Some generalized nomenclature is now required for trypanosome antigenic types so that a synthesis of available information develops naturally. At present different workers use different systems evolved *ad hoc* for their individual experimentation, and so far little comparison has been made between the antigenic type series accumulated by different workers. Lumsden *et al.* (1967)

proposed a system in which any given type is designated by a prefix and a number. The prefix is made up of initial capital letter indications of the laboratory concerned in designating the material and of the subgenus of *Trypanosoma* concerned, followed by the lower case letters "at" to signify antigenic type. It was desirable not to designate the material to lower than subgenus level as there is evidence that some variant antigens occur common to materials of different biological behaviour—*T. b. brucei* and *T. b. rhodesiense* (Cunningham and Vickerman, 1962)—and it seemed possible that the same antigenic type could occur in different "species". Examples of the system are:

ETat 3 —Edinburgh *Trypanozoon* antigenic type 3
LoNat 12—London *Nannomonas* antigenic type 12

Euphonious results seem usually easily obtainable and the code is conveniently and easily referable to a type collection. In its use a rule of priority should probably be observed, i.e. any new antigenic type recognized and published would constitute the "type type" and any other designation shown subsequently to be identical with it would be extinguished.

E. PURIFICATION OF TRYPANOSOME SUSPENSIONS

A major problem for biochemical and immunological work has for long been the preparation of a pure suspension of blood stream forms of trypanosomes. In the absence of any method for the cultivation of these forms outside the vertebrate host there is no alternative but to grow up populations in animals, and so the problem exists of separating the trypanosome population completely from blood components. Differential centrifugation or centrifugation on density gradients have been used but are not entirely satisfactory, losing much of the population or subjecting it to such insult that its behaviour is altered. A major contribution in this field is the development of an efficient method for using anion-exchangers for separating trypanosomes from blood components (Lanham, 1969).

The method depends on the fact that blood stream forms of some trypanosomes carry either a net positive surface charge or none, while blood components carry a net negative charge (Vickerman, 1968). By judicious adjustment of the molarity and pH of the suspending fluid the blood components are induced to attach to the anion-exchanger while the trypanosomes pass on. The method may also be applied to separate populations of different trypanosome species co-existing in the same animal (Lanham, 1969).

F. MEASUREMENT OF INFECTIVITY

The infectivity titration technique proposed for trypanosome suspensions by Lumsden *et al.* (1963) assumed that a single organism, if infective, would infect, if suitably introduced into a susceptible host. This assumption is likely not always to be a true one and, further, the technique is restricted in its practical application to trypanosome materials which declare their presence in the test host clearly, soon after infection. With materials which characteristically produce low level intermittent chronic parasitaemias, the recognition

of the infected host animals is too uncertain for its application. However, notwithstanding these and other disadvantages, the technique has continued to give useful information. Onyango *et al.* (1965) were able to show successive waves of infective trypanosomes at about eight-day intervals in a man infected with *T. b. rhodesiense* over a three-week period during which trypanosomes were only occasionally seen in thick blood film. Similarly, Cunningham and Grainge (1968) showed that the course of the parasitaemia was similarly periodic in an ox during a time when blood films only occasionally showed parasites.

There have been many other informative applications, e.g. for the measurement of the infectivity of metacyclic stabilates (Cunningham *et al.*, 1965b); to compare the effects of different rates of cooling to $-80°C$ on the infectivity of *T. brucei* (Cunningham *et al.*, 1965c); to measure the infective dose of organisms inoculated by individual *Glossina* when biting (Southon *et al.*, 1965). It has been extensively used by Wilson (1967) in a neutralization test to study antigenic variation in *T.* (*Nannomonas*) *congolense*, an organism which does not lend itself to agglutination procedures.

The method may be applied to examine factors which may affect the reproducibility of experimentation. As regards the mice used for the titration procedure, Lumsden *et al.* (1968) found that differences in age between 5 and 21 weeks were not influential. Neither was strain difference (Herbert and Lumsden, 1968a). And so long as the pH was suitably controlled, the infectivity of suspensions prepared from stabilates was maintained for up to about 8 h. Some unexpected factors of influence were revealed. There was indication that infectivity might increase during the 40 min or so following retrieval from deep freeze preservation. This effect, if confirmed, is of interest in relation to the suggestion of Stowell *et al.* (1965) that structural reconstitution of the nuclei of cells may take place at this time. Also, although there was no difference in infectivity according to two mouse strains examined by Herbert and Lumsden (1968a), there was a difference in virulence (see below).

Other methods of infectivity titration require to be explored, e.g. the "latent period" method used by Warhurst and Folwell (1968) to measure the infectivity of *Plasmodium berghei* infections to mice.

G. RECOGNITION OF ANTIGEN–ANTIBODY REACTIONS

The detection of antibodies to protozoa is discussed generally in Lumsden (1967), to which reference may be made for details of techniques. At present only those few techniques whose application has been studied recently in trypanosomiasis will be discussed.

1. *Agglutination*

Agglutination is mediated by the variant antigens and is indeed the reaction at present most widely used for their recognition. Convenient micro-tests are available, developed from that of Cunningham and Vickerman (1962). That they can be carried out with stabilate material contributes essentially to their reproducibility and reliability. They are limited, however, to trypanosome

species which can be grown up in populations concentrated enough to show agglutination, which implies over antilog 7 organisms per ml; they are thus practically confined to use with *T.* (*T.*) *brucei*. They have been extensively used, however, to study the serial appearance of different antigenic types in the course of extended infections (e.g. Gray, 1965a; Cunningham and Grainge, 1968). In most cases the antigens used have been grown up in mice without special precaution to arrange that the population is of a single antigenic type. Confusing cross-reactions have often arisen, probably because the test populations were of mixed antigenic types. Although they might contain only one population concentrated enough to show agglutination, others could be represented sufficiently to elicit antibodies of their specificity when inoculated into animals. More nearly monospecific materials, both of antigen and of antiserum, may be obtained if the precautions outlined in the section above on cloning are observed.

The timing of the operations for the preparation of specific antisera in mice by infection and chemotherapy is important. Lumsden (1969), using the stabilates of the antigenic types ETat 1 and ETat 4, and Berenil, showed that antibody titres are high only between about day 6 and 10 after inoculation. They fell off rapidly thereafter and were at very low levels by days 14–17. As the test is variant specific, agglutination is a meaningful result but absence of agglutination may be due simply to the absence of the appropriate antigen in the test.

2. *Lysis*

A micro-technique has been developed by Le Page (see Lumsden, 1967). The test is variant specific and is, usually, complement dependent. It has the advantage that it may be applied at much lower organismal concentrations than an agglutination test; or to trypanosomes such as *T.* (*N.*) *congolense* which tend to auto-agglutinate.

Clarkson and Awan (1969) describe a micro-technique applied to the detection of antibody to *T.* (*D.*) *vivax*, *T.* (*N.*) *congolense* and *T.* (*T.*) *brucei* in rats. Using 50% lysis as the end point, titres of 1/128, 1/2048 and 1/512 were found for sera of rats infected with the respective parasites, treated with Berenil on day 3 and bled at day 8 after infection. These results were obtained in the presence of guinea-pig serum as a source of complement. Inactivation of the guinea-pig serum complement, or its omission, resulted in abolition of reaction except with *T.* (*D.*) *vivax* in which case the titre was unaltered. Similarly, with sera raised in sheep against *T.* (*D.*) *vivax*, addition of guinea-pig complement did not affect the result. These authors suggest that *T.* (*D.*) *vivax* itself produces a complement-like substance.

3. *Neutralization*

Two techniques are described by Cunningham and Grainge (1968). In one, qualitative, antilog 3 mouse ID_{63} of the relevant trypanosome stabilate are incubated in undiluted test and control sera and groups of mice inoculated from each. In the other, quantitative, test, the incubated mixtures are titrated to give a measure of the neutralization activity of the serum.

Cunningham and Grainge (1968) applied the technique to sera obtained in succession from an ox infected with *T.* (*T.*) *brucei*, using as antigens stabilates of the original infecting population and of populations grown up from isolations made on days 14, 35 and 56 of the ox's infection. Results paralleled agglutination tests and it may be concluded that neutralization also is mediated by the variant antigens. Neutralization can be detected at antigen concentrations lower than can agglutination, or even lysis, as low as antilog 2 ID_{63} per ml. The test has been applied in this kind of situation for the study of antigenic change in *T.* (*N.*) *congolense* by Wilson (1967).

4. *Immunofluorescence*

Bailey *et al.* (1967) describe an indirect fluorescent antibody technique. The antigen used was a suspension of *T.* (*T.*) *brucei rhodesiense* harvested from heavily infected rat blood, separated by centrifugation from most blood components, and dried and fixed by heat on microscope slides. The test was carried out on small quantities of blood dried on filter paper. Five grades of fluorescence were estimated visually, of which the brightest and the next brightest were considered to represent "virtual certainty" and suggestiveness of infection, respectively.

Cunningham and Grainge (1968) applied a similar test to sera obtained serially from a bullock infected experimentally with *T.* (*T.*) *brucei*, using as antigen populations derived (with precaution to avoid antigenic variation) from the original stabilate used to infect, and from stabilates set up from the organisms circulating in the animal on days 7, 28, 56, 84 and 140 after infection. Sera obtained on days 6, 27, 63, 84 and 140 of the infection were tested. Except for that obtained on day 6, which was inactive, all the sera showed immunofluorescence to all the antigens. This result is in contrast to those provided by agglutination, lytic and neutralization techniques and indicates that by this technique immunofluorescence is mediated by the stable antigens.

5. *Complement fixation*

The antigen used in the complement fixation test proposed by de Raadt (1968) was disintegrated *T.* (*T.*) *b. rhodesiense* organisms. Seventy-four per cent of 110 sera from sleeping sickness cases, taken before treatment, fixed complement. This test also seems to be mediated by the stable antigens.

6. *Estimation of immunoglobulin M*

Much of the IgM produced by the host in trypanosome infections is probably not specific anti-trypanosomal antibody (Houba *et al.*, 1969), but the subject is conveniently included here.

Various methods for the estimation of IgM in the serum and cerebrospinal fluid are discussed by Lumsden (1967). Most commonly used has been the method of radial immunodiffusion; the material in which the IgM is to be estimated is introduced into a well in a plate of agar containing antibody to IgM (Fahey and McKelvey, 1965). The area of the precipitation zone developing is proportional to the IgM content (Mancini *et al.*, 1965). Cunningham

et al. (1967) have adapted the radial immunodiffusion method for use with blood samples dried on filter paper, an advantage for surveys away from good laboratory facilities. Another convenient and simple method is, by double diffusion, the titration of the serum to be estimated, in a succession of peripheral wells round a central well of anti IgM serum (Mattern, 1968).

Because of a lack of standard preparations of IgM much work may be related simply to pools of sera of healthy individuals. Standard IgM preparations will allow comparison of the results of different laboratories.

VI. Characteristics of Infections

Although a categorical distinction is not possible there is a broad distinction between two different kinds of trypanosome infection. There is, first, the short-term acute infection in which the host is simply overwhelmed by the reproduction of the parasite, and there is little doubt that the mechanism of death is simply the monopolization of the metabolites occurring in the blood of the host by the exploding parasite population. There are, however, variations even in this clear picture. There may be a difference according to antigenic type as to whether the host is overwhelmed in the first or in the second or subsequent parasitaemic wave, even in the smallest of experimental animals generally used—mice (McNeillage and Herbert, 1968). In some instances with some antigenic types the experimental mice seem to be able to overcome the infection altogether and survive. It is to be emphasized, however, that these differences are revealed only by sophisticated experimentation; a large inoculum of any antigenic type of *T.* (*T.*) *brucei* will overwhelm small animals. Second there is the longer term chronic infection in which the host may succumb to the high parasitaemia in some stage in the process but which most typically ultimately progresses to a state of cachexia in which slow degenerative pathological processes progress with parasite concentrations low; or it may even be difficult to demonstrate the presence of any. Partly these differences are due simply to the dynamics of the rate of reproduction of the parasite *vis-a-vis* the antibody production of the host. The smaller the animal, the more likely it is to be overwhelmed, as a given dose of parasites will reach lethal concentrations sooner in the smaller blood volume and correspondingly earlier in relation to immunological reaction. But characters inherent in the parasite also contribute to the differences. The difference between *T. b. gambiense* and *T. b. rhodesiense* is a case in point; this is likely to be inherent in the parasite as the difference is reproduced in laboratory animals. And parallel differences have been shown between different populations of single antigenic type (McNeillage and Herbert, 1968). It seems possible that such differences might be marked by a particular antigen or group of antigens.

In laboratory animals with high parasitaemias, daily microscopic examination will often show that the infection is composed of a series of waves of high parasitaemia, some days apart, separated by remissions. That a similar effect takes place in infections which do not reach levels recognizable by microscopy

has been shown for man and for the ox (Onyango *et al.*, 1965; Cunningham and Grainge, 1968). Thus the infection seems characteristically to be periodic and this periodicity is probably to be related to the succession of populations of different antigenic type. Gray (1965a) has contributed much information on the pattern of appearance of antigens in chronic infections. There is a tendency for a population of any antigenic type to revert, either on cyclical or on non-cyclical passage, to one particular type which Gray calls the "basic" type. Thereafter the pattern, in at least the early part of the series, seems again to follow a more or less stereotyped pattern. In an experiment involving a rabbit and three goats the first five variants arising were matched in each animal as regards type and order as judged by alterations in agglutinin titre. It is presumably this tendency to revert to the beginning of a more or less stereotyped series that accounts for the restriction of numbers of antigenic types circulating in the field, as is evidenced by the finding that the same antigenic type may be recovered from a given locality after a period of some years (Gray, 1966).

Similar results were obtained by Cunningham and Grainge (1968), who studied the successive appearance of antigens, and the agglutinating activity of the serum in oxen infected with *T.* (*T.*) *brucei*. They found that agglutinating antibody to the infecting antigen could be detected as early as day 6 after infection and was still detectable at day 250. As regards agglutinating activity to trypanosome populations isolated during the course of this long-term infection, sera could, in general, agglutinate populations isolated prior to, but not after, their taking. Occasional discordant results, of sera agglutinating populations isolated later in the infection, were ascribed to reversion of the trypanosome population to an earlier antigenic type during its maintenance in mice after isolation, rather than to a reappearance of an earlier antigenic type in the experimental ox.

Another point of interest is the effect discussed by Soltys (1957) and Gray (1965b) of the agglutinating titres obtained with the later appearing antigenic types being less than those obtained with the initial predominant antigens. Gray passed a clone in rabbits every 3–4 weeks and found reciprocal titres even against the homologous infecting populations to be rarely more than 640, while titres of over that value and up to 5120 were almost the rule in a corresponding situation but with antigen derived from weekly passages. Soltys explains the effect as "antibody resistance" on the part of the trypanosome population, but Gray suggests that the later developing antigens are less "prominent", develop more slowly and so elicit only a delayed antibody response. The phenomenon is an interesting one deserving further study.

VII. Immunodiagnosis

The application of immunological methods for diagnosis may conveniently be discussed under two headings related to practical applications, first, for the recognition of particular trypanosome populations, and second, for the recognition of infected hosts.

A. RECOGNITION OF TRYPANOSOME POPULATIONS

The recognition of populations of particular potentiality, such as capability to infect man or to exert a particular degree of virulence after establishment in the mammalian host, remains a central problem not only with regard to the salivarian trypanosomes but widely among the protozoa. The picture is typically of an assemblage of organisms morphologically similar which, however, is demonstrably diverse as regards its biological behaviour. The shortcomings of the purely morphological approach were discussed in the previous review (Lumsden, 1965). Identification of populations by their immunological behaviour seems an avenue of promise which demands systematic exploration.

The stable antigens do not seem likely to provide tools of this sort as it is clear that they are common to several populations of the same species and even to more than one species separable on morphological grounds. Most of the trypanosome populations so far examined appear able to produce a large number of different antigenic types and so an approach based on the variant antigens seems to offer only a daunting prospect. Nevertheless, these variant antigens are conveniently and certainly identifiable and it seems a possibility that any given population might be identifiable by the range of antigens which it could produce or which it might most fashionably produce in a comparatively short infection. An apt comparison might be with the classification of the genus *Salmonella* (Kauffman, 1954). However, the large-scale identification of antigenic types involving systematic and extensive storage of antigens and of antisera now passes beyond the scope of the individual lone worker who has traditionally contributed most in the past. There is need for concerted study based on a generalized nomenclature of antigenic type to be able to build up a systematic picture of trypanosome populations of particular potentiality. It may be that the distinction of populations of particular potentiality will be accomplished by a biochemical rather than by immunological methods, but until some such method is available for the classification of trypanosome populations it will hardly be ethical to experiment further in the inoculation of human beings; nor will epidemiological studies in the field progress much further.

B. RECOGNITION OF INFECTED HOSTS

"When only a few parasites are present in the body of the host or when the parasites are confined to some inaccessible tissue, diagnosis can be made more readily by serological tests; indeed such tests may offer the only possible means of diagnosis" (WHO, 1969). For a general diagnostic test to indicate infection with any species of *Trypanosoma* one tends to think mainly of tests based on the stable antigens, on the premise that a negative result with a test depending on a variant antigen may simply be because the particular antigen among many was not included in the test. However, this conclusion may not be true because with the proliferation of variant antigens in any long-term infection there is, presumably, an increasing chance that the sera of the host will react with quite a small number of test antigens. Cunningham (personal

communication) has provided an instructive comparison of the results of the agglutination test (Cunningham and Vickerman, 1962) with two parasitological methods—the examination of thick blood films and the inoculation of mice—as applied to the diagnosis of *T.* (*T.*) *brucei* infections in a group of cattle. For the agglutination test four different antigenic types were used. The numbers of animals indicated as infected, out of 152 examined, by the three methods were as follows:

By thick film examination	16
By mouse inoculation	31
By direct agglutination test	53
By all methods	68

Although the agglutination test does not necessarily indicate present infection but only past experience of the antigen, it seems useful, indicating 78% of all animals for which there was any evidence at all of infection. Of the 15 infected animals not discovered by the agglutination test, presumably because of omission of the appropriate antigenic type from the test, 7 were diagnosed by thick blood film and 8 by the inoculation of mice. Twenty-nine animals were indicated by the agglutination test alone.

Of tests based on the stable antigens, immunofluorescence and complement fixation have been tried out. Bailey *et al.* (1967) report on the correspondence between an indirect immunofluorescence test and parasitological evidence in a series of 130 healthy and 50 known infected people. Correspondence was generally good, but 8 of the 130 control sera (6%) showed fluorescence of the two highest grades and 4 of the 50 infected people (8%) did not. Two of the last were early cases. Despite these discrepancies (one would like to know if intensive parasitological examination of the healthy people whose sera fluoresced showed any evidence of infection), the test is clearly a useful one for field survey. Antibody activity persisted in blood specimens kept dried on filter paper for two weeks.

de Raadt (1968) found that 74% of 110 sleeping sickness patients, tested before treatment, reacted in a complement fixation test, while in a control group of 418 patients suffering from other diseases only 1·4% gave positive reactions. The results of the tests corresponded closely with those from the immunofluorescence test.

As a diagnostic aid, however, the estimation of the IgM in the serum and in the cerebrospinal fluid has attracted the most research and has been well established as a field survey tool. Mattern's observations (1962, 1964) that the serum and cerebrospinal fluid IgM levels were typically raised in *T.* (*T.*) *gambiense* sleeping sickness patients was briefly noted in the previous review (Lumsden, 1965). Since then the effect has been confirmed for serum IgM in *T.* (*T.*) *b. rhodesiense* sleeping sickness (Lumsden, 1966). Lumsden (1966) records also that although high IgM values occurred occasionally among patients under treatment in East Africa for tuberculosis, leprosy and syphilis, high values were not typically associated with any of these infections. He did, however, find that in "tropical splenomegaly", which appears to be associated

with long standing *P. malariae* infection (Marsden *et al.*, 1965), serum IgM levels were frequently raised.

Adaptation of techniques to deal with blood samples from finger prick (Cunningham *et al.*, 1967) offers the advantage of easy and rapid collection and of easy transmittal to a central laboratory where all specimens can be tested in optimum conditions. It also makes possible collection of specimens from primitive peoples likely to refuse venupuncture.

Cunningham *et al.* (1967) applied the technique to two populations, of 550 and 51 persons respectively, who were in contact with the sleeping sickness area in the south of the Busoga District, Uganda. Among the 75 people from the first group who showed elevated serum IgM, at least 9 showed anti-trypanosomal antibodies in an indirect fluorescent antibody test (Bailey *et al.*, 1967), and one man (but only after 7 days' blood slide examination) was found to be infected. Similarly, one of the two persons with raised serum IgM levels in the second group was found to be infected. Both these men were in good health.

There are other examples even more suggestive. Among 333 persons examined by traditional methods in a focus in the Republique de Côte d'Ivoire, 2 were found infected. Estimation of IgM carried out two days later discovered 33 people, including these two, whose serum IgM was raised. Among these, on further examination, 6 more were found infected. In the rest, the serum IgM levels diminished rapidly on specific drug treatment, as it did in the persons who were shown by microscopy to be infected (Bentz and Macario, 1963). Similarly, in Senegal, 62 persons were indicated as infected by IgM estimation, of whom only 3 would have been discovered by traditional means (Mattern and Peretti, 1967). These findings indicate a need for radical re-thinking of the epidemiology of sleeping sickness. It seems probable that, besides the persons found infected by microscopy, there may exist in the general population a large reservoir of people clinically unaffected who could be effective sources of trypanosomes to vector insects. Thus the "healthy carrier" situation may extend much more widely in Africa than Rhodesia, where it has been reported by Blair *et al.* (1968).

VIII. Immunopathology

So far, immunological studies on trypanosomiasis have been almost exclusively concerned with the humoral antibody aspect with applications for immunodiagnosis or immunization in view. Antigen–antibody combinations *in vivo* may have deleterious, as well as protective, effects and these may be important for an understanding of the pathology of the disease. The special propensity of trypanosome infections to stimulate IgM production is likely to be important in this context. Perhaps this high IgM production is to be related to the continuous antigenic stimulation by new antigens associated with the phenomenon of antigenic variation (WHO, 1969). Cell-mediated aspects of the immune response may also be important but have so far escaped study.

Basically the pathological process is inflammatory, with invasions of lymphocytes, histiocytes and plasma cells, progressing to fibrosis. In the brain,

demyelinization may occur in the vicinity of the infiltration; the critical change is from an inflammatory to a demyelinizing process, which appears irreversible even in the absence of organisms, and which may be immunologically mediated (WHO, 1969).

Some recent work is of interest. Virulence of the infection in mice may be related to antigenic type. McNeillage and Herbert (1968) and McNeillage *et al.* (1969a, b) found that virulence varied from one to another of a group of closely related antigenic types set up as clone populations from first variant populations of one *T.* (*T.*) *brucei* stabilate, or of a clone population derived from it. Six different antigenic types were studied, coded ETat 1–6. Using very small infective doses (1·3–3·8 ID_{63}) it was found that four types, ETat 2, 3, 5 and 6, were virulent, nearly always killing the mice in the first parasitaemic wave some 6–7 days after inoculation. Two others, ETat 1 and 4, were characteristically less virulent, the mean survival time of the mice infected with these types being 10–11 days. Mice tended to survive the first parasitaemic wave and die in the second. With the less virulent types too, some mice overcame the infection entirely and a complete self-cure seems to have occurred. These less virulent types also seem to be less infective, judging from the success rate in attempts to set up clone populations from them. For instance with ETat 1, only one attempt out of 23 was successful, while with the other types the success rate was usually 1 in 2 or 3 attempts.

Herbert and Lumsden (1968a) could detect no difference between two different strains of mice as regards the infectivity of two *T.* (*T.*) *brucei* stabilates for them. The method used could be expected to recognize only fairly large differences (about ten-fold) in infectivity, and the possibility that differences would be revealed by using closer dilution intervals and larger numbers of mice is not excluded. Nevertheless, although Herbert and Lumsden's experimentation showed no difference in infectivity related to mouse strain, it revealed a clear difference in virulence. With both stabilates, one mouse strain was more able than the other to overcome the first wave of parasitaemia and to survive to experience a relapse wave. This ability, consistent throughout the experimentation, resulted in the lowest dilutions of the titrations in highly significant differences in mean survival time. The cause of this difference is as yet unexplained. It could be due to differences in the capability of the two strains to produce antibody, to some difference in the host tissue fluids affecting the rate of trypanosome multiplication, or to external factors, such as the effect of other infections, e.g. *Eperythrozoon*, on the host, and so on. But the definition of such a difference offers a model for studies which could clarify our concepts of the mechanisms of pathogenicity of trypanosome infections.

IX. Immunization

First, there appear to be many examples of non-specific immunity to trypanosomes. *Papio* spp. (baboons) are reputedly unable to be infected with salivarian trypanosomes generally, and *T. b. brucei* is by definition "non-infective" to man. The criterion of non-infectivity is usually that of absence of clinical disease or parasitaemia distinguishable by microscopy after

experimental inoculation. Recent applications of serological tests for trypanosomiasis indicate that there occur in endemic areas persons who seem to be infected, but in good health, and in whom it is difficult or impossible to demonstrate parasites (Bentz and Macario, 1963; Mattern and Peretti, 1967; and see above). It must be entertained, therefore, that some of these apparently clear cases of non-specific immunity might have an element of acquired immunity in their causation. Acquired immunity without apparent infection by a human population in general also seems to be a likely explanation of the typical pattern of discovery of endemic foci of trypanosomiasis in Rhodesia—attention becomes drawn to an area, not because of cases among the indigenous population, but because of cases occurring in persons from *Glossina*-free areas paying short visits (Blair *et al.*, 1968). The factors deciding non-specific immunity and latency may be other than purely immunological—temperature of the ambient, food materials, host metabolites, etc. (Zuckerman, 1968).

As regards the induction of artificially acquired immunity this seems to be dependent upon the variant antigens. Recent attempts to raise immunity to trypanosome infections in mammals by means of vaccines have given more consistent results than many earlier ones because greater control over the antigenic stability of the materials used has been rendered possible by their viable preservation at low temperatures (Polge and Soltys, 1957; Cunningham *et al.*, 1963a). Immunization has been accomplished by the use of trypanosome suspensions inactivated by several means, with or without adjuvants and by the use of released trypanosome antigens (Soltys, 1957, 1964, 1965; Thillet and Chandler, 1957; Weitz, 1960; Dodin *et al.*, 1962; Johnson *et al.*, 1963; Seed, 1963; Gill, 1965). However, most of the successful immunization schedules reported have involved repeated inoculations of vaccine. Only Soltys (1965) and Herbert and Lumsden (1968b) have reported effective immunity elicited by single inoculations of vaccine.

Soltys (1965) used as vaccine a trypanosome suspension inactivated with β-propiolactone and found the experimental mice to be protected against challenge as soon as day 7 after vaccination. Herbert and Lumsden (1968b) succeeded in protecting mice with single doses of various kinds of vaccine-killed organisms, released antigens and formalinized whole infected blood, either crude or in water-in-oil or multiple emulsions. With the oil emulsion adjuvant vaccines, very small quantities of antigen were effective but immunity took some weeks to rise to a protective level. With large doses of formalinized antigen administered intravenously, however, protective immunity could be elicited in as little as 7 days. The formalinized vaccine administered subcutaneously was, however, ineffective. Perhaps Soltys' (1965) failure with formalinized vaccines may be attributed to his using the subcutaneous route or perhaps to the high concentration of formaldehyde (0·2%) which he used to inactivate the organisms. The formalinized vaccine administered intravenously provided a simple, rapid method of assessing the results of simultaneous immunization against several different antigenic types. Herbert and Lumsden (1968b) found that a mixed vaccine containing four antigenic types protected against all those types, but in experiments with heterologous pairs no cross-protection was shown.

This kind of approach is laborious and there is a school of thought which considers that as the protection is based on the variant antigens, it may not be possible to produce a polyvalent vaccine likely to be efficacious and which might not be nullified by the introduction of antigenic types not covered by the vaccine from other areas. These arguments are weighty but the approach based on vaccines prepared to individual antigenic types is a rational one and requires still to be pursued, particularly as Gray (1965b) has shown that the various antigenic types appearing in a chronic infection seem to diminish in antigenicity through the series as judged by the agglutinin titres to which they give rise. Experiment with a wider range of antigenic types is needed; a tentative comparison may perhaps be made with *Salmonella* infections in mice in which immunity seems to be associated with persistence in the tissues (Glynn, 1969). Also Gray (1966) has shown that given antigenic types may be repetitively isolated from a locality over extended periods, suggesting that the basic antigens circulating in an environment may be more circumscribed than might be thought. And there are the examples of specific acquired immunity operating in the field; indications that this occurs in man have been quoted above and a corresponding example exists with Ndama cattle.

Immunization by infection and treatment has been studied in cattle by Cunningham (1968) using a *T.* (*T.*) *brucei* stabilate and an infecting or challenge dose of antilog 6 mouse ID_{63}. Although the numbers of animals involved were small, only 12 in all, it was found that of 6 animals challenged 1–3 months after immunization only 1 became parasitaemic as judged by the inoculation of mice, while 5 of 6 control animals did so. This approach, being highly economical of antigen, could profitably be followed with stabilates of metacyclic trypanosomes representative of those circulating in field situations.

X. Conclusion

It is believed that the conviction expressed in the earlier review that immunology was the approach most likely to contribute fundamentally to our understanding of trypanosomiasis, has already been justified. One may point in particular to the application of serological tests in the field indicating that the silent reservoir of infection in the human population could be much greater than the numbers of people that declare ultimately as clinical cases. This enforces a radical change in epidemiological thinking. Besides this, we have acquired a great deal of information on the process of antigenic variation in the host, and on the relation between antigenic change and infectivity and virulence, which contribute towards a rational assessment of the possibility of artificial immunization and to an understanding of the mechanisms of pathogenesis.

It is to be expected that illuminating contributions will continue to flow from this kind of study, especially as techniques for the standardization, purification and characterization of trypanosome populations, essential preliminaries to precise immunological work, have also improved concurrently. It is hoped that immunological studies will contribute ultimately to what is the central

problem in studies in trypanosomiasis, and also in other pathogenic protozoa—the recognition of populations of particular biological capability among the assemblage of organisms of identical morphology. Little further progress will be made in the epidemiology of trypanosomiasis until this can be done.

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Relationships between the Species of *Fasciola* and their Molluscan Hosts

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I. The Ecology of the Snail Hosts

Recent studies have enlarged our knowledge of the snail hosts of both *Fasciola hepatica* and *F. gigantica* and have confirmed the former as being an amphibious animal best adjusted to temporary habitats and the latter as being truly aquatic.

A. Vectors of *Fasciola hepatica*

A series of studies by Boray (1964)* and Lynch (1965) in Australia have added greatly to our knowledge of *Lymnaea tomentosa*, now regarded as a synonym for both *L. brazieri* and *Simlimnaea subaquatilis* and as such apparently the only species of the genus in Australia and hence the only vector of *Fasciola.*

The snail is very widely distributed in New South Wales, Victoria and Tasmania and in certain localities in S. Australia and Queensland.

Water movement in the habitat to provide aeration and remove excreta seems to be essential. The main habitats are:

(1) *Black springs*—which are temporary or permanent and which provide water for swampy areas containing small holes, hoof-marks, etc., or long channels with slowly running shallow water.

(2) *Natural or artificial dams.* These are supplied by springs and small streams. If there is a permanent flow into the dam the snails are usually found around the mouth of the channel, otherwise they are distributed along the edges.

(3) *Irrigation channels, covered with water for short periods only.* In well established irrigation systems with adequate drainage, the snails were usually restricted to leakages and seepages from feeding and drainage channels.

* Readers should note also the recent review by Boray (1969). [Ed.]

In South Australia there are habitats of special interest along the margins of the Murray Lakes which have essentially been brought into being by the construction of barrages across the Murray delta. The habitats are of three types:
(a) *Ponds behind the lakes.* These fill after rain or when wind causes a rise in the lake level. They persist in the cooler months but normally disappear in the summer. With prolonged flooding, which sometimes occurs, the habitat can disappear as the pond assumes the characters of a permanent pool.
(b) *Banks of the lake.* In some areas the country is very flat and the banks of the lakes are indefinite, the presence of water in a particular area depending on the flow in the river.
(c) In other areas the banks have been eroded by wave action which is controlled by planting beds of *Typha.* This activity creates small habitats for populations of *L. tomentosa* near the banks.

In many respects *L. tomentosa* resembles *L. truncatula*. It is tolerant to a wide range of temperatures and resistant to low temperatures. Like *L. truncatula* the snail tends to leave the water if it becomes too hot and it can remain alive in aestivation for several months. There is more evidence of active burrowing, associated with the drying out of the habitat, than with *L. truncatula* (see Lynch, 1966).

On the whole the species is probably a little more aquatic in its habits than is *L. truncatula*. Water is the most important single feature in the ecology of both species and increases in populations tend to be related to increases in water supplies in the potential habitat. On the other hand, if water stays in the habitat too long the snail population will decline.

B. VECTORS OF *Fasciola gigantica*

McCullough (1965) has reported on the habitats of *L. natalensis*, the African vector of *F. gigantica*, in Ghana. The species is particularly common in the south-west of the country, including Ashanti, where the vegetation is primary or secondary tropical forest. The snail shows a marked preference for slow-flowing or standing water and is most often located in dams, deep ponds and sluggish streams, especially those with a well established aquatic plant life. It does not occur in fast-flowing streams or in large rivers; nor does it inhabit temporary or permanent swamps. In Ghana as in other parts of Africa and Asia (Kendall, 1965) it is a truly aquatic species which is not resistant to drying. McCullough suggests that it is less adapted to temporary drought than many of the other "species" which act as vectors for *F. gigantica*. None of these is, of course, resistant to drought as are the vectors of *F. hepatica*.

Under present conditions the areas where cattle and *L. natalensis* occur together are fairly restricted and the grazing habits of the local breeds of sheep and goats are not likely to put them in major danger. This probably explains why fascioliasis is not at present an important disease of livestock in Ghana. However, since 1950 many man-made habitats (dams, ponds, borrow-pits, etc.) have been built and have played an increasingly important role in the distribution of the snail. As there is an increase in the number of

sites favourable both for the survival of the snail and for the transmission of the parasite so will the danger of fascioliasis increase.

McCullough's comments underline the increasing danger arising from water conservation and utilization not only with regard to fascioliasis but also schistosomiasis.

II. The Range of Snail Species Parasitized

In the earlier review in Vol. 3 of *Advances in Parasitology* (Kendall, 1965) it was concluded that *F. hepatica* and *F. gigantica* were transmitted respectively by *L. truncatula* and its close allies and by the *L. auricularia* complex.

Boray (1966) has subjected a large number of species of *Lymnaea* to infection under laboratory conditions with the miracidia of *F. hepatica* and *F. gigantica* from different areas, having particular regard to the susceptibility of *L. tomentosa*. In general his results confirm those of previous workers. Some degree of susceptibility could be found in a number of snail species—including some not previously reported. Most were, however, susceptible only when young.

Of considerable interest was the discovery that although the European vector of *F. hepatica* (*L. truncatula*) was fully susceptible to infection with *F. hepatica* from both Europe and Australia, the Australian vector *L. tomentosa* was considerably less susceptible to the European strain of *F. hepatica* than to the Australian strain. On the assumption that *F. hepatica* was introduced into Australia at the time of European colonization this indicates the development of some degree of adaptation between the parasite and a new host—a phenomenon of which there was a further indication during laboratory passage of the European *F. hepatica* through *L. tomentosa*. Boray was also able to show that the Australian *L. tomentosa* was "fully susceptible" to *F. gigantica* from E. Africa, Malaya and Indonesia and he suggested it could, therefore, serve as a potential host for *F. gigantica*.

In general, Boray's paper (1966) differs from some previous literature in that it suggests the strong possibility of a multiplicity of snail vectors for the species of *Fasciola* and of the ready adaptation of the trematodes to new hosts. This has extremely important economic implications if a new vector occupies a different ecological niche from that of the accepted vector—thereby enlarging the geographical areas at risk and greatly complicating programmes of control.

The possibility of a trematode finding a new vector must, of course, be accepted—there are some well authenticated examples, of which that of *Fascioloides magna* in Czechoslovakia is one of the most striking (Erhardova, 1961), but the fact remains that in Europe it is extremely doubtful whether there is a well authenticated example of any snail other than *L. truncatula* acting as host for *F. hepatica* in the field. Certainly there are a few reports in the literature but the difficulty of species identification in *Lymnaea* cannot be over-emphasized.

Boray's evidence of the ready infection of *L. tomentosa*—presumably an original Australian species—with *F. gigantica* is of considerable interest but must be considered in the context of evidence that *F. gigantica* does not exist

in Australia, although opportunities for its introduction in the past would appear to have been as great as those which led to the introduction of two species of *Babesia*, of which one (*B. bigemina*) is typically tropical, as is *F. gigantica*, while the other (*B. argentina*) is at least semi-tropical in origin.

An extreme example of abnormal behaviour by *Fasciola* in an unusual host has been noted by Furmaga and Gundlach (1967), who confirmed the previously reported ability of *L. stagnalis* to become infected with *F. hepatica*, but observed also that cercariae might encyst within the hepatopancreas of the snail. Cercariae may, of course, encyst very rapidly if the shell of a snail containing a mature infection is broken and water allowed to enter, but assuming that the encystment had in fact occurred at some time before the time of examination, the observation is of interest as indicating an unusual reaction to an abnormal host.

The authors suggest that this may be a normal occurrence and that such snails with contained metacercariae may be a source of infection. Of this there is no evidence either direct or circumstantial, and the general question that arises is how far the exposure of snails in the laboratory, particularly when immature and to multiple infection, can give results which should be interpreted as indicating a stable host-parasite relationship in the field.

III. Host Resistance

Evidence discussed earlier (Kendall, 1965) suggested that although a preferred snail host was preferentially attacked others less favoured might be readily attracted and penetrated, particularly when young. The barrier to initial infection does not seem to be very strong.

Boray (1966), after confirming the relative lack of susceptibility of a number of *Lymnaeid* species to *F. hepatica*, hypothesized that the thickness of the epithelial layers of adult snails or their profuse mucus production might prevent the entry of parasites. He transferred young rediae of *F. hepatica* from young *L. stagnalis* (not a normal host) to the visceral haemocoel of adult *L. stagnalis*. The larvae died and there was evidence of tissue reaction and of encapsulation.

Rao (1966) compared the susceptibility of *L. rufescens* from Pakistan with that of *L. natalensis* from Africa to infection with miracidia of a West African strain of *F. gigantica*. As had been shown earlier (Kendall and Parfitt, 1959), *L. rufescens* was relatively insusceptible. Under the conditions of the experiment the behaviour of the miracidia of *F. gigantica* seemed to resemble the behaviour of the miracidia of *Schistosoma* described by Sudds (1960), in that after initial contact they remained close to the normal host but swam away from the abnormal host. It appeared that the miracidia came into contact with the snails by chance but that there was a chemotactic stimulation once contact had been made.

Miracidia seemed to take longer to penetrate *L. rufescens* than *L. natalensis*, the normal snail host of this strain of *F. gigantica*. Evidence both from the dissection of whole snails and from the examination of tissue sections indicated that the number and sizes of sporocysts developing in the two species of snails

were comparable but that there was considerable difference in the development of the rediae. Significantly fewer rediae developed in *L. rufescens*, their death or destruction apparently taking place soon after emerging from the sporocysts or parent rediae. No general tissue reaction to the presence of the rediae was noted but there was evidence of dead rediae being encapsulated and resorbed.

A further development of this line of work might be to make comparative studies on trematode development in a relatively insusceptible vector at different ages—from the very young snail in which development could be readily observed to proceed to maturity, to adults in which initial invasion in the absence of the true vector might be the only evidence of susceptibility.

Competition between parthenitae within the snail

Competition (probably for food) between the parthenitae of a single trematode species has been shown (Kendall, 1965) to inhibit the rate of development, and there was some evidence that the more acute competition, which could be expected to occur when one trematode species invaded a snail already occupied by another, might well result in the failure of the latter to become established and be one of the explanations of the comparative rarity of polyspecific infections.

Joe *et al.* (1965, 1966) have been able to demonstrate more direct antagonism between two species of larval trematodes (not *Fasciola*) in *L. rubiginosa*, consideration of which is particularly relevant to the present discussion because it acts as a host for *F. gigantica* in Malaysia.

Snails known to be naturally infected with a xiphidiocercaria were exposed to miracidia of *Echinostoma audyi*. Dissection of snails showed a decreasing incidence of sporocysts of the xiphidiocercaria. The *Echinostoma* rediae were believed to have eaten the sporocysts of earlier infections. *Echinostoma* rediae were actually seen to be attached to or nibbling on damaged sporocysts of xiphidiocercaria.

Presumably the larvae of trematodes within a snail are not markedly selective in the tissue they eat, although they do generally show a tendency to migrate to the hepatopancreas. It can be surmised that the originally indiscriminate consumption of dead and dying larvae may have evolved into the much more active habit demonstrated by Joe and his colleagues.

IV. The Survival of Metacercariae

This has important implications from the epidemiological point of view, and laboratory experiments have been carried out by Boray and Enigk (1964) using both *F. hepatica* and *F. gigantica* at temperatures between −20°C and +35°C and at controlled relative humidities.

Metacercariae of *F. hepatica* were no longer infective, although apparently viable after 12 h at −20°C. At −10°C most metacercariae retained their viability for 7 days and some were still infective at 28 days. Freezing in water at −10°C destroyed them, however, in 7–28 days. At −5°C some metacercariae were infective after 28 days but were non-infective after 56 days. If they were

kept at $-5°C$ for 12 h in each day and at $+10°C$ for the other 12 h a high proportion was infective after 70 days. Metacercariae of *F. hepatica* died when exposed to $+35°C$ for 14 days but a high proportion survived at $+30°C$. The results obtained at different relative humidities confirmed the view that a high relative humidity (90° or more) is necessary for prolonged survival, particularly at higher temperatures.

It was of interest that the metacercariae of *F. gigantica* were able to survive appreciably longer at higher temperatures than were those of *F. hepatica*, but they were more susceptible to desiccation.

Implications for the survival of metacercariae under field conditions

The observations of Boray and Enigk (1964) suggest that the metacercariae of *F. hepatica* are likely to survive all the usual winter conditions of both Europe and Australia and that infection may be contracted by grazing animals for many months after the cercariae have emerged.

In Europe a reduction in the number of parasites on the pasture as winter progresses is likely to result more from the natural wastage of metacercariae which have been washed away or into the soil where they are relatively inaccessible, than from their loss of viability or infectivity. Alternate freezing and thawing of the metacercariae did not appear to be markedly deleterious—a rather surprising observation. Temperatures above 30°C seem to be deleterious but, provided high relative humidity is maintained, high temperatures are unlikely to be critical under European conditions.

It was of interest that the metacercariae of *F. gigantica* were able to survive for appreciably longer periods at high temperatures than were those of *F. hepatica*, but that they were more susceptible to desiccation. *Fasciola hepatica* is essentially found in temperate climates (although it occurs in the Western Hemisphere under semi-tropical conditions) and it is transmitted by amphibious snails. *Fasciola gigantica* by contrast is a parasite of tropical areas and is transmitted by aquatic snails. Boray and Enigk (1964) suggest that their experimental results are in accord with the adaptation of *F. gigantica* to more aquatic conditions and to higher temperatures than in the case of *F. hepatica*.

Boray and Enigk further concluded that in Europe inadequately dried hay stored under cold and wet conditions could carry infection but that the risk from properly dried and stored hay was negligible—as it would be in any event under tropical and sub-tropical conditions.

V. The Epidemiology of Infection with *Fasciola*

Reference was made earlier (Kendall, 1965) to the theoretical considerations which led Ollerenshaw and Rowlands (1959) to propose a method of forecasting the incidence of fascioliasis in any particular year. Ollerenshaw (1966) has now reviewed this work and provided an assessment of the reliability of annual forecasts of disease incidence issued over the five years between 1958 and 1962.*

The broad principles originally established on the basis of records of incidence of acute disease and of climate in one particular part of Britain (Anglesey)

* See also Ollerenshaw and Smith (1969). [Ed.]

have been shown in general to apply throughout England and Wales. Fundamental weaknesses have always existed because, as had been known, the assessment of disease incidence has necessarily been based on rather unreliable data (fascioliasis in Britain is not a notifiable disease and there is no recognized channel for the regular collection of information on losses), and because considerable differences in climate may occur within areas which have had to be regarded as uniform from a meteorological point of view. Further discrepancies may occur when the forecast is being applied on a local basis because the incidence of disease in any particular area is dependent not only on climate but also on local conditions of geological structure, type of soil, topography of land, state of the drainage and density of stocking. Advice to farmers on a national scale needs, however, to be couched in fairly general and certainly unambiguous terms so that minor variations on a local basis are not of major importance. Establishing relationships between disease and climate seems to be becoming of greater interest not only with fascioliasis throughout the world but also with other diseases of helminth origin.

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Fascioliasis: the Invasive Stages in Mammals

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I. Introduction

Since our previous review was written (Dawes and Hughes, 1964) a vast amount of work has been published and in this short review, which should be read in conjunction with the previous one, we cannot discuss all such work but must select papers which seem to advance our subject, omitting much work because space is so limited.

Boray (1969) has produced a formidable review on experimental fascioliasis in Australia and no attempt will be made to review this work, but one of us made some editorial comments in the Preface and brief notes may be made here to qualify statements already written there. Recent work is extremely varied, as brief statements will indicate. Taylor (1964) published a book with abundant information about *Fasciola hepatica*, a monograph by Pantelouris (1965a) helps to satisfy a real need and gives copious references, Sinclair (1967a) has reviewed the subject of pathogenesis in respect of *F. hepatica* and other liver flukes, Brunsdon (1967) has considered the problem of liver fluke and its control in New Zealand, Smith (1965) has given a useful account of trematode metabolism and (1968) has reviewed research on the biochemistry of endoparasites. Lesser works of varied interest include infection by Ford and Lang (1967) of a ground squirrel (*Citellus tridecemlineatus*) with *F. hepatica* and consideration of the effects of hibernation of the host on the young flukes. Hibernation was induced by a temperature of 6°C and when this occurred 15 days after infection and therefore before the flukes had left the hepatic parenchyma, maturation and development were greatly retarded; the claim was made that when hibernation was induced 45 days after infection, flukes

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were lost from the bile duct. In reports of *in vitro* work, Wikerhauser and Svetnic (1967) attempted to keep immature and mature flukes alive in various cell-free media with and without mammalian cell cultures. They were able to keep immature flukes alive for a maximum of 14 days and adult flukes alive for a maximum of 5 days but there was no evidence of growth or development in any of the cultured young flukes. Hughes and Christofinis (unpublished data—in preparation) were able to maintain newly excysted *F. hepatica* using a tissue culture system or tissue culture media alone. Immature flukes were maintained for up to 108 days (Fig. 1) with regular fluid changes and also in some cases tissue culture changes. These flukes appeared to be in a state of "suspended animation" and, although 108 days old, never showed development beyond that associated with 8-day-old flukes from mice (approx. $0{\cdot}8 \times 0{\cdot}4$ mm). This stage of development was reached after only a few weeks in culture and the flukes did not develop further. The appearance and size of the young flukes was similar to those flukes obtained from mice 8–11 days after infection with X-irradiated cysts (Hughes, 1963; Dawes, 1964).

Pantelouris (1965b) noted that carbon-labelled glucose and galactose are taken up from the medium in which adult flukes were cultivated *in vitro*; when the hormones insulin, tri-iodothyronine or adrenaline were added to the medium, the radioactivity counts in wet tissue samples were greatly reduced for galactose, only adrenaline having this effect with respect to glucose. This finding was taken as indicative that host hormones might be an important factor in the metabolic compatability of host and parasite.

II. Excystment and Metacercarial Structure

Our knowledge of the excystment process and metacercarial structure in *F. hepatica* has been enhanced by Dixon (1964a, 1966a), who considered the physiology of excystment in great detail. The fine structure of the cyst wall was studied by Dixon (1965) and Dixon and Mercer (1964), and the morphology and histochemistry of the cyst wall and cercariae by Dixon (1965, 1966b). Excystment is considered to take place in two stages, activation and emergence. Activation is initiated by high concentrations of carbon dioxide, reducing conditions and a temperature of about 39°C. The stimulus of carbon dioxide has to be sustained for 5 min only, the reducing conditions for 30 min. The order in which these stimuli were applied was of no consequence. Emergence is "triggered" by bile. Changes in the staining reactions and fine structure of the cyst wall around the emergence hole suggested that an enzyme is secreted by the metacercariae which digests the ventral plug region. Dawes (1961, 1963) has produced evidence which would support this theory. Dixon compared conditions necessary for excystment *in vitro* with those conditions found in the gut of the host, and concluded that in ruminants activation takes place in the rumen and emergence takes place in the small intestine below the opening of the bile duct, whereas in non-ruminant hosts activation could take place in the stomach or the small intestine. He considered that bile may activate an enzyme secreted by the parasite and induce muscular movements. The excystment of metacercariae after injection into the peritoneal cavity as

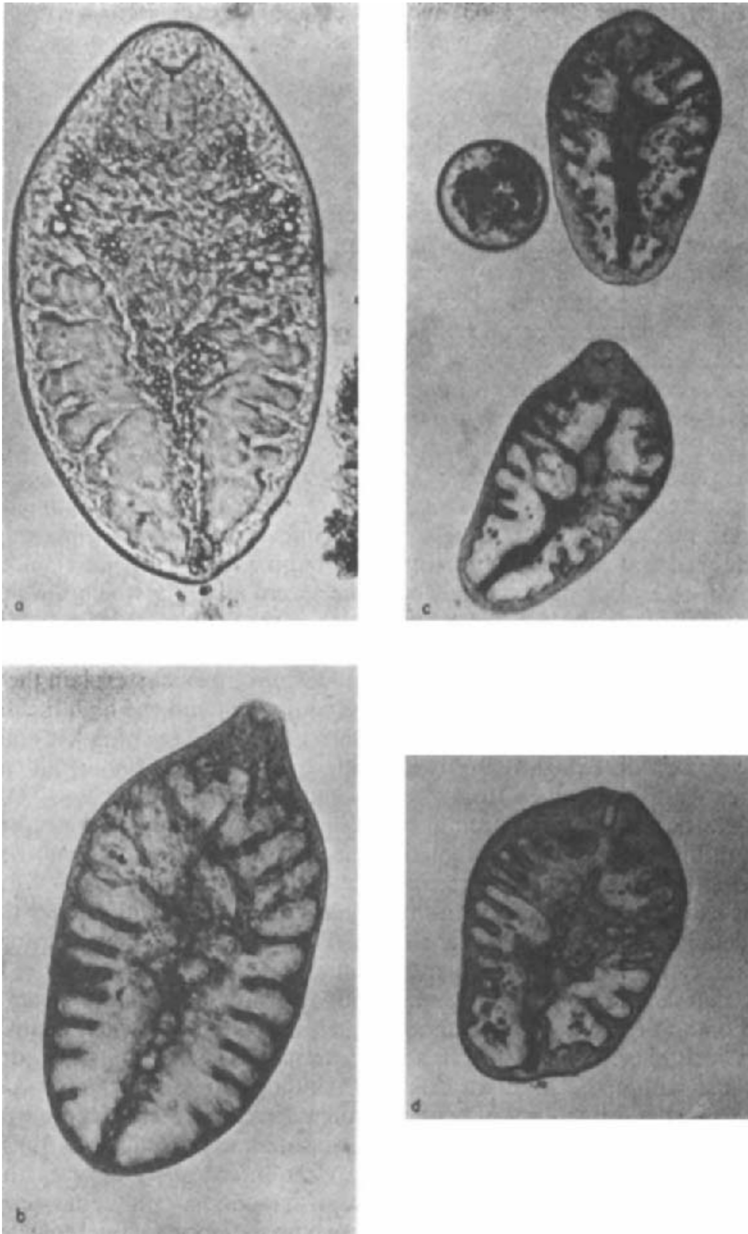

FIG. 1. Young *F. hepatica* from *in vitro* culture with approximate sizes (length × breadth). **A**, 42 days in culture (0·7 × 0·4 mm). **B**, 108 days in culture (0·8 × 0·4 mm). **C**, 42 days in culture. Two flukes (0·6 × 0·4 mm) with inner cyst for size comparison (0·2 mm). **D**, 42 days in culture (0·6 × 0·35 mm).

reported by Hughes (1959, 1963) and Dawes (1961) is explained as a response to continued stimulation from a relatively low concentration of carbon dioxide.

III. Fascioliasis in Cattle

Our knowledge of fascioliasis in cattle has been much improved lately and the papers of Ross and his co-workers in Ireland have helped our understanding of the host-parasite relationship in cattle. When cattle were infected with 200–1300 metacercarial cysts an increase in the infection level did not alter the percentage of the infection which became patent and a few flukes were inhibited in the damaged parenchyma, particularly in the ventral lobe where a preferential migration of the parasite occurred (Ross, 1964). Ross (1965) then compared the infection produced in cattle given a low level (LL) and a high level (HL) of infection. When the level of infection was increased to 2500 cysts, the number of flukes reaching the bile ducts was drastically reduced and at higher levels (5000 and 10000 cysts) many immature flukes were trapped in the liver parenchyma. The extent and severity of the fibrosis in the bile ducts in the HL infections were much less than in infections of 1300 or fewer cysts. Ross claimed that this observation (in conjunction with the absence of anaemia in the HL infections as compared with the severe anaemia which developed in the 1300 infection group and also the state of the bile ducts) confirmed that very few of the parasites in the HL infections reached the bile ducts. He considered that this inhibition phenomenon in HL infections may explain the rare occurrence of acute fascioliasis in cattle as compared with the high incidence in sheep. It is interesting to compare the percentage recovery of flukes and the disease process described by Boray (1967) from sheep given 200–10000 metacercariae with those which Ross (1965) obtained in cattle. Ross *et al.* (1966) state that the anaemia produced in calves appears to be attributable solely to the adult bile duct stages and that this anaemia appears to be haemorrhagic in type, although a varying degree of macrocytosis occurs.

Ross and Dow (1966) looked at the problem of acute fascioliasis in cattle in some detail and refer to the earlier work of Taylor (1949, 1964) concerning the fact that cattle show a particular resistance to infection with fluke. They stated that acquired immunity, acquired self-cure, preferential liver area migration and inhibition of immature flukes are all factors which contribute to this resistance. In HL infections, the immature flukes are trapped and eliminated within the liver parenchyma. Although these resistance mechanisms reduce the probability of acute fascioliasis in cattle, Ross and Dow point out that a syndrome exists, probably associated with immature flukes in cattle, in which death occurs 3–7 weeks after infection. It appears that a venous stasis and thrombosis are produced with secondary pulmonary emboli and secondary pneumonia. This syndrome was described by Dow *et al.* (1967); mural type thrombi were commonly found in calves in the first few weeks of infection and at about 7 weeks a diffuse oedema of arterial walls with gross swelling of the *tunica intima* was observed. Hypertrophy of the *tunica media* and internal fibrosis resulted in almost complete occlusion of the

affected vessel. Dow *et al.* also point out that the entry of the flukes into the bile duct resulted in proliferation of the epithelium, producing a glandular mucosa. The activity of the flukes resulted in destruction of the lining which became fibrotic and then calcified. Keck and Supperer (1967a, b) also describe the calcification process and bile duct changes in cattle.

Ross (1967a) described single experimental infections in 8–12-week-old calves with 200–15000 cysts. Histological examination of the parenchyma revealed the presence of trapped and disintegrated flukes. The number of flukes found in the bile ducts of cattle decreased as the infection increased above a threshold level of 1300–2500 cysts. Ross claimed that it is not realistic to refer to the life span of fluke in cattle except perhaps in an LL infection where no reinfection occurs—and that "spontaneous self-cure", a natural termination of a patent infection, may also result in cattle after calcification of the bile ducts. Ross (1967b) described the production of "acquired self-cure" in calves. Calves were given 200 cysts and challenged 18 weeks later with 300 cysts when the existing infection was eliminated. Kendall (1967) also showed in long-term experiments with sheep and cattle exposed to repeated reinfection with cysts, a marked difference in the host-parasite relationship in the two hosts. In sheep there was a build up of fluke's eggs and at death large numbers of parasites were found, but in cattle comparatively few fluke eggs were passed in the faeces and the numbers decreased with time to a low level. At slaughter, relatively few parasites were found in cattle.

This recent work in cattle has been reviewed briefly, but it provides an explanation for the findings of Montgomerie (1931) who stated "that a visit to almost any slaughter house makes it obvious that *F. hepatica* does not continue to infect cattle for a long period. The livers of 2½-years old cattle very commonly show evidence of there having been a severe infestation and yet no flukes can be found".

The fascioliasis/ostertagiasis complex in young calves was described by Reid *et al.* (1967), together with its differential diagnosis, treatment and prophylaxis. They showed how to differentiate this complex from Type II ostertagiasis in order that correct therapy could be given.

Dixon (1964b) compared sheep and cattle as hosts for *F. hepatica* after infection with metacercarial cysts derived from eggs of ovine or bovine origin. Irrespective of the host of origin, the prepatent periods and infectivity in these hosts were similar. The flukes in the sheep grew more rapidly, more uniformly and to a greater size, but cattle produced about twice as many fluke eggs per parasite per day as compared with sheep. Dixon gave the cattle or sheep only small numbers of cysts (i.e. 50 or 100) and the marked difference in cattle and sheep infection does not become apparent until much larger numbers are given, as has been already said in discussing the work of Ross (1967a).

IV. Immunity

In an effort to reveal naturally acquired immunity in rabbits, Ross (1966) challenged four rabbits with 50 cysts each, 24 weeks after an initial infection with 75 cysts each. A reduced "take" was observed in the challenge infection

and smaller fluke length was also recorded five weeks after challenge by comparison with single infections in control rabbits. Individual variation was high and the differences were significant only at the 20% level.

Kendall *et al.* (1967) infected rabbits with 10 cysts each and 12 weeks later gave each rabbit a further 20 cysts. The rabbits were killed 20 weeks after the initial infection and the number of worms recovered was smaller than could have been expected had the two infections been given separately. This apparent decrease in numbers was considered to be a probable result of inhibition of growth due to increasing numbers and the practical difficulties of recovering very small flukes. Thorpe (1965) has suggested that when large numbers of cysts are given to rats competitive inhibition occurs and there is delayed entry into the bile ducts.

Ross (1967c) investigated acquired immunity to *F. hepatica* in calves, sheep and rabbits. He injected extracts of 6-week-old immature flukes obtained from donor rabbits, and found retarded fluke growth in a subsequent infection 7 weeks after challenge. Following a previous experience of a natural infection of viable fluke implants, retardation and reduced take of the challenge infection was observed. However, considerable individual variation occurred and the level of significance between the treatment and control groups was low.

Sinclair (1968a) examined the effect of corticosteroid on the pathogenicity and development of *F. hepatica* in lambs. There was a highly significant ($P<0{\cdot}01$) difference in the mean length of (larger) flukes recovered from lambs which had been treated with corticosteroids compared with those flukes recovered from non-treated lambs. The administration of corticosteroid (dexamethasone) seems to modify the resistance mechanisms and the parasites grow more quickly. Increased pathogenicity produced in lambs varying degrees of inappetance, abdominal pain, mucosal pallor, weakness and dyspnoea. In one treated lamb, the clinical signs became so severe that the animal had to be killed 12 weeks after infection with 500 cysts.

The increased pathogenicity of the flukes in the corticosteroid-treated group was also reflected in the marked loss of live weight and anaemia in the lambs. As Sinclair remarked, the anti-inflammatory effect of corticosteroid therapy is well known, and his observations on sheep confirm the importance of the inflammatory response in the natural resistance of the sheep to *F. hepatica* and indicate that corticosteroid treatment may become a useful tool in elucidating the mechanism of such resistance.

Ross *et al.* (1967) demonstrated a considerable natural resistance to infection in pigs which resulted in immobilization, encapsulation and death of the parasite in the liver parenchyma between the second and fifth week after infection. They also describe the histology in some detail and compare it with that in other hosts. Natural resistance decreases in the order pigs, cattle, sheep.

Lang (1967) and Lang *et al.* (1967), using mice, claimed to have demonstrated acquired immunity to *F. hepatica* and affirmed that delayed (cellular) hypersensitivity plays a prominent role in this immunity. Lang (1967) used male mice 18–19 weeks old and cysts 24–80 days old. Mice were "immunized" on two occasions 60 days apart with 2 metacercariae per mouse on each occasion, and 40 days after the second stimulating infection the mice, together with

appropriate controls, were challenged with two cysts each. In all, 176 mice were used and Lang looked at the worm counts, location and size of the worms (but not the degree of development) and also at various host responses, including total body weight, total and differential leucocyte counts, spleen weights, serological response and liver histopathology.

To obtain a comparison of worm counts in the "immunized" and "non-immunized" mice, Lang killed groups of 12 mice, 20 and 40 days after challenge, and considered it a simple matter on the basis of size to differentiate the worms of the challenge and the stimulating infections! The 19 worms recovered 20 days after challenge were all in the liver parenchyma in the 12 control mice, whereas although a total of 41 worms were recovered from the immunized mice, 14 of these were considered to be from the challenge infection and six were found in the common bile duct, four in the liver parenchyma and four in the abdominal cavity. The four worms in the abdominal cavity were said to show evidence of an immune effect (two were dead and two appeared to be moribund) on the basis of greatly diminished activity in saline and disintegration of the cuticle. There was no significant difference in the number of worms from the challenge infections in the two groups, but their location differed and six of the 14 worms had already reached the common bile duct 20 days after challenge. At 40 days after challenge, the 12 immunized mice had 31 worms present, of which six were from the challenge infection. The 12 control mice had a total of 18 worms present in the bile duct. The difference between the six and the 18 worms found from the challenge infection in the two groups is significant at the level of $P=0{\cdot}001$ by the Students' "t" test. Deaths occurred in both "immunized" and control mice at about the same time after infection (i.e. 23–35 days), but the deaths were fewer in the "immunized" mice, 3/25 compared with 4/16 in the controls.

It is difficult from published results to see the fate of all 56 mice which were used in Lang's Group I (i.e. two "immunizing" infections followed by challenge). The fact that two lots of 12 mice were selected to be examined *post mortem* 20 and 40 days after challenge could possibly influence the results.

On the basis of the nature and timing of lymphocyte infiltration in (and the histopathology of) the livers, Lang suggested that delayed hypersensitivity may play a prominent role in the earlier migration of the worms to the common bile duct.

Lang *et al.* (1967) claim to have demonstrated immunity to *F. hepatica* in recipient mice given peritoneal exudate cells. Briefly, the experimental evidence was as follows: 10 male mice 24 weeks old each received $2{\cdot}75 \times 10^6$ peritoneal exudate cells from donor mice and 21 days later these mice, together with 10 control mice, were challenged with two cysts each. Those mice which had received exudate cells harboured eight worms at *post mortem*, 40 days later, whereas the eight control mice (two had died previously) had 12 worms. Lang *et al.* point out that statistical analysis by Students' "t" test of these results shows that the difference in worm count is highly significant ($P=0{\cdot}001$). Although statistically this may be so, using only 10 mice per group involves some risks and although a "take" of 40% of metacercariae given compared with 75% in the controls may represent a demonstration of the effect of delayed

hypersensitivity in producing an immunity in mice, it is felt that much larger groups of mice should be used to eliminate the variation in viability and "take" which is often evident in normal mice.

V. Anaemia and Fascioliasis

Sinclair (1964) examined the anaemia produced in sheep given 600 metacercarial cysts each. All the sheep developed various degrees of anaemia which began at about day 60. Blood and bone marrow examinations and studies on the metabolism of intravenously injected ^{59}Fe were used to compare the anaemia of fascioliasis with that produced by the daily removal of 60 ml of blood from control sheep. It was found that both anaemias were of the normocytic normochromic type, but that the production of erythrocytes was retarded in the infected sheep. Sinclair pointed out that the factors concerned in the control of erythropoiesis are imperfectly understood and that continuous production is known to be affected by the presence of raw materials in the blood which circulates through the bone marrow. Sufficient quantities of iron, copper, cobalt, vitamins and proteins are thought to be essential. He suggests that it is possibly the shortage of one of these which causes the anaemia, and concludes that haemorrhage is not the main factor. In further studies on iron metabolism in sheep, Sinclair (1965) suggests that the anaemia of fascioliasis is secondary to a disorder of reticuloendothelial function which leads to decreased erythrocyte production and increased erythrocyte destruction, and that the question of whether the adult fluke is predominantly a blood or tissue feeder is controversial. Sewell (1966) found the anaemia in Zebu cattle infected with *F. gigantica* was also normocytic and normochromic.

Obara *et al.* (1964), from their work on the *in vivo* uptake of Racobalamin-60 by *F. gigantica*, suggest that there might be a vitamin B_{12} deficiency in animals infected with liver flukes. Boray (1967) found that the intramuscular injection of vitamin B_{12} to sheep with subacute or chronic fascioliasis did not influence the Hb or PCV as compared with untreated infected controls. El-Hinaidy (1967) reviewed the literature on the nutrition of *F. hepatica* and quotes most of the relevant references starting in the late 1800s with Kuchenmeister.

Lutz and Siddiqi (1967) suggest that *F. gigantica* has a distinct haemoglobin of its own and that this pigment is a true haemoglobin and not a breakdown product of the host's haemoglobin. Todd and Ross (1966), using conventional chemical analytical techniques to examine the origin of haemoglobin in *F. hepatica*, came to the conclusion that the adult liver flukes are almost exclusively haematophagic. Symons and Boray (1967) followed the fate of ^{59}Fe in sheep infected with 4000 and 2000 cysts of *F. hepatica* and showed that iron was rapidly transferred to the bone marrow and thence to circulating erythrocytes and that erythropoiesis was greatly increased during infection. Histological examination of the site of fluke attachment to the bile ducts "often" revealed that the mucosa was missing and replaced by a blood clot. In the chronic form of the disease, they thought that the anaemia was due to ingestion of blood by the adult flukes. The senior author (B.D.) confirms complete abrasion of hyperplastic epithelium in rare instances but affirms

that a thick layer of fibrotic tissue invariably remains. Ulceration, or blood clots of the kind described, have never been seen in many hundreds of sections in numerous specimens of infected rats and mice. Never has there been clear demonstration by believers in blood feeding of the source by which this kind of food is obtained. In the hyperplastic, fibrotic state of the bile duct—a histological picture which must be seen to be believed—the acquisition of a blood meal by adult flukes is an almost impossible achievement. Most superficial blood vessels are occluded.

Holmes *et al.* (1967) used red cells labelled with ^{51}Cr in studies on the anaemia produced by fascioliasis in rabbits and sheep, and by comparing the radioactivity of a 24 h sample of faeces with that of the appropriate blood sample it is possible to get an estimate of the total amount of blood, or its breakdown products, which has entered the gut in that time. They found a good correlation in rabbits and sheep between the blood loss determined in this way and the degree of anaemia observed and suggest that this haemorrhage is one of the primary causes of the anaemia. Sinclair (1967b) criticized the findings of Holmes *et al.* (1967) and pointed out that in the sheep, labelled erythrocytes (^{51}Cr) lose 40% of the label to elution from the cells within 48 h and that most of it is excreted in the urine. In sheep which have been infected with *F. hepatica* this loss approaches 60% according to Sinclair and with it there is a corresponding increase in the radioactivity of the urine. In his own work also using ^{51}Cr, Sinclair found that following this early loss, the chromium survival curves of infected sheep paralleled those of control sheep, and he pointed out it is clear that in normal sheep the eluted chromium is mainly excreted via the kidneys, but he suspects that in fluke infected sheep a greater portion is excreted in the bile and thus one cannot be sure that all the faecal chromium represents a blood loss.

Sinclair stated that while there seems to be little doubt that some haemorrhage occurs from erosion of the biliary epithelium due to the activities of the parasite, he does not believe this to be the main cause of the anaemia of chronic ovine fascioliasis. Sewell (1967) supports Holmes *et al.* (1967) concerning the use of radiochromium as an erythrocyte label in normal and infected sheep; the apparent loss of blood per fluke per day was on average 0·5–1·0 ml, a figure greatly in excess of the estimated 0·2 ml per fluke per day previously reported by Jennings *et al.* (1956) and also that used by Sinclair (1964) in his unsuccessful attempt to mimic the effect of fascioliasis by repeated bleedings.

Sewell also noted that although Sinclair regards the apparent faecal blood loss as explicable by an increased elution rate, it has been shown that an alternative label, di-isopropyl-fluorphosphate ($DF^{32}P$), does not elute from normal sheep red cells and was used on the sheep studied using the ^{51}Cr technique. Using $DF^{32}P$ in normal sheep, the loss of the label bore a linear relationship with time, whereas the relationship was more nearly exponential in infected sheep. This finding may indicate a random loss of erythrocytes in the infected sheep, and Sewell believes that it may be due to destruction of erythrocytes, or a loss of blood by haemorrhage or similar means. The calculated blood loss was somewhat higher than that suggested by the ^{51}Cr method and the bile radioactivity could account for a daily blood loss by this route, of

the same order as the random loss suggested by the red cell survival curves. To Sewell it seems unlikely that we can explain these findings on the basis on which Sinclair criticized the ^{51}Cr findings, as $DF^{32}P$ is irreversibly bound to red cells of normal sheep, and may also be so in infected sheep. He considers that the apparent increased elution rate of ^{51}Cr in infected sheep could be explained in terms of a change from an almost linear red cell survival curve to an exponential type curve, and therefore the most likely primary cause of the anaemia is loss of blood into the bile, although this may be associated with an accelerated destruction of the numerous poikilocytes and immature erythrocytes in the peripheral blood.

Dargie *et al.* (1968) gave a full account of and extended some of their earlier work measuring the turnover of albumin by ^{131}I in normal and fluke-infected rabbits, providing much new information on the aetiology of the hypoalbuminaemia associated with the disease. Their infected rabbits were all in a hypercatabolic state with respect to albumin, and although the degradation rate was approximately twice that in normal rabbits, there was no dramatic fall in the body pool of albumin, indicating its greatly increased rate of synthesis in infected animals. Dargie *et al.* then pointed out that in view of their earlier evidence of a substantial loss of red cells or breakdown products into the gut (Holmes *et al.*, 1967) the most likely explanation of the observed hypercatabolism of albumin is an equivalent plasma leak into the gut. They support this suggestion by experimental evidence provided by the faecal excretion of polyvinyl pyrrolidone. Dargie *et al.* stated "It is tempting to relate the hypoalbuminaemia and the anaemia of fascioliasis to a direct loss of whole blood, due either to ingestion of blood by the flukes or haemorrhage caused by the flukes", but to obtain proof of direct loss of whole blood would require simultaneous determination of red cell and plasma loss by means of two distinguishable isotopic markers which are not reabsorbed. A possible system would be ^{51}Cr as a red cell marker and ^{95}Nb as a plasma protein label which is under consideration. Meanwhile, we must await patiently the solution of the difficult problems of the host's anaemia and the adult liver fluke's mode of nutrition. The controversy surrounding these problems has produced exciting as well as interesting information.

VI. Chemotherapy

It has never been our intention to review advances in chemotherapy of fascioliasis. This has been done by other writers (e.g. Gibson, 1964, 1965, 1967, 1969; Lämmler, 1964a, b, c, 1968). However, it is worthy of mention that the efficacy and safety of 11 established or new anthelmintics were tested by Boray and Happich (1968) using a standard chemotherapeutic test against both mature and immature *F. hepatica* infections in sheep. All the drugs examined were efficient against adult flukes and had a reasonable safety margin; they were effective against immature flukes as early as 4 weeks after infection but, unfortunately, only at potentially toxic doses. The conclusion was reached that improvement in safety was achieved in the treatment of acute and chronic fascioliasis by menichlopholan, nitroxynil and clioxanide. A full account of

this work (Boray, 1969) includes also a list of six drugs which are not recommended, i.e. not "satisfactory". Sinclair (1968b) considered that strategic anthelmintic medication is still probably the most practical way of tackling the problem of losses due to liver flukes. In his opinion, hexachlorophene and nitroxynil seem to be most useful in arresting mortality in sheep resulting from acute fascioliasis, and oxyclioxanide is most useful against adult flukes in cattle.

In the quest for chemical compounds active against liver fluke in sheep and cattle, the choice of an experimental host for the primary screen and the interpretation of the results obtained should be made with caution. Lämmler (1968) pointed out that some compounds may be highly effective in tests with rats or rabbits and then found to be useless in sheep. To put a finer edge on the matter, some compounds may be effectual in rats but ineffectual in rabbits. Boray *et al.* (1967) showed that Hilomid, a substance highly efficient in sheep, had virtually no activity in rats, and Tewari (1968) showed that clioxanide, a compound known to be effective against 4- and 12-week-old flukes in sheep, was ineffective against flukes of 4- and 19-week-old infections in rats. Rats also show a tendency to throw off long-standing experimental infections naturally (Hughes, Dawes, unpublished). Another important point is that the chemotherapeutic index of a compound in an experimental test animal often bears no relation to that in the large animal for which it is intended eventually. Of equal importance is the problem of host-specific side effects. Thorpe (1968), in comparing the enzyme histochemistry of immature and mature stages of *F. hepatica*, discussed the concept of basing anthelmintics on a differential and for preference irreversible inhibition of critical helminth enzymes and quotes the review of Saz and Bueding (1966).

The possibility that resistance may develop to compounds used against *F. hepatica* has been suggested by Dorsman (1967), who observed a partial resistance in mature flukes to hexachlorophene in cattle on farms where the drug had been used for several successive years. A dose of 15 mg/kg was eventually much less effective in cattle than was a dose of 12·5 mg/kg at the time when the drug was introduced in the field. Dorsman's research on the nature of this hexachlorophene resistance is continuing.

One of us has recently investigated the direct effect of drugs on *F. hepatica* and early results indicate that there may be advantages in seeking a drug of promise which does not necessarily kill flukes outright but interferes with the reproductive process, particularly egg formation. Bithionol (Actamer) was chosen because it is a drug of great promise in paragonimiasis (see Yokogawa, 1965, 1969) and because *Paragonimus* and *Fasciola* show common features of burrowing and tissue feeding during maturation and growth. Early work (Dawes, 1966a, b, 1967) revealed effects including vacuolation of the cuticle, widespread abnormality of the testes marked by almost complete failure of spermatogenesis and disorganization of the complex mechanism of egg-production, but little effect on the ovary, vitellaria or intestinal caeca. In later work (totalling 38 experimental infections plus controls) with (usually) 4–6-month-old rat infections (never less than 80 days but sometimes more than 250 days old), previous findings were confirmed and augmented (Dawes,

1968). Minimal dosage of the drug was 10 mg in suspension—and this was given once, twice or thrice with various intervening periods. A marked effect was seen in sections within 24 h of one dose of only 7 mg bithionol. The adverse effect on the testis tubules of the flukes at various periods was abundantly confirmed in serial sections of flukes removed from the bile duct or, in many instances, serialized sections of the entire bile duct and its flukes. Spermatogenesis was decidedly abnormal, even in less than 24 h, although at this early stage some previously formed sperms occur in the testes in small amounts. The full details are not available but cell cleavage at an early spermatocyte stage is deficient, and there is inhibited development of spermatids, which persist as comma-like forms. Complete failure of egg production is indicated by the presence in the first part of the uterus of masses of isolated vitelline cells and droplets of extruded secretion, as well as isolated oocytes. When treatment is extended, the uterus may be devoid of shelled eggs, and in some such cases the ovary and vitellaria show some signs of abnormality and death and disintegration of flukes sometimes occurs.

There was no claim that bithionol is a drug of choice in fascioliasis but it was suggested that there is some wisdom in studying the pathological effects of worms upon flukes, because this might lead to simpler and less expensive methods of control. It was taken as axiomatic that flukes threatened by drug action will first tend to sacrifice non-vital organs and functions and to conserve vital organs. When flukes are incapacitated to the extent of failure to produce shelled eggs, one important effect in fascioliasis has been overcome, i.e. the widespread scattering of eggs of the fluke on to the pastures. Perhaps this is the first effect to achieve, for immediate death of the flukes is not a priority when reproductive processes have been damaged. It is necessary to ascertain if the drug effects are permanent but it is clear that we must acquire much more knowledge about parasites than we now possess before setting out to exterminate them.

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Tick Feeding and its Implications

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Since the publication of the earlier review in Volume 3 of *Advances in Parasitology* (Arthur, 1965), further investigations on tick feeding have been made by a number of workers. The interplay between parasite and host in this intimate phase is fundamental in relation to such matters as trauma, allergy, toxins and ectoparasitic pathogenesis, and observations on the passage of fluids to and from ticks during the feeding process help us to understand how ticks transmit infections. In this short review it is proposed to consider (I) fluid flow interchange, (II) the formation of the "cement" and the "eosinophilic" zone around the mouth parts, (III) the characteristics of the lesion and associated histopathological changes and (IV) feeding and weight changes.

I. Fluid Flow Interchange

The most significant work on salivation and that of uptake of fluids is that of Gregson (1969) working on *Dermacentor andersoni*, in which he has used transillumination methods of observation, recorded on ciné film, and electronic monitoring of fluid flow. Within 10–15 min of the complete insertion of the tick's mouthparts into the skin, small transitory blood pools appeared at 5–10 sec intervals close to the hypostomal tip. Each was visible for 1/10 sec before being replaced by a small ejection of clear fluid from the tick. The flow of blood into this region increased 10–30 min later and the intervals between the establishment of the pools lengthened. Sucking pulses at the rate of five per sec were observed initially, each of which after about an hour lasted for 10–30 sec. The drainage of each blood pool was followed by a regurgitation of saliva, which radiated interstitially and became more sustained as feeding continued. During a resting period after each salivary ejaculation a new blood pool forms, the blood being sucked up when it reaches the mouthparts.

Spasmodically there is a "toying" with the blood during which it is sucked up from the tissues of the skin and returned. Comparable intermittent ejaculation of saliva into the host has been reported for *Hyalomma asiaticum* and *Ixodes ricinus* by Balashov (1965a), who suggested that the clear fluid-filled spaces frequently observed beneath the mouthparts result from the spurting of saliva into the lesion. A further significant movement of fluid observed in *D. andersoni* was an emission in several successive spurts of volumes of bright red blood through the oral aperture. This continuous rejection of blood suggests regurgitation from the gut, a contention supported by Tatchell and Moorhouse (1968), who found basophilic spheroids in the gut of *Boophilus microplus* and in the lesions they produce. Histochemically, these spheroids appear to be derived from the nucleoprotein of ingested leucocytes.

After partially fed females of *D. andersoni* have attached for about 2½ h, Gregson reported the establishment of a sudden haemorrhage which masked further visible observations on the pattern of saliva secretion and blood uptake. Tatchell and his collaborators (personal communication) have produced a ciné film of the actual sucking of a larva and this shows the movements of the pharynx and of the pharyngeal valve. Earlier, Balashov (1965a) commented on the inadequacy of our knowledge of tick saliva and of its secretion, emphasizing their significance in understanding the pathology of affected host tissue. The salivary secretion is of two different types and related to specific areas of the glands. Subsequently Balashov (1965b) observed sucking and secreting actions of ticks on depilated ears of mice and rabbits.

Gregson (1969) overcame the masking effect of blood on fluid flow in tissues during ejaculation of saliva and ingestion of nutrients by passing an electrical current through the mouthparts of the parasite to the host and recording on an oscilloscope the changes in conductivity that reflect the alterations in the food channel and the conductivity of the fluids within it. The presence of blood or saliva or their passing through indicates good conductivity by positive deflection on the oscilloscope. When the pharyngeal or neighbouring valves are closed, conductivity is poor and the 'scope registers negatively.

From the early stages of feeding to near repletion a series of discrete signals was monitored and these fell into the following categories:

(a) a baseline of inactivity, when the food passage is closed;
(b) a topline of inactivity, when the food passage is open;
(c) regular wave-like tracings of about half maximum amplitude, ending in an upward spike; usually of continuous and regular frequency, with repetitive groupings;
(d) irregular wave-like tracings, similar in amplitude to those of (c) but of continuous and usually faster frequency; usually ending in an upward spike, but without the pattern being immediately repeated;
(e) downward spikes originating from the topline; of increasing or varying amplitude and of regular or varying frequency;
(f) upward spikes originating from a baseline, usually of regular amplitude but of regular or irregular frequency;
(g) erratic and continuous tracings, varying in amplitude and frequency and with no top or baseline.

As far as possible, correlation of the oscilloscope traces (Fig. 1) with the movement of tissue fluids around the feeding ticks was achieved by positioning the tick and the source of food under a binocular microscope and arranging it in circuit with an oscilloscope. Similar experiments involved feeding ticks with their hypostomes in capillary tubes partially filled with blood or their own secretion with the electrical current passing through the fluid to the tick.

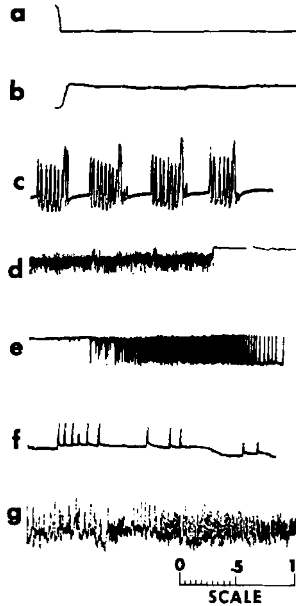

FIG. 1. Oscillograph recordings of various aspects of tick feeding (after Gregson, 1969). For full explanation see text.

The (c) tracing is characteristic of the rhythmical sucking of blood and the terminal spike of each phase coincides with the salivary secretion. A continuous ejaculation of saliva is interpreted as a topline from which, upon the apparent cessation of its flow, successive downward spikes occurred. The tick's abundant secretion when (d) was monitored corresponds with an active rapid ingestion of blood and occurs when a substantial haemorrhage has been established. The downward spikes from the topline in (e) were rarely apparent visually in Gregson's hamster pouch preparation, except that vibration signals preceding their appearance were accompanied by tremors in the tissue fluid in the vicinity of the tick's mouthparts. In one preparation, however, the downward spike was associated with the intake of blood. The co-ordination of the upward spikes of (f) with the visible effects of fluid movements is not entirely clear, and Gregson assumed that each was a dilation of some part of the food canal and frequently the spikes were associated with the ejections of saliva. Occasionally, the tracings showed short toplines followed by the (f) pattern of

a brief grouping of upward spikes, and this was accompanied by ejection of blood from the tick. Gregson interprets this as being probable regurgitation. Irregular combinations of sucking and secreting are indicated by the pattern shown by (g) and correspond with the "toying" process.

The records of Tatchell and his colleagues (personal communication) are substantially similar to those of Gregson, but using *B. microplus* as the tick and the cow as a host. The mains "hum" has been largely eliminated by using a battery-operated oscilloscope together with a 0·005 μF capacitor. By this means they have been able to show the movements of the pharyngeal valve as well as the pharynx.

In Gregson's paper are given a series of tracings covering feeding over periods of time, and this is a major break-through at a technical level but leaves many questions unanswered and, as he admits, more observations are required to correlate both the visual and structural movements with oscillographic records. Gregson also recognized possible sources of error in the techniques employed; for example, short circuiting could cause drift in the oscillographic records, and the changing volume and contiguity of the contents of the food channel and of the salivarium may be sufficient to cause fluctuations in the flow of electricity through them. The nature of the fluid is also significant, for its conductivity influences the relative magnitude of the tracing and in this respect the increased rate of salivation towards the end of the feeding has significance. Both Gregson (1967) and Tatchell (1967a), working with *D. andersoni* and *B. microplus* respectively, have shown that an added function of saliva is to remove excess salts and water, and its salt content in *B. microplus* is 30% higher than that of the host's blood towards the end of the feeding period. Thus, assuming ionic equivalence, the conductivity of saliva would be greater than that of host's blood and the replacement of blood in the buccal cavity would be expected to register positively on the oscilloscope and vice versa.

What does emerge, however, is that from the recording of electrical impulses it is clear that the overall pattern of feeding is more complex than a simple alternation of sucking of blood and ejection of saliva, although these are discrete initially. Later there is a suggestion of mixed sucking and salivation interspersed with longer periods of ejaculation. At about the fifth day the dilations of the salivarium suggest increased salivary secretion, a feature also noted by Balashov (1965a) during the final rapid engorgement of *H. asiaticum* and *I. ricinus*.

Anticoagulants in saliva of some species have been reported by Pavlovsky and Stein (1927), Foggie (1959) and others, but according to Tatchell and Moorhouse (1968) the need for an introduced anticoagulant is slight where heavily infiltrated lesions such as those produced by *B. microplus* occur. Tatchell has shown that the saliva of this species lacks an anticoagulant. Under these circumstances the release of host heparin from damaged tissues suffices, and the increase in salivation towards the end of feeding is therefore not necessarily associated with the prevention of blood clotting, but rather is a means of rapid removal of water to concentrate the blood meal at a time when its uptake is very rapid.

II. THE CEMENT AND EOSINOPHILIC ZONES

Gregson (1967) fed *D. andersoni* experimentally on the extruded pouches of hamsters and, using transillumination techniques, recorded the results on ciné film. Attachment to the pouch was enhanced if the ticks either had been pre-fed for 5 days on sheep or if they had begun to feed on a rabbit. During penetration the first trophic movements seen were the sweeping and cutting motions of the digits of the chelicerae, followed by the thrusting movements of the cheliceral shafts through the stratum corneum. This was accompanied by the ejaculation of cement as a milky white secretion from the tick's salivary glands. The bulk of the secretion forms a cone on the surface of the skin (Gregson, 1937, 1960) but there is also an invasion of the outer skin layers and the secretion flows around the penetrating mouthparts. When transilluminated, the cement is seen to spread outwards in the host tissues as an opaque cloud. The piston-like action of the chelicerae occasionally pulled up the cement around the trophi and, as it hardened rapidly, formed a sheath around the embedded mouthparts. This cement sheath is frequently referred to as an "eosinophilic" zone.

On the basis of the distribution of the cement Saito and Ohara (1961) recognized two types of tick feeding. The "*Haemaphysalis*" type is characterized by an abundance of cement on the surface of the skin and around the mouthparts with superficial penetration by the mouthparts. Included within this group are *D. andersoni* and *B. microplus*. The "*Ixodes*" type has relatively little cement both on the skin surface and around the mouthparts. Moorhouse (1969) has catalogued the distribution of the so-called cement in a few species representative of most ixodid genera and suggests that this may be a criterion for separating the ixodid genera. Some species of *Ixodes* (e.g. *I. ricinus* and *I. holocyclus*) lack cement of salivary origin and the mouthparts are inserted deeply into the skin tissues, and are probably held *in situ* by transformed host tissues in much the same way as fine reticulin fibrils succeed tissue damage inflicted by parasitic flatworms. This may result from stimulation by a factor of tick secretion (see p. 281). Under these circumstances one can postulate that only in such species does the saliva contain other secretions having proteolytic or cytolytic properties which will effect tissue destruction below the region of anchorage. In other genera the mouthparts are enclosed in saliva-derived cement and the depth of insertion is characteristic for each species (Moorhouse, 1969). The mouthparts of *Amblyomma*, *Hyalomma* and *Aponomma* are fully inserted and the superficial cement is minimal, whereas in *Haemaphysalis*, *Dermacentor*, *Boophilus* and *Rhipicephalus* the cement is mainly superficial and the mouthparts do not penetrate the dermis. This reaches its zenith in the genus *Haemaphysalis* where insertion is just beyond the epidermal surface. In superficially attaching genera of the latter group active digestion of surrounding tissues would weaken the attachment. Accordingly, such genera would be expected to have less active salivary secretions, to place less reliance on salivary anticoagulants to maintain blood flow, to rely on host tissue reactions for their source of nutrients and on the release of such pharmacologically actively released host substances as histamine (see p. 285) to cause capillary dilatation, increased flow rate of the blood and increased permeability of the capillaries.

It seems, therefore, in accord with this concept that rapid feeders such as the argasids would secrete anticoagulants and would rely on vigorous breakdown of tissues for their nutrients, and this appears to be true for these soft ticks (Lavoipierre and Riek, 1955; Howell, 1966). Of the more superficially feeding ixodid genera the saliva of *B. microplus* (Tatchell and Moorhouse, 1968) and *D. andersoni* (Gregson, quoted by Tatchell, 1969b) do not have anticoagulants. Moorhouse (1969) also suggests that the evolutionary trend in feeding in the family Ixodidae is towards the more superficial positioning of the mouthparts and this, whilst reducing the area of tissue damage, places the more highly vascular outer layers of the dermis beneath the mouthparts.

No histochemical analysis of the cement produced by *D. andersoni* has been made, but according to Saito and Ohara (1961) the cement in "*Haemaphysalis*" types is laid down as an inner, or periosteal, layer which ensheaths the rostrum and invades the adjacent host-connective tissue and the outer layer or "cover" cement which lies peripherally and mostly above the surface of the skin. This duality of the cement in other ixodid ticks has also been reported by Saito *et al.* (1960). In *B. microplus* the cement surrounds the mouthparts as an irregular cone which adheres firmly to the epidermis, and where males and females attach and feed in close proximity both secrete and the cones may become confluent (Moorhouse and Tatchell, 1966). When the mouthparts have assumed their definitive position the cement may infiltrate between the stratum corneum and the rest of the epidermis. Successive cement secretions extend under the stratum corneum and consolidate the sheath around the trophi. Meanwhile the superficial cone builds up and its outer layer thickens during the intermediate period of feeding. Secondary production of cement, which occurs towards the end of feeding, is distinct from that of the cone and associated with the development of cavities formed after collagen destruction in dermal lesions below the mouthparts. This also occurs in *Rhipicephalus* ticks.

Histochemically, the cement of *B. microplus* consists of at least two components, a cortex and an internum. The cortical layer may be a lightly tanned carbohydrate containing protein, probably sclerotized by the quinone tanning of adjacent sulphydryl groups, and the internum is probably lipoprotein (Moorhouse and Tatchell, 1966). Both layers are defined in the cone during the first 24 h after attachment, but the mode of cone formation requires elucidation. The secondary cement is constitutionally predominantly allied to that of the cortex of the cone. The two major cement components in this species may be derived from types of salivary gland cells whose secretory droplets stain similarly to these components of the cement cone, which stabilize rapidly after secretion.

The establishment of dermal lesions of the skin subsequent to tick bite by *B. microplus* results from collagen destruction, but by induced leukopenia the dermal collagen appears to remain unaffected (Tatchell and Moorhouse, 1969) and ticks fed on such hosts produce viable eggs. In skin tissue affected by the bites of the larvae of *I. ricinus* an incipient "eosinophilic" zone appears around the mouthparts after about 30 min of attachment and is accompanied by slight oedema, and the connective tissue fibres around the mouthparts are a little more widely separated than in normal tissues. The zone is pronounced after

3 h attachment, is largely fibrillar and ultimately lacks cells. Up to 24 h after attachment by ticks the eosinophilic material may even cover the tips of the embedded larval mouthparts, being very dense at about 9 h, but becoming diffuse after 18 h. Characteristically the earliest change in the skin, apart from the rupture of small capillaries, is a compression and re-orientation of the connective tissue fibres and fibroblasts to the area immediately surrounding the mouthparts, and incipient necrosis of the nuclei in contact with the tick's mouthparts is characteristic. Stevens (1968) has shown and illustrated, by collagen specific stains, that the increase in "eosinophilic" zone material is at least in part due to the aggregation of connective tissue. These observations are not new, for Pavlovsky and Alfeeva (1941) reported on the re-orientation of fibres at the site of bite of *I. ricinus* on cows and subsequently (1949) in the skin around the mouthparts of *Hyalomma.* Saito and Ohara (1961) also reported collagen fibres of the dermis "embedded" in the cement plug around the trophi of the immature stages of *Haemaphysalis*, *I. persulcatus persulcatus* and *I. japonensis.* When sections are digested with collagenase at 37°C for 3 h the dermal collagen is destroyed, as is most of that in the eosinophilic zone. Prolonged digestion for 6 h almost completely removes the collagen-staining material in the eosinophilic zone, although some substances remain at the site of the zone. The abundance of collagen in the plug around the trophi suggests that it is not due merely to aggregation of fibres, but that there may be active synthesis of it. Fibroblasts are generally believed to be responsible for secreting collagen, but these are noticeably absent from the eosinophilic zone. Collagen is, however, a highly polymerized and relatively insoluble substance, and it is possible that enzymes in the salivary secretion of *I. ricinus* may polymerize the collagen precursors in the skin.

When penetration by the mouthparts occurs in regions containing elements other than connective tissue these are broken down and replaced by eosinophilic substance which is less homogeneous than that derived from connective tissue. This implies that the pattern of the eosinophilic zone in the skin is a factor of host age and condition and the depth of the lesion. Stevens (1968), for example, showed that in the skin of a one-day-old rat the nuclei of the hair peg adjacent to the mouthparts are pycnotic, with no well developed eosinophilic zone. In 8–24-day-old rats the hair follicles are closely packed and in wounds in such areas the eosinophilic zone is not as homogeneous as in wounds involving only connective tissue. Initially the nuclei and the cytoplasm of the sheath cells are broken down and replaced by strongly eosinophilic fibres. Subsequently the hair shafts stain intensely with eosin and become fibrous. Penetration into the striated muscle of the panniculus carnosus also results in conversion to eosinophilic zone substance, whilst the cell membranes of the fat cells of the panniculus adiposus are broken down, the contents of the adjacent cells coalesce and the nuclei become pycnotic and disappear. Again, conversion of fatty tissue into eosinophilic material is not as rapid as in connective tissue.

Examination of the wounds produced by more than 150 larva eand 14 nymphs of *I. ricinus* led Stevens to the conclusion that the eosinophilic zone consequent on their bite was to a large extent produced by the deposition of

collagen, and that this followed the gradual breakdown of skin structures. Apparently, eosinophilic zones in different genera of ticks may have different origins, although all workers are agreed that the prime purpose is to hold the tick in the feeding position (Arthur, 1965). Moorhouse (quoted by Tatchell, 1969b) proposed that the cement evolved to complete the channel formed by the mouthparts and to direct the saliva more effectively into the tissues without simultaneously digesting the tissues lateral to the mouthparts and hence weakening the attachments.

III. The Characteristics of the Lesion and Associated Histopathological Changes

Studies by Stevens (1968) and Arthur (unpublished) show that the tips of the mouthparts of all stages of *Ixodes* ticks may lie in a wide variety of tissues including dermal connective tissue, white and brown fatty tissue, striated muscle, beds of capillaries, venules and arterioles, loose connective tissue and the mesenchymal cells of future cartilage. All these tissues show evidence of histolysis and the fact that their fluid derivatives are drawn towards the mouthparts. The conclusion to be drawn is that *Ixodes* ticks feed from such tissues as are available at the tip of the mouthparts, and these may range from relatively non-cellular tissue such as loose connective substance, to highly cellular materials such as striated muscle or brown fatty tissue. This is confirmed by examination of the contents of the tick's diverticula. Haemorrhages are not a constant feature associated with the lesion at an early stage and even when present are quite small, but extensive haemorrhages do usually occur at the later stages of feeding, particularly as a result of nymphal feeding, by which time tissue damage is extensive. The feeding of several ticks in close proximity frequently induces an intense haemorrhagic condition, especially when the tissues penetrated are particularly vascular. In such cases the capillaries and other blood vessels near the mouthparts were grossly dilated and haemorrhagic.

In her work (Stevens, 1968) on feeding larvae and nymphs of *I. ricinus*, a cellular reaction is usually not a prominent feature of the wounds either beneath the level of the tip of the mouthparts or in the dermis surrounding the eosinophilic zone. Occasionally it may become very marked, and possibly this is due to secondary infection.

Histopathological changes in the skin consequent on adult feeding by *B. microplus* have been studied by Tatchell and Moorhouse (1968), who noted a general uniformity of reactions. During feeding, except for the final stage of engorgement, small spaces filled with regurgitate are present below the mouthparts and this agrees with observations of regurgitation of diverticular contents (Gregson, 1969). As the tick continued to feed, and up to 24 h before detachment, there was a progressive infiltration around the mouthparts of neutrophils and lymphocytes and sometimes a few fibroblasts were also present. At all times the capillaries in the vicinity of the mouthparts were dilated and haemorrhagic and as feeding continued these changes were noted in the deeper local blood vessels. The intensity of infiltration was accompanied by the disappearance of collagen bundles of the dermal tissues and the establishment

of a cavity and a surrounding zone of heavily infiltrated collagen. Tatchell and Moorhouse thus consider that collagen is digested in the formation of the lesion in *B. microplus*, whilst on the presently available evidence for *I. ricinus* larvae and nymphs our evidence suggests that it is synthesized in a manner which is not yet explicable and that it forms the basis of the eosinophilic zone (see also Stevens, 1968). The fluid-filled cavity produced by *B. microplus* contained some leucocytes and haemorrhage from the damaged blood vessels and these could have a deep-seated origin in the dermis, but as the final rapid engorgement proceeded the leucocytes, many necrotic, were depleted. In some instances the widespread precipitation of the tissue nuclei which occur in earlier infiltrations below the mouthparts persisted and extended the area of degeneration. The eosinophils also increased in number and were most numerous in the periphery of intense infiltration. Monocytes and histocytes showed intense phagocytic activity until the deposition of the secondary cement, but thereafter monocytes decreased in number.

The lesions produced by the male of *B. microplus* are very small when compared with those produced by the females. They lack true cavities and the collagen is infiltrated mainly with neutrophils (a condition reminiscent of the immature stages of *I. ricinus*). Haemorrhages occur beneath the mouthparts, but as males attach close to females, these may be of female origin. Characteristically, there is deposition of nuclear precipitate with a similar flow pattern to that of other stages.

From their work on *B. microplus* Tatchell and Moorhouse (1968) suggested that specific vascular damage results from the saliva of the tick while tissue damage is caused by the host response—an hypothesis based on the finding that collagen destruction beneath the mouthparts was preceded by an intense infiltration of polymorphonuclear neutrophil leucocytes. By the administration of nitrogen mustard to dogs parasitized by *Rhipicephalus sanguineus*, Tatchell and Moorhouse (1969) induced a leukopenia whereby the number of circulating leucocytes was very substantially reduced. Commensurate with this was the fact that in the skin of such animals bitten by ticks polymorphonuclear leucocytes were rare, but heavy infiltrations around the mouthparts characterized untreated tick-infested hosts. The latter too, towards the time of full engorgement, had well established cavities below the mouthparts, due to destruction of the collagen, whereas in treated dogs cavities were either absent or insignificant and dermal collagen appeared to be unaffected. No secondary cement was laid down in the nitrogen-mustard treated dogs. The ticks from both sets of dogs oviposited normally and produced viable offspring. This experiment is thus consonant with the above authors' earlier concepts in respect of *Boophilus*.

In the earlier review (Arthur, 1965) it was stated that the degree of penetration of the mouthparts into the skin reflects the varying lengths of the trophi of the tick and this is true for ticks having long hypostomes (Stevens, 1968) and for *Hyalomma anatolicum anatolicum* (Snow, 1967). *Boophilus* does not conform to this (Tatchell and Moorhouse, 1968), but nevertheless the quantity of cement and the extent of the lesion produced by each stage appears from their figure to be related to the respective sizes. The lesion produced by the larva of this species develops within the papillary layer of the dermis and may involve some

of the superficial capillaries; that of the nymph extends into the reticular dermis, including the blood vessels of the reticular plexus; and that of the adult passes deeply into the reticular dermis affecting some of the larger blood vessels. The overlying epidermis and hair follicles are not damaged nor is there epidermal proliferation and hyperkeratinization (Tatchell and Moorhouse, 1968) such as referred to by Pavlovsky and Alfeeva (1941), Berlin (1957) and Hoeppli and Schumacher (1962) in *B. microplus*.

During the first 5 h of feeding of larvae of *B. microplus* on zebu and shorthorn cattle, pycnosis of prickle cells adjacent to the trophi of the ticks occurred and the dermal capillaries showed dilatation, though this did not invariably accompany tick bite (Tatchell and Moorhouse, 1968). All these changes could happen with 5 min of release of the larvae on to the host. Degeneration and distortion of the nuclei of the papillary layer of the dermis followed, and oedema was fairly typical. These changes persisted for 24 h accompanied by increased incidence and blockage of the capillaries by neutrophils and lymphocytes. They also accumulated in the dermis in the vicinity of the trophi. The lesions enlarged further in 48 h and the dominant cells within them are neutrophils, eosinophils and lymphocytes, and within 72 h extravasation from dilated capillaries into enlarging lesions occurred in most cases. Dense accumulations of neutrophils and lymphocytes replaced the connective tissue fibres of lesions in the papillary layer of the dermis so as to form a pus-filled cavity.

Similar but larger lesions were produced by the nymphs and leucocytes were abundant, but they did not block deeper vessels as happened in larval feeding. These lesions involved the loss of sweat and sebaceous glands and phagocytosis of materials by histocytes and monocytic leucocytes.

Feeding by *I. ricinus* and other British species of this genus (Arthur, 1962; Stevens, 1968) and of *H. anatolicum* (Snow, 1967) produces hyperplasia of the epidermis surrounding and extending beyond the mouthparts. This apparently is not so in *B. microplus*. Exposure of the skin of rats to the bites of immature stages of *I. ricinus* leads to the destruction of the epidermis around the area of entry, with some invasion by neutrophils and degenerating dermal fibroblasts. Slight haemorrhages may occur immediately below the Malpighian layer, having their origin in the superficial capillaries, and there is evidence of pycnotic fibroblasts at the haemorrhagic edges; 9 min after attachment there is little change in the host tissue beyond compression of the connective tissue fibres and fibroblasts immediately around the mouthparts, and those further away are slightly re-orientated; 15 min after attachment the nuclei in contact with the mouthparts are pycnotic and more closely packed than in uninfected tissue.

The breakdown of tissues commences with those nearest the mouthparts whether these be muscle, adipose tissue or chondroblasts, and oedema is associated with histolysis as early as 3 h after attachment. The oedematous fluid may contain a few red blood cells and remains of fibroblasts and connective tissue fibres. A frequent, but by no means invariable feature of the wounds is the formation of a haemorrhage, and where this is extensive it may seep to the underlying tissues, but unless a dense capillary bed is penetrated haemorrhages do not occur after short periods of attachment. At 3, 6, 18 and 48 h after the larvae have attached, the changes at the tip of the hypostome vary from

negligible damage to extensive histopathological changes, but the changes after 48 h are invariably marked. Nymphal feeding produces marked effects in the skin after 48 h. Tatchell and Moorhouse (1968) were unable to relate changes in either the frequency or appearance of mast cells to the feeding of the ticks, but Stevens (1968) noted that these cells migrated to the region penetrated by the trophi of immature stages of *I. ricinus*. They occurred in greater numbers than in unaffected skin, extending to beneath the epidermis, where they do not normally occur. The membranes of the cells break down and the granules release to some distance from them, where they show either reduced staining reactions or appear as diffusely staining patches or clusters of fibrils. These mast cells occur frequently in the eosinophilic zone and throughout attachment they, or their "ghosts", and the released granules occur distal and lateral to the "eosinophilic" zone. Because of the build-up of mast cells in the skin at such sites it is not surprising to find them or their "ghosts" in the tick's gut soon after attachment. Subjection of connective tissues to trauma produces an oedema. This is associated with the liberation of histamine from the granules released from mast cells and is a possible contributory factor to the increased flow of tissue fluids, although pharmacologically active components of the saliva could produce the same effect. The presence of disintegrating mast cell granules in and around the wounds, caused by feeding larvae and nymphs of *I. ricinus*, and in their gut diverticula correlates well with the oedema, which is a distinctive feature of many wounds produced by larvae and nymphs.

IV. Feeding and Weight Changes

The course of feeding in *I. ricinus* is made up of the following three phases (Sutton and Arthur, 1962; Arthur, 1962, 1965):

(i) The carry-over of blood from the previous instar and/or the imbibition of small quantities of blood released from superficial capillaries ruptured during the penetration of the skin. This will vary considerably for each individual but in any case would only be a minor contribution, but might be relevant in the earliest stages of feeding by *B. microplus* as shown by Seifert *et al.* (1968).

(ii) The non-blood fraction forms a major component of the food for varying lengths of time even up to half a day from full engorgement. Tatchell and Moorhouse (1968) recognize this feeding phase in *B. microplus*, dividing it into the earlier ingestion of tissue fluids and later to tissue fluids and large numbers of leucocytes arising from the skin reaction. These leucocytes remain recognizable in the caeca of the tick's gut.

(iii) Finally, blood is predominantly ingested, presumably because it is derived from the circulation under pressure and is a more readily available source of nutrients than "non-blood".

This third stage is also recognized by Tatchell and Moorhouse (1968) in respect of the nymph and the adult of *B. microplus*, although larvae of this species had a very low "tick haematocrit" and were usually of a creamy-yellow appearance when fully engorged, presumably because they may be principally tissue exudate feeders. Even so, in intense local concentrations engorged

larvae may ingest sufficient erythrocytes to assume a dark purple colour (Seifert *et al.*, 1968).

By labelling the erythrocytes and plasma of a British and Brahman steer with ^{51}G and ^{125}I respectively, Seifert *et al.* (1968) established a relationship between the weight of *B. microplus* and its dietary intake. Fully engorged ticks took up twice their own weight of blood components and included both the labelled red cells and plasma. However, other blood components such as leucocytes, which were unlabelled, may form a not insignificant part of the blood meal. Fully fed females which had detached themselves from the host contained more red cells per individual and generally also more plasma than apparently fully engorged ticks removed from the host. Moreover, the latter group weighed less, and had lower concentrations and contents of blood than did the former, suggesting that the terminal stages of engorgement (i.e. the last few hours or even minutes) are significant in the final concentration of the meal. Information on the feeding of males of this species shows that their weights approximate to those of half-engorged nymphs, with a lower blood content and a high proportion of red cells. In the matter of weight this is not inconsistent with the observations of Snow and Arthur (1966) in *H. anatolicum anatolicum*. Females of *Rhipicephalus appendiculatus* also absorbed greater quantities of nutrients than did males, and the body weight of male ticks, under the experimental conditions of temperature and humidity, was directly related to the period of attachment on the host, until a maximum was reached after 7–9 days when they roughly doubled their weight (Joyner and Purnell, 1968). In contrast to females, whose rate of engorgement increases greatly during the final phase of attachment, as in other species (Arthur, 1962), the males of *R. appendiculatus* show a steady increase in weight to the maximum. After this there was a falling-off in weight which may be attributed to the utilization of energy reserves in wandering and mating by male ticks which have ceased feeding. Males and females less than five weeks after moulting were less inclined to attach than older ticks.

Using *in vitro* techniques, developed by Purnell and Joyner (1967), *R. appendiculatus* was offered whole blood, serum or plasma in capillary tubes placed over the mouthparts, and the volumes absorbed over periods of 24 h measured. In these experiments it was unnecessary to spread the palps (cf. Gregson, 1938; Chabaud, 1950) to induce the tick to feed, but it is extremely doubtful whether this actually happens during natural feeding on the host. The other complication, apart from the need to compensate for the loss of liquid due to evaporation in the tubes, is that regurgitation of blood from the tick's alimentary tract occurs (Gregson, 1969) and this may alter the height of the column from time to time. Qualitatively, the results of Purnell and Joyner (1967) and Joyner and Purnell (1968) show that the volumes of both plasma and serum consumed were greater than either heparinized or defibrinated blood. This is not in accord with the observations of Gregson (1955), who observed that heparinized blood was more actively consumed than plasma by *D. andersoni*.

The relationships between feeding and other physiological processes in most ticks still remain virtually an unexplored field. The contribution of Joyner and Purnell (1968) on *R. appendiculatus* is a useful starting point, and their

results show agreement with that for other ixodids (Arthur, 1962) in that the rate of engorgement of females increases greatly during the final stage of engorgement, and this is so also in *B. microplus* (Kitaoka and Yajima, 1958; Tatchell and Moorhouse, 1968). They also show that there is an intermediate phase between the end of the first four days of attachment and the commencement of rapid engorgement. In *I. ricinus* the male does not feed, whereas in *Boophilus*, *Hyalomma*, *Rhipicephalus* and *Dermacentor* the males require a meal before mating to complete spermatogenesis. Differential feeding rates in species whose males require a blood meal for this purpose were established for *D. andersoni* by Gregson (1943). He showed that female ticks which had mated fed more actively than did unmated ticks, and completed engorgement. The length of the feeding period is considerably increased in *D. andersoni* when mating occurs late during the attachment period and the females which detach are stunted (Gregson, quoted by Sonenshine, 1965). Oviposition and hatching are also curtailed, the former being reduced in direct proportion to the prolongation of their engorgement period. In *D. variabilis*, however, even though the feeding time was increased due to delayed mating, there was no reduction in size of the females, nor was there evidence of reduced viability of the eggs laid by females with prolonged feeding time (Sonenshine, 1965). More recently, Snow (1967) has shown that as the pre-mating attachment period of females of *H. anatolicum anatolicum* increased, so too did the duration of the feeding period. This increase was linear for about eight days, beyond which the graph curves. Females which mated immediately after attachment became fully engorged, on average, after about 5¼ days, whilst those which mated 12 days after attachment fed for 13¾ days. Unmated females attained an average weight of 182·1 mg after 15 days feeding and just over 200 mg after 20 days, but they never engorged fully. The weight changes due to feeding in females of *H. anatolicum anatolicum* mated at known times after attachment show that the increase in weight before mating occurs corresponds, expectedly, to that of virgin females, but that immediately after mating the rate of feeding was intensified to full engorgement. Moreover, the longer the delay in mating subsequent to female attachment the greater was feeding accelerated. Thus, females mated on the second day after attachment increased in weight from 12 to 25 mgm in the 24 h after mating, and in the same time those mated on the 7th day increased from 70 to 88 mgm, and those mated on the 12th day from 142 to 208 mgm. The intervals between the periods of mating are the same (i.e. 5 days) but females detached more rapidly (2–2½ days) when mating was long delayed (12 days) as compared with earlier matings (4½ and 4 days respectively) at 2 days and 7 days. This difference in timing may possibly be attributed to the higher food base-line from which mated females begin, as food material is continually being accumulated in virgin females.

Arthur and Snow (1966), Snow and Arthur (1966) and Arthur (1969) have examined other associated aspects in *H. anatolicum anatolicum*. The ovary of the unfed *H. anatolicum anatolicum* is small with numerous egg precursors visible on its surface. The development of the egg proceeds during feeding and on its cessation the ovary contains eggs of various sizes. During the pre-oviposition period a proportion of the eggs continues to increase in size so that

on the penultimate day of this period the eggs have a mean diameter of about 0·58 mm and pass into the oviduct on the last day of preoviposition. The remainder continue to develop daily throughout the oviposition period and, as they do so, are laid.

When the tick is feeding the weight of the ovary increases by about eight times and during the preoviposition period by nearly 200 times (Snow, 1967). During the first 8 days of egg laying there is a more or less constant diurnal decrease in ovarian weight of 3·7 mgm, but subsequently the loss in weight *per diem* becomes less due to decreasing fecundity. Digestion proceeds throughout the feeding, preoviposition and oviposition periods, but the nutrient substances absorbed during the period of attachment on the host are used principally for metabolic purposes and in Ixodides for cuticle synthesis. Little is available for egg maturation at this stage in *H. anatolicum anatolicum* (Snow, 1967; Arthur, 1969) and in *B. microplus* as shown by Kitaoka (1961) and in *Argas persicus* by Tatchell (1964). The preoviposition period is characterized by a slow rate of digestion of blood (3·4%–28%; mean 11%) in *H. anatolicum anatolicum*, accelerating slightly from the first to the last day, and this shows a parallel with the number of eggs laid on the first day (*c.* 10%). The rate of digestion from the first to the sixth day of oviposition, on the other hand, is rapid and coincides with the high productivity of the eggs, and the peak, evident under controlled conditions, is probably attributable to the accumulation of eggs in the oviduct and to the rapidly increased availability of nutrients. This may also be responsible for the increased size of the eggs within 0–48 h of the peak. After the sixth day of egg laying the rate of digestion slows down appreciably and the ovary becomes increasingly exhausted of eggs.

Periodically accounts of variation in the sizes of ticks are reported in the literature (e.g. Cunliffe, 1913, 1914; Robinson, 1915; Arthur, 1949; Haarløv, 1962), and Arthur and Snow (1966) have analysed the variability of this parameter within the progeny of a single female and have followed such variants through from one developmental phase to the next. Plotting the lengths of unfed larvae from single females produces two peaks, which is suggestive of two groups of individuals within the population, and this has been substantiated statistically. Feeding of these larvae either on a group basis or as a whole population confirms bimodality of distribution which in *H. anatolicum anatolicum* have peaks at 0·40–0·45 mgm, and at 0·55 mgm when ticks are grouped into 0·05 mgm weight classes. When these engorged larvae moult to nymphs and are grouped into 0·05 mg weight classes the relationship between the larvae and nymphs is linear, i.e. lighter gorged larvae produce correspondingly light unfed nymphs and heavier gorged larvae yielded correspondingly heavy unfed nymphs. A similar linear association is noted when weights of unfed nymphs are plotted against their fully fed weights.

Adults emerging from these nymphs, irrespective of sex, have a linear weight association when plotted against those of their engorged nymphal progenitors. In *H. anatolicum anatolicum* both males and females are 0·6 to 0·7 times the weight of the gorged nymphs from which they emerged, which is a little higher than is observed in the transition from gorged larvae to nymphs. More significantly, however, gorged nymphs weighing about 12 mgm and equal to about

0·22 mgm when unfed gave rise to male ticks. Thus on average their consumption of nutrient host fluids is of the order of 11·78 mgm. Gorged nymphs of around the mean weight of 16 mgm (or 0·27 mgm when unfed) yielded female ticks. This involves an intake of about 15·7 mgm or some 33% more than for potential males. At extreme ranges unfed nymphs (male) of 0·19 mgm ingest about 9·5 mgm of food, whilst nymphs of 0·31 mgm unfed weight (giving rise to females) take in about 18·69 mgm—a difference of nearly 60%. The individuals intervening between the two peaks gave rise either to males or females and represent those at the higher levels of the "male curve" and those at the lower levels of the "female curve". Wharton (unpublished data) observed a possible similar relationship in *B. microplus*. Accordingly, the currently accepted concept of the larvae and nymphs of other species forming homogeneous populations requires revaluation, and it may be a source of some marked discrepancies in the estimation of the sizes of immature ticks. The attributes of sexuality in the immature stages are doubtless reflected in this size factor and determine the quantity of blood ingested. This may act as a controlling factor in transovarial and trans-stadial transmission of pathogens and may be a profitable field for further investigation.

The mechanism of detachment of the fully engorged ticks from the hosts still requires exposition, although Wharton and Utech (1969) have given us a clear picture of the timing of this in *B. microplus*. When detached the wound in the host heals rapidly and according to Tatchell and Moorhouse (1968) in *B. microplus* the cement remains *in situ* and its central canal becomes occluded by a dense mass of necrotic neutrophils. Mitosis occurred within the Malpighian layer, becoming continuous beneath the cone and isolating both the cement and its inclusions from the underlying healing lesion. Fibroblasts migrated to the lesion and the normal processes of wound healing followed. Snow (1967) observed that at about 72 h after detachment of *H. anatolicum anatolicum* the area beneath the affected tissue was subject to histogenesis of the epithelial tissue. The mass to be rejected consisted of the cement, necrotic cells including infiltrated leucocytes, and pus formations. The rejection was completed at about the seventh day after the tick had detached, leaving a cup-like depression in the skin. By differential growth the surface of the skin once more became smooth and there was no evidence of the lesion 15 days after detachment. In the region of tissue growth fibroblasts were visible and in the early stages of repair a number of leucocytes were present in the healthy tissue. Soon after the epithelial layer had been reformed the leucocytic invasion eased, probably due to the removal of the antigenic stimulus.

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