ADVANCES IN PARASITOLOGY

Volume 6

Ben Dawes

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Advances in
PARASITOLOGY

Edited by

BEN DAWES

Department of Zoology, King's College,
University of London, England

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CONTRIBUTORS TO VOLUME 6

RONALD ELSDON-DEW, *Amoebiasis Research Unit and Institute for Parasitology, Durban, South Africa* (p. 1)

*C. HORTON-SMITH, *Near East Animal Health Institute, Beirut, Lebanon* (p. 313)

*CLAY G. HUFF, *Naval Medical Research Institute, Bethesda, Maryland, U.S.A.* (p. 293)

OTTO JÍROVEC, *Zoologický Ústar Přirod Fakulty University Karlovy, Prague, Č.S.S.R.* (p. 117).

NAFTALE KATZ, *Instituto Nacional de Endemias Rurias, Centro de Pesquisas René Rachou, Belo Horizonte, Minas Gerais, Brazil* (p. 233).

FRITZ KÖBERLE, *Faculty of Medicine of Ribeirão Prêto, University of São Paulo, Brazil* (p. 63)

*JOHN E. LARSH, JR, *Schools of Public Health and Medicine, University of North Carolina, Chapel Hill, and School of Medicine, Duke University, Durham, North Carolina, U.S.A.* (p. 361)

*J. LLEWELLYN, *Department of Zoology and Comparative Physiology, The University, P.O. Box* 363, *Birmingham* 15, *England* (p. 373)

*P. L. LONG, *Houghton Poultry Research Station, Houghton, Huntingdon, England* (p. 313)

BRIAN MAEGRAITH, *Department of Tropical Medicine, Liverpool School of Tropical Medicine, Liverpool, England* (p. 189)

J. PELLEGRINO, *Instituto de Biologia, Faculdade de Filosofia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil* (p. 233)

MIROSLAV PETRŮ, *Zoologický Ústav Přirod Fakulty University Karlovy, Prague, Č.S.S.R.* (p. 117)

*D. POYNTER, *Research Division, Allen & Hanbury's Ltd., Ware, Hertfordshire, England* (p. 349)

*W. P. ROGERS, *Waite Agricultural Research Institute and Department of Zoology, The University of Adelaide, Adelaide, South Australia* (p. 327)

*R. I. SOMMERVILLE, *Waite Agricultural Research Institute and Department of Zoology, The University of Adelaide, Adelaide, South Australia* (p. 327)

* Authors in the section "Short Reviews".

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PREFACE

As a teacher of parasitology I advocate using about one half of the available time on the Protozoa and the remainder on the various groups of helminths amongst which my main interests lie. This half and half apportioning of effort seems reasonable also in respect of *Advances in Parasitology*, although once more in this volume the main emphasis is on protozoological research. However, the reviews on amoebiasis, American trypanosomiasis and trichomoniasis are deeply concerned with pathological features of the host–parasite relationship, and a review on liver involvement in mammalian malaria also indicates my belief that this area of parasitological effort merits our closest attention in the future. Pathological tissue formations may represent the repair of damage caused by parasites, and sometimes they are a provision whereby parasites are nourished and sustained in their hosts. Sometimes they arise as a result of biological agencies operating far from the site of parasitism, and they may appear cryptic also because they persist after the parasites which evoke them have disappeared from their former locations. Doubt and difficulty may arise because we fail to investigate pathological developments in parasitism from the earliest manifestations that can be recognized. Another important research area is chemotherapy, and the remaining full review is concerned with its experimental aspects in schistosomiasis. In this series now and out of 35 full reviews, 12 concern Protozoa, 6 Trematoda, 3 Cestoda, 6 Nematoda, 1 Acanthocephala and 7 deal with general topics which overlap taxonomic groups and include vectors. Out of 37 contributors, 17 operate in the U.K., 7 in the U.S.A. including Hawaii, 4 in Australia, 2 each in Japan and Brazil and one each in Canada, Czechoslovakia, Israel, Poland and South Africa. I am just as anxious that the word of authority shall be global as that coverage shall be comprehensive.

An innovation in this volume is the introduction of short reviews by the contributors to the first volume, all of whom had something to say in bringing these reviews up to date and gave unanimous support to my wish to conserve freshness throughout the series. One full review was sacrificed to this end but in spite of the precaution this volume is larger than usual. I hope readers will realize that this is chiefly due to the size of full reviews of immense interest and importance, will pardon this aberration and will accept the assurance that it will not recur.

Ronald Elsdon-Dew states "much modern thinking carries a legacy of the misconceptions of the past" before making a long historical approach to the perplexing features of amoebiasis. It is my belief also that some reliable data tend to lie buried in the original literature of the past and can be rescued from oblivion with advantage, as is indicated by some clear statements which emerge in this review. When compiling his WHO pamphlet on amoebiasis Elsdon-Dew was disappointed with many shortcomings of effort made in this field of study (see p. 20), and in this review he has responded well to my insistence that we should learn from him why and where in the past mistakes

were made and how in the future we can avoid making them. It would be ludicrous to paraphrase his essay by going over his many considerations and close scrutiny of expert opinions about parasites which are almost ubiquitous. He considers the incidence of amoebiasis in some detail for three continents at least and concludes that invasive amoebiasis is less widespread than is generally believed, many notorious areas producing few cases, although important foci occur in Mexico, West and South Africa and the whole of South-East Asia. We have to remember, however, that reports tend to emanate from places where medical schools exist and hospitalization is good. We are told that the medical concept of amoebiasis is confused because parasitologists "do not agree as to what will be called *Entamoeba histolytica*", which is defined carefully along with *E. hartmanni*, a form which never invades the tissues and is never histolytic in action. Disease can be established only by finding *invasive* haematophagous trophozoites. The term amoebiasis, in Elsdon-Dew's opinion, should be confined to the harbouring of *E. histolytica*, and the term "invasive amoebiasis" denotes invasion of the host's tissues. The condition in which amoebae live in the gut as commensal forms is "lumenal amoebiasis". The normal or *minuta* cycle is commensal and productive of cysts which are necessary for the transmission of the parasites to new hosts. This occurs when *E. histolytica* confines its attention to the lumen of the bowel, but for some reasons as yet unknown the amoebae may go "beyond the bounds of hospitality, and invade the tissues", undermining the mucosa and adopting the haematophagous habit. Such amoebae may be carried to the liver in the portal circulation, causing "that focal necrosis commonly called an amoebic liver abscess", sometimes spreading directly or else by means of metastasis and reaching remote sites such as the brain. Even the genitalia may be invaded but "all lesions are focal, the amoeba itself being the active agent, and there is no conclusive evidence of any specific remote effect". Invasive forms which have penetrated beyond the bowel "have forfeited all chances of a sustained posterity", because they do not produce cysts which are essential for survival in an external world. The conclusion that invasive amoebiasis is not as widespread as some persons would believe is based on the use of the liver abscess as an index, but there is a need for more definitive surveys. The hope is expressed that if and when antigens become freely available, serological methods might yield comparable estimates of the invasiveness of *E. histolytica* on a worldwide scale.

Fritz Köberle deals with a dangerous parasite affecting some millions of persons in the New World, and especially with pathological aspects of Chagas' disease, or American trypanosomiasis, as manifest in Central Brazil. The parasite, *Trypanosoma cruzi*, was discovered 60 years ago by Chagas in the intestines of blood-sucking triatomid bugs commonly found in the primitive dwellings of Brazil. Within about 5 years of this discovery numerous acute and chronic cases of human disease became known and one form was seen to affect the nervous system. Some investigators failed to find comparable cases elsewhere in South America, and Köberle has made reference to bitter criticism of Chagas, periods of "oblivion" and of "rediscovery", and to a growing recognition of the importance of this disease, but only after change from a

broader original concept to the limited concept of chronic chagasic myocarditis. The application of serological methods, and especially complement fixation, has revealed the full significance of the trypanosome in producing the commonest infectious disease of South America. In this review the general pathology of *T. cruzi* infection is first considered briefly, with mention of various forms of infection, which is usually acquired in early life and affects infants more severely than adults. The transmission of the trypanosome from bug to man is achieved during the bloodsucking act in the night and the usual portal of entry is the ocular conjunctiva, the primary complex being known as the Romaña sign. Within a few weeks the parasites occur in the blood vascular system and invasions of various cells occur, notably muscle and glia cells. In man the cycle of *T. cruzi* involves trypanosomes and leishmanias, and these two forms are described in detail. The inflammatory reactions are discussed and the final cause in the acute stage is designated the parasites, though not necessarily complete and intact parasites. Certain degenerative lesions are also considered and in the acute stage the disintegration of nerve cells is noted. In chronic cases there is a marked diminution in the numbers of ganglion cells and this suggests the presence in leishmanias of a neurotoxic substance which eventually is liberated and acts locally when these forms break down. As the parasite shows a preference for muscle cells, the most intensive destruction of nerve cells is in the autonomic system and related to all the hollow, muscular organs including the heart. The failure of acute Chagas' disease to produce severe sequelae in the skeletal musculature is attributed to the absence of nerve cells in striated muscles. Degeneration within the nervous system occurs mainly during the acute phase of the disease, when the parasitaemia is intense. In a letter dated 14 June, 1968, after correcting page proofs, Köberle stated, "In the last months we have been able to reproduce 'Chagas' cardiopathy' without leishmanias and trypanosomes, simply disturbing the sympathetic-parasympathetic equilibrium of the nervous regulation of the heart. These results serve as evidence for my neurogenic theory".

After considering the concepts of American trypanosomiasis in older and newer senses, the technique of ganglion cell counting in various organs and detailed information about acute and chronic phases of the disease, the ground is well cleared for the enormously interesting discussion of Chagas' syndromes which follows: with aperistalsis and enteromegaly, cardiopathy and cardiomegaly, exo- and endo-crinopathies, encephalopathies and myelopathies. Even when we seem to have reached the climax of this theme, Köberle indicates that he has not exhausted the extraordinary symptomatology of American trypanosomiasis. The chagasic patient is primarily a victim of the triatomid bug that bites him and infects him with the trypanosome, but the ultimate parasitic condition is cryptic and, having organic lesions in various parts of his nervous system, the patient becomes a neuropath and his symptoms may sometimes be regarded simply as psychoneurotic. We are interested to know that some features of the chronic ill health of Charles Darwin, who probably acquired Chagas' disease when travelling in South America, were closely similar to those of known chagasic patients in that country. Also that the earlier knowledge about the syndrome megaoesophagus was gained more than 300 years

ago by Sir Thomas Willis, who worked mainly on the brain and nervous system and whose papers were sometimes illustrated by engravings made from drawings by Sir Christopher Wren, designer of St Paul's Cathedral in this city of London.

Otto Jírovec and Miroslav Petrů are concerned with *Trichomonas vaginalis*, a flagellate protozoan once regarded as a harmless commensal but now known to be a serious pathogen in both male and female organs of the human genital system, particularly the vagina. A vast original scientific literature and many comprehensive articles on this ubiquitous parasite and the disease trichomoniasis belie the fact that very little of this knowledge has gone into textbooks of parasitology, or even of gynaecology. The review starts with a detailed account of morphology in which both light- and electron-microscopy play their part, and with reproduction and the controversial cyst problem. After discussing the systematic position of *T. vaginalis* as an independent species, there are details of methods of culture *in vitro* and on solid media. Another section on the biochemistry of this parasite contains more information on metabolism than is usual in accounts of Protozoa, and valuable cytochemical data emerge. Serology and immunobiology have been mainly of theoretical interest in trichomoniasis but are assuming greater practical importance in modern institutions, and the Complement Fixation Reaction (CFR), Agglutination and Agglomeration Reactions (AR), the Intradermal Test (IDT) and other serological reactions are discussed in relation to *T. vaginalis* infections. This species of protozoan is not homogenous, although all known strains have agglutinogens in common; an example is given of 8 distinct serotypes which were found amongst 19 strains, and recent modifications of this picture are noted in detail. The experimental introduction of *T. vaginalis* in axenic cultures into laboratory animals by various modes of transmission—intravaginal, peritoneal and subcutaneous routes—have given persistent infections in hamsters, guinea pigs, rats and mice, and much has been learned about the pathogenicity of *T. vaginalis* for various kinds of cell cultures (HeLa cells, chick-liver cells, fibroblasts, etc.). In all matters mentioned so far we are given proof that the senior author's 30 years' experience of research on *T. vaginalis* and trichomoniasis does indeed "provide justification for critical appraisal" of various problems. However, absorbingly interesting problems remain to be discussed, namely trichomoniasis as a clinical entity, not merely in adult women and girls but also in men. This section contains useful information and ideas for parasitologist and clinician alike. There is more: the very practical matter of diagnosis of infection of men and women with *T. vaginalis*, the chemotherapy of vaginal trichomoniasis and the epidemiology of the disease all come in for detailed discussion, and there is a valuable appendix containing details of media for the cultivation of *T. vaginalis*. Undoubtedly, Jírovec and Petrů have produced one of the most illuminating reviews in the scientific literature on trichomoniasis.

Brian Maegraith's concern with liver involvement in acute mammalian malaria follows up a physiological approach to pathology which revealed that disturbances arising in the host are not specific to malaria but occur in other acute medical states. Changes in tissues may arise locally from histotoxic

effects or from circulatory disturbances or altered vascular permeability. Circulating toxins may also produce specific effects and shock may induce non-specific circulatory failure. The erythrocytic (E) phase of the life cycle is generally understood to be more important than the exoerythrocytic (EE) phase in these considerations of pathogenesis. The review is concerned mainly with *P. knowlesi* infections in rhesus monkeys, where the subject has been studied most carefully. Biochemical evidences of hepatic dysfunction are considered first, the pathological lesions of *P. knowlesi* malaria are then related to those of other forms, and finally such lesions are discussed separately. Lesions appear sooner and are more advanced in lytic infections than in infections characterized by shock effects, modifications arising as a result of severe haemolysis. The lesions of lytic infections are considered in fine histological detail, electron microscopy supplementing light microscopy. Attention is then focused on lesions in malarial shock and findings are summarized. The chief lesions in the liver show a central zone of sinusoidal dilatation and congestion followed by cellular degeneration and necrosis that affects parenchymal cells and, later on, Kupffer cells and other adjacent tissues. Such lesions are unevenly scattered and may be more pronounced near the surface. The most notable feature of degenerating liver cells in the central lobular zone is the swelling and ultimate destruction of mitochondria. The pattern seen in early stages resembles that seen in carbon tetrachloride poisoning. An entire section is later devoted to the pathogenesis of liver lesions, which are considered in respect of intrahepatic circulatory disturbances, liver blood flow, sympathetic hyperactivity, other circulatory factors, humoral factors responsible for biochemical lesions, and fatty degeneration. Immune and sensitivity reactions are also considered. A final comment indicates the commonness of hepatic dysfunction in mammalian malaria, and the involvement in *P. knowlesi* malaria of two pathogenic factors—stagnation of blood and anoxia due to redistributed intralobular flow, and the presence of an as yet unspecified cytotoxic substance which inhibits the respiration and oxidative phosphorylation of mitochondria of the polygonal cells. The simple concept finally outlined of initiating factors starting off a chain reaction of pathogenic physiological effects and related processes involving hormone balance, membrane permeability and other effects which may become irreversible and thus indicate the great importance of time as a factor in these reactions, is "a good basis for promoting research" and, in my opinion, a concept which must be kept in mind by all students of parasitic diseases.

José Pellegrino and Naftale Katz point out that since tartar emetic was used as the first effective drug against schistosomiasis fifty years ago, progress in the chemotherapy of schistosomiasis has been limited by comparison with that in other forms of helminthic disease. Only a few active compounds investigated have reached the clinical stage and proved their usefulness as antischistosomal agents. The routine screening of many chemical compounds involves great expense and this may be inhibitory. However, as a result of such work 30 years ago Miracil A was discovered and to date possibly one quarter of a million chemical agents have been tested, mostly on *Schistosoma mansoni*, the experimental fluke of choice. This quest has established that a schisto-

somicide must be more than just active against the parasite. The ideal drug should be without side effects and toxicity to human hosts, highly active against all three human schistosomes (i.e. *S. haematobium* and *S. japonicum* as well), inexpensive to produce, effectual by oral administration as well as injection, and rapid in effect so as to avoid distressing early symptoms of disease. A simpler aim is to seek drugs which prevent infection (prophylactics), prevent oviposition and thus interrupt transmission (suppressants), and destroy adults (curative drugs). In this review Pellegrino and Katz deal thoroughly with a very extensive programme carried out during two decades at Belo Horizonte on the chemotherapy of *S. mansoni*. Maintenance of the life cycle of *S. mansoni* in the laboratory calls for three sections dealing respectively with the culture of snails (*Biomphalaria glabrata*), the infection of snails, and the infection of laboratory animals (mice, hamsters, monkeys). As a visitor to Belo Horizonte I was immensely impressed by the techniques involved, and my requests for details which will benefit researchers elsewhere and activate potential workers have been admirably satisfied. Other sections deal with the different approaches for the assessment of antischistosomal activity, drug testing *in vitro* and *in vivo* and quantitative methods. There is a section devoted to drug testing by the oogram method, "a simple, sensitive and reliable criterion for drug screening" which indicates changes in the numbers and characters of eggs and has proved helpful in the study of relapses. This method involves the use of press preparations of the intestinal wall and the use of a projection microscope for the counting and classifying of schistosome eggs contained therein. The oogram changes are directly related to disturbances of the normal egg-laying process, examination of which indicates numbers of eggs and percentages of viable eggs in various stages of development. If any drug induces oogram changes, including abnormal eggs, dead eggs and egg shells, it qualifies for further evaluation by means of other criteria for the assessment of antischistosomal activity. Other sections of the review deal with preclinical trials and the development of new chemical schistosomicides. Drugs belonging to several groups have been developed during the last fifty years. Many of those known up to the early 1960s have been reviewed previously and are mentioned by name. However, new compounds have come into the picture during recent years and these are considered briefly, emphasis being placed on niridazole and hycanthone, which have proved to be remarkably active in schistosomiasis mansoni.

The six short reviews which bring the contributions of Volume 1 up to date will no doubt be welcomed by many readers who recognize the rapid growth of parasitology. My ardent wish to conserve original freshness has been generously supported by the contributors but I can only mention very briefly what they have tried to do. Clay G. Huff divides his short review on avian malaria into an introductory piece and sections concerned with parasitology, cultivation, biochemistry and physiology, exoerythrocytic stages, fine structure and immunity, with a final section on *Haemoproteus* and *Leucocytozoon*. Sections are subdivided when necessary so that the parasitology section deals with the effects of temperature and ultrasonics, susceptibility, penetration and other features relating to sporozoites, the effects of association with virus, the

cause of death, and epidemiology. Biochemical and physiological matters concern enzyme action, metabolism, and haematin pigments. Fine structure is about erythrocytic and exoerythrocytic stages, merozoites, sporozoites, ookinetes and exogenous stages. Much new information of great value is succinctly provided. In a short review on coccidia and coccidiosis, P. L. Long and C. Horton-Smith (order of authorship reversed) also provide a number of sections, dealing with the life history of *Eimeria* species, the biological characters of *Eimeria* in the fowl, factors affecting reproduction and pathogenicity, the effect of parasitism on the host, and immunity to *Eimeria* infection. Reviewing again the infectious process and early parasitism with respect to nematodes, W. P. Rogers and R. I. Sommerville deal with stimuli which induce development, internal mechanisms which govern development, and factors which affect development directly. Writing on the subject of parasitic bronchitis, D. Poynter deals with *Dictyocaulus viviparus* in the pasture, treatment, *D. viviparus* antigen, vaccination and pathology. In dealing with experimental trichiniasis, J. E. Larsh Jr had to limit consideration to new methods for separating stages of the life cycle (the use of thiabendazole and mythyridine), new knowledge on hypersensitivity (immediate and delayed), and new concepts of the mechanism of acquired immunity. Recent evidence lends support to a working hypothesis formulated previously indicating that delayed hypersensitivity is responsible for the expulsion of adult worms, a phenomenon which may play some part in immunity to other tissue-invading nematode parasites. Writing about larval Monogenea, J. Llewellyn notes that relatively few new forms have appeared in recent years, and his effort is concerned with some aspects of larval biology and development, host-finding, invasion routes, and post-larval development. Some new conclusions are formulated about monogenean trematode species and their interrelationships.

I am grateful to all these parasitologists, new and old friends alike, for furnishing valuable information and ideas about their respective areas of research. My aim to teach dynamically by clear exposition of events at the wave fronts of parasitological research with medical as well as veterinary interest and economic importance prompts me to request particular kinds of knowledge and experience which will stimulate biologists to greater effort. My requests have again been well received and well met, for which I am also very grateful. I wish to thank also members of staff of Academic Press for great effort behind the scenes to produce a good and useful book; it is gratifying to have such care so generously bestowed.

King's College
University of London
Strand, London, W.C.2

Ben Dawes
Professor of Zoology (Parasitology)
May 1968

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The Epidemiology of Amoebiasis

RONALD ELSDON-DEW

Amoebiasis Research Unit and Institute for Parasitology, Durban, South Africa*

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I. INTRODUCTION

There has been, and indeed still is, a great deal of confusion about the epidemiology of amoebiasis. The first difficulty is semantic, as there are several differing concepts as to the meaning of the word. What is amoebiasis? The didactic meaning implies a host inhabited by an amoeba, whether it be *Entamoeba histolytica*, *E. coli*, *E. gingivalis*, *Iodamoeba bütschlii*, *Endolimax nana* or any other, including those species of *Hartmanella* which have been shown to invade man. Current practice has confined the term to hosts harbouring *E. histolytica*, but some authors have further restricted the meaning to those with disease. But, as will be seen, even the disease needs definition, for whilst some observers have a "tight" concept of this, others consider that all those harbouring the parasite are diseased. As the information sought is some concept of the

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distribution of the parasite and its effects, a way must be found around this Tower of Babel and of establishing some criteria for reasonable deductions from the enormous mass of literature available.

A second difficulty lies in diagnosis, both in the laboratory identification of the parasite and in the clinical recognition of the disease. No parasite has been more often wrongly labelled than has *E. histolytica*, and to few organisms has such a wide gamut of clinical presentations been attributed.

The differentiation of the Rhizopoda of the human bowel is by no means as easy as is commonly imagined, and the unbridled enthusiasm of the uninitiated is to be matched only by the careful caution of the expert. Whilst the least said the better about the all too common practice of leaving the identification of the parasite to inadequately trained technicians, even experts disagree on the interpretation of certain criteria, the "small race" and *Entamoeba hartmanni* being a case in point.

On the clinical side, too, there has been much misunderstanding, unfortunately fostered by the sophism of some pedants of the past. The over facile use of the label at one time led to the comment that "Amoebiasis is the regular resort of the diagnostically destitute". Worse, of course, was the wilful, but lucrative, misuse of the diagnosis, often without any attempt at laboratory confirmation. Iatrogenic amoebiasis became common in some areas, creating an amoebophobia, not only in the individual patient, but also in communities, in some of which the label became a social status symbol!

Small wonder then, that appreciation of the situation has been and still is confused. Much modern thinking carries a legacy of the misconceptions of the past, and for this reason, an historical approach must be made to each of the perplexing features.

II. The First Thirty-five Years, 1875–1910

In 1919, Dobell said:

> It is truly astonishing, in reading the works on the intestinal amoebae of man, such as Behla's and the larger medical and zoological treatises published at the end of the last and the beginning of the present century, to observe the blindness which appears to have descended upon everybody who studied the subject at this period. Instead of illumination, darkness followed: and the twentieth century began with a period of nearly a dozen years of chaos.

Dobell considered that Schaudinn was chiefly responsible for this period of confusion. Just as Schaudinn's report of the entry of the sporozoite from a mosquito directly into an erythrocyte confused the malarial picture for many years, so too did some of his observations on the amoebae, observations which Dobell described as "so incredible that it is difficult to believe that they were not sheer inventions". But who would dare to question?

The nineteenth century was indeed productive in the field, and I would recommend as a salutary experience that all investigators read "Massenhafte Entwickelung von Amöben im Dickdarm" by Lösch (1875), not only for an accurate description of the clinical condition, autopsy findings and the parasite, but also as a model of investigative technique and deduction. Despite what

many would have considered more than adequate evidence, he did not assume that the amoebae were the cause of his patients' condition, nor did he presume to italicize his name for the parasite.

> Da die von mir beschreibene Amöbe, so viel mir bewusst, überhaupt mit keiner der bisher bekannten Formen volkommen überinstimmt, so scheint es mir gerechtfertigt, dieselbe bis auf Weiteres mit einem besonderen Namen zu bezeichnen und nach ihrem Fundorte etwa *Amoeba coli* zu nennen.

Other names from that era worthy of mention are Koch and Gaffky (1887), Kartulis (1887b), the mythical O Uplavici (Hlava, 1887) and Osler (1890). The masterly description by Councilman and Lafleur (1891) has never been surpassed. For example, they appreciated that the so-called liver abscess is not inflammatory, but "caused by necrosis, softening and liquefaction of the tissue. In these abscesses, the amoebae are not associated with any other organisms." They referred to the parasite as *Amoeba dysenteriae*.

According to Quincke and Roos (1893): "Not only by injecting stool containing amoebae into the rectum can one produce dysentery in cats, but also by feeding them with stool containing cysts." They distinguished amoebae other than "*Amoeba coli*, Lösch" by their lack of pathogenicity in cats. These they called "*Amoeba coli mitis*", found in mild dysentery; and "*Amoeba intestini vulgaris*", found in the stools of healthy people.

Casagrandi and Barbagallo (1897) described *Entamoeba coli* from healthy people, thus establishing the generic name *Entamoeba* as distinct from *Endamoeba* Leidy, 1879.

Huber (1903) demonstrated quadrinucleate cysts, but was told by Schaudinn that these belonged to another species, which he himself had already seen and which he proposed to call *Entamoeba tetragena*. Schaudinn (1903) went on to distinguish between the *Entamoeba coli* found in healthy people, and the haematophagous amoeba he called *Entamoeba histolytica*. His further observations are described by Wenyon (1926) thus:

> In his description of the development of *E. histolytica*, Schaudinn described a method of reproduction by bud formation. The nucleus was supposed to give off chromatin material into the cytoplasm in the form of granules, which collected in groups on the surface of the amoebae. Small cytoplasmic buds, each containing a group of chromatin granules, were formed. These buds were described as becoming enclosed in very resistant capsules, forming spores, which were much smaller than the cysts of *E. histolytica* as they are now known. Schaudinn claimed to have produced infection in cats by means of these spores after complete drying, a procedure which is known to kill immediately the cysts of *E. histolytica*. Recent investigations have failed entirely to confirm Schaudinn's statements, so that it is safe to conclude that the budding process and spore formation as described by him do not take place.

Schaudinn made other peculiar observations, such as schizogony in *E. coli*, but nevertheless his name *Entamoeba histolytica* has now been universally accepted for the amoeba causing dysentery in man.

It was the name "*tetragena*" which caused confusion. Such was the stature of Schaudinn that his concept that the quadrinucleate cysts were those of some species other than *E. histolytica* remained unquestioned for a dozen years.

Huber (1909), Hartmann (1908) and others accepted that *E. tetragena* was a separate species. Viereck (1907) considered that *E. tetragena* was a strain of *E. coli*; Elmassian (1909) coined the name *Entamoeba minuta* for the trophozoites he found associated with quadrinucleate cysts.

Craig (1905) considered that Councilman and Lafleur (1891) had priority for their name and that the correct title should be *Entamoeba dysenteriae*.

Thus, in 1910, there were four names, *dysenteriae*, *tetragena*, *minuta* and *histolytica*, for what is now considered to be a single entity. The title *E. dysenteriae* is still used by some French writers, and the word *minuta* has a descriptive function. Despite the other vagaries in the proponent's descriptions, the accepted name for the parasite is *Entamoeba histolytica* Schaudinn, 1903,

III. Tetragena and Minuta

A. 1910–1920

It was Walker (1908, 1911) who showed that the names *tetragena* and *minuta* applied to phases of *E. histolytica*, and that this species has a similar development to that of *E. coli.* Walker and Sellards (1913) showed the difference in pathogenicity to man between the two species. Though their article is frequently quoted, it is only too apparent that it has not always been read, for there are a number of points, appreciation of which would have led to a better understanding of the relationship between *E. histolytica* and man, and many of the false trails subsequently followed might have been avoided.

There were three groups of patients in this classical study, all being fed gelatin capsules containing amoebae of varied origin and species. Of those given amoebae derived from water and other sources in Manila, none became colonized; of twenty prisoners given *E. coli*, seventeen were infected but none developed dysentery; of twenty given *E. histolytica*, seventeen were infected at the first attempt, a further one at a second attempt, and two remained refractory. Of the eighteen successful infections, only four developed dysentery, some after a considerable period. The "flow-chart" of the various strains employed is instructive, and is reproduced in Fig. 1.

It will be seen that strain A, which was derived from a case which had had a spontaneous attack of dysentery 2 months before, was apparently virulent in cases [2] and [30], but not in 29. On further passage from the dysenteric case [2] it gave rise to no further trouble in three cases 31, 32 and 33. After trouble-free passage through 29 and subsequently through 36, it caused dysentery in [41], but not in 42 or 44, and it failed to "take" in (43). Strain B, derived directly from a case of acute dysentery, colonized but caused no trouble in either of the two victims. Strain C, taken from a liver abscess at post-mortem, colonized one of two subjects, without initiating dysentery. Strain D, derived from a woman who had no history of dysentery, colonized all four subjects, causing dysentery in one. It is noteworthy that none of the dysenteric subjects had been infected from active cases, though these were apparently able to initiate infection.

Walker and Sellards point out that the amoebae fed were accompanied by the other organisms of the bowel, and that therefore the small proportion of

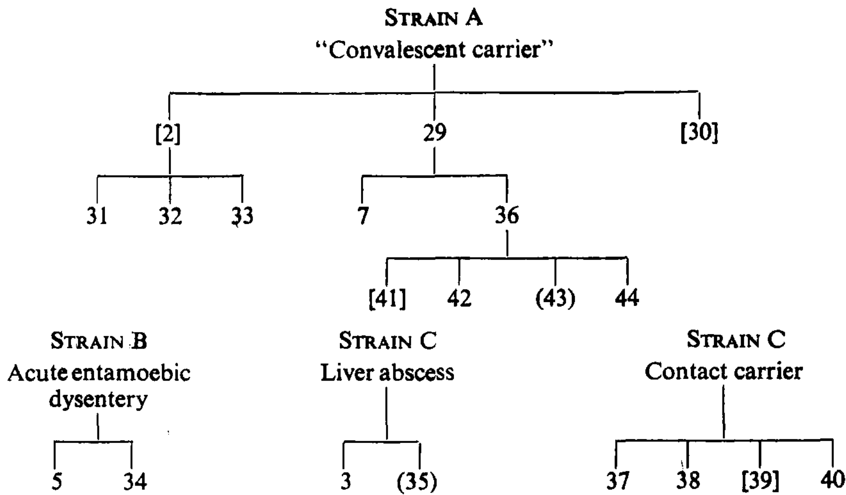

FIG. 1. Strain flow-sheet based on Walker and Sellards (1913). Numbers in brackets indicate dysentery and those in parentheses failure to colonize.

dysenteries attained has no bearing on the aetiology of the disease, but is evidence of its frequent latency. They go on to say:

> To what extent this latency, which is characteristic of entamebic dysentery, is due to the chronicity of the ulcerative process, or whether or not the inability of *Entamoeba histolytica* to penetrate the healthy epithelium plays a part in it, cannot at present be definitely answered. In the latter case, the entamoebae might be conceived to live as commensals in the intestine of their host, and only when there occurred some depression of the natural resistance of the host or of its tissues, or some inflammation or actual lesions of the intestine, were the entamoebae able to penetrate the intestinal epithelium, become tissue parasites and produce the characteristic lesions of entamebic dysentery.

Working in Sumatra, Kuenen and Swellengrebel (1913), speaking of what was then called *Entamoeba tetragena*, said:

> Wir unterscheiden in ihrem Lebenszyclus drei Stadien, das Histolytica-stadium, das Minuta-stadium und das Cystenstadium. Im ersten Stadium ist die Amöbe ein Gewebesparasit, und fundet sich in den Geschwüren des Darmes, und in den Wanden der Abscesse der Leber und andere Organe. Im Minuta-stadium, zu dem es in einem Bruchteil der Fälle kommt, wird sie in fäculenten Stühlen vorgefunden und stellt die saprozoische Phase der *E. tetragena* dar; Sie can sowohl gefunden werden, wenn noch Reizerscheinungen von der Seite des Darmes vorhanden sind, als auch in ganz normalen Stühlen von ausscheinend gesunder Personen. Das dritte, das Cystenstadium, tritt meistens zusammen mit den Minuten-stadium auf; die Cysten besorgen die Verbreitung der Art. Das Auffinden von Amöben und deren Cysten bei gesunden oder scheinbar gesunden Personen soll also nicht der kritiklosen Angabe Veranlassung geben, daß alle in Darme des Menschen gefundenen Amöben pathogen sind.

At the Réunions d'Information sur l'Amibiase in Paris, Swellengrebel (1961)

explained that while Walker and Sellards had regarded the *E. minuta* of Elmassian as a phase of *E. histolytica* intervening between the tissue stage and the formation of cysts, he and Kuenen had retained the name *minuta*, not as a different species, but to indicate that there is a particular stage in the evolution of *E. histolytica* which is independent of the tissue phase. Whereas Walker regarded the *minuta* forms as descendents of tissue forms, and the absence of pathology in some cases as being due to an adequacy of host defence, Kuenen regarded these small amoebae as being commensals with a variable tendency to metamorphose into tissue forms. The probability of invasion would depend both on the resistance of the host, and the inclination of the particular amoebae to undergo metamorphosis. This inclination would be enhanced by rapid passage from host to host, such as occurs in warm climates where the standard of hygiene is low.

Other observers had, independently, reached similar conclusions. Mathis and Mercier (1916) state:

> The fact that the *histolytica* form is only to be found in the muco-sanguineous stools of the acute dysenteric crises of amoebiasis, suggests to us that this form plays no part in the evolutionary cycle of the parasite. On the other hand, the pre-cystic and cystic forms seen in *tetragena* are suitable for transmission. Contrary to other opinions, we do not think these *tetragena* forms are modifications of the *histolytica* form. They arise from *tetragena* forms which have persisted in the intestine even through the dysenteric crisis.

In an editorial comment on the republication as a monograph of the series of papers by Wenyon and O'Connor (1917), Dobell (1918), referring to the appearance of *E. histolytica* cysts in apparently healthy hosts, wrote:

> The explanation of all these observations is now so obvious that it is almost difficult to believe that they ever appeared problematic; and it turns simply upon the proper appreciation of the habits of the amoeba. *E. histolytica* is—unlike most parasitic amoebae—a tissue parasite. It lives in and upon the living tissues of its host, and it can exist in no other way. This is the most important fact about the amoeba—as far as man is concerned. Now a parasite which feeds upon its host may obviously do so to a greater or less extent. The ideal condition for host and parasite alike is a state of equilibrium like that between Prometheus and the eagle—the former regenerating sufficient tissue each day to compensate for the ravages of the latter. The "natural" condition of a man infected with *E. histolytica* is similar. His amoebae feed, grow, and multiply at the expense of the living tissue lining his large intestine. . . . A human being in this Promethean state of equilibrium is now called a "carrier": and there can be little doubt that this is the "normal" or most common condition of infection of human beings with *E. histolytica* in nature.

In his classical work, "The Amoebae Living in Man", Dobell (1919) waxed almost lyrical in his condemnation of the concept that the *histolytica* phase plays no part in the normal transmission cycle. He felt that Kuenen and Swellengrebel

> hold peculiar views. . . . In the ordinary carrier of *E. histolytica* all the phases actually exist simultaneously. The cysts are in his stools; the precystic amoebae are in the lumen of the gut—from which they can easily be obtained at any time

> by the administration of a purgative; and the tissue-invading forms are in the ulcers in his gut wall—from which they could be obtained by scraping the ulcers, but not, as a rule by the administration of purgatives.

Linking the conclusions of Mathis and Mercier with those of Kuenen and Swellengrebel, Dobell concludes:

> I find nothing to support their view, and do not understand why they do not adopt the simple, obvious, straightforward and consistent interpretation of Walker and most other observers!

Thus, in 1920, there were two schools of thought, and as will be seen the schism has not even now been entirely resolved. The Swellengrebel school might well be termed the "commensalist", and that of Dobell, to use his own analogy, the "Promethean". The commensalists were, practically speaking, confined to continental Europe, whereas English-speaking workers followed Dobell, and the British and American schools were Promethean in outlook.

The two schools differed basically on the status of the cyst-passer, the Prometheans holding that this was a disease state and the commensalists insisting that this was not necessarily the case. The repercussions will be discussed later.

B. 1920–1960

Once it was appreciated that the quadrinucleate cysts of tetragena type were those of *Entamoeba histolytica*, the almost world-wide distribution of this parasite was established. Not only was it found in people who did not show any sign of disease, but also in countries where its disease manifestations were, to say the least, very rare.

Reichenow (1926), after quoting the prevalence of the parasite as reported from Asia and Africa, and from Northern Europe, goes on to say:

> Diese Tatsachen haben nichts Auffälliges, wenn man sie von dem Standpunkt aus betrachtet, daß *E. histolytica* gewöhnlich als harmloser Kommensale im Darminhalt lebt; sie sind aber schwer erklärlich, wenn wir diese Amöbe als einem obligatorischen Gewebsparasiten ansehen.

He, himself, was a carrier, possibly having been infected when he was in the Cameroons some 8 years before. He examined his own stools daily for some 6 months, and was struck by the enormous numbers of cysts.

> An vielen Tagen waren sie so zahlreich, daß in dem in der üblichen Weise hergestellten gefärbten Präparat etwa jedes Gesichtfeld der Immersion eine Zyste aufwies. . . . so bleiben es immer noch Millionen von Amöben, die an einem Tage die Darmwand zur Enzystierung verlassen haben, und diese können doch auch wieder nur ein Teil der Gesamtmasse sein, die sich dort auf Kosten der Gewebes ernähren würde, ohne die geringste Funktionsstörung des Darmes zu verursachen.

He thereupon dosed himself with "Karlsbader salz", washing out numerous "Minutaformen". Thereafter there was a considerable and prolonged drop in the production of "Histolytikazysten". He felt that this was incompatible with the concept that the *minuta* amoebae arose from *histolytica* forms

undisturbed by the treatment. He also mentioned *minuta* forms containing bacteria.

In a later paper, Reichenow (1931) says:

> Auch *E. histolytica* ist meist harmlos: eine herabgesetzte Reaktionsfähigkeit des Darms, die in der Regel durch Einflüsse der warmen Klimas auf den Organismus bewirkt wird, führt zum Gewebsparasitsmus dieser Art der je nachdem ob geringere Läsionen oder größere Geschwüre erzeugt werden, Enteritis leichterer Form oder Dysenterie bedingt. Die von solchen Amöbiasiskranken ausgeschiedenen, von der Gewebsform herstammenden Zysten bilden Stämme von erhohter Virulenz, die auch bei weniger disponierten Personen zum Gewebsparasitsmus übergehen können.

This last remark implies that amoebae which have gained experience of life in the tissues can have progeny capable of reaching a new host!

Brumpt (1926) said:

> Aussi, n'est-ce pas sans un certain étonnement que l'on remarquait les extraordinaires variations géographiques de virulence de l'amibe dysentérique produisant expérimentalement 20 fois sur 100 le syndrome dysentérique aux Philippines, 2 ou 4% d'après les statistiques médicale aux Indes et en Indochine, et une fois seulement sur plus de 3 ou 4 millions de porteurs en Angleterre.

He postulated that there are three species of amoebae parasitic in man which produce quadrinucleate cysts—*Entamoeba dysenteriae*, *E. dispar* and *E. hartmanni*, the last having cysts with a diameter less than 10 μ.

> L'*E. dispar* présente des kystes de 10 à 16 μ tout à fait comparables à ceux de l'*E. dysenteriae*. Pour differencier ces deux amibes, il suffit de donner un purgatif salin aux porteurs et d'injecter les selles liquides fraîches à de jeunes chats de 400 a 600g. en ayant soin de collodionner l'anus de ces animaux. Quand il s'agit de l'amibe dysentérique, le chat meurt avec les lésions habituelles des la maladie. S' il s'agit au contraire d'un cas à *E. dispar*, le chat présente une infection parfois intense, généralement de faible durée et, s'il succombe, l'intestin ne presénte aucune lésion macroscopique et ses parois ne sont pas épaissies.

According to the Brumpt concept, there are two species of morphologically identical amoebae producing cysts of 10–16 μ diameter from small non-haematophagous trophozoites—the *E. dysenteriae* of the tropics, which can be invasive and cause dysentery, and the *E. dispar* of temperate zones, which is always harmless.

With the advent of the Boeck and Drbohlav (1925) method of cultivation, Dobell carried out extensive studies (Dobell and Laidlaw, 1926a, b; Dobell, 1927, 1928; Dobell *et al.*, 1952), being particularly interested in the associated bacterial flora. He also studied the intestinal protozoa of monkeys (Dobell, 1928, 1931), comparing these with those of man. This experiment convinced him that, contrary to his previous views, *E. histolytica* could feed on bacteria, both *in vivo* and *in vitro*, and ultimately he said:

> It appears almost certain now that *E. h. hominis** sometimes lives in man as a harmless commensal—just as *E. h. macacorum** typically does in its natural hosts.

* Dobell's designations *hominis* and *macacorum* refer to the origin of the strains of *E. histolytica* concerned.

Unfortunately this change of view did not receive anything like the publicity given the Promethean concept.

Hoare (1952a) had the opportunity of re-examining specimens from case H of the Wenyon and O'Connor (1917) series, and from a number of other carriers and "chronic infections". He examined 670 amoebae from seven cases. Of these, 51 % had ingested bacteria, 22 % had "undetermined inclusions" and none were haematophagous. He paraphrased the famous statement by Dobell (1919) thus:

> The position of those who regard *E. histolytica* as an obligatory tissue parasite is untenable even on *a priori* grounds, for it is inconceivable that an organism feeding exclusively on tissue elements and erythrocytes in the human host should completely change its habits in other mammalian hosts and in culture, and become a feeder on bacteria.

In his review, Hoare (1952b) concludes:

> A critical analysis of the available data leaves no doubt that, in symptomless carriers, as well as in latent periods of chronic infections, the amoebae live in the lumen of the gut and feed—among other faecal elements—on bacteria. Apart from their food habits, these trophozoites (known as *minuta* forms) can be distinguished from the haematophagous amoebae found in dysenteric cases by their smaller size. Since they are the only amoebae capable of producing cysts, the *minuta* forms represent the essential stage of *E. histolytica*, whereas the haematophagous amoebae have no place in its normal life-cycle.

Unfortunately this statement by Hoare came too late to stem the conflagration initiated by Dobell's Promethean concept, the flames of which were fed by uncritical but vociferous writers. The embers still smoulder!

IV. Large and Small

There was another confusing issue! Wenyon and O'Connor (1917) working in Egypt stated:

> A feature of the *E. histolytica* infection which the examination of a large series of cases has impressed on us, is the variation in the size of the cysts . . . but it appears that various strains of *E. histolytica* occur. There is one which produces very small cysts, associated with correspondingly small "minuta" forms of amoebae. The cysts are seven to ten microns in diameter, and have the same characters as the larger cysts.

They give (p. 160) a number of cyst measurements from different cases. The means and deviations are as follows: R. 7·68 μ, σ 0 × 72; K. 9·22 μ, σ 1·01; C. 11·76 μ, σ 0·99; M. 12·90 μ, σ 1·14; F. 12·84 μ, σ 1·11; H. 15·10 μ, σ 1·70. Analysis suggests that there are at least two populations present, one with a diameter of the order of 8 μ.

Von Prowazek (1912) had described an intestinal amoeba with cysts from 6 to 8 μ in diameter, which he called *Entamoeba hartmanni*; Kuenen and Swellengrebel (1917) described *Entamoeba tenuis*, which they later regarded as being *E. hartmanni*; Brug (1918) described *Entamoeba minutissima* from cases in Sumatra, which he later regarded as synonymous with *E. tenuis* and *E. hartmanni*.

Dobell and Jepps (1918) studied "The diverse races of *Entamoeba histolytica* distinguishable from one another by the dimensions of their cysts". They measured 500 cysts from each of seven different patients, and constructed size-distribution curves for each case. On the basis of the differing modes they obtained, they concluded:

> *Entamoeba histolytica* is a collective species. It comprises a number of distinct races, strains or pure lines, distinguishable from one another by the size of the cysts which they produce. How many such distinct races exist is still undetermined; but we have demonstrated at least five.

Luckily they present their raw data, and it is possible to apply modern statistical techniques to them. Figure 2 shows the effect of pooling some of their cases. These are plotted on a probability grid, on which a straight line indicates a normal spread from a single population.

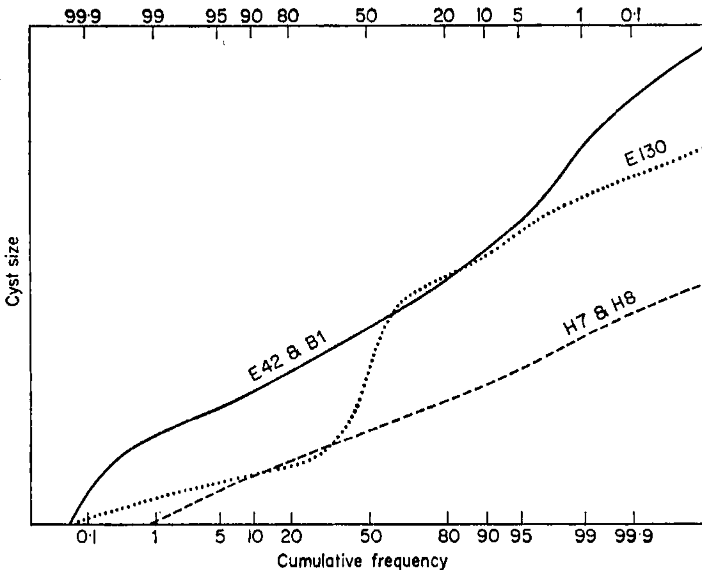

FIG. 2. Data from Dobell and Jepps (1918) plotted on probability scale. It will be seen that some mixtures [E42 and B1] and [H7 and H8] form relatively straight lines, whilst E130 from a single case shows two populations.

Malins Smith (1918) constructed a size-distribution curve based on the measurements (in iodine) of a total of 1000 cysts obtained from 30 different cases. He obtained a bimodal curve with peaks at 7·1 μ and 12·2 μ. He says: "the cysts of this species divide themselves naturally into two strains, differing only in size."

In his classical work, Dobell (1919) is literally scathing in his comment on Smith's work, but to the present writer his remarks reveal some lack of appreciation of statistical method. He was similarly critical of the observations of von Prowazek, of Kuenen and Swellengrebel, of Brug and of Woodcock 1917), being apparently more concerned with the terminology these authors

happened to use than with the facts they recorded. He was emphatic that *E. histolytica* could have cysts ranging in size from 5 to 20 μ, depending on the strain. He relegated *E. hartmanni* to the status of a synonym of *E. histolytica* and of a *pro parte* synonym of *E. coli.*

Wenyon (1926) did the same, though he states: "It cannot be regarded as finally established that the races of *E. histolytica* which produce the small cysts are able to give rise to amoebic dysentery."

When two observers of such eminence as Dobell and Wenyon adhered so firmly to what has become known as the "unicist" theory, it is small wonder that the English-speaking world followed. This concept has been repeated from textbook to textbook. Perhaps the widest publicity was given by Craig and Faust (1943), whose "Clinical Parasitology" had several editions and many reprints. They say;

> The cysts [of *E. histolytica*] vary greatly in size, measuring from 3·5 to 30 microns in diameter, some strains producing cysts measuring from 3·5 to 12 microns in diameter, and others measuring from 12 to 20 microns in diameter.

As this book was the *vade mecum* of several generations of medical parasitologists, it is not surprising that *E. hartmanni* went into the limbo.

The "pluralists" were practically confined to continental Europe. Brumpt (1949) reaffirms his acceptance (*vide supra* Brumpt, 1926) of the species rank of *E. hartmanni* by giving an illustrated description. The species is also accepted by Neveu-Lemaire (1943) and by Piekarski (1954).

It was the publication by Sapero *et al.* (1942) of their statistical study, not only of their own extensive survey of some 3000 cysts from over 280 cases, but also of the data given by Dobell and Jepps, and those of Malins Smith, which "demonstrated the existence of two significantly different races, a large and a small." Their figure (p. 196) is a beautiful demonstration, and should convince the non-mathematical without further explanation. It is here reproduced (Fig. 3). They were cautious in drawing their conclusions, stating:

> These observations clearly indicate the importance of clarifying the respective roles of the two races of *E. histolytica.* The suggestion exists that there might possibly be a distinct species. Such a suggestion however is premature, as there is great need for further evidence on the various apparent differences exhibited by the races of the organisms.

Thereafter, a number of observers began to report large race and small race, either being unaware of, or not caring to be drawn into, the controversy on taxonomy. Hoare (1949), while quoting extensively from Sapero *et al.*, states:

> However, in view of an overlap in their dimensions, there is more justification for regarding them as subspecies rather than as species. If this course is adopted, the small race should bear the name *E. histolytica hartmanni*, whilst the large race becomes *E. histolytica histolytica.*

Hoare gives the dimensions of the cysts of *E. histolytica* as 5–20 μ, and those of *E. coli* as 10–30 μ.

Freedman and Elsdon-Dew (1958, 1959) showed that the size of trophozoites in culture varies with differing bacterial associates, and thus felt it was unwise to contrast the sizes of trophozoites from different environments. They therefore

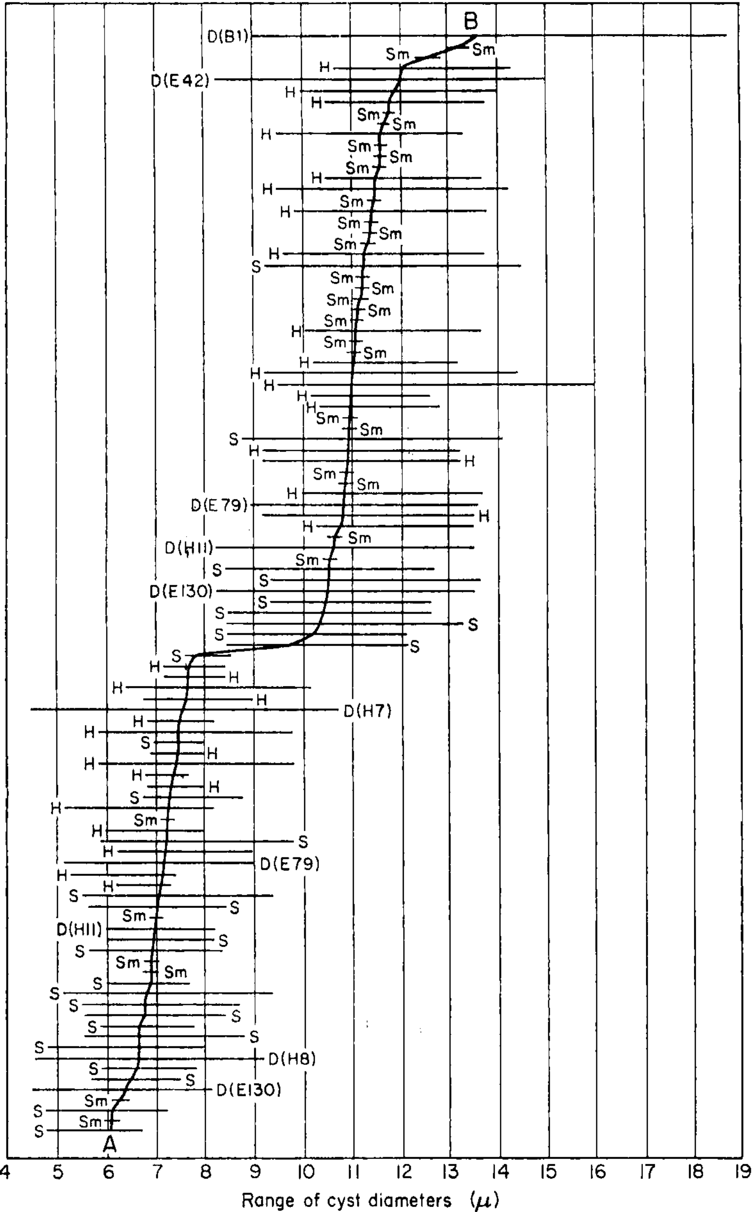

FIG. 3. The figure from Sapero, Hakansson and Louttit (1942). The horizontal lines show the range of cyst diameters (measurements on the basis of wet-fixed, stained specimens) obtained by independent workers in each of 99 individuals harbouring *E. histolytica*. The line AB passes through the mean diameter of each strain. Note that the 9 μ line divides all strains into two series, a larger with average diameters from 10–14 μ, and a smaller from 6–8 μ. The mean and range of sizes are largely similar within each series; the strains belonging to different series are markedly dissimilar. [D, Dobell and Jepps; S, Sapero; H, Hakansson; SM, Malins Smith (mean diameters only).]

grew amoebae from different sources together in the same test tube, with a joint flora, and then constructed size-distribution curves. Using a strain of *E. histolytica* isolated from a liver abscess (Freedman *et al.*, 1958) into monoxenic (*Clostridium welchii*) culture as a criterion, they grew this together with other strains, some from liver and some from stools, both dysenteric and apparently normal. Whereas strains from invaded cases gave unimodal curves when grown together with the standard strain, some strains isolated from faeces gave bimodal curves. When these small strains from faeces were grown together, only unimodal curves were obtained. They felt that this genetic constant was sufficient to warrant species rank for *Entamoeba hartmanni*.

Burrows (1957, 1959), in detailed studies of the morphology of intestinal amoebae, states:

> *Entamoeba hartmanni* may be distinguished from the dwarf forms of *E. histolytica* by the smaller size of the trophozoites; by the smaller and more *E. coli* type of nucleus; by the smaller sizes of the cysts; by the smaller percentage of nuclear diameter to cyst diameter; and by the diffuse type of cyst vacuolation.

(It should be redundant to emphasize that Burrows bases his criteria on populations, and not on individual amoebae, the distinction of which is frequently impossible.) For more extensive detail, and also for the differentiation of *E. histolytica* from *Entamoeba polecki*, with which it may also be confused, see Burrows (1965).

Using a fluorescent antibody technique, Goldman *et al.* (1960) contrasted *E. histolytica* and *E. hartmanni*. They state:

> In our opinion the results justify the conclusion that the small Entamoeba differs significantly from *E. histolytica* in its antigenic make-up, and that this difference confirms the concept that the organism belongs to a different species, *E. hartmanni*.

Though Faust *et al.* (1962) do not go the whole way, they say:

> In the present state of confusion, perhaps a satisfactory solution is to refer to the two racial types as *E. histolytica* var. *histolytica* for the large race, and *E. histolytica* var. *hartmanni* for the small race, in the meanwhile having an "open" [their quotes] mind on this question of a small race and its appropriate designation.

Hoare (1961) was by this time prepared to accept that the small race warranted species rank, for, at the Paris Réunions d'Information sur l'Amibiase, he said:

> Il est maintenant établi que le complexe *histolytica* comprends au moins deux espèces—(1) *E. hartmanni*, anciennement considérée comme n'étant qu'une petite race de *E. histolytica*; (2) *E. histolytica* proprement dite, représentant la grande race.

It would seem well founded that there are two sizes of cysts found in the *histolytica* complex, with 10 μ as a dividing line for the mean size of a population. While the "pluralists" wish to call the small cysted amoeba by a specific name, the "unicists" do not agree that cyst size and such other differing features as have been described are sufficient to warrant species rank, and insist on keeping the small race within the species *E. histolytica*.

However, the very name *histolytica* implies at least potential pathogenicity, and as the "small" amoebae do not, from all accounts, invade or dissolve the

tissues and their clinical importance lies merely in their distinction from amoebae which might do this, their designation should not suggest this potential lest some clinician be induced to institute drastic measures.

V. The Clinical Picture

Whilst argument at the academic parasitological level as to whether the *E. histolytica* complex comprises one or more species or whether it can be a commensal does but little harm, the answer to these questions is of paramount importance to the clinician confronted with a case. On him rests the decision as to what action is to be taken on the result of a stool examination. If he be inadequately informed, his actions may have unfortunate sequelae. It is perhaps the responsibility of the parasitologist to ensure that the significance of his findings is adequately appreciated by those who will act on them. This admonition is all the more necessary for *ex cathedra* statements likely to influence the opinions and actions of many. One might well paraphrase Hoare's quotation from Dobell (1918), and say: "When one pedant tells a tale emphatically and often enough, it is apt to be believed in preference to the many observations of more experienced, but less vociferous men."

The frank manifestations of amoebic dysentery and amoebic liver abscess, so adequately described by Councilman and Lafleur (1891), should present little difficulty in diagnosis to the experienced clinician. For the less experienced, many standard textbooks on tropical medicine give a reasonable account, but for more detailed information, see Wilmot (1962).

It is the interpretation of the finding of cysts in the absence of overt manifestation which is the main difficulty.

A. Iatrogenic Amoebiasis

Unfortunately, Dobell's earlier Promethean concept was perpetuated on the other side of the Atlantic, particularly by Craig, and later by Faust and others. Craig (1927) said:

> In every carrier of this parasite there must be numerous foci of infection present in the mucous membrane from which toxic material is absorbed, and whether symptoms are produced or not depends on the resistance of the individual carrier.

Faust (1941) carried out parasitological examinations on necropsy material from 202 accident cases in New Orleans, and found *Endamoeba histolytica* in 13. Lesions described as amoebic were found in 7 of these. In 3 cases, all without lesions, only a single cyst was found. In one case (62), though cysts and trophozoites were found in the bowel contents, they could not be found in the extensive superficial erosion, which was therefore not classed as amoebic. In the remaining two cases without lesions (9 and 137) amoebae were found at all levels. (*En passant*, it is of interest to note that, of the cases with lesions, 4 had cysts under 10 μ in diameter.) Though Faust interpreted these findings as indicative of damage to the bowel, Hoare (1952b) quotes the paper as indicative of "an intact mucous membrane".

In their widely read "Clinical Parasitology", Craig and Faust (1943) say:

> Thus, strictly speaking, a healthy carrier of *E. histolytica* does not exist, for in all carriers cytolysis and necrosis of at least the superficial layer of the mucosa are continually occurring.... Carriers should be treated, whether symptoms are present or not.

Craig (1944) divides the clinical manifestations of amoebic infection into five classes, and goes on:

> In the writer's experience the symptomatology of amebiasis in Class II cases, or carriers with symptoms, is confined to the gastro-intestinal tract, and the nervous and circulatory systems. The symptoms most commonly observed in the gastro-intestinal tract are constipation, very slight and evanescent diarrhea, colicky pain in the lower abdomen, gaseous distension of the abdomen after eating, gaseous eructations, a capricious appetite, and slight nausea before or after eating. The symptoms most often noted in the nervous system are headache, usually frontal in type; aching in the arms and legs, especially on waking in the morning; somnolence or disturbed slumber; poor memory; lack of ambition; backache or mental depression. The symptoms noted in the circulatory system are irritable pulse, arythmia, vaso-motor disturbances, as flushing of the skin and excessive perspiration of the hands and feet, and tachycardia.

D'Antoni (1952) says:

> Amebiasis, unfortunately, has no typical clinical picture, which is one reason why it is so difficult to present to both physicians and medical students. I am increasingly impressed, however, with the frequency with which certain special symptoms appear....

His subsequent headings are fatigue, headache, nervousness, low-grade afternoon pyrexia and such hepato-biliary symptoms as epigastric fullness after meals, epigastric distress, belching, nausea and sometimes vomiting.

A cross-section of any clinical practice would reveal a high proportion of patients presenting with at least part of such a wide gamut of symptomatology, and, in the stools of some of these, quadrinucleate cysts of one species or another, real or imaginary, would be found. The sequel is obvious—in the mind of the Promethean-indoctrinated, busy physician, the symptoms and the cysts are associated. If, perchance, the symptoms are ameliorated after anti-amoebic therapy, the association is established. If not—"The amoeba is difficult to treat".

The spiral goes further—once the physician has become amoeba-conscious, he attributes the lack of laboratory confirmation to the highly publicized difficulty of finding the parasite, and he treats anyway. Alternatively, if he sends a large enough series of specimens to a "pepper-pot" laboratory, sooner or later some object resembling an amoeba will be found.

Though emetine is said to be specific for the amoeba, this does not exclude other beneficial activity, This is certainly true of other anti-amoebic drugs such as chloroquine. Unpleasant therapy will, *ipso facto*, cure many a neurotic, and, where the criteria of disease are so vague, a successful "test of cure" means little—or nothing!

Unfortunately, there are unscrupulous physicians to whom a diagnosis of amoebiasis is lucrative. Sir Philip Manson-Bahr was wont to recount the story of one "Emetine Joe", who ran two limousines on that drug. "The Story of San Michele" (Munthe, 1929) might be quoted as another example. In investigating the amoebiasis position in an American city which must be nameless, the present writer was driven to say: "Amoebiasis is apparently a disease of those who can afford it!"

The literature is replete with examples of false association of the amoeba with some clinical manifestation, so much so, that even one who is only too aware of the depredations of the parasite is moved to spring to its defence. Suffice it to quote a few instances.

1. *Rheumatoid Arthritis*

Ten cases of chronic amoebiasis in which rheumatic complaints predominated are reviewed by Zinneman (1950), who concludes: "In every case of rheumatoid arthritis, arthralgia or myalgia, it seems worth while to keep examining stools for protozoan parasites". Four cases of recurrent polyarthritis secondary to chronic intestinal amoebiasis are reported by Rappaport *et al.* (1951), who suggest that this is "due to sensitisation of [*sic*] a toxin elaborated by the ameba".

Rinehart (1952) says that his "findings strongly suggest an etiologic relationship between most cases of rheumatoid arthritis and infection with *E. histolytica*". In a later paper (Rinehart and Marcus, 1955) he states: "Nearly all (95 per cent) of patients with rheumatoid arthritis are infected with *E. histolytica*". He contrasts this with a finding of 30% for normal persons—in Oregon!

2. *Ocular Manifestations*

Because *E. histolytica* was found in 37·5% of 64 cases of chronic chorioretinitis, as against 8% in cases with other eye conditions and in 9% of normal persons, Vrancic and Mimica (1959) suggest that *E. histolytica* might play some role in this condition. Though parasites were not demonstrated in the faeces, oedema of the macula was attributed to amoebiasis by Eggers (1959) because of the prompt response to therapy.

A case of ulcerative keratitis and relapsing acute glaucoma with cysts of *E. histolytica* and ova of *Ascaris lumbricoides* in the stool, was reported by Jiminez-Quiros *et al.* (1961). They say:

> After the anti-parasitic treatments the symptoms disappeared completely; however, reinfection with either of the two parasites caused new allergic manifestations. The permanent cure of ocular [keratitis and glaucoma] and extra-ocular [angioneurotic oedema, rhinitis, asthma] manifestations corresponded to permanent elimination of the parasites.

3. *Thoracic Manifestations*

Though extension to the thorax of an amoebic process in the liver is well recognized, some reports are open to question. The finding of trophozoites in

the sputum of nine cases, and of cysts in that of another, as reported by Webster (1960), is indeed strange in a series of 48 cases of "hepatic amoebiasis" in none of which an abscess pointed elsewhere.

Remote pulmonary effects are discussed by Carri (1948), who felt that respiratory allergy might be caused by *E. histolytica.* Peyron (1948) based a thesis on a single case with cysts of *E. dysenteriae* in the stools, whose Loeffler's syndrome was cured by 8 days of emetine. Gelin (1949) found that 69 of 80 cases examined in Oran had "Précardialgie" and 73 had hypotension. The high frequency with which subjects with chronic colitis present with precordial pain was noted by Perroni (1952). More recently, Hamilton and Lutwyche (1960) describe a case "worth recording as an example of respiratory symptoms due to a latent amoebiasis". Questioned, they replied (1961): "We assumed it to be an allergic phenomenon."

4. *Allergy*

According to Radke (1952b) "We have learned that one should have one's diagnostic suspicion of amebiasis raised by patients with chronic allergic disorders, such as migraine, urticaria and neurodermatid." The manifestations of *E. histolytica* are divided into allergic and non-allergic by Lyon (1958), who justifies the variability of the former by citing that Bozicevich (1950) had to use poly-antigens for the complement-fixation reaction!

The opening gambit to a paper by Banerjee (1958) on the evaluation of an amoebicide reads: "In various skin conditions such as leucoderma, internal toxic dermatitis, intractible urticaria, proritis, etc., amoebic colitis has been found as a factor causing much difficulty in the management of the cases."

5. *Other Amoebae*

Some authors go so far as to attribute symptomatology to amoebae other than *E. histolytica.* Rothman and Epstein (1941) state: "The most common symptoms were constitutional, i.e. extreme tiredness and weakness . . . of local nature were abdominal discomfort and diarrhoea . . . From a clinical point of view all forms of amoebae appear to have a pathogenic role." Kasliwal and Sogani (1958) considered that such symptoms as "change of behaviour", palpitation, and vague abdominal pain were a result of infection with *Entamoeba coli.* In an address to the Texas Society of Gastroenterologists and Proctologists, Golding and Black (1959) conclude: "We treat all non-pathogenic amoebae. To date, the majority of these patients have shown clinical improvement."

It is painfully apparent that many "diagnostically destitute" physicians have seized upon the amoeba as a convenient scapegoat for their inability to find the real cause of the patient's symptoms. If perchance, an amoebicide, probably aided in no small part by the *Vis medicatrix Naturae* was followed by improvement, the doctor's self-deception was enhanced.

Adams (1953) was provoked to comment:

> To me it seems entirely unwarrantable . . . to ascribe to an *E. histolytica* bowel infection, apparently limitless indeterminate symptoms remote from the

> abdomen. One's credulity is stretched beyond the limit when anything from headache and visual disturbance to sexual disorders and rheumatoid arthritis have been attributed to an otherwise symptomless *E. histolytica* infection. . . .

The examples quoted thus far have little or no justification, but there is another widely used diagnosis, in which it is not quite so easy to exonerate *E. histolytica*.

B. AMOEBIC HEPATITIS

In the Lettsomian Lectures, Rogers (1922) showed that there is, prior to the development of frank liver abscess, a stage of painful hepatomegaly associated with fever and a leucocytosis, and that abscess formation could be averted by the use of emetine. According to Rogers and Megaw (1946):

> Every case of hepatitis or hepatic congestion should be assumed to be amoebic in nature unless there is clear evidence that some other cause exists. Unexplained fever with leucocytosis of the characteristic type should be suspected to be due to amoebiasis, even in the absence of a history of dysentery and of entamoebae in the stools. Sometimes there are no definite signs of liver involvement; in such cases the results of a short course of emetine treatment are so striking that there can be little doubt of the nature of the disease. For these cases the term hepatic amoebiasis would be more appropriate than amoebic hepatitis, which conveys the suggestion that the condition is necessarily associated with the obvious manifestations of hepatitis.

Rogers described two forms—acute and chronic. The acute form described was almost certainly due to a hidden, developing abscess, and for such emetine is life-saving.

Whether the chronic form Rogers encountered was a similar, but much slower process is not clear. He says:

> This also commences with fever, which is often the only symptom for weeks, and the discovery of the true nature of the case usually depends on a blood examination revealing a moderate degree of leucocytosis, often with little or no increase in the proportion of the polymorphonuclears.

Such a finding, *per se*, does not warrant a specific diagnosis, but in a population in which frank amoebic dysentery and amoebic liver abscess are prevalent, it might justify at least the trial of emetine.

Commenting on 1 000 cases of amoebic dysentery encountered in eastern India during World War II, Payne (1945) reported liver abscess in 2·8 % and amoebic hepatitis in 53·9 %, and states:

> Hepatitis was so common that it came to be considered part of the disease. Usually it was mild—discomfort in the right hypochondrium and over the lower ribs and back, with tenderness and slight hepatic enlargement. Whether these symptoms are due to bacterial "showers" from the ulcerated gut, as some believe, or to amoebic emboli, they responded immediately to emetine.

This quotation illustrates the well-known fact that, in frank amoebic dysentery, there is not uncommonly a tender hepatomegaly, which in a few cases goes on to abscess formation. Payne also raises the controversial point—the mechanism of the hepatic disturbance.

In frank dysentery the use of systemic amoebicides is mandatory, but it is not in this situation that difficulty arises! Amoebic liver abscess may appear without antecedent dysentery, and indeed, in the apparent absence of *E. histolytica* in the gut. For this reason any tender hepatomegaly might well come under suspicion, but the only other criterion with any pretence to specificity is the response to emetine.

Thus there arose the concept of "amoebic hepatitis" (a term Rogers deplored) with the diagnosis based on a tender liver responding to emetine. This diagnosis became popular, even in areas where frank amoebic dysentery is uncommon, and where amoebic liver abscess is, to say the least, rare. The literature is full of articles on the subject, and it would be odious to quote examples of what might almost be termed "wishful thinking".

As a justification for such a clinical picture there evolved the postulate of a diffuse amoebic infiltration of the liver, without abscess formation. However, with the exception of the report by Doxiades *et al.* (1961), there has never been pathological proof of such a condition, despite intensive search, not only in autopsy material (Kean, 1955; Roach, 1959) but also in liver biopsy in appropriate cases (Kean, 1957; Powell *et al.*, 1959).

Powell *et al.* (1959) found that dysenteric cases treated with tetracycline and di-iodohydyoxyquinoline (known to be ineffective in the prevention of liver abscess) were relieved of their tender hepatomegaly to the same degree as those who were given emetine or chloroquine in addition.

It is not necessary to assume that invasion of the liver by amoebae is the cause of the condition. There is evidence of hepatic enlargement in non-amoebic bowel conditions such as ulcerative colitis (Pollard and Block, 1948; Kimmelstiel *et al.*, 1952; Bargen, 1956). Elsdon-Dew (1964a), in the Theobald Smith Memorial Lecture, stated:

> The enlargement of the liver that receives this label occurs in many conditions in which the continuity of the colonic mucosa is broken, and is probably consequent on an increased load placed on the barrier function of the liver.

Nevertheless, the diagnosis "amoebic hepatitis" is still in all too common use, providing as it does a convenient pragmatic label. There is variation in the size of a normal liver from person to person and even from time to time; and most livers are tender if sufficient pressure be applied, and especially to the "punch" test advocated by some. Even if the laboratory could find them, the parasites are not always present, so why bother to have the stools examined; a leucocytosis is not always present, so why confuse the issue with a blood-count; if the patient is in bed, emetine will not do much harm, and it might work. Such casuistry is unfortunately self-perpetuating!

Sufficient has been said to indicate the wide gamut of conditions which have been carelessly attributed to *Entamoeba histolytica*, when, in point of fact, this parasite is usually focal and frank in its manifestations. It is a strange feature that the frequency of such parodies is usually in inverse proportion to the observer's experience of the genuine disease.

Iatrogenic amoebiasis rapidly becomes a disease of communities. The disease becomes "fashionable" for both doctor and patient, and the latter may find it

a convenient excuse for his inability to cope with his environment. Such iatrogenic amoebiasis is often endemic, but epidemics may arise on the arrival of some new technician or doctor, or when the disease is given undue publicity such as contributed in no small part to the Chicago "outbreaks".

Nevertheless, in some communities, the depredations of *Entamoeba histolytica* cannot be gainsaid, but the picture is, for the most, that painted so long ago by Councilman and Lafleur (1891). There are authenticated reports of invasion of the skin, the cervix and other organs, but in all these the amoeba is to be found at the site of the pathology. Such unusual manifestations are, as a rule, only encountered in areas where both amoebic dysentery and amoebic liver abscess are prevalent.

VI. Geographical Distribution

A. The Parasite

At the request of the World Health Organization, Elsdon-Dew (1964b) reviewed the available literature on the prevalence of the parasite, but, as little reliance could be placed on the majority of the data, he restricted circulation. A high proportion of the reports were based on such other activities as routine hospital examinations, and in few was there any attempt at representative sampling of the populations concerned. Criteria obviously differed from author to author, a variety of techniques was employed, and it was apparent that not only was the competence, but sometimes even the *bona fides* of some observers in question. The writer has made repeated attempts to establish some order in this chaos, but is forced to admit failure. Burrows (1961) attempted the use of mathematics on the data from the U.S.A., with unconvincing results.

The parasites seem to have been found in most parts of the world and in most peoples examined, with the exception of the Eskimo (Brown *et al.*, 1950; Laird and Meerovitch, 1961) and of the Poles (over 90000 examinations with a nil report). Reports of prevalence of over 50% in apparently healthy people are shown in Table 1.

The Table is given for interest only. The high results may have been due to the assiduity of the workers. Lawless *et al.* (1956) examined the same eighty subjects from an Egyptian village on six occasions over a period of 2½ years. The percentages given for total *E. histolytica* (large and/or small) were 66, 59, 65, 53, 52, 61, but over the whole period, in only one of the subjects did they fail to find one or other form, and they found both in over 80%. Though the authors modestly attribute the low (*sic*) finding at the individual occasions to technical inadequacy, another explanation is that the parasites circulate through the population. The South Bend factory workers had been exposed to faecally contaminated drinking water. Though the figures for Mexico may seem high, there are other reasons for suspecting that this is an endemic area.

From earlier remarks, it will be appreciated that the confusion between large and small entamoebae with quadrinucleate cysts invalidates any attempt to use extant surveys as data upon which to base the apparent distribution of *Entamoeba histolytica* (*sensu stricto*). Even if *E. histolytica* and *E. hartmanni*

TABLE I

Reported prevalence of over 50%

Author	Group
Lawless *et al.*, 1956	Nile agriculturalists
Chandler, 1954	Egyptian villagers
Kuntz and Lawless, 1958	Egyptian children, 2–4 years
Bray, 1958	Harbel, Liberia
Amaral and Pires, 1947	Sao Paulo, prisoners
Earle, 1950	Guayaquil, food handlers
Williams and Thomas, 1930	Haiti, food handlers
Thomas, 1951	Bahamas, schoolchildren
Bustos, 1935	Mexico, country children
Santos-Zetina, 1940	Mexico, Merida
Domingues-Rojas, 1953	Mexico, Villa Vicenzo
Markell and Chavez-Nunez, 1956	Mexico, Chiapas
Brooke *et al.*, 1955	U.S.A., South Bend factory workers
Berberian *et al.*, 1952	U.S.A., Wassaic mental defectives
Hemming *et al.*, 1945	India, prospective food handlers
Epstein, 1933	U.S.S.R., Kola peninsula
Piekarski, 1949	Germany, Bonn, soldiers

are regarded as a single group, it is still impossible to attain valid comparison. Some concept of the relative prevalence of the large *E. histolytica* and the small *E. hartmanni* may be gained from Table II. It must be emphasized that contrasts should not be made between author and author. It should be reasonable to draw comparisons between observations in the extensive series by Kuntz and his colleagues, and between those by Jones and Eyles, who were co-authors, as were Norman and Brooke. Subject to the usual reservations, it would seem that, in stable populations, *E. hartmanni* is commoner than *E. histolytica.*

Table II also reveals that *E. hartmanni* is widely distributed. If indeed this organism has been labelled as *E. histolytica*, and the patient treated by some member of the Promethean school, there must have been a lot of unnecessary therapy, much of it not without danger. However, it is the writer's experience that, possibly because of its small size, many laboratories do not find it. Certainly many laboratories do not report *Endolimax nana*, either because of ignorance or because they do not see it! This failure to find *E. hartmanni* may thus have been a blessing in disguise.

In general it would seem that the subjectivity of stool examination is so great as to invalidate any conclusions on the available data. Until such time as sufficient, competent, objective personnel can use standard method and criteria on adequately sampled populations, any attempt at mapping the faecal protozoa is doomed to failure.

TABLE II

Reports of large and small E. histolytica

Author	Place, etc.	No. examined	Percentage Large	Percentage Small
	Africa			
Kuntz *et al.*, 1958b	Egypt	1 408	19·9	30·4
Colless, 1958	Liberia	100	37	32
Gaud *et al.*. 1943	Morocco	518	14·0	0·4
Kuntz *et al.*, 1955	Bahr el Ghazal	79	24·1	21·5
English, 1945	Zambia, Africans	509	31·2	2·2
Angelini, 1944	Italians *ex* East Africa	100	20	9
	Asia			
Kuntz *et al.*, 1958a	Turkey	249	14·0	21·2
Kuntz, 1960	Bengal	295	13·9	27·8
Kuntz and Wells, 1962	Sarawak	1 089	1·6	4·7
Kuntz and Lawless, 1960	Philippines	488	11·6	16·6
Kuntz *et al.*, 1959	Formosa, U.S. soldiers	116	5·2	2·8
	dependents	184	2·2	2·2
Kuntz, 1964	Leyte	304	6	10
Kuntz, 1967	Thailand, Chinese evacuees	197	3	14
	North America			
Rossien *et al.*, 1954	New York, T.B.	264	1·5	1·9
Avery, 1946	Washington, D.C.	19 512	1·8	1·9
Brooke *et al.*, 1955	Indiana, survey	600	1·3	2·5
Harper *et al.*, 1957	Indiana, mental hospital	110	17·3	11·8
	college students	120	1·7	3·3
Colvin *et al.*, 1947	California veterans	14 744	2·0	1·6
Brown and Garber, 1960	California military	18 604	2·9	3·2
Eyles *et al.*, 1953	Tennessee, Fayette	2 657	6·2	6·9
Jones *et al.*, 1954	Tennessee, New Hope	322	17·1	6·2
Brooke *et al.*, 1954	Georgia	400	3·0	7·0
Norman and Brooke, 1955	Georgia	377	3·7	7·8
Jones *et al.*, 1953	Mississippi, Negroes	935	5·6	5·1
	South America			
Gimenez, 1951	Argentine, La Plata	891	0·6	0·4
	Australia			
Bearup *et al.*, 1949	New South Wales, children	416	0·2	0·5
	soldiers	2 386	0·7	0·6

TABLE II—*continued*

Author	Place, etc.	No. examined	Percentage	
			Large	Small
	Europe			
Woodruff and Bell, 1960	London, patients	72	91·7*	22·2*
Bell and Woodruff, 1960	London, patients	20	85*	35*
Sautet *et al.*, 1948	Marseilles	200	13·0	0·5
Sigalas *et al.*, 1949	Bordeaux, children	120	9·7†	34·5†
Andrieu *et al.*, 1949	Toulouse	217	8·3	0·5
Bach, 1949	Bonn 1922	435	3·9	2·1
Bach, 1949	Stade 1932	496	1·8	1·2
Bach, 1949	Beuthen 1932–35	433	1·4	0·9
Bach, 1949	Saar 1943–44	115	0·9	2·6
	1947–48	213	6·6	1·4
Bach, 1949	Saar, foreign workers	351	8·3	4·8
Bach, 1949	Stade 1932, mental patients	504	9·5	2·2
Piekarski, 1949	Bonn, soldiers, 1944	?	26·4	30·9
	soldiers, 1947	?	13·5	33·7
Piekarski, 1954	Bonn	?	9·5	12·1
Pöhn, 1951	Frankfurt, patients	165	2·4	0·6
Angelini, 1944	Venice	400	4·8	3·5
Bucco and Chieffi, 1954	Naples, amoebiasis	69	62·3*	40·6*
Mazzitelli, 1959	Naples, cyst-passers	84	58·3*	41·6*
Simic, 1935	Skoplje, children	110	40·0	12·7
Simitch and Petrovitch, 1955	Yugoslavia, children	7 000+	30·6	0·6
Savateen, 1941	Crimea, G.-I. cases	401	6·0	3·9
	children		16·8	12·9

* Presumably percentage of cases with *E. histolytica*.
† 11/120=9·16%; 41/120=34·16%; 42/120=35·0%.

B. THE DISEASE

The extreme variety of opinion as to what constitutes the disease immediately clouds any attempt at establishing a valid geographical distribution. In an attempt to obtain clarity, the writer visited a number of laboratories and hospitals in a number of countries. His experience is best illustrated by an oft-repeated conversation:

"Amoebiasis is a great problem in our country!"
"Do you see much amoebic liver disease?"
"Oh, yes—hundreds of cases every year."
"Do you prefer aspiration to open surgery for abscess?"
"We have not had an amoebic liver abscess in five years."

Another example—where a University Hospital had indicated that they saw very few cases of amoebiasis, a high proportion of the Rotarians in the town had been treated for the disease!

1. *Official Records*

These are no better. Even the exclusion of unlikely manifestations does not improve the picture, as there seems to be a tendency to "dump" all dysenteries not proved otherwise into the amoebic class.

In 1958, the World Health Organization published the returns for Bacillary Dysentery and Amoebiasis for the years 1946–1956. These data have been summarized and analysed in Table III, which gives the mean annual incidence, the mean number of deaths per annum, the mortality rates and the ratio of bacillary dysentery to amoebiasis in each area.

If one is to take these incomplete returns as an index of the medical importance of *Entamoeba histolytica*, the parasite must be a major hazard to man in many parts of the globe. French West Africa and Colombia apparently have about a hundred cases a day. However, the case mortality seems to be low, French West Africa giving 3·6/000 and Colombia making no return. In contrast, the case mortality in the U.S.A. is 33·5/000 and that for Japan 56·5/000.

The common concept that bacillary dysentery is commoner than that due to amoebae is not borne out by the official returns. However, the ratio varies a great deal, even for adjacent territories where the epidemiology should be similar. Contrast Mozambique with Tanganyika and Zanzibar; Laos with Malaya; and the Ryuku Islands before and after the Treaty of San Francisco of 1951.

Perhaps some indication of the true situation is given by Baylet (1959), who quotes the official returns for Senegal as showing 4248 cases of amoebiasis as against 323 of bacillary dysentery for the same period. He examined stools from 1441 patients with "troubles entéritiques" with the results set out in Table IV.

He comments: "Les Shigelles sont rencontrées dans le pathologie dakaroise dix fois plus souvent que l'amibe pathogène." He gives as reasons for the misconception: a "hang-over" of ideas from operations in the Far East;

> l'opinion du malade qui n'a entendu parler que de l'amibiase et tient à son diagnostic personnel, lequel lui semble devoir expliquer les troubles multiples et variés qu'il ressent, tout en espérant sinon pension, tout au moins possibilité de cures thermales; les infirmiers "spécialisés" sont parfois incapable de faire le départ entre une amibe hématophage et ses homologues non pathogènes et parfois même un macrophage; du fait de cette difficulté réelle, le medecin a tendance à se passer d'un examen long et dispendieux ou, s'il le demande, à le discuter surtout s'il est négatif, pour adopter une solution de facilité qui satisfasse en même temps son client; injection d'émétine.

A similar situation almost certainly holds in other territories, and little real information is to be gained from official returns, except some idea of the "popularity" of the diagnosis in some areas.

TABLE III

Reports to the World Health Organization for the years 1946–1956
(*Epidem. vital Statist. Rep.* **11**, 97–134)

	Bacillary dysentery			Amoebiasis			Ratio
	Cases per annum	Deaths per annum	Case Mortality per mille	Cases per annum	Deaths per annum	Case Mortality per mille	B/A
Africa							
Angola	346	3·0	8·9	1 781	23·4	13·2	0·19
Cameroun	71	2·8	39·8	3 772	29·5	7·8	0·18
Congo Belge	3 555	96·8	27·2	19 008	176·3	9·3	0·19
Ethiopia	4 321	6·5	1·5	5 465	12·4	2·3	0·79
French Equatoria	323	7·5	23·6	9 185	65·7	7·2	0·03
French West Africa	452	6·0	13·3	33 940	121·7	3·6	0·01
Ghana	1 138	27·0	23·8	1 205	27·2	22·6	0·94
Kenya	6 074	81·0	13·3	4 138	23·0	4·6	1·48
Madagascar	1 837	11·3	60·6	2 483	26·1	10·5	0·75
Morocco (French)	3 127	—	—	25 373	—	—	0·12
Mozambique	79	1·7	21·5	3 991	29·3	5·6	0·02
Rhodesia & Nyasaland	171	2·5	30·5	360	3·3	10·2	0·47
Ruanda-Urundi	2 667	96·5	36·2	4 647	59·0	12·7	0·57
Sierra Leone (I/P)	43	5·9	10·8	136	11·2	7·5	0·31
Spanish West Africa	26	—	—	65	—	—	0·36
Tanganyika	2 768	27·8	10·1	1 647	19·1	11·6	1·70
Uganda	1 965	25·4	13·6	1 482	9·4	6·7	1·32
Zanzibar	194	2·5	12·7	129	2·5	18·3	1·5

TABLE III—*continued*

	Bacillary dysentery			Amoebiasis			Ratio
	Cases per annum	Deaths per annum	Case Mortality per mille	Cases per annum	Deaths per annum	Case Mortality per mille	B/A
America							
British Guiana	114	4·6	35·9	377	19·3	46·6	0·30
British Honduras	9	0·3	—	28	2·0	81·1	0·34
Canada	527	—	—	32	—	—	16·3
Colombia	5 352	—	—	53 549	—	—	0·10
Costa Rica	40	—	—	2 995	—	—	0·01
Ecuador	22	2·5	17·2	1 682	27·1	17·0	0·01
Guadeloupe	2	—	—	36	—	—	0·41
Mexico	2 031	4·6	206·2	17 221	423·7	25·7	0·12
Netherlands Antilles	215	—	—	52	—	—	9·9
Panama	28	—	—	19	—	—	1·5
U.S.A.	20 686	323·6	14·2	4 135	140·0	33·5	5·0
Venezuela	1 224	49·5	39·8	10 725	100·0	9·3	0·12
Asia							
Bahrein	585	—	—	529	—	—	1·11
Federation of Malaya	744	65·3	42·9	1 812	114·3	57·1	0·41
Hong Kong	380	21·7	64·4	191	9·2	41·4	1·99
Iraq	6 863	0·5	0·7	14 719	9·5	0·6	0·04
Israel	2 715	—	—	1 614	—	—	1·70

Japan	88 957	1 019·2	115·6	410	23·1	56·5	217·1
Laos	157	2·3	14·9	13 384	44·0	3·6	0·01
North Borneo	78	0·6	7·4	339	8·7	25·7	0·23
Philippines	725	178·8	247·0	2 040	375·2	183·9	0·36
Ryukyu Islands *ante* '52	29	4·0	161·0	1 493	16·4	10·1	0·01
post '52	59	13·8	235·3	88	3·3	37·0	0·67
Sarawak	240	—	—	1 175	—	—	0·20
Singapore (I/P)	32	14·0	396·7	158	15·8	96·4	0·20
Turkey	222	8·1	36·5	166	14·8	55·7	1·34
Viet Nam	2 351	18·1	7·7	18 515	53·3	2·9	0·13
West New Guinea	335	6·0	12·2	170	2·3	8·8	2·22
Europe							
Greece	295	2·4	6·8	439	0·6	0·6	0·66
Italy	159	29·2	168·5	300	30·6	97·6	0·53
Netherlands	902	20·1	22·3	76	1·0	15·7	11·82
Portugal	70	12·5	177·2	1	0·7	400·0	49·24
United Kingdom	—	35·3	—	—	10·9	—	—
Oceania							
Australia	317	12·3	35·6	36	4·4	112·1	8·81
Fiji	143	—	—	26	—	—	5·48
Gilbert & Ellice Islands	216	—	—	121	—	—	1·78
Guam	9	—	—	10	—	—	0·91
Hawaii	133	1·9	14·3	15	1·3	85·9	9·01
New Zealand	198	3·3	18·7	35	0·3	9·0	5·63
Papua & New Guinea	489	—	—	7	—	—	70·00

TABLE IV
La dysenterie dakaroise
(*selon Baylet*, 1959)

	Selles à macrophages		
	Glairo sanglantes	Non-glairo sanglantes	Selles pâteuses
Numéro	219	255	967
E. dysenteriae	7·8%	—	0·2%
Shigelles	77·2%	11·3%	5·0%
Formes *minuta* et des kystes		30/1,441 = 2·1%	

2. *Clinical Aspects*

Review of the sources of literature on the clinical aspects should give some concept of the geographical distribution of the disease, but so much has been written (and so little said) that it would be both tedious and unedifying to cite more than the few examples necessary to outline the situation. A high proportion of clinical reports is based on drug trials and Table V is derived from the available articles on this aspect for the year 1960.

It is apparent that, for the most part, the criteria employed for the selection of cases for experimental therapy leave much to be desired. Many authors seem to regard the presence of the parasite as adequate justification for the administration of an untried therapy, and some (Chatterjee, 1960) even treat in its absence. Though some seem to recognize that cyst-passers may be asymptomatic, the variety of symptoms attributed to the parasite makes one marvel that any escaped. It is likely that some authors use haematophagy as a criterion for a vegetative form, but only one (Elsdon-Dew, 1960) specified the phenomenon as a *sine qua non*. Routine sigmoidoscopy is apparently carried out in but few places. It is small wonder that little reliance can be placed on the majority of drug trials, and it is eloquently evident that few writers were prepared to condemn the material under trial. Geographically, the evidence can only be taken to indicate that the physicians in question are "amoeba-conscious", and, though there may be some basis for their suspicions, such impressions may well be questionable.

Hospital records often come under similar criticism. For example Kasliwal and Gambhir (1960), in an article on the incidence, diagnosis and management of amoebiasis, give the figures for the S.M.S. Hospital at Jaipur. Here the label is apparently applied to over 30% of some 70 000 medical outpatients *per annum*, implying about 70 cases a day! If, as they suggest, they examine at least three specimens on consecutive days, the laboratory must indeed be both busy and efficient. They refer to typical symptoms (dysentery or diarrhoea), to uncharacteristic symptoms (asyndromic) and to so-called healthy carriers. Amongst the variety of symptoms they quote are "distressing flatulence", "irritability" and "sexual weakness". This article was not specifically selected, but is unfortunately typical.

TABLE V

Drug trials in amoebiasis reported in 1960

Author	Place	No.	Criteria: Laboratory	Criteria: Clinical, etc.
		Africa		
El Sheikh	Cairo	52	V	Dysenteric
		28	C	Symptomatic
Salem and Morcos	Alexandria	4	V	Dysenteric
		13	?	Chronic symptomatic
		10	C	Asymptomatic
Marsden	Lagos	151	?	Intestinal amoebiasis
		17	V	Acute dysentery
Shaldon	Nigerians in London	4	V	Acute dysentery, two with visible ulcers
Elsdon-Dew	Durban	1 880	H	Dysentery with ulcers
		Asia		
Chatterjee	Calcutta	69	VC	Chronic amoebiasis
		23	N	
Ghosh and Gupta	Calcutta	66	VC	Intestinal amoebiasis
Parekh and Patel	Bombay	118	C	
		7	V	Variety
		10	VC	
Mehta *et al.*	Bombay	24	VC	?
Shah *et al.*	Bombay	87	VC	Clinical amoebiasis
Tripathy and Kar	Cuttack	13	V	Dysenteric
		60	C	Chronic amoebiasis
		25	?	Amoebic hepatitis
		Americas		
Faigenbaum and Monlezun	Santiago	33	VC	Chronic amoebiasis
Niedmann	Santiago	46	VC	Not stated
Donckaster and Donoso	Santiago	55	VC	Not stated
Dooner	Santiago	179	VC	Chronic amoebiasis
Canedo-Acosta	Mexico City	68	VC	Chronic amoebiasis
		13		Acute amoebiasis
		Europe		
Woodruff and Bell	London	18	C	Cyst-passers
		49	C	
		5	V	Intestinal amoebiasis
Bell and Woodruff	London	20	C	Cyst-passers

V = Vegetative; C = Cysts; H = Haematophagous; N = Negative.
All references are 1960.

Obviously, sharper "focus" is required, and preferably on some aspect of amoebic disease which is unequivocal. Dysentery, being caused by such a variety of agents, is no criterion. Liver abscess is probably the only clearly defined condition, though here too, confusion with so-called amoebic hepatitis must be avoided. No doubt many cases of true abscess will have been called amoebic hepatitis, but, if the amoeba is an important pathogen in an area, frank abscesses or their complications should be manifest, however efficient the therapy.

Review of such literature may be biased, in that those areas in which amoebic liver abscess is common tend only to publish either unusual complications or the results of therapeutic trials. On the other hand, where the condition is rare, there is a tendency to report isolated cases.

Table VI is a compilation from the literature available. An attempt has been made to exclude reports where there was no definitive evidence of abscess such as the aspiration of "typical" pus, or rupture of the abscess onto the thorax or peritoneum. The diagnosis "amoebic hepatitis" was not *per se* acceptable. It would seem that, in some areas, physicians are reluctant to attempt aspiration, but what they do with a pointing abscess does not emerge. In our hands, this procedure is not only of diagnostic use, but is a valuable therapeutic measure.

A feature of the Table is the apparently low incidence of abscess in some areas in which, by repute, amoebiasis is a common disease.

Africa

Judging by the well-documented high prevalence of *E. histolytica* in Egypt (Kuntz and Lawless, 1958; Lawless *et al.*, 1956), the occurrence of abscess seems to be small. This may be a false view, as autopsies are not commonly performed in that country. The figures from North Africa are also low, and it would seem that the bad reputation of Tunis, Algeria and Morocco has, in no small part, been based on troops with previous service in Indo-China. Coirault *et al.* (1955) report fifteen verified cases from Oran—but only one of these was indigenous. Corcos *et al.* (1956) comment that they and Cattan had encountered only eight liver abscesses in 15 years. Though Morocco reports (see Table III) some 25 000 cases of amoebiasis per annum, it seems strange that the literature should produce, after 1950, only a single report of a liver complication.

Liver abscess is apparently by no means uncommon in Senegal, and reports certainly do not reflect the official records so condemned by Baylet (1959). With the establishment of medical schools in Nigeria, the picture for that country is becoming clearer. The paper by Olatunbosun (1965) gives an insight into infantile amoebiasis.

The incidence of abscess appears to be very low in the Congo, which may follow a failure to report. The official figures are so high that manifestations sufficiently bizarre to inspire case reports should not be uncommon.

The picture for South Africa is well defined, but this reflects not only the incidence of the disease, but also the enthusiasm of the workers. Amoebiasis now seems to be more common in Mozambique than it was some twenty years ago (Elsdon-Dew, 1950).

TABLE VI

Geographical distribution of amoebic liver abscess

Author	No.	Remarks
		AFRICA
		Egypt
Debbas, 1953	6	Children, *aet.* 6–7
El-Din and Yassin, 1956	1	Pericardial rupture
Magill and Killough, 1958	5	Abscesses in 100 positive stools?
Abd-el-Hakim and Higazi, 1958	8	
Abd-el-Ghaffar, 1960	2	+ 1 in spleen
Ibrahim and Abd-el-Wahab, 1963	9	In 11 cases photoscanned
Razzak, 1965	3	Hepatoscan
		Tunisia
Corcos, 1953	6	In 12 years
Corcos *et al.*, 1956	8	In 15 years
		Algeria
Thiodet *et al.*, 1950	2	
Coirault *et al.*, 1955	1	Only indigenous case in large series
Lebon *et al.*, 1956	1	
		Morocco
Perard and Roux-Berger, 1926	46	Casablanca
Huber, 1950	1	Seen in Germany
Delanoe, 1960	1	Pericarditis
		Senegal
Sohier, *et al.*, 1953	2	
Sohier, 1957, 1958	8	Cases treated with continuous drainage
Senecal *et al.*, 1957	4	Children
Armengaud *et al.*, 1962	16	3 treated surgically
Payet *et al.*, 1963	23	Encountered in 3100 liver biopsies
Payet *et al.*, 1964	37	*Abcès collectés* in 104 hepatic amoebiasis
		Sierra Leone
Rowland, 1963	17	Radiographic study
		Nigeria
Jeliffe, 1951	1	
Falconer, 1958	1	
Downie, 1964	1	Child
Adi, 1965	76	Of 90 aspirates in 120 cases
Olantunbosun, 1965	4	Autopsies on 14 children with amoebiasis
Essien *et al.*, 1965	20	In 921 autopsies
Alele, 1966	3	In 30 cases of hepatic amoebiasis
		Republic of the Congo
Lambillon and Beheyt, 1949	49	*Abcès vrai*, Kivu, 1938–1945
	73	*Abcès vrai*, Leopoldville, 1941–1948
Valcke, 1950	5	Seen in Belgium
Limbos, 1950	1	Left lobe
Limbos *et al.*, 1963	2	In 5 cases of hepatic amoebiasis

TABLE VI—*continued*

		Chad
André, 1961	3	Biliary fistulas
		Ethiopia
Lanzo, 1966	10	Asmara
		Somalia
Solier and Dejou, 1939	1	Seen in Paris
		Kenya
Macdougall, 1960	10	Children
Nevill, 1962	1	Encountered in therapeutic trial
Elsdon-Dew, 1964b	7	450 bed hospital, 1958
Mullan *et al.*, 1967	5	In assessment of serology
		Mozambique
Torres, 1964	10	In cancer biopsy survey
		Rhodesia
Osburn, 1944	1	
Gelfand, 1966	55	Pus found in "most"
		South Africa
Johannesburg		
Chatgidakis, 1953	2	Children under 18 months
Keeley *et al.*, 1962	10	Use of biopsy technique
Durban		
Wilmot *et al.*, 1952	5	Trial of chlortetracycline
Gordon *et al.*, 1955	5	Trial of camoquin
Norris and Beemer, 1956	1	Pericarditis
Wilmot *et al.*, 1958a	35	Trial: Emetine vs Chloroquine
Wilmot *et al.*, 1958b	6	Trial: Erythromycin
Lamont and Pooler, 1958	250	Clinical study
Walt, 1959	16	Children
Wilmot *et al.*, 1959	48	Chloroquine vs Emetine and Chloroquine
Powell, 1959	31	Biochemical findings
Scragg, 1960, 1961	100	Children: *E. histolytica* in liver pus
Powell *et al.*, 1965a	360	Proved abscess } Evaluation of serology
	168	Clinical abscess }
Powell *et al.*, 1965b	60	Emetine vs Chloroquine vs Dehydroemetine
Powell *et al.*, 1966b	50	Trial: Niridazole
Scragg and Powell, 1966	28	Children, trial therapy
Powell *et al.*, 1966c	15	Trial: Niridazole
Kallichurum, 1966	3	Pathological study of venous thrombosis
Powell *et al.*, 1966e	10	Preliminary trial: Metronidazole
		ASIA
		Syria
Huard *et al.*, 1933	1	Tunisian in Marseilles *ex* Syria
Solier and Dejou, 1939	1	Seen in Paris

TABLE VI—*continued*

		Israel
Schorr and Schwartz, 1951	49	Radiological study
Heichman, 1953	4	
De Vries *et al.*, 1956	29	
Efrati, 1957	6	
Ravina *et al.*, 1960	1	In 5 children with fatal amoebiasis
Alkan, 1961	8	Left-sided abscesses
Marberg and Czerniak, 1964	2	In 5 hepatoscans
		Saudi Arabia
Elsdon-Dew, 1964b	1	317-bed hospital in 1958
		Iraq
Fawzi, 1950	1	Bagdad
		West Pakistan
Ahmad, 1960		Comment on "cryptogenic abscess"
		East Pakistan
Islam *et al.*, 1960	17	With pus, in 50 cases of hepatic amoebiasis
		India
Charles, 1908	42	42152 dysenteries in Indian jail
	72	23516 dysenteries in native army
	860	5581 dysenteries in British army
Payne, 1945	8	Of 700 European troops with amoebiasis
	20	Of 300 Indian troops with amoebiasis
Reddy and Thangavelu, 1948	1	Child
Carter and Korones, 1950	1	Pericarditis in Indian seaman in Boston
Sharma *et al.*, 1951	2	Bangalore
Tribedi and Roy, 1951	1	Pericarditis. "Liver abscess commonplace"
Chatterjee and Roy, 1952	1	On chlortetracycline
Patel, 1953	11	Bombay
Purandare and Deoras, 1955	105	162 liver abscesses in 12686 autopsies, 1926–53
Self, 1955	1	Seen in London *ex* Assam
Wagle *et al.*, 1958	1	Child
Bawa and Wahi, 1960	8	In 35 cases of hepatitis
Sarma and Nambiar, 1960	1	Rupture into peritoneum. Madras
Kasliwal and Sethi, 1960	43	In 1934 admissions in 10 years
Kasliwal and Gupta, 1961	26	In 50 clinical abscess + 10 pyogenic
Raghavan *et al.*, 1961	42	In 194 hepatitis in 10 years
	25	Autopsy cases
Kapoor *et al.*, 1961	10	Bombay. Criteria not clear
Chhuttani *et al.*, 1963	24	In 135 cases of "abscess"
Konar and Mandal, 1963	3	Trial of dehydroemetine
Merchant and Shikaripurkar, 1964	8	In 23 cases. Trial of dehydroemetine
Kapur, 1965	1	Pericarditis in child
Kasliwal *et al.*, 1966	5	Assessment of serology

TABLE VI—*continued*

		Ceylon
Wijerama, 1939	48	In 4070 admissions in 21 months
Singha, 1959	5	Cases with peritoneal rupture
Jayaratne and De Silva, 1960	1	Child
Paul, 1960	203	Surgical cases, 1936–1959
		Thailand
Harinasuta, 1950	9	
Buri *et al.*, 1955	3	Pericarditis
Viranuvatti and Biseshurarit, 1955	2	Erythromycin
Sadun *et al.*, 1956	17	Chlortetracycline and oxytetracycline
Elsdon-Dew, 1964b	47	14899 admissions in 1958
Jarumilinta, 1966	15	Trial of Niridazole
		Malay States
Shaumugaratnam, 1941	1	Chinese child
Da Silva, 1950	13	57 hepatic abscesses in 5000 autopsies
		Indo-China
Huard, 1950		Morbidity (*per mille*):
		South Vietnam 1863–72 17·0
		1914–44 0·7
		North Vietnam 1918–30 1·49
		1938–44 0·51
Ravina *et al.*, 1950	1	Seen in Paris
Crosnier and Darbon, 1952	1	Seen in Val-de-Grace. Pulmonary
Grizaud, 1952	10	Surgical *ex* Indo-China
Swynghedauw and Salembier, 1953	1	
Ouary *et al.*, 1953	1	Seen in Marseilles
Berty *et al.*, 1954	1	Seen in Bordeaux
André and Cornet, 1955	12	Seen in Marseilles
Coirault *et al.*, 1955	14	Evacuated to Oran cf.
André, 1956	409	1949–55
Besseige and Carloz, 1956	2	Autopsy. Saigon
Lestrade and Guérineau, 1956	3	Children
Hollender and Grénier, 1957	1	Pericarditis
Roumagnac *et al.*, 1958	4	In 109 cases. Opposed to aspiration
Cornet, 1958	24	Surgical
André, 1961	9	Hepato-colic rupture
Elsdon-Dew, 1964b	9	416-bed hospital, 1958
		Indonesia
Smitskamp, 1952	18	Referred cases. Djakarta
Roovers and Van Steenis, 1951	4	Seen in Amsterdam
Schaible, 1955	34	Treated with Resochin
Schaible, 1956a	11	Treated with Resotren
	5	Treated with Resotren comp.
Schaible, 1956b	7	Treated with Atebrin
Elsdon-Dew, 1964b	0	200 hepatitis in 4500 out-patients, 1958

TABLE VI—*continued*

		Philippines
Stransky and Dauis-Lawas, 1948	1	Child
Freedman and Cleve, 1950	5	
Tupas and Dauis-Lawas, 1951	2	Children
Sison *et al.*, 1951	15	2647 autopsies
Braun and Stonehill, 1953	1	Rupture into peritoneum
Samson, 1958	4	
Villano *et al.*, 1963	73	+46 hepatitis. In 8 years
		Korea
Ludlow, 1917	30	
Ludlow, 1926	3	Children
Pelikán and Trapl, 1958	11	+7 hepatitis, +4 pleural syndrome
Cook-Sup, So, 1959	11	
Lieske and Kim, 1960	18	+7 pyogenic
Lieske *et al.*, 1960	14	Use of contrast medium
Elsdon-Dew, 1964b	1	3824 patients in 1958
Dorrough, 1967	22	In 5619 admissions
		China
Sweet, 1934	1	Child
Tan and Liu, 1944	6	In 20 autopsies on amoebiasis
Yang *et al.*, 1963	13	+13 pyogenic. Evaluation of ultrasonics
Wang, 1964	95	+39 pyogenic. Evaluation of ultrasonics
Meng *et al.*, 1965	239	1955–65
		NORTH AMERICA
		United States
Koszalaika *et al.*, 1949	7	Overseas
	3	Home
Spellberg and Zivin, 1948	3	In 50 cases of amoebiasis in veterans
Hays *et al.*, 1948	1	Home
	6	Overseas
	2	Doubtful
Walters *et al.*, 1944	2	Overseas
Harris and Wise, 1952	2	Overseas
	1	Home
Radke, 1952a	42	AFIP autopsies (reports cysts in tissues)
Smith *et al.*, 1955	1	Child
Kean *et al.*, 1956	56	AFIP material
Burnside and Cummins, 1959	1	Child
New York State		
Emmett, 1949	1	Origin uncertain
Conan, 1948	4	Overseas
	2	Home
Conan *et al.*, 1950	2	Home (? above)
Berberian *et al.*, 1951	1	Pericarditis. Never left Albany
Bargen, 1951	3	Thoracic complications

TABLE VI—*continued*

Pennsylvania		
Diamond and Scribner, 1956	1	Post-traumatic scapular abscess
Missouri		
J. Mo. Med. Ass. (1950) **47**, 228–230	1	Overseas
Hoadley, 1959	1	Liver to lung to brain. Autopsy
Indiana		
Lemaistre *et al.*, 1956	3	South Bend outbreak
California		
Sloan and Freedman, 1953	1	Overseas
Jordan, 1955	5	Overseas
Delaware		
Mick, 1955	1	?
Virginia		
Slaughter, 1895	1	Child
Johnston, 1897	17	Surgical
Tennessee		
Webster, 1960		10 pulmonary cases. "Cysts in sputum"
Kentucky		
Radke, 1951	1	Trophozoites in sputum
Radke, 1952c	1	*Ex* South Pacific
North Carolina		
Smith and Ruffin, 1946	13	+12 hepatitis. 134 amoebiasis in 16 yrs
Verner, 1948	1	Lung lesions
Alabama		
Hogan, 1958	16	1897–1947
Georgia		
Tillman and Galambos, 1960	1	Communicating with bile duct
Dorrough, 1967	23	In 582545 admissions. 1950–63
Louisiana		
Ochsner and DeBakey, 1943	263	In 20 years
	1 920	In 5250 autopsies on amoebiasis
Texas		
Patterson and Lawlis, 1956	20	All from rural areas
May *et al.*, 1967	15	1943–66
		Mexico
Haro y Paz, 1952	120	3135 admissions
Salas-Martinez *et al.*, 1953	3	Children
Pena y de la Pena *et al.*, 1953	23	
Staines, 1952	2	
Torroella *et al.*, 1956	14	Children
Ramos, 1956	159	1939–51
	38	1952–55
Biagi, 1958	1	
Biagi and Navarrete, 1958	79	

TABLE VI—*continued*

Salas *et al.*, 1958	17	Children. Autopsy
Sepulvida *et al.*, 1959	28	Acute absçess } 13 deaths
	46	Chronic abscess } 13 deaths
Flores-Barroeta *et al.*, 1959	98	2202 autopsies
Pasquel *et al.*, 1961	14	*Puncion* } In 47 cases
	4	Bronchial emptying } In 47 cases
Toussaint *et al.*, 1962	1	2 cases hepatopneumography
Villegas *et al.*, 1962	1	Of 2 cases with cerebral abscess
Gonzalez-Mendoza *et al.*, 1962	6	Autopsies
Elsdon-Dew, 1964b	28	3354 admissions, 1958
		Guatemala
Herrera-Llerandi, 1966	8	In 67 cases of thoracic amoebiasis?
		Honduras
D'Antoni, 1948	1	Child
Schapiro, 1956	6	Comments on rarity in zone of high infection
		Costa Rica
Céspedes, 1958	13	In 3220 autopsies
		Panama
Struve, 1950	50	Gorgas Hospital 1920–45
		Jamaica
Hayes and Ragbeer, 1966	4	8 autopsies with amoebiasis
		Puerto Rico
Santiago-Stevenson *et al.*, 1947	2	14497 admissions 1942–45
		Martinique
Fournier, 1953	1	Seen in Marseilles
		Cuba
De Velasco-Guzman *et al.*, 1952	1	
Martin-Jiminez *et al.*, 1956	1	
Moreno *et al.*, 1956	1	Child
Fernandez de la Arena y Sante, 1956	5	Children under 2 months old
Martinez, 1960	19	Cases from local literature
		Curacao
Van Raalte, 1949	2	
Hartz, 1950	9	In 2283 autopsies
		SOUTH AMERICA
		Cayenne
Castile, 1951	6	1945–50
		British Guiana
Neal, 1892	1	Boy *aet.* 5. Left lobe

TABLE VI—*continued*

		Venezuela
Razetti, 1913	2	Children
Vizcarrondo *et al.*, 1959	2	Children
		Colombia
Cortes-Mendoza, 1956	47	Of 728 cases of amoebiasis
		Peru
Carvallo, 1920	1	Child
		Chile
Pizzi and Céspedes, 1949	20	5000 autopsies. Santiago
Jarpa, 1952	2	
De la Maza and Guzman, 1953	6	
Armas-Cruz *et al.*, 1955	22	1947–54
Boero and Shurmann, 1964	39	7110 autopsies in 11 years. Concepcion
		Brazil
De Oliviera *et al.*, 1950	3	Pleuro-pulmonary. Sao Paulo
Zamot and De Brito, 1952	1	Brain metastasis in an Esthonian
Pontes and Da Costa, 1953	1	Rio de Janeiro. *Ex* Bahia
Da Silva and Torres, 1957a	1	725 autopsies and 145000 admissions. Rio
Da Silva and Torres, 1957b		Comment on rarity in Brazil
		Argentina
Ayas and Carlos-Arauz, 1950	1	Into right lung. Buenos Aires
Pastorino *et al.*, 1953	1	Pulmonary
Carpanelli *et al.*, 1957	1	Rupture into peritoneum
		Uruguay
Cardeza, 1948	36	(Unconfirmed)
	EUROPE	
		Britain
Critchley, 1948	1	Home. Possibly pyogenic
Lowe, 1949	1	Home. Subphrenic
Johnstone, 1949	1	Home. Empyema
Wright, 1958	2	Overseas
Paulley, 1961	1	Home. Doubtful!
	1	Polish. Anchovy sauce
Mullan and Williams, 1965	1	Episodes over 48 years
Wright, 1966	6	2 pleuro-pulmonary
McTaggart and Seaton, 1967	1	Home
		France
Bertrand-Fontaine *et al.*, 1948	1	Surgical. Never out of Paris
Siguier *et al.*, 1951	7	Overseas
Moulin, 1953	1	Overseas. Rupture into I.V.C.

TABLE VI—*continued*

		Germany
Huber, 1950	1	Home
	1	Russia
	1	Rotterdam
Möhr and Schwarting, 1953	1	China and North Africa
		Italy
Buccelato, 1947	1	Child
Musitelli, 1949	8	Bergamo
Soragni, 1949	1	Child. Modena
Scotti and Tripodi, 1950	1	*Ex* Africa
Gambardella and De Michele, 1951	1	Naples
Ruggieri and Cali, 1951	3	Catania
Scaffidi and Sangiorgi, 1952	10	Catania
Scaffidi and Li Volsi, 1954	7	Catania. Chloroquine trial
Mene and De Gaetano, 1955	1	Never out of Italy
	OCEANIA	
		Hawaii
Williams *et al.*, 1961	3	

Asia

Perhaps medical enthusiasm is also responsible for the number of reports from Israel. Ahmad's (1960) report from West Pakistan is quoted, as his remarks on cryptogenic liver abscess imply that amoebic liver abscess is not uncommon in that area. The mass of reports from India (culled from an enormous literature on amoebic hepatitis) indicates that invasive amoebiasis is a problem. If one is to contrast later reports with those of Charles (1908) there seems to have been a considerable reduction in the incidence of liver abscess. Though this may, in part, have been due to the wholesale use of emetine, there may have been other factors.

Amoebic liver abscess is common in Thailand—the Chulalongkorn hospital returning 47 cases in one year (Elsdon-Dew, 1964b). The report from Singapore by Da Silva (1950) is interesting by virtue of the high proportion (43/52) of non-amoebic abscesses—twelve were due to Ascariasis. In reply to a questionnaire (Elsdon-Dew, 1964b), the General Hospital at Singapore reported 25 proved amoebic abscesses for the year 1958.

Indochina has always been considered as a major focus of amoebic liver abscess, so much so that French authors refer to the hepato-trophic amoeba of the Extreme-Orient. The figures given by Huard (1950) not only indicate the high incidence, but also reflect the impact of emetine. He also comments on a rise in incidence during the Franco-Vietnam war. The Hôpital Grâll reported only 9 cases for the year 1958 (Elsdon-Dew, 1964b).

The picture from Indonesia is confusing. Whereas of 136 cases of hepatic amoebiasis at Balige in Sumatra, Schaible (1955, 1956a, b) classed 42% as

abscess, some of which required surgery, Schmidt (Elsdon-Dew, 1964b) from Bangkinang some 200 miles away, returned 200 cases of amoebic hepatitis in one year, but no abscesses in three years. Smitskamp (1952) from Djakarta, reported on 18 proved cases, referred to him because of obscurity in diagnosis. Some of these seem to have been remarkably chronic.

The Philippines are, of course, a classical area for amoebiasis. There are no reports available for Japan, but Korea seems to have a number of cases. The literature from China is scanty, but what there is suggests that invasive amoebiasis is prevalent in some areas.

North America

There are no reports of amoebic liver abscess from Canada or Alaska. The picture for the United States is confused by the high proportion of cases drawn from overseas, and it is not always possible to glean the origin of cases from the published reports.

From the North-Eastern States, autochthonous cases are reported by Conan (1948), Berberian *et al.* (1951) and by Bargen (1943). Of course, the classical description by Councilman and Lafleur (1891) came from this area. From Mid-Western states, the lack of recent reports is a contrast to the plethora which arose after the 1933 "epidemic" in Chicago, the aetiology of which is critically analysed by Boyd (1961). The South Bend outbreak (Lemaistre *et al.*, 1956) produced three abscesses, one of which was unrelated to the contamination. The Western states produce no autochthonous cases of amoebic liver abscess, despite some authors' predilection for the diagnosis of "amebiasis".

Study of the figures for the South-Eastern states is instructive. For Virginia, it was necessary to draw on nineteenth-century records. For Tennessee, very much in the amoebiasis literature, the only report is that of Webster (1960), who found cysts in sputum! The origin of the cases reported by Smith and Ruffin (1946) from North Carolina is not clear. Alabama only had 16 cases worth reporting in 50 years. Dorrough (1967), whose experience in Korea obviously made him critical, reviewed the findings on 23 cases in 13 years at Atlanta, Ga.

No one has written on amoebic liver abscess in Louisiana since the monumental work by Ochsner and DeBakey (1943). The cases from Texas are well authenticated, a proportion being in Mexicans.

Mexico shows a high incidence of amoebic liver abscess, and the common occurrence of other complications. Most reports, naturally, come from Mexico City, the altitude of which (7000 ft) differs from other areas of high endemicity. The comment by Schapiro (1956) on the rarity of abscess in the allegedly highly endemic Honduras raises doubt on the reliability of the faecal examinations on which the allegation was based. Panama is frequently cited as the origin of cases seen in the continental United States, yet the Gorgas Hospital reports only fifty cases in 25 years.

In the Caribbean, Cuba has always featured in the amoebiasis literature, yet the apparent incidence of abscess in adults is small, reports being confined to its occurrence in children.

South America

Reports from the Caribbean coast seem to be sparse. There is but a single report available on the incidence of amoebic liver abscess in Colombia, but infection with *E. histolytica* is common in Cali (Faust, 1958). Yet both amoebic dysentery and amoebic liver abscess do occur in this area (Elsdon-Dew, personal observation). In Chile, the large number of reports reflects the activity of the Medical School, which is also revealed by the number of drug trials carried out (see Table V).

Brazil seems to be a large country to put under one heading, yet, despite the parasitological orientation of the medical schools, there is a paucity of reports on amoebic liver abscess. The comments of Da Silva and Torres (1957b) from Rio de Janeiro are all the more surprising in view of the high incidence of typhoid fever, and the notorious "favelas" of that city. The Argentinians attribute their apparent immunity to their high-protein diet—certainly they do not see much invasive amoebiasis.

Europe

Here too, most of the observations are on returned travellers. The only area with a high reported incidence is Sicily, which is the home of a great deal of migrant labour.

Oceania

Despite the numerous cases classified as *ex* Pacific in American publications, there is only one report available from Hawaii.

In an evaluation such as this the bias must not be forgotten. Reports are most likely to come from areas with good hospitalization, and particularly from those with medical schools.

It would appear that invasive amoebiasis is much less widespread than is generally believed, for many areas, notorious by repute, do not produce enough cases of amoebic liver abscess to stimulate publication. Some areas warrant attention. Mexico is the main focus in the Americas, the West Coast and Natal the known points in Africa, and the whole of South-East Asia is affected. It would seem worth while to collect more definitive data, possibly by direct returns from pathologists carrying out autopsy examinations.

VII. Present Beliefs and Semantic Definitions

The preceding sections have indicated the necessity for strict definitions of the terms used in referring on the one hand to the parasite, and on the other to its relationship to man. At present, parasitologists do not agree as to what will be called *Entamoeba histolytica*, and the medical concept of the disease is confusing in the extreme.

Dobell was, rightly, scathing in his condemnation of Schaudinn, whose pontifical pedantry misled so many, but subsequent events have shown that Dobell's championship of the Promethean concept has had even wider

repercussions. Had his subsequent rejection of this theory received the same publicity as did his exposition of it, much subsequent confusion and no little harm would have been averted. Similarly, his contemptuous comment on the observations of entamoebae with small tetranucleate cysts undoubtedly frightened those who might have dared disagree.

The Promethean concept was uncritically promulgated by prolific American writers, with the result that infection with *E. histolytica* was given a significance out of all proportion to its real meaning. Those who teach should avoid dogma lest it ultimately be interpreted as sophism.

It is perhaps Hoare who has done most to put the *histolytica* complex back into proper perspective. As a result of his teachings, informed opinion has swung back to the views of Kuenen and Swellengrebel, who, for over 50 years, remained, as far as the English-speaking world was concerned, voices crying in the wilderness. Some may recall the famous occasion when the commensal phase of *E. histolytica* was being discussed at an international Congress in Lisbon, and Swellengrebel startled the audience by asserting: "*Entamoeba histolytica* has not got a commensal phase!" In the astonished silence which followed, he concluded: "It *is* a commensal!"

Unfortunately, even in these days of the rapid dissemination of information, the teachings of Hoare have still not reached the majority of clinicians who have to deal with the parasite, or, for that matter, the laboratories who have to make the diagnosis. Good news, obviously, travels slowly.

To outline present belief, the terms used must first be defined.

A. THE PARASITE

1. *Entamoeba histolytica*

The *minuta* trophozoites (10–20 μ in diameter when rounded) are characterized by purposeful movement, showing clear, hyaline pseudopodia when they become sluggish. The granular endoplasm may have food vacuoles containing bacteria or debris. The single nucleus (3–5 μ) has a slightly eccentric karysome, from which an achromatic network extends to the nuclear membrane, which has a chain of closely packed, but relatively even chromatin granules. These are the forms found in cyst-passers, and are presumably their progenitors. From their diet (there is barely room for a red blood cell) and their association (they are seldom found in active dysentery) they are probably commensal. The almost spherical cysts (10–20 μ) have a greenish tint in the unstained condition, and, by appropriate "optical section", some details of internal structure may be discerned. The uninucleate cyst usually has an ill-defined glycogen mass (best seen with iodine staining) in contrast to the deeply-staining sharply-demarcated mass of *Iodamoeba bütschlii*. Refractility may reveal the chromatoidal bars, which are better seen in haematoxylin-stained preparations. These rod-like structures have rounded ends, in contrast to the acicular chromatoids of *E. coli*. The quadrinucleate cyst must be distinguished from that of *Endolimax nana* which is smaller and oval (9–12 μ × 5–8 μ) and has condensed nuclear chromatin. Like the *minuta* trophozoites, the cysts, which are essential for transmission to a new host, are seldom seen in acute dysentery.

In dysentery, the trophozoites are larger (up to 50 μ when rounded) and many are *haematophagous*, sometimes having enormous numbers of condensed, contracted erythrocytes in the endoplasm. In fresh specimens, they are even more motile than are the *minuta* form, and a preparation of mucus from the edge of an ulcer may be literally seething. The purposeful, directional movement is striking, and the amoebae may drag a tail of detritus behind them, suggesting a cytopyge. If visible at all the clear ectoplasm of the finger-like pseudopodia is rapidly filled by the endoplasm. In stained preparations the nuclei are larger, and the chromatin more delicate than in the *minuta* form. The minute karyosome is usually central. In forms from the liver, haematophagy is not as common and motility not nearly as marked. As a name distinctive from *minuta*, and descriptive of their activity, these amoebae might be termed *invasive*. Whether such forms can, by reverting to *minuta* forms, give rise to cysts, is not known.

2. *Entamoeba hartmanni*

Whether or not the finer morphology of these amoebae differs sufficiently from that of *E. histolytica* to warrant species rank, they apparently never invade the tissues and are not histolytic—a distinctive name is thus preferable to one which implies this potential. The trophozoite is small (5–14 μ), active with clear pseudopodia, and has a granular endoplasm sometimes containing bacteria. In the unstained condition it cannot be distinguished from the trophozoites of *Endolimax nana* (though the pseudopodia differ) or from *minuta*. The nucleus is small in proportion (2–5 μ), and the tiny karyosome appears eccentric. While the majority have finely distributed chromatin like that of *minuta*, many will have clumps, described as crescents or otherwise, depending on the angle of view. The cysts range from 4–10 μ in diameter, the majority being well below 10 μ, which forms a practicable dividing line for distinguishing populations from those of *E. histolytica*. The glycogen, when present, is dispersed, presumably in a number of vacuoles, and the deeply-staining chromatoids are, as might be expected in so small a cyst, almost coccal in appearance. According to Burrows (1965), the nuclei are disproportionately small.

Thus, the distinction between the cysts and *minuta* forms of *E. histolytica* on the one hand, and the corresponding stages of *E. hartmanni* on the other, is, apart from size, not based on easily definable morphological characteristics, and is certainly beyond the competence of the ordinary laboratory technician. The common presentation—cysts—does not present the same difficulty, as size is easily determined provided an adequate number of cysts is encountered. A mixed population may necessitate the construction of a size-distribution curve, which, on probability paper, will usually settle the question. However, the presence of either of these forms does not imply that the patient is diseased. Microscopically, this can only be established by the finding of the *invasive* haematophagous trophozoites.

B. THE HOST–PARASITE RELATIONSHIP

1. *Amoebiasis*

Concession will have to be made to popular usage, and this term confined to the harbouring of *Entamoeba histolytica*. Just what term should be applied to hosts harbouring other amoebae is doubtful—Rhizopodiasis seems clumsy. Distinction must be made between hosts harbouring *E. histolytica* as a commensal, and those in whom the amoeba has invaded the tissues, though both states may be co-existent.

2. *Lumenal Amoebiasis*

This implies that the amoeba is living in the gut as a commensal. As these *minuta* forms are the progenitors of the cysts, the term is possibly synonymous with *cyst-passer*. If this is the only form present, the host will, as far as the amoeba is concerned, be healthy.

3. *Invasive Amoebiasis*

This term should be used to indicate that the amoeba is invading the host tissues. This is the disease state, and *may* be manifest by the passage of *haematophagous* trophozoites. Such a host may or may not be a cyst-passer, but only if he harbours the *minuta* form in addition.

VIII. RECENT ADVANCES

As some recent advances may have an impact on our appreciation of the host–parasite relationship, they are here included for the information of non-medical parasitologists.

A. THERAPY

There have been radical changes in therapy over the last 20 years. Though emetine held pride of place since its value was established by Rogers in 1912, it was by no means the complete answer, for, though symptomatic cure was often attained, it often failed to relieve ulceration and the parasites persisted in a high proportion of cases. Relapse was common, and the development of "chronic amoebic dysentery" was a regular sequel. The extremely rigorous treatment, including retention enemata and the like, given to patients in the early forties is evidence of the contumacy of the parasite.

The evaluation of various forms of therapy has always been clouded by the differing criteria used by various workers in the selection of trial subjects, and in the assessment of results. However, the Amoebiasis Research Unit, in Durban, where frank amoebic dysentery and amoebic liver abscess are common in the African (Elsdon-Dew, 1946, 1949), was able to lay down strict criteria for diagnosis and assessment, and to select uniform material for comparative trials.

1. *Dysentery*

After disappointing results with emetine and di-iodohydroxyquinoline used singly and in combination, and with such other drugs as carbasone and E.B.I.,

Armstrong *et al.* (1949) followed the suggestion of Hargreaves (1945) and used a combined therapy of penicillin, sulphasuxidine, emetine and di-iodo-hydroxyquinoline, but gave the antibacterial drugs for a week before instituting anti-amoebic therapy. On sigmoidoscopy at 7 days they found to their surprise that not only did all the ulcers show evidence of healing, but amoebae could not be found in 49 of the 59 cases. After the amoebicides, 5% still showed ulcers, but in no case could amoebae be found at 27 days. They then tried the antibacterial drugs individually and in tandem, and found considerable amoebicidal action, which they attributed to action on symbiotic bacteria (Armstrong *et al.*, 1950). Thereafter they tested a series of antibiotics (Elsdon-Dew *et al.*, 1952), concluding that the wider the antibacterial spectrum the greater the likelihood of successful removal of the amoebae. Though the tetracyclines effected a very high immediate cure rate, there was a significant proportion of relapses, and they felt that amoebicides should be used in addition. This view was strengthened by the finding (Wilmot *et al.*, 1952) that chlortetracycline was ineffective in the treatment of amoebic liver abscess. They came to the conclusion (Powell *et al.*, 1960; Wilmot, 1962) that the approach should be three-fold, using tetracycline for the intramural forms, a quinoline aimed at intralumenal forms, and emetine or chloroquine to preclude hepatic invasion.

A single drug active in both intestinal and hepatic amoebiasis is desirable. Powell *et al.* (1966b, c) found that niridazole was active in both bowel and liver, but gave rise to electrocardiographic changes, and, in the prolonged dosage required, some patients developed temporary confusional states. A preliminary trial of metronidazole (Powell *et al.*, 1966e) suggests that this drug, when given in adequate dosage, is an effective direct-acting amoebicide in both bowel and liver, and it has little significant toxicity.

2. *Hepatic Amoebiasis*

It was in amoebic liver abscess that emetine completely changed the prognosis. Prior to the work of Rogers, the mortality was indeed high, but he showed that emetine and aspiration gave far better results than had the heroic surgical and other procedures previously used.

Emetine held undisputed sway till Conan (1948) tried chloroquine—a 4-aminoquinoline developed for the treatment of malaria—on 6 cases of "hepatic amoebiasis" and obtained "clearcut disappearance of symptoms and signs, *viz.* fever, anorexia, enlargement and tenderness of the liver". Murgatroyd and Kent (1948) had a dramatic success in a case with liver abscess which had proved completely refractory to intensive emetine therapy. Emmett (1949) also had success with the treatment of a liver abscess complicated by a sinus and skin involvement. Harinasuta (1950, 1951) conducted a comparative trial, with 45 cases each on chloroquine and on emetine. There were six deaths and ten relapses on chloroquine, as against four deaths and two relapses on emetine. He concluded that the low toxicity and ease of administration of chloroquine conveyed advantages, and that it might be used where emetine is contra-indicated.

The A.R.U. in Durban have conducted a series of trials, using as a diagnostic

criterion the aspiration of bacteriologically sterile characteristic pus. Having previously (Wilmot, 1949) established that a single 10-day course of emetine was inadequate, Wilmot *et al.* (1958a) compared the standard emetine treatment (1 grain × 10 days, 14 days rest, 1 grain × 6 days) with chloroquine treatment over 29 days. Two cases on chloroquine had to be given emetine therapy, and another four relapsed within 6 weeks, thereafter being cured by emetine; there were no relapses on emetine. Feeling that the two drugs might be combined, Wilmot *et al.* (1959) contrasted chloroquine in higher dosage with a combined course (emetine × 10 days, chloroquine × 29 days). There were no relapses on the combined course, and 5 out of 24 on the chloroquine course.

The advent of the less toxic, synthetic dehydroemetine led to a preliminary trial (Powell *et al.*, 1962). This showed that 80 mg of the synthetic had a parallel activity to 1 grain (=65 mg) of emetine, in both dysentery and in liver abscess. After further comparative trials, Powell *et al.* (1965b) conclude: "Dehydroemetine in a dosage of 80 mg daily for ten days in combination with chloroquine is the [to date] preferred routine treatment of amoebic liver abscess."

There are indications that Metronidazole may prove to be the drug of choice for both dysentery (see p. 45) and abscess.

B. SEROLOGY

In recent years there has been a re-awakening of interest in serological diagnosis of amoebiasis. Much of the confusion in the past arose from the use, in the evaluation of techniques, of sera from patients whose clinical and parasitological status was inadequately established, and from a lack of appreciation of the commensal state and of the persistence of antibodies.

A variety of techniques is currently under trial in a number of laboratories. An International Evaluation Programme was initiated at the International Congresses of Tropical Medicine and Malaria at Rio de Janeiro in 1963, the collection and distribution centre being the Communicable Diseases Centre at Atlanta, Ga.

The biggest series of observations has come from Durban (Maddison, 1965; Maddison *et al.*, 1965), where, because of its sensitivity and simplicity, the gel-diffusion precipitin test is preferred. This test has the advantage of revealing the non-specific systems which may appear when antigens are as heterogeneous as are those prepared from parasitic animals (Maddison and Elsdon-Dew, 1961). Different antibodies are involved in haemagglutination and precipitation (Maddison *et al.*, 1965). The precipitin test was positive in 92% of 400 cases of acute amoebic dysentery, as against 16% in miscellaneous non-dysenteric diseases (Powell *et al.*, 1966d). Positive results were obtained in 96% of 360 patients with amoebic liver abscess proved by the aspiration of characteristic pus, and in 90% of 168 patients in whom a clinical diagnosis of the condition could be made although aspiration of the liver was either not indicated or did not yield pus. In contrast, of 373 patients with other conditions, but in whom the possibility of amoebic liver abscess arose in differential diagnosis, only 12% were positive (Powell *et al.*, 1965a).

Applying the technique to a series of 16 different population groups, Maddison *et al.* (1965) found that there was correlation between the prevalence of antibodies and the incidence of invasive amoebiasis. Of African blood-donors in Durban, where invasive amoebiasis is common, 9% showed antibodies, whereas their Johannesburg counterparts showed none. Only 40% of asymptomatic cyst-passers gave positive results.

The general interpretation is that antibodies are produced to amoebae in parenteral contact with the tissues. These antibodies persist after the parasites have disappeared—for how long has not been established. A cyst-passer who has not had parenteral contact with his amoebae may have no antibodies, whereas a patient with no amoebae may have antibodies reflecting previous invasion by amoebae.

The clinical value of the test lies in the exclusion of invasive amoebiasis in the aetiology of some of the obscurer manifestations, being particularly useful in left-sided liver abscess, pericarditis (Macleod *et al.*, 1966) and in infections at the base of the right lung. The test must be interpreted in the light of the background (noise) of antibodies in the general population; for example, a positive result has much less significance in the Durban African, where the background is of the order of 15%, than it would have in his Johannesburg counterpart.

As the test is an index of invasion, its use as an epidemiological tool is obvious. Not only does it reveal the pathogenicity of the parasites in an area, but a serological test is free of the inevitable subjectivity of stool survey.

A major application should be to exonerate *Entamoeba histolytica* in many of the bizarre conditions for which it has been blamed. It is to be hoped that such tests will become more readily available, as their use will help to put amoebiasis into proper perspective.

IX. Gaps in our Knowledge

Figure 4 illustrates our present knowledge, with the gaps queried. The normal or *minuta* cycle is commensal, and is responsible for the production of the cysts necessary for transmission to new hosts. Under some circumstances there may be an aberration, and the amoebae invade the bowel mucosa and beyond, causing the diseases known as amoebic dysentery and amoebic liver abscess.

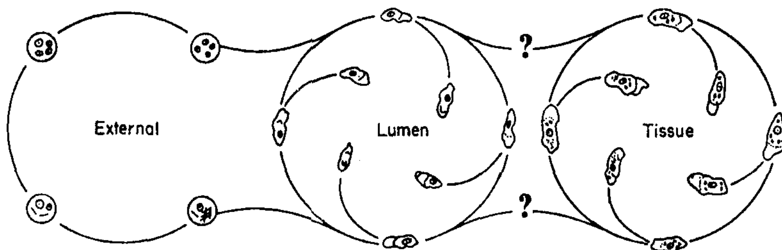

Fig. 4. The cycles of *Entamoeba histolytica*.

Whilst *Entamoeba histolytica* has been reported from most parts of the world, it is mainly in the warmer areas that tissue invasion occurs. But, even in these areas, there are many apparently healthy carriers of the protozoan. Obviously, other factors are concerned. These may be inherent in the "strain" of amoebae concerned; some other organisms may play a part; the status of the host, nutritional or otherwise, may be important; finally, such mechanisms may act in concert.

According to the observations of Neal (1957), there is considerable strain variation in virulence for the intracaecally inoculated rat, even when the amoebae have been recultivated after encystment. Virulence lost on prolonged culture can be restored, but only to some strains, by passage through the hamster liver (De Carneri, 1958). By the intrarectal inoculation of kittens, and sealing of the anus, Brumpt (1926) was able to distinguish between what he called *E. dysenteriae* and *E. dispar*, and he considered the latter completely non-pathogenic to man. Such experiments smack of artificiality, for not only are abnormal hosts being used, but also abnormal methods of infection. The only animals harbouring *E. histolytica* naturally are primates, and in African monkeys (*Cercopithecus*) (Elsdon-Dew and Maddison, 1965) and in baboons (*Papio*) (Powell and Elsdon-Dew, 1961), the amoeba remains a commensal, despite considerable provocation.

Enhancement of virulence by rapid passage from host to host is a common phenomenon, and it is certain that in most areas where invasive amoebiasis is prevalent, hygiene is such that amoebae must pass from host to host, not only frequently, but rapidly. As a measure of the rapidity of faecal transfer, Brooke *et al.* (1955) suggested the use of a "combined amoebic prevalence rate". Applying this to population groups in Durban, Powell *et al.* (1966a) showed that this index was much higher in the African than in the Indian. Though the evidence is inferential, it is suggestive. However, such rapid transmission of contamination might equally well be favourable to some other living agent.

If such tendency to invade is inherent in the strain of amoebae, and is enhanced by passage, this implies "experience" by the parasite, an experience which can only be gained by previous sojourn in the tissues. But can we be sure that invasive amoebae can return to the *minuta* cycle, and thereafter produce cysts with the appropriate genetic constitution? With a few questionable exceptions, there is general agreement that amoebae do not encyst in the tissues, and certainly the amoebae in a liver abscess have no chance of posterity. Dobell (1952) was able to induce encystment of amoebae *in vitro* by the addition of certain specific bacteria. As the amoebae studied had all originated in the faeces, there is a possibility that some *minuta* forms were available. Other authors, too, have devised techniques to stimulate encystment, but thus far we have had no success with amoebae derived from the liver. Have such amoebae possibly forgotten how to encyst?

The success of anti-bacterial therapy in the treatment of acute amoebic dysentery (Armstrong *et al.*, 1950) suggests that the flora of the bowel may play some part. Even antibiotics used in the cultivation of amoebae will eliminate these parasites from some cases of dysentery. The wider the spectrum, the more efficient is the therapy, but such agents are ineffective in amoebic liver

abscess (Wilmot *et al.*, 1952), the contents of which are, primarily at least, bacteriologically sterile (Maddison *et al.*, 1959). Histology of the amoebic ulcers suggests that the bacterial component is small, the main feature being a necrosis (Roach, 1959). It would seem that in the bowel wall, the bacteria are providing some substance necessary for the amoebae, but that in the liver this is provided by the host. *In vitro*, too, amoebae are usually grown together with some living associate, which apparently provides the essential factor. What this is, is not known.

The status of the host may be a determining factor. It has been postulated (Elsdon-Dew, 1949) that diet may play some part, but to the Zulu "inyanga" (witch-doctor) the dysentery is known as "isigwebedhla"—the disease of the strong young men—an astute clinical observation (Elsdon-Dew, 1946). Nevertheless, there may be some inapparent dietary defect which may favour invasion. The association of "tropical liver" with alcoholic excess was stressed by nineteenth-century workers (Karunaratne, 1941).

It is apparent that there are many gaps in our knowledge. Possibly the main bar to progress is the lack of a small laboratory animal which would reflect the position in man, and in which virulence could be assessed without operative interference.

X. Conclusion

The confusion surrounding the epidemiology of amoebiasis has arisen in part from a failure to appreciate the nuances in the host–parasite relationship, and in part from faulty diagnosis, not only of the parasite, but also of the host's reaction to infection.

As indicated at the outset, the primary necessity is semantic. Even if the term Amoebiasis is confined to parasitization by *Entamoeba histolytica* (*sensu stricto*), there is still need to distinguish the two forms of relationship, though, of course, these may be concurrent.

On the one hand, *E. histolytica* may confine its activity to the bowel lumen, being a commensal like the other Rhizopoda of the gut, in which state the cysts necessary for transmission to new hosts are produced. Perhaps a good name for this type of relationship would be "Lumenal Amoebiasis", but the term "Cyst-passer" basically has the same meaning.

On the other hand, for some unknown reason, the amoebae may go beyond the bounds of hospitality, and invade the tissues. They may undermine the mucosa, and feed on their host's blood. They may be carried by the portal blood stream to the liver, where they cause that focal necrosis commonly called an amoebic liver abscess. Though metastasis to such remote sites as the brain may occur, direct spread from lesions in the liver and gut is much more common. Bizarre manifestations such as invasion of the genitalia are known, but all lesions are focal, the amoeba itself being the active agent, and there is no conclusive evidence of any specific remote effect. This is the disease state, which is aptly described by the term "Invasive Amoebiasis".

The invasive state is as abnormal for the amoeba as it is for the host. Certainly those parasites which have penetrated beyond the bowel wall have

forfeited all chances of a sustained posterity. Such amoebae do not produce the cysts essential for survival in the external world. Thus, the passage of cysts is evidence only of lumenal amoebiasis, and the only microscopic criterion of invasion is the finding of haematophagous amoebae. In the absence of such an observation, the decision as to whether or not there has been invasion must rest on other evidence, not the least of which is the clinical acumen of the physician concerned.

Serology now provides additional information, but this too must on no account be viewed in isolation, but must be assessed in the light of the overall picture. A positive response may be a relict of previous parenteral contact with amoebae, and be no reflection of the patient's present status. Such a result should be evaluated both on the clinical presentation and on the prevalence of amoebic anti-bodies in the general population. On the other hand, a negative serological result is good evidence that a suspect lesion is not amoebic in origin, particularly when such lesion is remote from the bowel. Thus in deciding on the institution of active, possibly dangerous anti-amoebic therapy, the clinician must not be blinded by laboratory reports, but must retain a proper perspective, interpreting reports and clinical findings as a whole.

The term *sensu stricto* with reference to the parasite was emphasized, as distinction must be made between the "large" and "small" races. Though some taxonomists are reluctant to regard the latter as a distinct species, it has never been shown to invade, and its appellation should not imply this potential. After all it is the action arising from the use of a word which is important. The name *Entamoeba hartmanni*, which has been acceptable to many for a long time, should therefore be used.

It is painfully apparent that, for the most part, parasitological diagnosis is in the hands of the inept, with the result that in very few places can any reliance be placed on published figures, or for that matter, on reports on individual cases. Had the labelling of the parasite been based on haematophagy, as in the long past, much mis-diagnosis and subsequent misery would have been avoided. Parasitology has always been the Cinderella of the clinical sciences, and faecal protozoology, despite its importance, the ashes. There is no real cure for this sad state of affairs, other than appropriate education, training and informed supervision.

It has been shown that available data—either parasitological or clinical—give but little information on the distribution of amoebiasis. The most reliable is that based on the occurrence of amoebic liver abscess, though this too is clouded by differing outlooks on the condition. Those in everyday contact with such abscesses, and *ipso facto* with amoebic dysentery, do not regard these with the same awe as do those encountering sporadic cases. Nevertheless, the present review has shown that, using liver abscess as an index, invasive amoebiasis is not nearly as widely spread as some would believe.

There is, however, need for more definitive surveys. With the lack of adequately trained personnel, faecal protozoology offers but little chance of a reliable finding, and other approaches will have to be used. On the clinical side, autopsy returns on the prevalence of amoebic liver abscess might be unequivocal. If and when the antigens become freely available, serology, being

objective, would, if based on uniform sampling procedures, give comparable estimates of the invasiveness of *Entamoeba histolytica* in various parts of the world.

In general, amoebiasis is no exception to the rule that uncritical adherence to the dicta of the great is apt to mislead. In malaria the story of the direct entry of the sporozoite into a red blood cell retarded progress for many years, and the false concept took a lot of destroying. Similarly, some *ex cathedra* statements on bilharziasis delayed appreciation of the true situation. So, too, in amoebiasis! Dobell, possibly disgusted by the prevalent spate of species-splitting, refused to recognize that there are two races within the *histolytica* complex, and at the same time promulgated the Promethean theory. Though he himself discarded the latter, it was highly publicized by Craig and others, with the unfortunate result that a totally false picture was constructed, the amoeba being blamed for almost everything except pregnancy. Thus many a patient must have been subjected to unpleasant if not dangerous therapy for a parasite which was not responsible for his complaint.

It is for parasitologists to appreciate that their findings may be misinterpreted, with results far beyond their control, and for clinicians to realize that a positive laboratory report is not a licence to stop thinking.

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Chagas' Disease and Chagas' Syndromes: The Pathology of American Trypanosomiasis

FRITZ KÖBERLE

Faculty of Medicine of Ribeirão Prêto, University of São Paulo, Brazil

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I. INTRODUCTION

Advance in our knowledge of American trypanosomiasis during the last fifteen years has reoriented this illness from an exotic and rare disease into the most frequent and dangerous plague of the American continent, "which contains an estimated minimum of 7 million infected individuals" (WHO Report,

1962). One may suppose that this *minimum* does not express the actual reality, because more than 80% of these persons acquired the infection during their early years of life, i.e. many years ago. However, in many areas where Chagas' disease is still endemic, poor conditions of life have not changed essentially during recent decades. In fact, we are informed that in Santiago del Estero, N. Argentina, more than 200 cases of acute Chagas' disease were recorded during six recent months (October 1966–March 1967) (Rohwedder, 1967). Thus, Chagas' disease is still a real and serious problem "in several aspects, including the socio-medical" (WHO Report, 1962).

This account of the pathology of American trypanosomiasis is based mainly upon personal material derived from nearly 1000 autopsy cases and several hundred biopsies. The pathology of the infection here discussed represents the aspects of the American trypanosomiasis seen in Central Brazil.

The history of Chagas' disease is full of unnecessary and disagreeable polemic which I do not wish to reawaken. Therefore, I will avoid quarrels and shun meaningless discussion of different opinions which ran contrary to my interpretation, because many former opponents now agree with my concepts.

The differences of opinion are based on marked regional variations in manifestations of *T. cruzi* infection. I do not know the causes of those variations, and it would be profitable to study them and to explain the various aspects of Chagas' disease.

It seems to me that the polymorphic form of Chagas' disease in Central Brazil represents the illness in *all* its multiple and variable facets.

II. Periods in the History of Chagas' Disease

Chagas' disease—like all really great and important discoveries—has its own and very peculiar history, called by Magalhães (1944) a "tragicomedy, which embittered and destroyed the life of one of our greatest compatriots". In this history it is useful to distinguish the following six periods.

A. Discovery

Chagas in 1908 discovered in the intestine of the blood-sucking triatomas—which were common in the primitive huts of the Brazilian hinterland—an unknown trypanosome, which he named "*Schizotrypanum cruzi*" in honour of his teacher. The presence of this parasite in bugs suggested to him the possible existence of an infectious disease in animals and man. Soon afterwards he also found the parasites in the blood of domestic animals (dogs and cats) and in the blood of a sick child with a high temperature (1909). Two years later—in August, 1911—he reported his discoveries to the "Academia Nacional de Medicina" (Rio de Janeiro) in a precise description of the acute and chronic phases of the disease as well as of its different forms, repeatedly calling his discovery a "new realm in Pathology". Among several publications which appeared later, there is an outstanding report on more than 200 cases of the so-called central nervous system form (Chagas, 1913).

B. DENIAL

However, this important discovery by Chagas was violently combated and even denied, probably because it passed the comprehension of Chagas' contemporaries. The campaign became intensified when Kraus (1915) could not find any human case of Chagas' disease in N. Argentina, although a great number of infected bugs were found in hut dwellings. Chagas rejected Kraus' report but the attacks and doubts against his discovery continued, and he was called "a man who searches in the jungle for diseases which do not exist".

C. OBLIVION

After 1920, Chagas' disease was simply forgotten and for more than 10 years it disappeared from Nosology as an infectious disease of public health importance.

D. "REDISCOVERY"

Mazza (1934) reported many acute cases of Chagas' disease found in N. Argentina, exactly where Kraus failed to find any human case of this disease. Like Chagas, Mazza was criticized for "discovering new diseases instead of treating the many already existing ones", but he and his colleagues persisted in their investigations and were able to record more than 1 000 acute cases before 1944, stressing the importance of the disease as a Public Health problem. Due to Mazza's research, investigations on once forgotten Chagas' disease were reinstated in South America.

E. MUTILATION

Although the disease became recognized as both real and important after Mazza's "rediscovery", it emerged much changed from the original description. The concept became limited to the "chronic cardiac form" called "chronic chagasic myocarditis". The remaining forms described by Chagas were almost all discarded. Even so it was considered a rare disease because, until 1944, 45 cases of chronic chagasic myocarditis were recorded in Brazil and, until 1948, only 134 outside this country (Laranja, 1954).

F. REHABILITATION

The real significance of this plague was proved as a result of improvements introduced in the technique of the complement fixation test (Davis, 1943; Freitas and Almeida, 1949) and its wide application, and also through the new concept on the pathogenesis of American trypanosomiasis (Köberle, 1956). Nowadays Chagas' disease is no more an "odd exotic parasitic disease" but the commonest infectious disease in South America, representing, as Chagas wrote in 1918, a "problem of highest importance of the American continent".

It is now realized that Chagas was completely right in predicting his discovery as "a new realm in Pathology", and it is just this new area which will be discussed in this review.

III. General Pathology of *Trypanosoma cruzi* Infection

A. Transmission and Portal of Entry

The possibilities of human infection are listed below.

1. *Infection through contaminated feces of* Reduviidae

After the blood-sucking act (chiefly on exposed regions of the body during the night) the bug expels semiliquid contaminated feces, plus the metacyclic forms of the trypanosomes, near the wound of the bite. As the wound itches, scratching follows and the contaminated feces are rubbed into the small puncture produced by the insect or into an abrasion of the skin, reaching the subcutaneous tissues. Or the parasites may penetrate even through the intact mucous membranes, commonly the ocular conjunctiva. These are by far the most usual sites of infection.

2. *Diaplacentar infection of the fetus*

This kind of infection was first clinically reported by Dao (1949), pathologically confirmed by Gavaller (1951) and further recognized by many other observers.

3. *Infection through the maternal milk*

Infection through the maternal milk was only once reported (Mazza *et al.*, 1936).

4. *Infection through blood transfusion*

This is a very important mode of transmission (Freitas *et al.*, 1952), as blood donors in endemic areas are frequently infected.

5. *Infection through other contaminated insects*

Contaminated insects other than triatomas (mosquitoes, bedbugs, and ticks) may infect animals (Brumpt, 1913; Mayer and Rocha Lima, 1914; Alcântara, 1966).

6. *Infection in laboratory*

Infection in the laboratory is accidental, and fortunately a rare event.

B. Primary Focus and Primary Complex

The infection is generally acquired during the first years of life and it usually has a more severe development in infants than in adults. Both sexes are equally infected (Viana Martins *et al.*, 1940; Laranja *et al.*, 1948; Romaña, 1963).

Once introduced into the connective tissue of the skin or mucous membrane, the parasites cause a circumscribed local inflammatory reaction, and through lymphatic propagation, almost simultaneously, elicit an acute regional lymphadenitis. This primary complex develops after a short incubation period

of about one week, and lasts for approximately one month. Rarely, an exulceration occurs at the site of the primary lesion.

In most cases the portal of entry is the ocular conjunctiva. The unilateral edema, conjunctivitis and pre-auricular lymphadenitis are known as Romaña sign (Fig. 1).

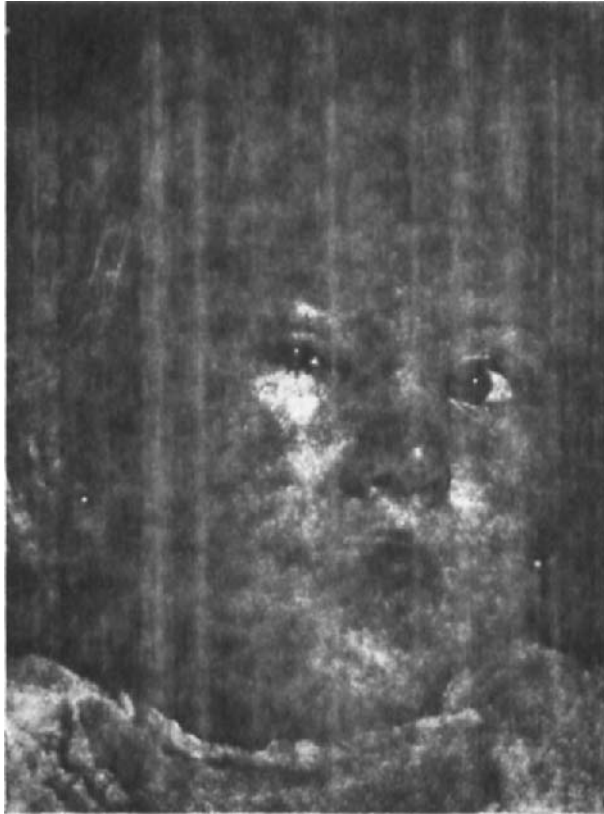


FIG. 1. Romaña sign. (By courtesy of Professor J. L. Pedreira de Freitas.)

C. GENERALIZATION

Soon after the formation of the primary complex, even during the first or second week of infection, the parasites penetrate into the blood stream, producing a septicemic state, with hematogenic metastatization. Any kind of cell may be parasitized (Vianna, 1911; Mayer and Rocha Lima, 1914) but the muscle and glia cells are specially invaded.

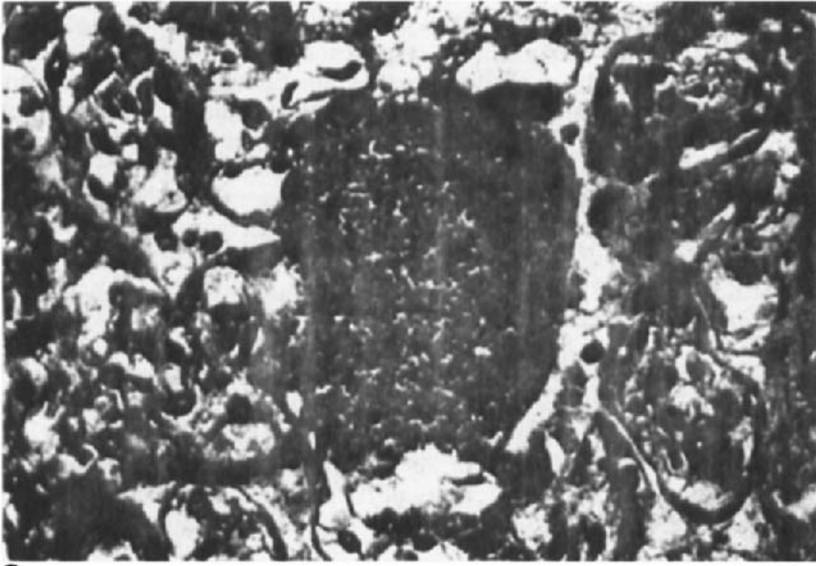
D. LOCAL REACTIONS

In the evolutionary cycle of *T. cruzi* in man, two different forms are distinguished: (a) the trypanosome or blood form and (b) the leishmania or intracellular form.

A

B

FIG. 2. A, B and C (facing page). Parasitism of human tissues in acute *T. cruzi* infection. A—Striated muscle fibers of the esophagus. B—Heart muscle. C—Placenta.


C

The trypanosomes circulate in the blood stream and after a certain period of time, invade the tissue cells. We do not know (1) how long the trypanosomes can or must circulate in the blood stream, (2) for what reason the parasites leave the circulation, and (3) how they select preferentially the muscle and glia cells. As the trypanosome needs carbohydrates for its survival, it seems likely that a product originating from its metabolism reaches the blood, acting as a trigger which informs the parasite of the proximity of a cell containing glycogen. Certain investigations indicate that the trigger information is received as soon as the parasite enters the venous circulation. It is at this level that the trypanosome leaves the capillary, actively perforating its wall and finally the plasmatic membrane of the host cell, entering by its posterior end (opposite to the free flagellum) (Kollert, 1960). At this posterior end of the trypanosome a vesicle is present (Meyer *et al.*, 1958) which probably contains an enzymatic substance capable of promoting lysis of the plasmatic membrane of the host cell, facilitating penetration. Once in the interior of the host cell the trypanosome transforms immediately into the intracellular leishmania form. Binary division follows and the parasite multiplies during a period of 5 days (Romaña and Meyer, 1942; Kollert, 1960) in a cyst-like cavity (pseudocyst) within the invaded cell (Fig. 2).

Mayer and Rocha Lima (1912) observed that the development of the leishmania forms within the host cell is uniform. However, in the final phase, before the transformation into trypanosomes occurs, a slight difference in size appears among the parasites and at the same time a small amount of fluid accumulates inside the pseudocyst. This feature was recently confirmed by electronmicroscopy (Tafuri, 1967). This fluid seems to originate from the intracellular

material after liquefaction through enzymatic substances derived from the parasites. The presence of a certain amount of liquid is apparently necessary for the early maturing mobile forms of the parasite. This final stage of transformation from the leishmania to trypanosome forms is variable and never occurs simultaneously in all the parasites inhabiting a pseudocyst. Usually only a small fraction of the parasites reach complete maturity, and as soon as this occurs they leave the pseudocyst using their non-flagellated pole to perforate the cell membrane. Very rarely there is a simultaneous transformation of leishmania forms into trypanosomes. As soon as the first trypanosome has burrowed from the pseudocyst the transformation of leishmania into trypanosome ceases due to the entrance of interstitial fluid into the ruptured pseudocyst. All leishmania forms not yet transformed into trypanosomes are non-viable outside the pseudocyst and ultimately disintegrate (Romaña and Meyer, 1942).

The two described forms of parasites act differently during the disease. The trypanosomes which leave the pseudocyst and penetrate into the blood stream, are destined to maintain the infection, burrowing into new host cells, where the intracellular cycle is reinstated; the leishmania forms, not yet transformed into trypanosomes, remain in the vicinity of the ruptured pseudocyst, disintegrate and become responsible for the local lesions.

1. *Inflammatory reactions*

Inflammatory reactions are detected in the vicinity of the ruptured pseudocyst, but they have different features and characteristics according to the state of development of the disease.

In the acute phase the cellular infiltrates consist of neutrophils and sometimes almost exclusively of eosinophils. As the disease progresses there is a decrease of polymorphonuclears and a gradual increase of monocytes, lymphocytes and plasma cells. Later on there appear histiocytes which are transformed into epithelioid cells, sometimes multinucleated giant cells of the Langhans type. According to the modification of the cellular components there is also a corresponding change in the patterns of the infiltration. The infiltrates are always localized around the ruptured pseudocysts but in the acute phase they show a certain tendency to propagation associated with the presence of a leucomonocytory reaction without sharp demarcation. The inflammatory foci may show confluence, giving the impression of a diffuse phlegmonous infiltration which may make it difficult to recognize the primary multifocal origin. As the quantity of monohistiocytes rises, chiefly when epithelioid and giant cells increase in number during the chronic phase of the disease, there is a distinct delimitation of the inflammatory reaction, resulting finally in the formation of granulomas (Fig. 3A, B).

As occurs in other so-called specific inflammations, we also find in the *T. cruzi* infection inflammatory reactions in accordance to the immunobiological state of the parasitized organism. This condition of the organism, through the production of antibodies, is responsible for the pattern, composition and extent of the inflammatory response. However, Chagas' disease does not produce

peculiarities of the inflammatory reactions from the standpoint of the principles of general pathology.

During the acute phase, with a high parasitemia and accentuated parasitism of the different tissues (or organs), the inflammatory foci are extremely frequent and impressive, while in the chronic phase they are extremely few and small, their detection calling for careful examination of many histological sections.

The evolution of the acute septicemic phase toward the chronic pauciparasitic phase results from the mobilization of the specific defense of the host, establishing an immunobiologic equilibrium between the micro- and the macro-organism. We can imagine that in Chagas' disease, as in other chronic infections, there may occur a disturbance of this equilibrium of internal as well as of external origin, causing a recrudescence of the infection. Such an exacerbation of a latent *T. cruzi* infection was involuntarily produced by Wolf *et al.* (1953) administering cortisone to Rhesus monkeys recently imported from India. Although not proved such recrudescences may occur in man.

The final cause of the inflammatory reactions in the acute phase is undoubtedly the parasite.

Arguments against the inflammatory reactions being due to the presence of the parasite are:

(a) Large number of pseudocysts filled with hundreds of parasites are found not to produce any reaction in the surrounding tissues.
(b) Even after the rupture of a pseudocyst there may be numerous leishmania forms of the parasite in its vicinity and within the interstitial spaces, equally without any inflammatory response.
(c) Parasites are almost never seen in the centers of inflammatory foci.

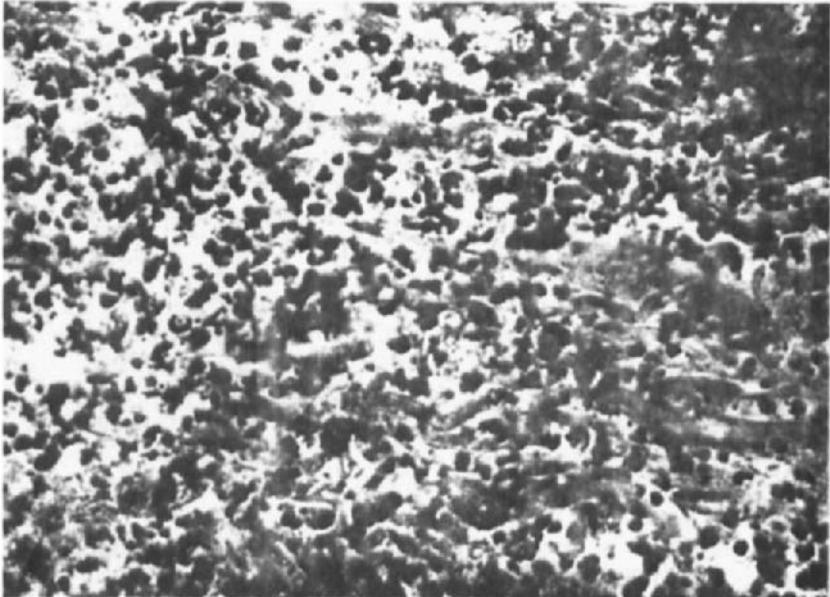
In fact, all arguments presented "against" the parasitic origin of the inflammation are in favor of it. It is not the intact parasite—inside or outside the pseudocyst—that evokes the inflammatory reaction. This reaction is due to the products of parasite disintegration only. "Intact" parasites cannot be found in the inflammatory foci because they have been destroyed.

2. *Degenerative lesions*

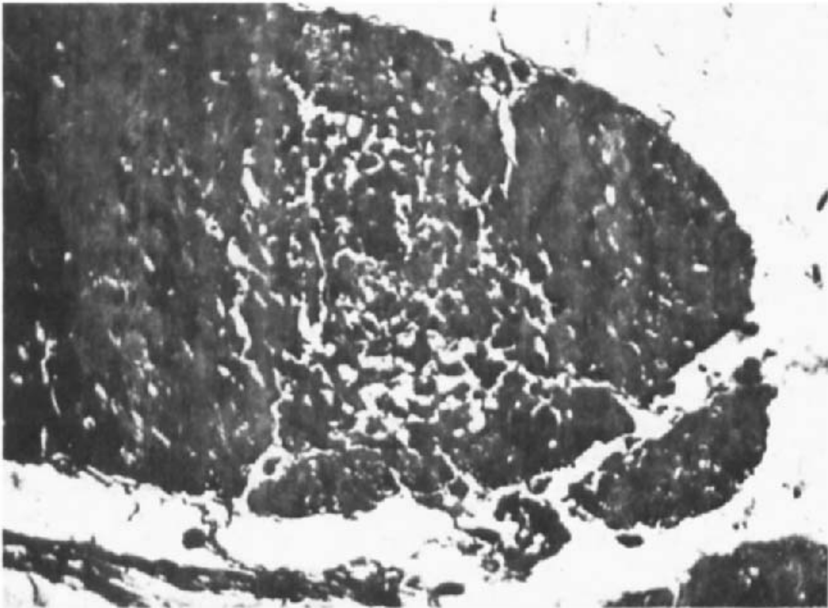
In addition to these well known inflammatory reactions, degenerative lesions may be observed after the rupture of a parasitized host cell and following the disintegration of the remaining leishmania forms. These features were rarely observed and therefore their pathogenetic significance was not recognized. Nevertheless, they exactly provide the clue to the interpretation of this disease which Chagas (1911) termed "a new world in Pathology".

In his first report on the pathological findings in Chagas' disease, Vianna (1911) described in the central nervous system "a more or less marked alteration of ganglion cells and their disintegration in the vicinity of ruptured pseudocysts". Fat necrosis around ruptured parasitized host cells in subcutaneous and peritoneal adipose tissue was reported by Mayer and Rocha Lima (1912). Mönckeberg (1924) mentioned "severe lesions of nerve and ganglia" in the heart of dogs experimentally infected with *T. cruzi*.

Whereas all these observations were made in the acute phase of experiment-

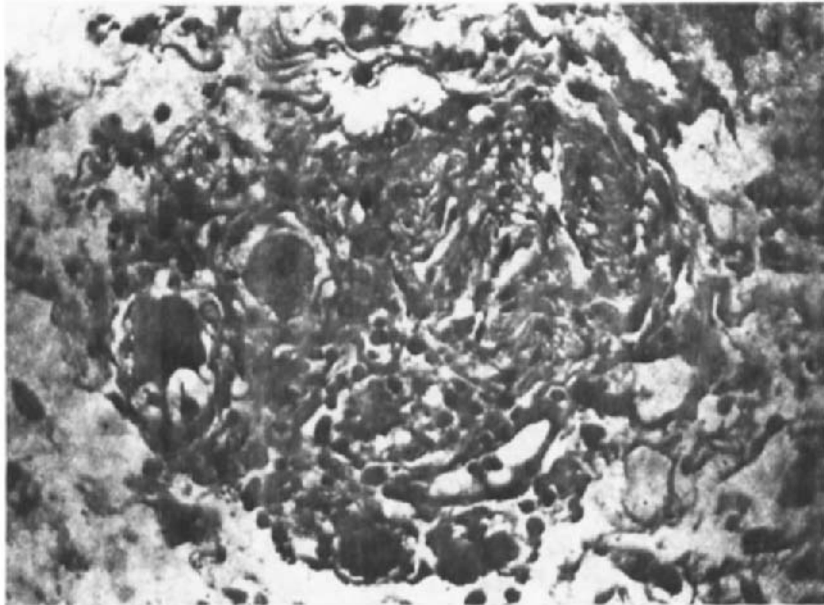


A

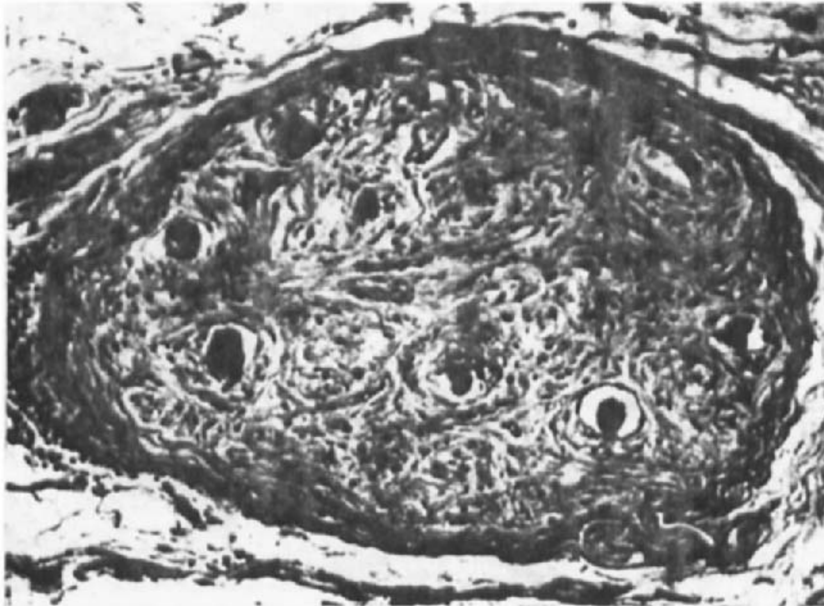


B

FIG. 3. Inflammatory lesions and degenerative changes in human cases of *T. cruzi* infection. A—Myocardium with acute inflammatory infiltrates containing leishmania forms. B—Chagasic granuloma in the esophagus. C—Severe degenerative lesions of ganglion cells of an autonomic ganglion of the heart in acute Chagas' disease. D—Autonomic ganglion of the heart with marked numerical diminution and degenerative lesions of ganglion cells.



C



D

FIG. 3—*continued.*

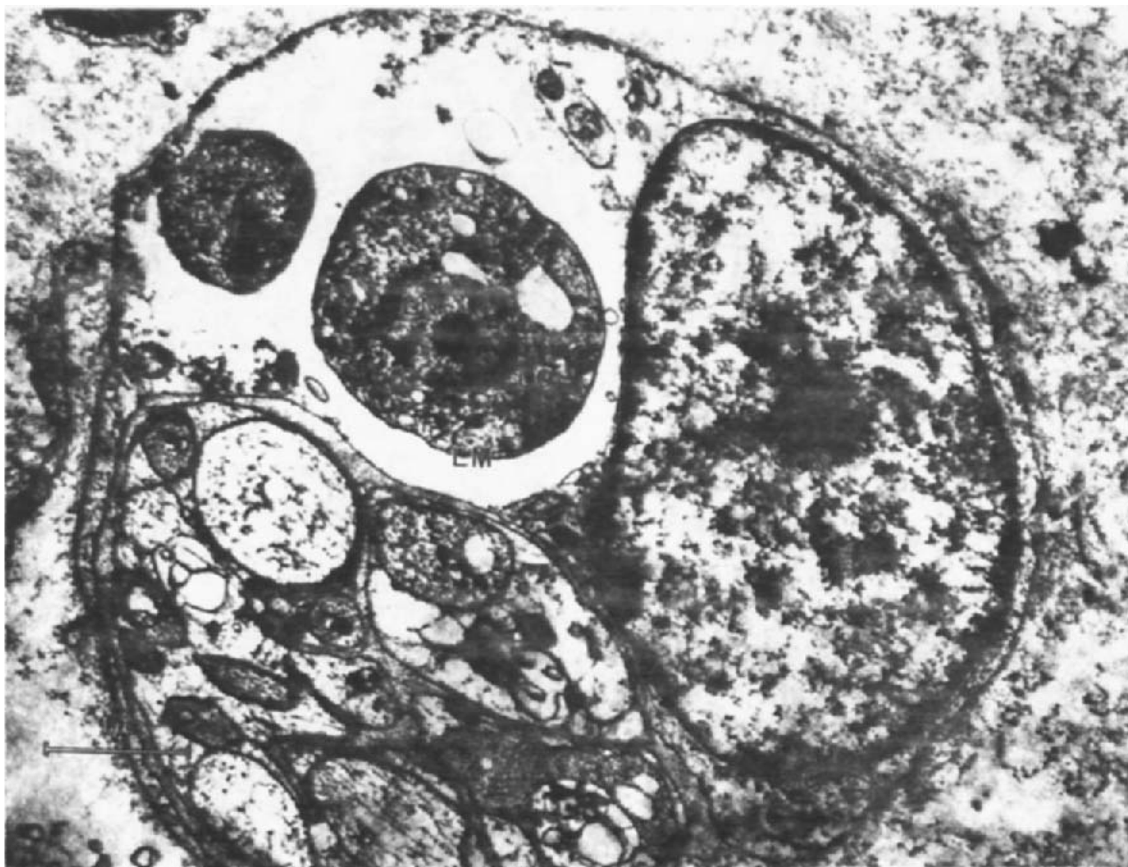


FIG. 4 A. For legend see p. 76.

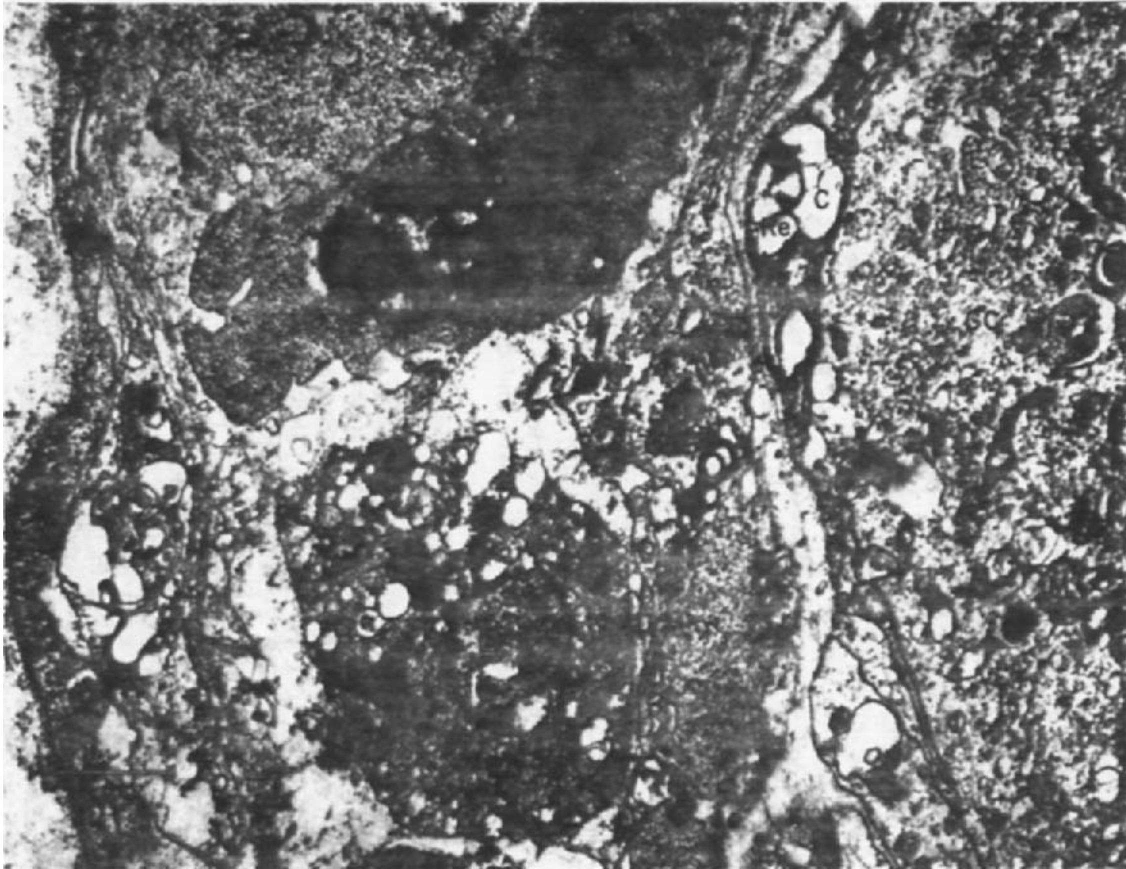


FIG. 4 B. For legend see p. 76.

ally infected animals, through examination of human material from chronic chagasic cases I arrived at the conclusion that in this case there is a marked diminution of the number of ganglion cells in the central and/or peripheral nervous system (Köberle, 1956) (Fig. 3C, D). These findings were experimentally confirmed in animals (Köberle, 1957), suggesting the presence of a neurotoxic substance inside the leishmania forms (Köberle, 1957). This neurotoxic substance is liberated from the leishmania forms after their destruction and acts locally at a short distance only. Tafuri (1967) studied by electronmicroscopy the autonomic ganglia of the heart of mice in the acute phase of the infection, and observed severe lesions of the Schwann cells and ganglion cells. These lesions suggested to him a neurolytic action of the leishmania forms after their disintegration (Fig. 4).

Like the inflammatory reactions, the degenerative lesions are also more frequent and severe in the acute phase of the disease. However, there is a little difference in respect of the time of their appearance. The degenerative alterations of the ganglion cells appear a little sooner than the inflammatory reaction. Once the inflammatory reaction has begun, it is difficult to verify changes in the nerve cells because they are hidden and covered by the infiltration of inflammatory cells. Therefore, the best opportunity to examine the degenerative alterations of the ganglion cells is immediately after the rupture of the pseudocysts, when the inflammatory reaction is still undeveloped. We must admit that the parasitism of the host cell and the inflammatory reactions after the rupture of the pseudocysts are much more impressive than the degenerative changes of the ganglion cells. Sometimes it is difficult to disclose the damage to ganglion cells, which are destroyed and disappear rapidly without any reaction of their satellite cells.

Since the parasites show a preference for the muscle cells, we find the most intensive destruction of ganglion cells where there is intimate connection between muscle tissue and autonomic ganglion cells. This is true of the heart and all the hollow muscular organs. In spite of the sometimes intensive parasitism of the skeletal muscle and the presence of a resulting accentuated and diffuse acute myositis, it is astonishing that the acute Chagas' disease does not produce severe sequelae of the skeletal musculature. This is perfectly explained, however, by the fact that in the striated muscle there are no nerve cells which could be damaged or destroyed.

These degenerative changes of the nervous system occur mainly or exclusively during the acute phase of the disease, when the parasitism is extremely high and a protective barrier is not yet formed by the organism, delimitating the process and avoiding its propagation. Alcântara (1959) has shown that

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FIG. 4. Electronmicrographs of a parasitized autonomic ganglion of the heart of mice with acute *T. cruzi* infection. A—Parasitized Schwann cell (*SC*) with its nucleus (*SCN*); two leishmanias (*L*) with nuclei (*LN*), cytoplasm (*LC*), cytoplasmic membrane (*LM*) and blepharoplast (*BL*); vacuolization of the cytoplasm around the parasites. B—Leishmania (*L*) in disintegration into the cytoplasm of a Schwann cell (*SC*), with enlargement of the endoplasmic reticulum cysterns (*ReC*); severe alterations of the ganglion cells (*GC*) with tumefaction of mitochondria (*M*) and pronounced lysis of ergastoplasm (*E*). (By courtesy of Dr. W. Tafuri.)

80% of the ganglion cells of the heart may be destroyed during the acute phase in experimentally infected rats.

Investigations carried out in serial histological sections demonstrate that the neurotoxic substance is active only at a short distance and in high concentration, since only the ganglion cells in the immediate vicinity of a ruptured pseudocyst display severe alterations, and even lysis of cells occurs in the presence of many leishmania forms in disintegration. Nerve cells at a short distance from the burst pseudocyst remain completely unchanged. We suppose that the neurotoxic substance which can damage all types of cells—especially nerve cells—is probably an endotoxin or an enzyme.

3. *Allergic reactions*

Allergic reactions in the different organs are found in chronic Chagas' disease as in most other chronic infectious diseases. Muniz and Azevedo (1947) demonstrated allergic manifestations in the heart after inoculation of dead trypanosomes. Necrotizing arteritis was reported in human and experimental material (Köberle, 1957; Brito and Vasconcelos, 1959; Okumura *et al.*, 1960, 1962). The significance of these allergic reactions in the development of the disease is not yet understood.

IV. Concepts of American Trypanosomiasis

Throughout the historical development of ideas about Chagas' disease, there have been disputed problems about its very existence, its importance in Public Health and its manifold aspects. Many physicians working in endemic areas were inclined to attribute several disturbances of the digestive tract—e.g. megaesophagus and megacolon—to American trypanosomiasis. These attempts, however, were strongly rejected by nearly all other investigators, who argued that these "diseases" exist in countries where Chagas' disease was never reported. Meaningless discussions thus continued without palpable results through three decades, because it was impossible to find a common denominator for such different views. As Prado (1959) stressed, "a missing link" in the chain of our knowledge about Chagas' disease has to be searched for, in order to explain the discrepancies between the various attempts at interpretation.

A. Older Concept

For almost half a century Chagas' disease was considered to be a morbid entity beginning with an acute septicemic phase, sometimes fatal, but usually passing after some weeks into a prolonged chronic phase. Since the acute manifestations of the disease disappeared, apparently without sequelae, and further manifestations emerged many years later, these were attributed to the repeated inflammatory foci, caused by intermittent parasitic metastatization. Thus the only form admitted after the period of "rediscovery"—the cardiac form—was interpreted as a "chronic myocarditis" (Fig. 5A).

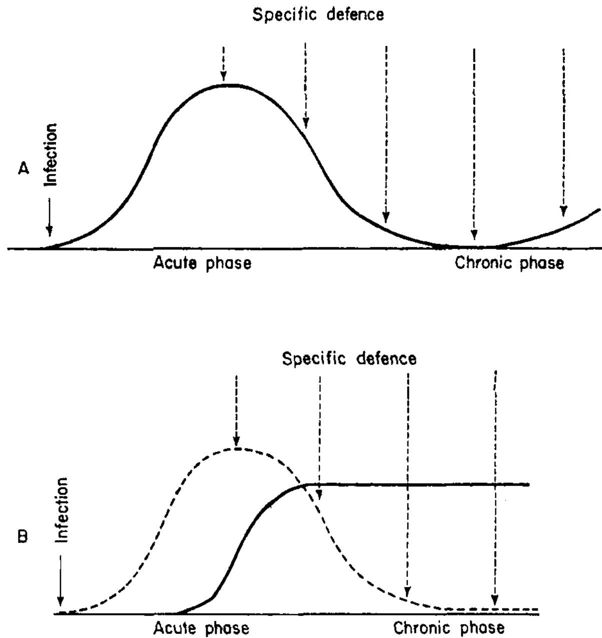

FIG. 5. A—Old concept of *T. cruzi* infection: the curve indicates the parasitism and parasitemia in the acute and chronic Chagas' disease, showing the decrease of the inflammatory reactions with the increase of the specific defense. B—New concept of *T. cruzi* infection: interrupted line represents the parasitism and parasitemia; the full line indicates the destruction of ganglion cells.

B. NEWER CONCEPT

The analysis of our study of the general pathology of *T. cruzi* infection suggests that American trypanosomiasis embodies two different pathological processes.

The initial process is the parasitic infection with its typically local inflammatory reaction, which subsides apparently without any important sequelae in the various organs. Torres (1941) reported that no other disease causes such intense myocarditis as acute Chagas' disease and heals without vestiges. However, this is only an erroneous impression based on a superficial examination, because marked sequelae—the beginning of a new process—are found, after the acute myocarditis, in the autonomic nervous system of the heart, being easily overlooked. As the sequelae comprise a numerical diminution of the ganglion cells, the pathologist has to search for something that has disappeared. Similar lesions occur in the most varied territories of the central and peripheral nervous system. Therefore, we may—*cum grano salis*—compare the acute Chagas' disease with poliomyelitis. In this disease there is also an acute inflammation with degenerative lesions and destruction of the ganglion cells, responsible for the post-poliomyelitic paralytic phenomena. The difference between

poliomyelitis and Chagas' disease consists only in the fact that the virus infection heals whereas the parasitic infection continues in latent form.

Hence we ought to distinguish in American trypanosomiasis according to the classic concepts of the Greek Medicine of "Nosos" and "Pathos", i.e. between Chagas' disease and Chagas' syndromes (Fig. 5B):

(a) *Chagas' disease*, in the strict sense, that is, infection by *T. cruzi* showing an acute septicemic phase, which passes after the development of the specific defense of the host into a chronic phase, with rare parasites in the circulating blood and in the body tissues;

(b) *Chagas' syndromes*, sequelae of Chagas' disease, also called "late manifestations" of the disease, which develop as the result of the extensive destruction of ganglion cells in the peripheral and/or in the central nervous system during the acute phase.

According to our new concept on *T. cruzi* infection, all the so-called "late manifestations", which were until now considered to be the expression of the chronic Chagas' disease, represent in reality Chagas' syndromes and, as they are caused by a numerical reduction of the ganglion cells, are essentially *neuropathies*. The symptomatology of these neuropathies has to be evaluated mainly through functional methods. The extension and intensity of such functional derangements depend not exclusively, but principally, on the degree of the numerical reduction of ganglion cells. This more or less marked denervation must be confirmed by quantitative and comparative studies on material obtained from normal and chagasic cases. Such investigations are easy, but very tiresome, requiring thorough examination of great numbers of histological sections.

1. *Quantitative study of the nervous system in man*

In the study of the nervous system in man the following methods were employed.

(a) *Ganglion cell counting in the digestive tract.* Rings 3–4 mm thick were taken at different levels of the digestive tract and embedded in paraffin as a whole or divided in segments 2 cm long. Serial histological sections—each 7 μ thick—were cut and every seventh section mounted and stained with H.E. As the ganglion cells never have a diameter larger than 50 μ, with this technique the double counting of cells is avoided. Twenty sections were taken from each block, completing a total width of approximately 1 mm. The ganglion cells of the myenteric and/or submucous plexuses were counted in the twenty selected sections of every block. In that way the total number of ganglion cells in each of the plexuses—(in a ring 1 mm thick)—of the hollow muscular organ was obtained.

(b) *Ganglion cell counting in the respiratory tract.* Since the distribution of the ganglion cells in the respiratory tract is highly irregular, it was necessary to count the ganglion cells in a ring 1 cm thick, using the same methods as above.

(c) *Ganglion cell counting in the heart.* In the heart the number of ganglion cells was counted in the posterior wall of the right atrium, in a strip taken between the superior and inferior venae cavae.

(d) *Ganglion cell counting in the autonomic ganglia.* The ganglion was entirely cut in sections 7 μ thick and the counting was performed in every seventh section.

(e) *Ganglion cell counting in the central nervous system.* In the central nervous system the counting of ganglion cells was accomplished with the same technique in pre-established areas.

2. *Quantitative study of the nervous system in animals.*

(a) *In mice and rats.* Due to the smaller diameter of the ganglion cells in these animals (average of about 24 μ), histological sections 8 μ thick were obtained and each third section was selected for counting.

(b) *In cats and dogs.* In these animals the same method used for human material was applied.

V. The Phases of Chagas' Disease

A. Acute Phase

1. *Symptomatology*

After an incubation period of 1–2 weeks, the disease begins with tiredness, headache, abatement and fever of variable intensity, reaching 38–40°C. Temperatures above 40°C (104°F) are usually rare. The fever may be continuous or recurrent, lasting 4–5 weeks and then falling gradually to normal. An edema of elastic consistency may appear during the second week. It may be localized (chiefly in the face) or generalized, sometimes suggesting an acute nephritis, and subsides spontaneously with the other symptoms. Generalized lymphadenopathy and moderate hepatosplenomegaly are frequent, and gastrointestinal symptoms (vomiting and diarrhea), cutaneous rash and meningoencephalitis may be present. Nevertheless, the most frequent signs appear in the cardiovascular system: tachycardia (without correspondence to the intensity of the fever), cardiac enlargement, hypotension and heart failure. The electrocardiogram shows sinus tachycardia, low voltage, prolonged P–R interval and primary T-wave changes. This occurs in about half of the cases. The other half shows no EKG alterations (Laranja *et al.*, 1956). Hematological examination reveals leukocytosis due to marked lymphocytosis with relative neutropenia. In the late stage of the acute phase the number of eosinophils may be high.

The severity of the acute disease is extremely variable. Formerly—in Chagas' epoch—the mortality rate was about 50%, whereas today it is less than 5%. Death is caused either by encephalomyelitis or myocarditis.

All these acute manifestations described by Chagas (1916) were confirmed by numerous other investigators. Nevertheless we must emphasize the following symptoms, which Chagas reported but could not explain satisfactorily.

(a) *Tachycardia.* Tachycardia is always higher than one would expect it to be in relation to the fever. Sometimes the frequency is extremely high and continues increased after the remission of the temperature during the recovery. This finding was confirmed by all researchers and yet Laranja *et al.* (1956)

stressed that "the tachycardia very often only manifest or become more intense, when the fever and the other acute phenomena have disappeared".

(b) *Coughing.* Bronchial irritation associated with abundant mucus secretion is also related.

(c) *Dysphagia.* We quote Chagas:

> Another symptom which we observed during the acute infection of adults, is an accentuated dysphagia for solid food and sometimes for liquids too. The swallowing of solid food requires the help of a gulp of water. The patients say that the food stops in the esophagus and produces extreme pain. Even the swallowing of liquids and water may be difficult, sometimes impossible. In this case only a trick can help, namely the ingestion of small amounts of fluid. This phenomenon, without satisfactory pathogenetic explanation, may have some relation to the dysphagia known as "Mal de Engasgo", another endemic disease widely encountered in the interior of Brazil, and according to our observations, specially in those regions where Trypanosomiasis is frequent. Could the "Mal de Engasgo" be another component of the Brazilian Trypanosomiasis, and could that dysphagia of the acute forms represent the initial phase of the syndrome? (Chagas, 1916).

(d) *Diarrhea.* This condition is frequent, very obstinate and cannot be explained either by bacterial or by parasitic intestinal infections.

I emphasize the above four symptoms because they demonstrate impressively the rarity of Chagas' observations.

2. *Pathology*

The pathological findings in fatal cases of acute Chagas' disease were described in detail by Chagas (1911), Crowell (1923) and other authors so completely that nothing need be added.

(a) *Macroscopic findings.* The main macroscopic findings are generalized edema, especially in the face, generalized swelling of the lymph nodes, serous effusions in the body cavities, hyperemia and edema of the brain, spinal cord and their meninges, eventually punctiform hemorrhages in the brain, dilatation of the heart with flabby, friable and spotted myocardium, serofibrinous pericarditis, acute swelling of the liver and acute infectious swelling of the spleen.

(b) *Microscopic findings.* In all organs or tissues are found variable quantities of parasitic pseudocysts and acute inflammatory foci, containing numerous eosinophils. In most cases the heart is the most frequently and intensively damaged organ. It may show a diffuse multifocal myocarditis, sometimes of a phlegmonous type and of an intensity not yet seen in any other known infectious heart disease (Torres, 1941). In the central nervous system forms of the disease the inflammatory infiltrations are more prominent in the brain, spinal cord and leptomeninges. The liver shows a parenchymatous or fatty degeneration and there is an acute splenitis. Parasites are seldom found in the liver or the spleen.

These pathological features in the acute stage of Chagas' disease correspond to a typical form of a septicemic and metastasizing process; all possible variations of intensity and localization can be found.

B. Chronic Phase

1. *Symptomatology*

As the acute phase of Chagas' disease subsides, the chronic phase arises and parasites become extremely scarce in the blood and tissues due to the mobilization of the specific defense of the host. The patient appears to be normal for a long period, 10–20 years (Laranja *et al.*, 1956). However, the diagnosis may still be established by the finding of parasites in the circulating blood (by means of xenodiagnosis) or the highly specific complement fixation test of Guerreiro-Machado.

As already mentioned, acute recurrences of the disease have been provoked in animals but, so far as we know, are not confirmed in man. Such confirmation is needed because if acute stages do not recur, the chronic chagasic infection would be the best protection against a new infection, and to cure it would be unnecessary and might even be harmful.

2. *Pathology*

As in most chagasic cases the acute phase passes into an apparently asymptomatic chronic phase; we find in such instances—if the patient dies in consequence of another fatal illness or an accident—no characteristic features at autopsy. However, painstaking examination of many histological sections will reveal some chronic inflammatory foci or granulomas and, exceptionally, a pseudocyst may be detected. An eventual diminution of the number of ganglion cells in the central and/or peripheral nervous system can be displayed only by quantitative studies of serial histological sections. These lesions of the nervous system, as we have already explained, are determined in the acute phase of the disease, and are not attributable to the chronic phase.

VI. Chagas' Syndromes

Under the term "Chagas' syndromes" we understand all pathological phenomena caused by the diminution in a number of ganglion cells, which may become manifest immediately following the acute disease or after an apparently asymptomatic period of many years. We must emphasize that the denervation is the ***absolutely indispensable*** element for the appearance of these syndromes, but its development depends on the conjoint action of many other factors, among which the most important are (a) localization and intensity of denervation, (b) sensitivity of the affected organ to denervation and (c) solicitation and overloading of the damaged structure.

For the analysis of any syndrome and its evolution, we have to evaluate the participation of each of the mentioned factors and, when possible, a quantitative study should be carried out.

Concerning the *localization*, it is evident that the manifestation depends on the damage of the corresponding part of the nervous system, be it situated in the organ itself, in its vicinity, or in the central nervous system.

The *intensity* of the *denervation* alone has real value only when related to the sensitivity of the affected organ. In fact, I have established different limits of

tolerance to denervation in various hollow muscular organs (Fig. 6) and verified that a certain denervation may produce severe functional disturbances in one organ, while the same denervation may not provoke functional repercussions in another organ.

In the same way we can understand that the functional disturbance becomes more evident, and occurs sooner, the more frequently and intensively the organ is solicitated or overloaded. Some syndromes thus appear immediately after ganglion cell destruction, whereas others become manifest only after many years. Chagas' syndromes may be explained according to Cannon's law of denervation, which is of the utmost fundamental importance in the hierarchy of the organization of life. In a way, they represent an experiment elaborated by Nature, confirming, in a grandiose example, the validity of Cannon's law!

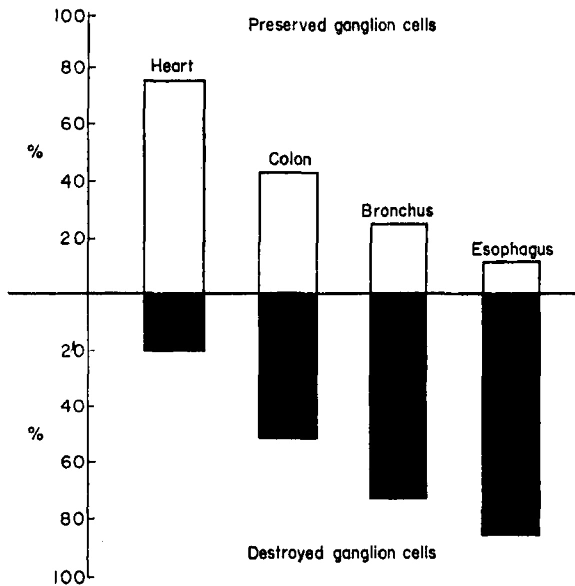

FIG. 6. Limits of tolerance to denervation.

Of all these Chagas' syndromes, the cardiopathy is by far the most frequent and most important. Nevertheless, I will begin my presentation with the disturbances of the motility of the digestive tract, since the pathogenic mechanism is relatively simple and better elucidated than is the very complex pathogenesis of chagasic cardiopathy, which is still problematical.

A. APERISTALSIS AND ENTEROMEGALY

The disturbances of the motility of the hollow muscular organs—so frequently observed in chagasic patients—have been termed "aperistalsis" (Brasil, 1956). For such alterations and their morphological consequences, more than one hundred different terms exist in the medical literature, among

which the most common ones are: achalasia, cardiospasm, idiopathic dilatation, idiopathic hypertrophy; megaesophagus, megacolon, megacystis, etc.; dolichoesophagus, dolichocolon, etc.

About thirty different theories have attempted to explain the pathogenesis of this morbid condition. Such dilatations are relatively rare outside South America and most cases are diagnosed in advanced stages of development. Since the etiology of the lesion in cases outside South America was unknown, it was impossible to trace its evolution and study its pathogenesis. However, once the chagasic etiology of such dilatations of the hollow muscular organs in Brazil (Köberle, 1956) had been established, it became feasible to study the motility of these various hollow organs in all phases of their evolution in a great number of chagasic patients. In fact this was the first step towards elucidating its pathogenesis.

The final stage of the altered motility has its morphological expression in a hypertrophy of the muscle wall, followed by dilatation of the organ. All these dilatations in various organs—e.g. megaesophagus, megacolon, megaureter, etc.—are denoted by the term *enteromegaly*.

Table I shows the incidence of enteromegaly with its distribution in the different organs, and also the various combinations of local manifestations in 800 autopsy cases. Five hundred of these cases showed only chagasic cardiopathy, which was always the cause of death. Three hundred cases disclosed dilatation of one or more hollow muscular organs. The great majority of these enteromegalic manifestations have been found in the digestive tract, and megaesophagus and megacolon were the most frequent alterations (Fig. 7).

1. *Digestive tract*

The denominations megacolon (Mya, 1894) and megaesophagus (von Hacker, 1907) as well as other similar terms are used to denote all such hypertrophies and dilatations of a hollow muscular organ in which neither roentgenological nor gross examination can reveal any obstruction which could explain the dilatation of the organ.

In those cases in which a cause could not be appointed as the origin for the dilatation, many attempts were made to demonstrate an obstacle, because it seemed to be impossible that a hollow viscus could become dilated in the absence of an obstruction. Among these dilatations, the megacolon was best known, after its description by Hirschsprung (1888), who interpreted his impressive findings in newborn infants as a possible malformation of the large intestine. Later on, Mya (1894) introduced the term "megacolum congenitum", defining the lesion as local gigantism, thus avoiding explanation in terms of the presence of an obstacle in the distal portion of the large intestine. His interpretation was accepted and used to explain similar dilatations of other hollow organs; von Hacker (1907) coined the term megaesophagus, suggesting an analogous malformation in the initial part of the digestive tract. Finally, Dalla Valle (1920, 1924) demonstrated that the huge dilatation of the colon is due to an obstruction consisting of a distal aganglionic segment in the rectum. It is noteworthy that Hirschsprung mentioned in his first report that the dilatation did not include the rectum, which seemed to be normal. With the discovery

TABLE I

Chagasic syndromes in the hollow muscular organs of 800 autopsy cases

C	500
C—MC	72
C—ME	52
C—MC—ME	50
C—BR	23
MC	22
MC—ME	13
ME	12
C—MC—BR	6
C—MD	4
C—ME—MG	4
C—MC—ME—MD	4
C—MC—ME—MD	4
C—ME—BR	3
BR	2
C—MGb	2
C—ME—MD	2
C—MC—ME—MG	2
C—ME—BR—MD	2
MG	1
C—MG	1
C—MCy	1
C—MU	1
MC—BR	1
MC—MG	1
ME—BR	1
ME—MG	1
ME—MGb	1
MD—MJ	1
C—MC—MGb	1
C—MC—MCy	1
C—MC—MJ	1
MC—ME—MG	1
C—MC—ME—BR	1
C—MC—ME—MJ	1
C—MC—MD—MGb	1
C—ME—MG—MD	1
MC—ME—MD—MJ	1
C—MC—ME—BR—MG	1
C—ME—MG—MD—MU	1
	800

C—cardiopathy; MC—megacolon; ME—megaesophagus; MG—megagastria; MD—megaduodenum; BR—bronchiectasis; MGb—gallbladder; MJ—megajejunum; MCy—megacystis; MU—megaureter.

of Dalla Valle, i.e. the existence of an aganglionic segment, representing the responsible functional obstacle of the intestine, the theory of a congenital malformation was confirmed, but the malformation was represented by the *narrow* distal segment, and not by the dilated part. Thereafter the denomination of megacolon was not justified, because the dilatation denotes only a secondary alteration and not the primary lesion. However, the denomination of megacolon was dangerous as well as erroneous, because it induced surgeons to remove the dilated part (to no purpose), leaving the responsible segment in the body, promoting an obligatory recurrence after an anastomosis was achieved.

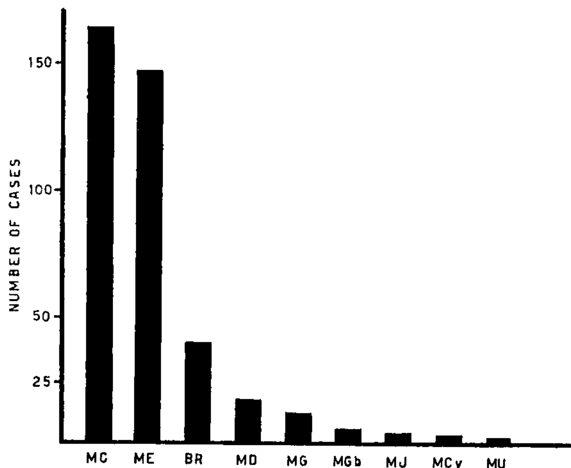

FIG. 7. Frequency of "megas" in 800 autopsied chronic chagasic cases: MC—megacolon, ME—megaesophagus, BR—bronchiectasia, MD—megaduodenum, MG—megagastria, MGb—megagallbladder, MJ—megajejunum, MCy—megacystis, MU—megaureter.

The existence of an aganglionic segment was confirmed by several investigators, and Bodian *et al.* (1949) insisted on the replacement of the misleading term "megacolum congenitum" by the correct denomination of "Hirschsprung's disease". Unfortunately the terms "megacolum congenitum" or "aganglionic megacolum" are still used, giving rise to misinterpretations. Some authors still use the terms "symptomatic" or "organic" megacolum, for dilatations of the intestine caused by organic obstruction (Lee and Bebb, 1951; etc.). These names should not be used, because they are inadequate and call attention only to a secondary phenomenon and not to a responsible original lesion.

The term megacolum, in my opinion, should be reserved for dilatations of the intestine, without a responsible obstacle, in spite of the fact that many authors do not believe in the existence of a so-called "idiopathic dilatation". I quote the following part of Lee and Bebb's article to illustrate the general opinion about this problem:

> The greatest stumbling block to a proper interpretation of the observed facts has been that in so many cases no recognizable organic obstruction could be demon-

strated. That form of megacolon secondary to obstructing stricture or tumor has long been recognized and has been termed symptomatic or organic megacolon. It has never presented any problem, and yet the pattern is so similar to that of the so-called "idiopathic" type that, in retrospect, it seems almost incredible that the latter could have been attributed by so many distinguished observers—including Hirschsprung, Osler and Finney—to a disorder intrinsic within the dilated and hypertrophied bowel itself. If such an interpretation were correct, it would represent the only instance known to medical science of a hollow viscus becoming chronically dilated and *hypertrophied* in the absence of obstruction.

A phenomenon analogous to that referred to with regard to the megacolon occurred with the megaesophagus.

The megaesophagus was considered primarily as a malformation, the dilated part being the pathological one. Hurst and Rake (1930) verified the absence of ganglion cells in the myenteric plexus of the non-dilated segment of the cardia, denominating the functional disturbance achalasia, i.e. non-opening of the cardiac sphincter. It seemed, therefore, that the mechanism of the evolution of the dilatation would be—as in Hirschsprung's disease—an absence of ganglion cells, an aganglionic segment. If this were true, the term megaesophagus should also be abandoned, because the dilatation would represent only a consequence of the achalasia of the cardia.

I believe in the existence of a dilatation and hypertrophy of the hollow muscular organs without obstruction, as a result of an "intrinsic disorder within the dilated and hypertrophied bowel itself". These dilatations could be called "idiopathic" not in the sense that the cause is unknown, but in the sense that the cause resides in the altered part *itself*, represented by severe lesions of the intrinsic nervous system.

This disturbance of the motility is called aperistalsis and the morphological consequences are megaesophagus, megagaster, megaduodenum, etc. The histological substratum of aperistalsis is a more or less marked diminution of the number of ganglion cells of the myenteric plexuses. This diminution has to be verified through quantitative and comparative studies. To show the increasing reduction of nerve cells and its consequences, I shall use the esophagus as a representative model for all types of hollow muscular organs.

(a) *Normal esophagus.* We know today that the peristaltic movement of the esophageal musculature requires only a normal function of the myenteric plexus and of the vagus nerve. The myenteric plexus occupies, in the transversal section, about 5% of the total mass of the esophagus (Köberle, 1963). Ganglion cells rarely appear in this plexus below the level of the cricoid cartilage. They continuously increase in number up to the middle portion and then maintain approximately the same number up to the cardia (Fig. 8). Comparing the three columns we note this uniformity of the distribution of ganglion cells in the inferior half of the esophagus, furthermore a diminution in column B which is still more in column C. It suggests that there may be a decrease in the number of ganglion cells with age. Figure 9 demonstrates very clearly that there is a physiological disappearance of the ganglion cells in the esophagus with increasing age; the numerical diminution of nerve cells occurs uniformly in the entire length of the organ (Köberle, 1963).

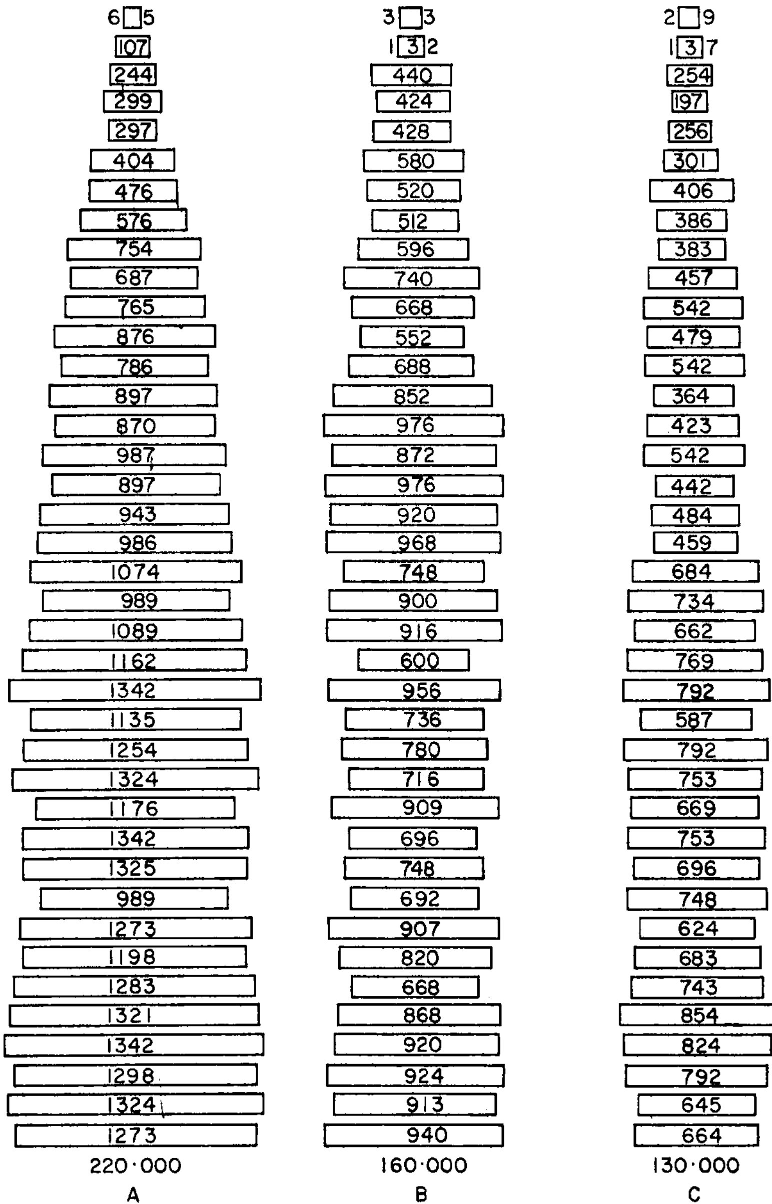

FIG. 8. Innervation of the normal human esophagus. A—20 year-old man. B—60 year-old man. C—75 year-old man.

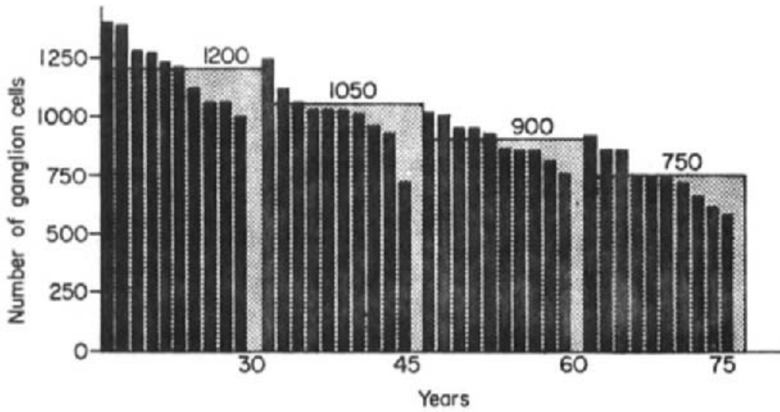

FIG. 9. Physiological diminution of ganglion cells in the esophagus with increase of age.

(b) *Presbyesophagus.* In old age the diminution of nerve cells is more pronounced and can fall beneath 50% of the number found in youth (Fig. 10). It is very probable that such a marked diminution of the intrinsic nervous system may have functional repercussions. The difficulties in swallowing in aged people are generally well known and have been studied systematically by Piaget and Fouillet (1959) in 100 individuals ranging from 63 to 95 years of age. Similar disturbances have been described by Turano (1959), Lauar *et al.* (1959) and Molitor (1962). Soergel *et al.* (1964), studying 15 persons ranging from 90 to 97 years of age, reported tertiary contractions, delay in emptying and moderate dilatation, indicating an insufficiency and disorganization of the motor function of the esophagus; they called this syndrome "presbyesophagus". The physiological numerical reduction in nerve cells is without doubt

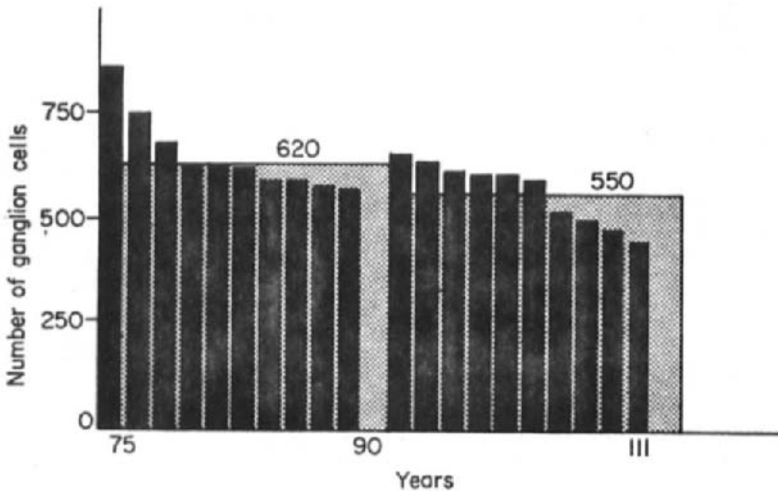

FIG. 10. Presbyesophagus with marked diminution of the number of ganglion cells.

responsible for the altered peristalsis and the difficulties in deglutition found in old people.

(c) *Chagasic esophagus.* As a matter of routine in my Department, the ganglion cells in the lower third of the esophagus are counted according to the method already described. Thus, we have counted the ganglion cells in the esophagus of more than 200 autopsied chagasic cases. From our material will be shown only the representative finding in 40 chagasic esophagus apparently normal at gross examination. Grouping the findings of nerve cell counting according to the patient's age, disclosed a very remarkable phenomenon. We expected to find a more marked diminution of the number of ganglion cells with increasing age of the group, as in normal cases. However, exactly the reverse was found, i.e. an increase in the average number of nerve cells with age (Fig. 11). These singular results are perfectly comprehensible because they

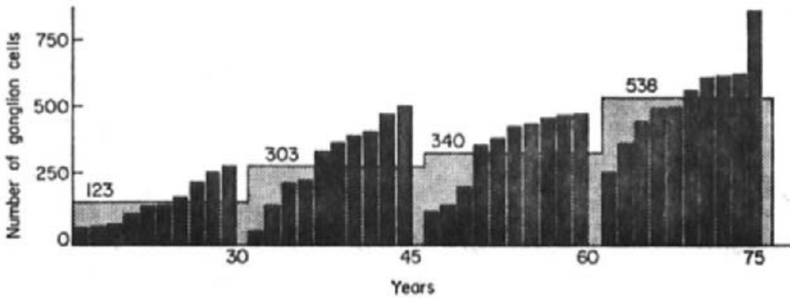

FIG. 11. Chagasic esophagus. Increase of the number of ganglion cells with age.

show only that the chagasic patient will die sooner, the more pronounced his ganglion cell destruction has been. In spite of the fact that all these patients died in consequence of an associated chagasic cardiopathy, the nervous system of the esophagus expressed eloquently the more or less severe damage of the nervous system as a whole.

Most cases are derived from autopsy material. Unfortunately only a few cases occur in which, during life, the motility of the esophagus was graphically recorded; therefore we are not able to correlate the intensity of the denervation with the clinically verified functional disturbance of the organ. We know, through numerous clinical investigations, that the motility of the esophagus is altered in 14–80% of chagasic patients (Rezende and Rassi, 1958; Godoy and Haddad, 1961; Vieira and Godoy, 1963; Morales Rojas *et al.*, 1961; Iñiguez-Montenegro, 1961; Pessôa and Mesquita, 1964; Haddad and Godoy, 1963). These individual results depend in part on the method employed but, evidently, also on the regional differences of the disease.

(d) *Chagasic megaesophagus.* The megaesophagus is so frequent in certain areas of Brazil that the clinical manifestations are well known to the people, and several terms to characterize the disease and symptomatology are in use: "Mal de Engasgo", "Entalo", "Embuchamento", etc.

The most frequent symptoms according to Rezende (1966), expressed as occurring in percentage of patients, are:

Dysphagia 99%, Regurgitation 57%, Odynophagia 52%, Erructation 41%, Singultus 38%, Sensation of plenitude 32%, Coughing 26%.

Pyrosis and loss of weight are also very frequent, occurring in about 70% of the cases (Rezende, 1966).

X-ray examination shows dilatation of the organ mainly in the lower part. Advanced cases also show elongation of the organ, i.e. megadolichoesophagus. The functional study reveals retention of the contrast, which passes gradually at long and irregular intervals through the cardia into the stomach. The Mecholyl test gives positive results, except in cases of extreme dilatation with secondary degenerative changes of the musculature of the organ.

Gross examination shows the characteristic dilatation and hypertrophy of the musculature, which can reach 26 times the normal weight. The mucous membrane is generally thickened, with patchy leukoplakia most marked in the lower part. In cases with severe stagnation, small erosive lesions or a diffuse esophagitis are found in this same localization. Cases with very prolonged evolution show a predisposition to carcinoma, which may occur in 10% of the cases (Camara-Lopes, 1962). All these alterations are limited to the thoracic part of the organ, while the abdominal portion does not disclose any gross changes.

On microscopic examination the various layers of the organ show different alterations. The epithelium of the mucous membrane is thickened and may disclose cornification of its superficial layer. Leukocytic infiltration and erosions of the epithelium are secondary changes occurring in advanced cases. These inflammatory infiltrations may penetrate the muscularis mucosae, sometimes the submucosa, but never the tunica muscularis propria. Hypertrophy of the muscularis mucosae is frequent. However, the most impressive feature is, without any doubt, the hypertrophy of the tunica muscularis propria. The increase in thickness of this muscular mass is always much more evident in the inner layer than in the outer layer. While normally the ratio between the former to the latter is 0·9:1·0, it may reach 1·0:6·0. The reason for this difference lies in the fact that the inner circular layer is much more distended than the outer longitudinal layer, due to the dilatation of the organ. Quantitative studies of the number of muscle cells in normal esophagus and megaesophagus demonstrated that the increase of musculature is the consequence of a hyperplasia (Pereira and Gonçalves, 1958). In this muscle layer small localized lympho-histiocytic infiltrations are sometimes seen, especially near the myenteric plexuses. Typical granulomas with giant cells are very rare. Proliferations of the preganglionic nerve fibers, giving rise to features very similar to those found in traumatic neuromas, may occur. However, the most striking and important finding is a *negative* one, i.e. the absence of more than 95% of the ganglion cells of the myenteric plexuses (Fig. 12). The decrease in number of nerve cells is found uniformly along the whole extension of the esophagus, including the abdominal part also, which appears completely normal at gross examination (Fig. 13).

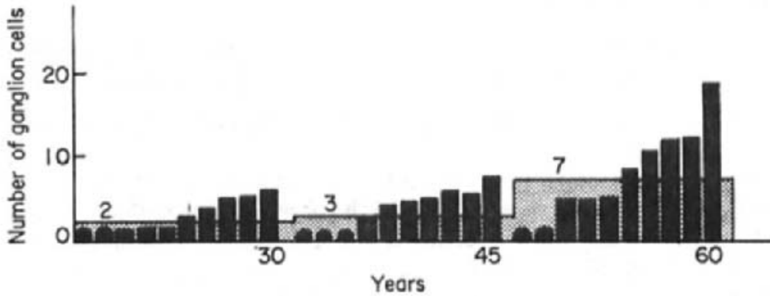

FIG. 12. Chagasic megaesophagus with loss of more than 95% of ganglion cells.

The consequences of the destruction of the ganglion cells of the myenteric plexus of the esophagus are as follows: (1) loss of the coordinated peristalsis, (2) supersensitivity of the denervated musculature. This phenomenon, occurring in a denervated hollow muscular organ, was called by Brasil (1956) "aperistalsis".

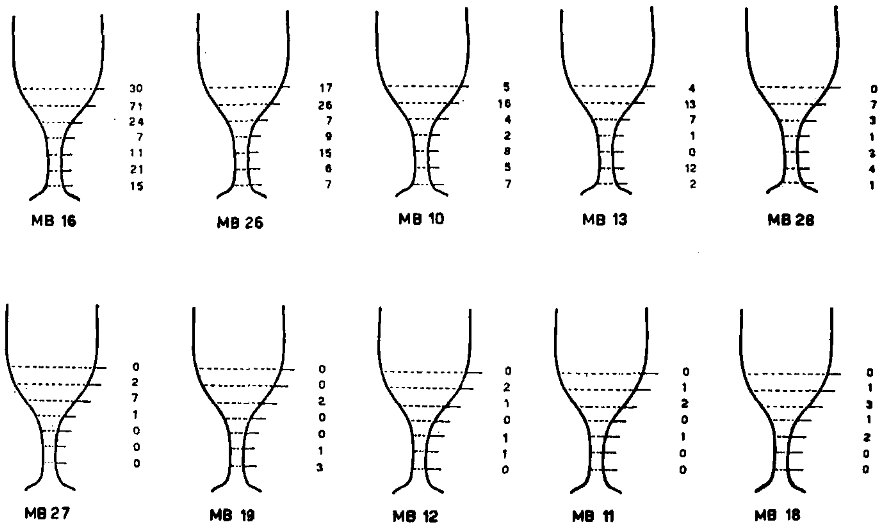

FIG. 13. Abdominal portion of ten cases of megaesophagus, showing the same denervation as the thoracic portion of the esophagus.

The absence of ganglion cells of the plexus does not, therefore, mean absence of the peristalsis only. In conformity with Cannon's law, the denervated structures become supersensitive to any stimuli, especially to the adequate substance which in the present case is acetylcholine. Therefore, the Mecholyl test is positive in chagasic esophagus and in chagasic megaesophagus (Vieira and Godoy, 1963; Vieira, 1964; Pessôa and Mesquita, 1964), sometimes in an

impressive manner, inducing diffuse and severe spasms of the organ, which occasionally need an urgent application of atropin. It is understandable that the transport of the contents in a hollow organ becomes slower, irregular and sometimes arrested, when the peristaltic movement is severely disturbed and partially substituted by uncoordinated non-propulsive contractions. Obviously there is a retention or temporary stagnation of the contents, with prolongation of the emptying time. All these phenomena are responsible for the dilatation of the organ, which implies a distension of the muscle fibers. This stretching leads to hypertrophy of the muscle, causing more powerful contractions and making still more difficult the passage of the contents. Through this vicious circle of change, monstrous degrees of muscle hypertrophy and dilatations of the organ finally occur. In this way the uncoordinated peristalsis associated with supersensitivity of the denervated structure *per se* induce a dilatation of that aperistaltic hollow organ, through retardation and stagnation of the contents. Therefore, I do not find it "incredible that a hollow viscus becomes chronically dilated and hypertrophied in the absence of obstruction" (Lee and Bebb, 1951). In chronic chagasic patients we can find dilatations and hypertrophies of hollow organs without a sphincter (megaduodenum, megajejunum, megaileum, megatrachea, etc.), which prove that a sphincter is not indispensable for the occurrence of a "mega" formation.

Most authors have attributed the megaesophagus to the presence of a "hindrance" at the level of its terminal portion: "spasm of the lower esophagus" (Zweigel, 1733), "cardiospasmus" (von Mikulicz, 1904), "hiatospasmus" (Lotheissen, 1926; Jackson, 1922), "phrenospasm" (Jackson, 1922), "fibrosis of the terminal esophagus" (Mosher, 1933), "compression by the aorta" (Handford, 1888), "compression by the diaphragm" (Sauerbruch and von Hacker, 1906), "compression by the liver" (Mosher, 1922), "compression by the lung" (Mosher and McGregor, 1928), "achalasia of the cardia" (Hurst and Rake, 1930), etc.

Of all these, the only mechanism that really exists is the achalasia, i.e. the non-relaxation of the lower esophageal sphincter. Since in the chagasic esophagus the reduction of the number of ganglion cells is the same in the thoracic portion as in the abdominal portion of the organ, an aperistalsis is also present in its abdominal portion. This aperistalsis of the terminal esophagus in fact represents an achalasia of the lower esophagus sphincter, because it does not relax at the exact moment when the thoracic portion of the esophagus contracts. As already stated, there is an aperistalsis of the entire esophagus, including its abdominal portion and the lower esophageal sphincter. The most important lesion is the denervation of the thoracic esophagus and this can be illustrated by two facts: first, many patients with aperistalsis refuse the diagnosis of an obstacle at the end of the esophagus, emphasizing the disfunction of the thoracic portion ("the force is absent", "the food is not driven forward", "the food is not pushed from above", etc.); and secondly, there are cases of megaesophagus in which the cardia functions regularly. These cases always show a slight dilatation of the lumen only. These facts demonstrate on the one hand, that a "hindrance" represented by an achalasic sphincter is not essential for the development of a dilatation in an aperistaltic hollow organ, but show

on the other hand, that a hindrance contributes markedly to the development of monstrous dilatations in cases of advanced megaesophagus.

Other factors also play important roles in the pathogenesis of megaesophagus and other "megas": (1) nature of the contents, (2) time and (3) psychic factors.

Concerning the nature of the contents the consistency of the ingesta is very important, because the transportation of solids needs a better coordinated peristalsis than the movement of liquids through a lumen representing a high overload to the damaged organ. I believe that the increasing consistency (reduced liquidity) of the contents of the esophagus is an aggravating factor in the development of the megaesophagus. This is well illustrated by the patients with megaesophagus who frequently drink large quantities of liquids to aid the passage of the solid ingesta through the organ. The temperature of the ingesta is also of some importance. Generally, either very hot or very cold food increase the difficulties in swallowing, and perhaps the associated abundant salivation constitutes a type of auxilliary mechanism for the deficient deglutition. The significance of the temperature of food and the extraordinary frequency of the dysphagia in chagasic patients is indicated by the stereotyped answer to the physician's inquiry, "But, Doctor, who doesn't have difficulty when eating cold rice?"

With regard to time, it is established that the development of the dilatation is generally a slow progression, taking many years to reach the final stage of megadolichoesophagus (Rezende, 1963). However, a very rapid development of the megaesophagus, corresponding to a severe degree of denervation, may be seen, though rarely. Thus Rezende (1963) observed an acute case of Chagas' disease in a three-year-old child, who immediately after the acute disease had severe difficulties in swallowing and in defecation. Eight months later a surgical operation (cardiomyotomy after Heller) was performed and repeated twice without evident results. The child died after the third operation, about eighteen months after contracting the acute disease. The ganglion cell counting at three different levels of the esophagus (upper, middle and lower portions) gave, as a maximum value, three ganglion cells in a ring of 1 mm thickness. This case demonstrates two important facts: (1) the destruction of the nerve cells occurs during the acute phase of the disease, and (2) the nearly complete denervation may immediately produce a severe dysphagia and in only a few months a marked megaesophagus.

The effect of the psychic factors on the motility of the normal esophagus was convincingly proved by Faulkner (1940), and in cases of "cardiospasm" by Wolf and Almy (1949). In chagasic cases with or without megaesophagus, these psychic influences become strikingly evident, and nearly every patient outlines his own history referring to the aggravation of his sufferings by different emotional conditions. There is one very impressive case of a young man who presented temporarily slight dysphagia, and complained: "Whenever my mother-in-law visits us, I can't even swallow coffee!" Anybody who knows the importance of coffee for a Brazilian will appreciate the great significance of this psychic trauma (Rezende, 1963). Many chagasic patients claim that their esophagus functions like a "barometer" of their mood, and nearly all confirm

that the dysphagia is first observed immediately after emotional stress. Even in non-chagasic cases outside of South America the influence of such stresses is so regularly found as the trigger of the first complaints, that the so-called cardiospasm is by many authors considered to be a psychosomatic disease (Carvalho, 1950; Hoff, 1953; etc.).

(e) *Chagasic megacolon.* The same reduction in the number of ganglion cells with increasing age is found in the colon as in the esophagus (Fig. 14). In my opinion, this finding explains the frequent constipation present in old people. Similar disturbances of the motility of the large intestine with no

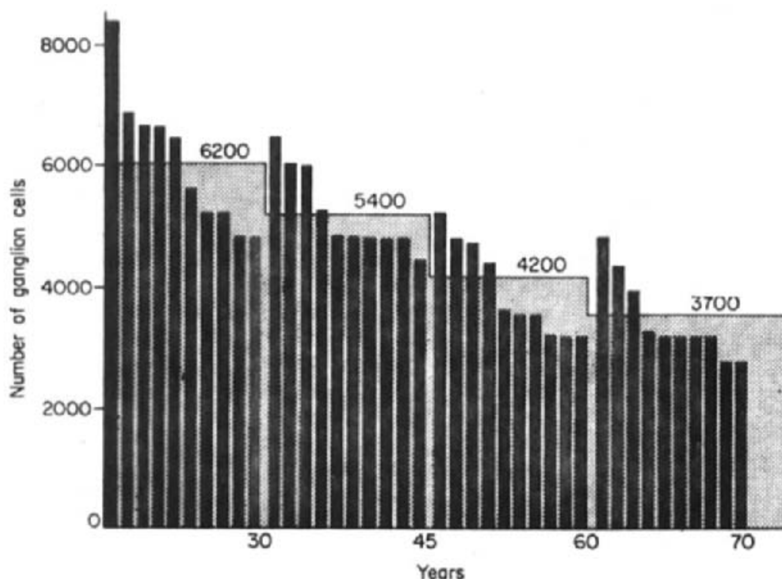

FIG. 14. Physiological diminution of ganglion cells in the colon with increase of age.

dilatations are frequently observed in chronic chagasic patients whose complaints are meteorism, irregularity of peristalsis and difficulties of defecation. These cases without dilatation of the colon, but with a remarkable reduction of the number of nerve cells, can be registered under the term of chronic chagasic colopathy. If the reduction of ganglion cells surpasses a critical limit—55%—dilatation and hypertrophy of the colon are initiated, leading to a megacolon or megadolichocolon (Fig. 15).

The main symptom of megacolon is a difficulty or an impossibility of evacuation. Patients who do not defecate for 2–4 months are not rarities and in some cases retention of feces may even last 5–6 months. It is remarkable that many such patients feel no discomfort, do not complain about indisposition and come to the hospital because of some complication. These complications are (1) volvulus of the dilated sigmoid loop, (2) formation of fecaloma with decubital ulcer, (3) perforations of this ulcer associated with peritonitis, and (4) very rarely, primary perforation without previous ulceration. The macroscopic findings at autopsy or during the operation are impressive because of

the dilatations, which may sometimes reach gigantic dimensions with a capacity of 30–40 liters. The thickness of the intestinal wall attains about 0·5 cm and the length of the dilated colon may surpass 2 meters.

It is evident that a denervated organ becomes dilated only upon functional solicitation. This can be demonstrated frequently in cases of megacolon which need a transversostomy to reduce the enormous dilated sigmoid before the

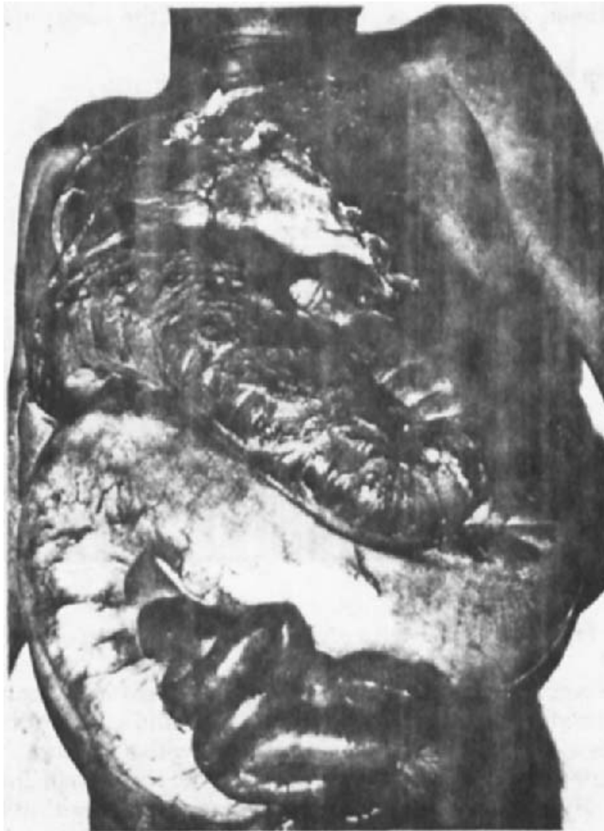

FIG. 15. Megadolichocolon in a chagasic patient.

definitive sigmoidectomy. When the surgeon, some weeks later, sends the surgical specimen to the pathologist, the previous dilatation has almost subsided and only the thickening of the muscle layer (which may reach 1·0 cm or more) indicates that we are dealing with a megacolon.

Figure 16 shows the different degrees of denervation in 40 chagasic cases with and without megacolons. The denervation is also uniform in the entire length of the intestine and the appendix, a finding completely different from the alterations encountered in Hirschsprung's disease (Köberle, 1957, 1962; Costa

1963a, b; Brandão and Köberle, 1964). The supersensitivity of the denervated large intestine to cholinergic drugs was clinically demonstrated (Vieira *et al.*, 1964b).

(f) *Other "mega" formations.* As was shown in Fig. 6 and Table I, the incidence of "megas" in other segments of the digestive tract is relatively rare in spite of the sometimes marked denervation in this part (Costa and Alcântara, 1965, 1966). The liquid contents in the stomach, small intestine and biliary tract, may militate against marked dilatation.

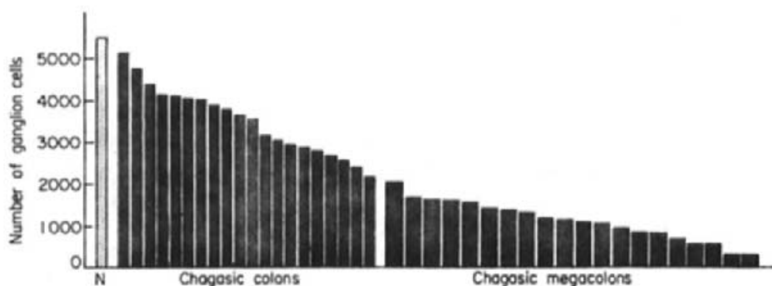

FIG. 16. Results of ganglion cell counting in normal colons (*N*—normal average), in chagasic colons without macroscopic alterations, and in chagasic megacolons.

2. *Respiratory tract*

In the respiratory tract, in cases of chronic chagasic patients, dilatations of the trachea (megatrachea) and bronchiectasis are also found (Köberle, 1959). It seems remarkable at first sight that even hollow organs, transporting gaseous contents and without any sphincter, may be the seat of dilatations after denervation. However, these findings demonstrate that even in the respiratory tract, where air is apparently passively transported, its movement—inflow and outflow—is controlled by precise nervous coordination of the muscular contraction of the trachea and bronchi walls. The neurogenic bronchiectasis in chagasic patients is well pronounced but never very impressive. Therefore, it is easily overlooked at autopsy. Even in my Department—where we always search for respiratory manifestations of Chagas' disease—the frequency of bronchiectasis is about 5 %, while a previous bronchographic study in chagasic patients in our University Hospital reaches 45–65 % (Souza, 1966).

Investigations into disorders of the motility of the bronchi gave a much higher incidence of "aperistalsis" of the bronchi (Godoy, 1963). The Mecholyl-test demonstrates clearly the supersensitivity of the denervated bronchi (Manço *et al.*, 1967).

In apparently asymptomatic cases the percentage of bronchopathy was 29 %, in cases of megaesophagus 50 %, and in cases of megaesophagus and megacolon 80 %.

The results of ganglion cell counts indicate that chagasic bronchopathy is characterized by a numerical diminution of less than 75 % and that bronchiectasis occurs with a reduction higher than 75 % (Köberle, 1962).

3. *Urinary tract*

In our autopsy material aperistalsis of the urinary tract with megacystis and megaureter is very rare. We have found only three cases of megacystis and two cases of megaureter. Clinically the urinary bladder of the chagasic patients better supports an abrupt instillation of fluid than the normal one, as revealed by cystometry (Ciconelli, 1963).

Ganglion cell counts have not been made in human beings. The diminution in number of ganglion cells of the hypogastric plexus in experimentally infected rats is about 45 % (Ciconelli, 1964).

B. CARDIOPATHY AND CARDIOMEGALY

Chronic chagasic cardiopathy is the most frequent Chagas' syndrome and also the commonest cause of death in chronic chagasic patients. In our autopsy material, of nearly 1 000 cases, 87 % of the deaths were due to cardiopathy, with higher incidence in males (about 66 %) (Fig. 17).

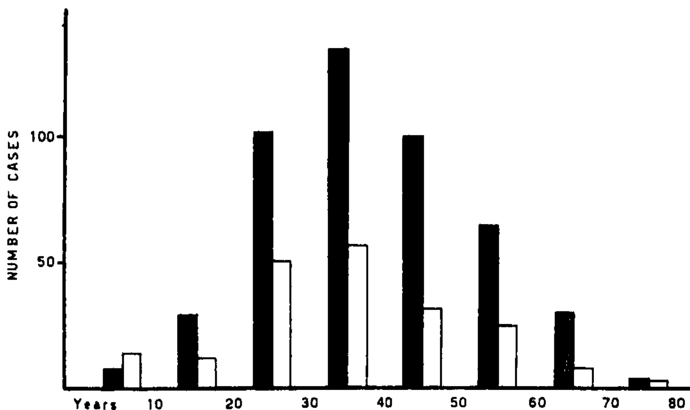

FIG. 17. Frequency of cardiopathy in males (black) and females (white) according to age.

1. *Symptomatology*

The cardiopathy was characterized by Chagas as a heart disease with changes in the formation and conduction of stimuli, constituting "a new chapter in human pathology". In his publications with Villela (1922), the following symptomatology was reported:

(a) Tachycardia and bradycardia.
(b) Disturbance in the pathway of the conduction:
  (i) Retardation of the conduction (prolonged P–R interval)
  (ii) Partial block
  (iii) Total block (dissociation of the rhythm of the atria from the ventricles).
(c) Premature beats
  (i) Atrial extrasystoles
  (ii) Ventricular extrasystoles.

(d) Atrial tachysystolia ("atrial flutter").
(e) Paroxystic tachycardia (atrial, ventricular and nodal).
(f) Complete arhythmia (atrial fibrillation).
(g) Alternans.

The rural people of the endemic areas have long known this peculiar heart disease with its characteristic rhythm alterations, and even name them: "Baticum" (palpitations), "Vexame do Coração" (heart irritation), "Coração de Boi" (ox heart), etc. They are also well aware of the rough symptomatology which consists of attacks of an inexplicable "shooting" of the heart beats, dizziness, blurred vision, loss of consciousness, and episodes of heart standstill. All these symptoms are frequent in young people, and sudden death occurs in a great number of cases. Oddly enough, in certain regions where "Baticum" is common, barkeepers do not sell alcoholic drinks upon trust for fear of losing their money. Because of the frequency of sudden cardiac death in young men, there are villages in endemic areas officially named "Recanto das Viuvas" (Widow Villages). Football teams always have reserves because not infrequently some of the players die suddenly during the match.

The cardiac manifestations appear generally after a long asymptomatic period varying from 10 to 20 years. We know very little about this latent period, because the patients have no complaints and do not seek medical assistance. From a group of 57 patients observed during 10 years after the initial acute infection, 40 showed a normal EKG and 17 developed EKG abnormalities which were transient in 5 and permanent in 12 (Laranja *et al.*, 1956).

In chronic Chagas' heart disease signs and symptoms do not appear always simultaneously. Thus we can find cases with advanced cardiomegaly and severe EKG changes with no subjective complaints. The initial symptoms are palpitations, a certain precordial discomfort—different from angina pectoris—and dizziness. In a later stage, paroxysmal tachycardia, marked bradycardia and episodes of multifocal extrasystoles are common. Sometimes bradycardia may cause temporary cardiac standstill and epileptiform seizures. Right heart failure is more common than left heart failure and not secondary to the latter (Lima and Rassi, 1962).

The systolic blood pressure is normal or lowered; and the pulse pressure is sometimes markedly reduced.

The EKG findings are very characteristic of the chronic chagasic cardiopathy and very probably permit diagnosis. Another characteristic feature is the "mutability" (Brasil, 1953) of the EKG alterations, the great variations occurring mainly in conduction system. In the different Latin American countries where Chagas' disease is endemic, the various types of EKG changes and its incidence are very similar.

Among these EKG changes, ventricular extrasystoles (60·67 %), right bundle branch block (43·20 %) and A-V blocks (17·93 %) are the most frequent (Lima and Rassi, 1962).

Cardiac death may occur from cardiac failure, but the most characteristic death is the sudden and unexpected one. Chagas himself had reported that in Lassance (Minas Gerais, Brazil), where he discovered the disease, almost every family lost one or more of its members by this typical sudden heart death. One

would expect that sudden death would occur during excessive physical effort—such as football, or any other overwork—but it is strange that in most cases this type of death takes place under emotional stress. It is very common for the cardiac patient to come to the hospital in a decompensated state; he is treated and compensated, and when he is just about to leave the clinic he falls down and dies at the hospital door.

2. *Pathology*

The gross pathology of the chronic chagasic cardiopathy is variable from apparently normal findings to enormous cardiomegalies. However, a special preparation is essential to demonstrate the characteristic lesions. In order to obtain a good anatomical specimen we use the following method:

(i) Removal of the heart by sectioning the blood vessels as near as possible to the pericardial sac.
(ii) Careful washing of the heart chambers in the direction of the blood stream, with running water, removing all blood.
(iii) Ligature of all pulmonary and caval veins.
(iv) Injection of 5% formalin solution, at a pressure of about 100 mm of Hg, through the aorta with the canula introduced beyond the aortic ostium into the left ventricle. When the left heart chambers are well distended, the canula is removed. Now the coronary system is injected. Ligature of the aorta follows. The right heart chambers are injected in the same way and the pulmonary artery tied. The distended heart is immersed in a tub containing 5% formalin solution for 24 hours.
(v) The fixed heart is divided by a frontal section, beginning at the apex and proceeding to its base, into anterior and posterior halves. This section accompanies the margo obtusus and the margo acutus, dividing the mitral and tricuspid ostia, and also the corresponding atria, into anterior and posterior parts.

(a) *External inspection of the heart.* Chagasic hearts may appear quite normal in size and form. However, the great majority show marked alteration of size and frequently changes in form too. We may find all degrees of enlargement of the organ, predominantly of the right heart and strikingly of the right atrium. Monstrous cardiomegalies can be found. The heart can become almost spherical with separation between the right and left apex (cor bifidum), and bulging of the conus arteriosus and circumscribed swellings of the apical region, more frequently on the left than on the right side. Sometimes there are either large white plaques in the pericardium or tiny white granules along the coronary vessels (fibrous pericarditis in rosary). Thinning of the muscle wall of the ventricles and principally of the right atrium is very characteristic. Transillumination shows the translucent areas very distinctly (Fig. 18).

(b) *Internal inspection of the heart* (Fig. 19). In the great majority of cases the chagasic heart shows dilatation of all chambers, predominantly on the right side, and—almost always excessively pronounced—of the right atrium, which appears translucent. The thickness of the wall of the ventricles is variable,

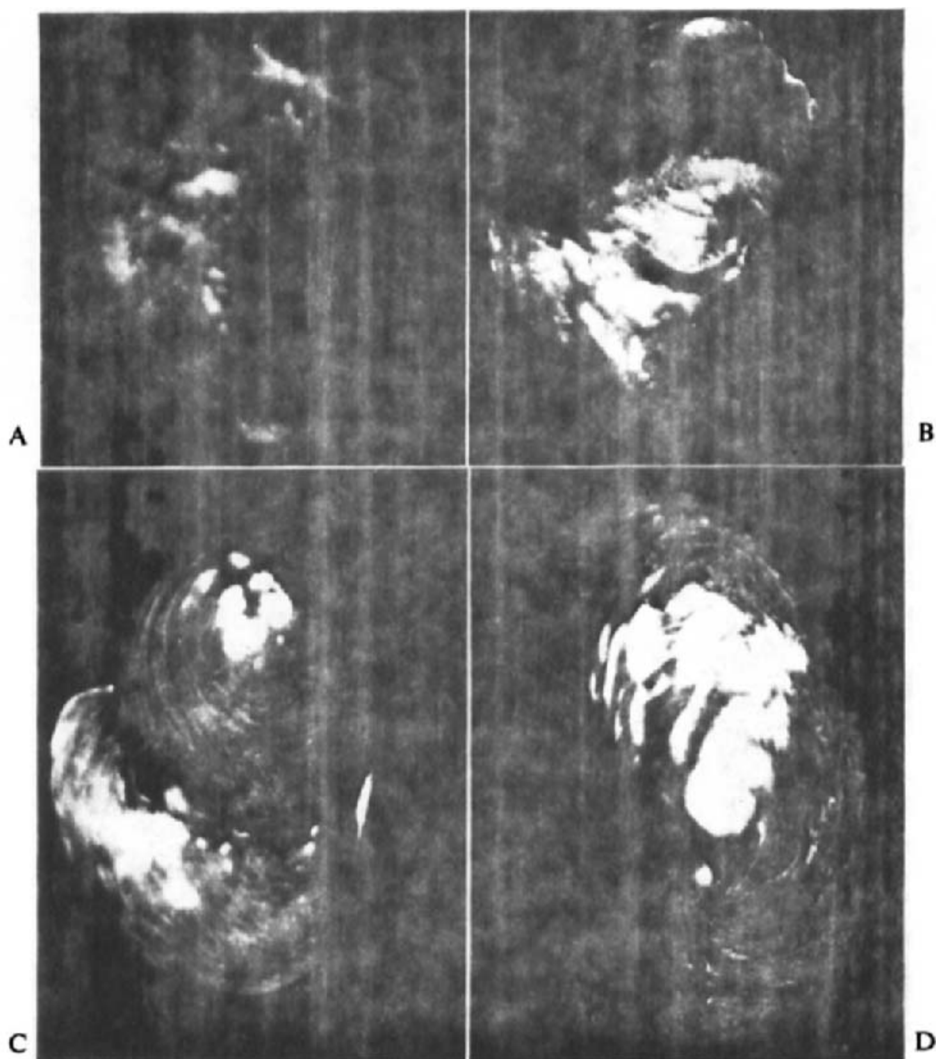

FIG. 18. Transillumination of chagasic hearts, showing the thinning of the muscle wall. A—cor bifidum with aneurysm at the left apex and thinning of the conus arteriosis. B—thinning of the inferior part of the anterior wall, right ventricle. C—aneurysm at the left apex, thinning of the wall of the conus arteriosus. D—small aneurysm at the left vortex and large aneurysm of the right ventricle.

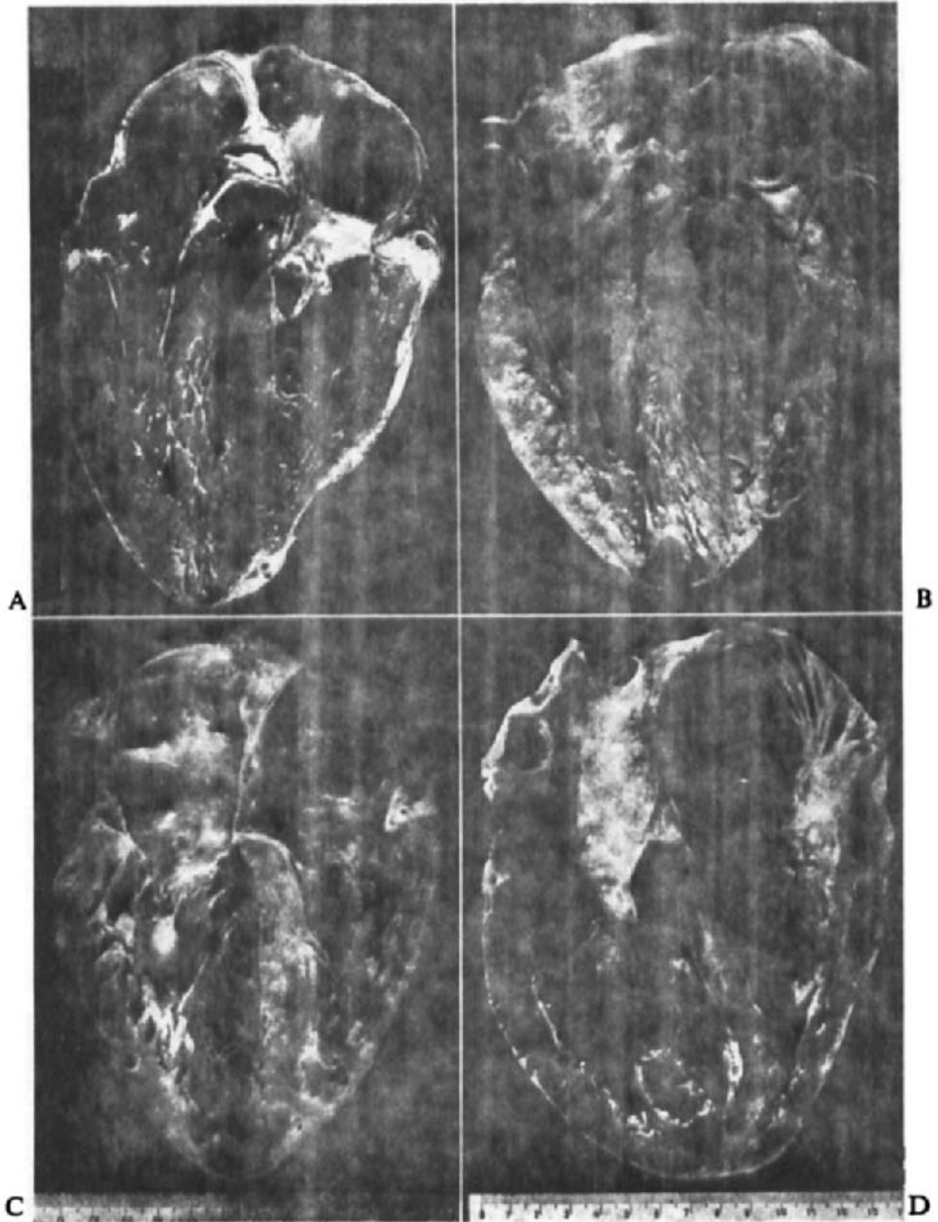

FIG. 19. Frontal section of chagasic hearts. A—small aneurysm at the left apex and slight hypertrophy of both ventricle walls. B—moderate aneurysm at the left apex and beginning aneurysm at the right ventricle, global hypertrophy and dilatation of the right atrium with thrombosis of the auricle. C—aneurysm of the left ventricle. D—cardiomegaly with global dilatation and parietal thrombosis in the left ventricle and right auricle.

according to the degree of the dilatation of the cavities, and to the secondary degenerative lesions of the myocardium. More than half of the chagasic hearts disclose a very odd alteration at the apex, which seems to be almost pathognomonic for the Chagas' cardiopathy, since it has never been found in any other cardiopathy. This lesion consists of a thinning and bulging of the apical region, mainly on the left ventricle, and it is known variously as: area of infarct in the apex of the left ventricle (Montes Pereja *et al.*, 1938), ventricular aneurysm (Romaña and Cossio, 1944; Moia *et al.*, 1955; Köberle, 1959), fibrous nodule of the apex (Ferreira-Beirutti, 1947), thinning of the apex (Freitas and Lima, 1950; Capriles *et al.*, 1962), necrotic lesion of the apex (Carvalhal *et al.*, 1954), apical lesion (Andrade, 1956), fibrosis of the apex of the left ventricle (Laranja *et al.*, 1956), atrophic lesion of the apex (Mignone, 1958) and vorticillar lesion of the apex (Raso, 1964).

I have adopted the term "aneurysm" for this lesion.

The diameter of this aneurysm never surpasses 5 cm; its wall may consist of the endocardium and pericardium only and then appears translucent. Similar aneurysmatic dilatation may occur less frequently at the right apex and rarely in the posterior wall of the left ventricle. Thrombosis of the aneurysm is very common. Even without aneurysm, extensive mural thrombosis in the lower part of the left ventricle may be seen, and in cases with cardiomegaly a massive thrombosis of the right auricle is almost obligatory. All phases of thrombi organization and final hyalinization can be found in the same heart. Thrombosis in the left auricle is uncommon. The presence of thrombi explains the so frequent thromboembolic phenomena occurring in the pulmonary and/or in the systemic circulation (Nussenzweig *et al.*, 1953; Andrade, 1959).

The myocardium of the lower part of the ventricles shows small fibrotic scars, rarely occupying large areas (as after myocardial infarction). Marked atrophy and slight fibrosis on the trabecula carneae of both apical regions, as well as in the papillary muscles of the left ventricle in cases of accentuated cardiomegaly, are frequent.

Extensive thrombosis of the lower half of the left ventricle almost completely replaced by fibrous tissue may give the impression of an endomyocardiofibrosis. However, while in the latter condition the fibrotic lesions tend to reduce the cavity beginning at the apex, in the former there is a tendency to enlarge the cavity in the apical portion.

The coronary arteries are dilated and with no arteriosclerotic alterations.

(c) *Histological findings.* On microscopic examination the myocardium shows hypertrophic muscle fibers intermingled with some atrophic muscle fibers and generally very discrete interstitial fibrosis. In some instances the fibrotic foci may appear to a greater extent, especially in the lower part of the ventricles and in the wall of the right atrium. Small inflammatory foci of monohistiocytic type and rare granulomas indicate the persistence of the parasitic disease. Beside these inflammatory reactions there may be found—not always but frequently—areas of intense lymphohistiocytic infiltration, sometimes containing certain numbers of eosinophils. Sarcolemma sheaths filled with inflammatory cells can be seen inside these infiltrates. Nests of leishmania may (rarely) be found in the center of the circumscribed or granulomatous inflam-

matory foci, but never in these large, more diffuse lymphohistiocytic infiltrates. It seems, therefore, that these latter reactions are not directly connected with the parasites. Parasitic pseudocysts are extremely rare and found only through the examination of a great number of histological sections.

The conduction system shows the same inflammatory and fibrotic lesions found in the myocardium (Andrade and Andrade, 1955; Köberle, 1957; Torres and Duarte, 1961; Oliveira, 1967).

The parasympathetic ganglion cells of the intrinsic cardiac nervous system reveal a marked numerical reduction (Köberle, 1956, 1957, 1959; Mott and Hagstrom, 1965; Reis, 1965).

Figure 20 shows the same phenomenon already demonstrated in the esophagus, i.e. an increase with age in the average number of ganglion cells.

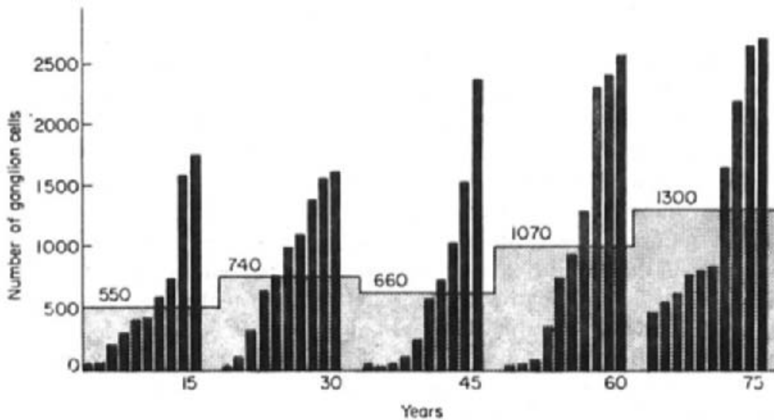

FIG. 20. Results of ganglion cell counting in 50 cases of Chagas' cardiopathy, showing an increase of the number of ganglion cells with age.

3. *Pathogenesis*

The symptomatology and pathology of the chronic chagasic cardiopathy are, without doubt, very unusual. In spite of the impossibility of explaining either the symptoms or the morphological findings as an inflammatory process of the myocardium, this heart disease was long, and is still regarded by many authors as a "chronic myocarditis". I consider the chagasic cardiopathy to be a neurogenic heart disease caused by the destruction of the ganglion cells in the heart.

As in other hollow muscular organs, we find in the chagasic heart a dilatation and hypertrophy without any plausible explanation. The blood pressure is surely not the cause of this hypertrophy because it is normal or lowered in chronic chagasic patients. Nor do the cardiac valves show alterations. The chagasic heart is deprived of its nervous control, losing the capacity of adaptation to the changes required by the environmental influences on the circulatory system as a whole. This incapacity of adaptation results in an increase of the residual blood in the heart chambers, i.e. a gradual dilatation of the cavities,

and therefore a distension of the heart muscle fibers. This distension finally leads to a hypertrophy of heart musculature. The same vicious circle of dilatation and hypertrophy prevails as was mentioned when discussing the pathogenesis of megaesophagus. Therefore, we are facing a "neurogenic dilatation and hypertrophy" of the heart.

Costa (1963a) demonstrated that the hypertrophy of the chronic chagasic heart is a consequence of its denervation. In his investigation, normal, chronic chagasic and atropinized rats were studied at rest and after being submitted to forced swimming during 3 weeks. In the group at rest no significant differences of the heart weight was noted. However, after swimming a significant difference of the hypertrophy of the heart was observed in all groups of rats (normal, chagasic and atropinized). The hypertrophy of the heart in the chagasic group was more marked than that of normal rats and still more marked in the atropinized group. This experiment demonstrates clearly that the hypertrophy of the heart is in accordance with the degree of its denervation, partial in the chagasic and complete in the pharmacologically denervated rats.

In most cases the hypertrophy and dilatation of the right heart is more pronounced than that of the left, and mainly the dilatation of the right atrium. This is understandable because the right heart is the first to receive the peripheral blood inflow and because it is not able to adapt itself to the variations of input. In most cases the symptomatology of the chagasic cardiopathy begins during the third decade, although the denervation occurred generally in the first years of life. We believe that the extraordinary capacity of adaptation of the infant's heart may explain this relatively long, asymptomatic interval.

The most impressive and characteristic morphological finding is the so-called aneurysm of the apex, already observed by Chagas (1916). There are three principal hypotheses attempting to explain the mechanism of its formation: (1) hypoxemic, (2) inflammatory and (3) mechanical.

Hypoxemic mechanism. The thinning of the wall is caused by ischemic necrosis or necrobiosis of the musculature. This ischemia was explained through lesions of the coronary vessels (Laranja *et al.*, 1951; Carvalhal *et al.*, 1954), associated with other factors. "In the enlarged heart, the vascular lesions and the dynamic factor usually operating in this condition (reduced systolic and pulse pressure, diminution of the capacity of the myocardium to raise systolic pressure, occurrence of frequent ectopic inefficient contracture) may result in coronary insufficiency" (Laranja *et al.*, 1956). Andrade and Andrade (1955) considered the parietal thrombosis in the apical region, which occludes the Thebesian veins, the cause of the ischemic necrosis at the apex. Köberle (1957, 1962) explained the hypoxemic necrosis through a relative coronary insufficiency. However, an analysis of 400 cases revealed that the hypoxemic alterations do not increase with the increase in weight of the muscular mass. So it seems more probable that the hypoxemia is a consequence of the disturbed innervation of the heart vessels. A definite proof of this mechanism does not yet exist.

Inflammatory mechanism. This hypothesis was used by Moia *et al.* (1955) and Mignone (1958) to explain the thinning of the wall. The destruction of the muscular wall by the inflammatory process is the primary factor involved. Local substitution by fibrous scar tissue follows, lessens resistance and favors the development of the apical lesion.

Mechanical hypothesis. According to Raso (1964) various conjoint factors (inflammation, denervation, diminution of muscular tonus) contribute to the formation of the "vorticillar lesion". The decisive factor would be a mechanical one causing the disappearance of the muscle at the vorticillar region through dislocation of the spino-spiral and bulbo-spiral muscle bundles. After this separation the endocardium becomes herniated and fuses with the epicardium.

Nowadays we believe that none of the above-mentioned theories explains satisfactorily the apical lesion. In fact, the "apical aneurysm" is not exclusively found at the apex, but sometimes in the anterior wall of the right ventricle as well as in the posterior or lateral wall of the left ventricle. Anyhow it is difficult to imagine a dislocation of the muscle bundles at these localizations.

Besides this very distinct apical lesion, there is another one, which sometimes is evident at first sight, and sometimes is only revealed after removal of the subepicardial fat tissue. It is the separation of the musculature of the left and right ventricle, originating an aspect of "cor bifidum". It seems to me that this separation into left and right heart is the morphological expression of the independent function of each ventricle, which results from the denervation. In about 50% of the cases of chagasic cardiopathy there is a right bundle branch block, which evidently disturbs the synchronous pulsation of the heart. Besides this, it was shown that the intact sympathetic innervation of the heart is essential for the synchronous contraction of the two ventricles (Priola and Randall, 1964). Recent studies of chagasic patients with and without cardiopathy, carried out in our University Hospital by Amorim *et al.* (1967) using several specialized methods and techniques, demonstrated that the chagasic heart without apparent cardiopathy behaves as a parasympathetic denervated organ. Hearts of chagasic patients with cardiopathy revealed a parasympathetic and sympathetic denervation. We have found that in chagasic patients with cardiopathy there are severe lesions and a numerical diminution of ganglion cells in the stellate ganglia.

The most characteristic EKG findings in chagasic cardiopathy are "alterations of the formation and conduction of stimuli" (Chagas and Villela, 1922). It is astonishing that in spite of this very categoric and precise characterization of the chagasic heart disease, no systematic studies of the conduction system of the heart have been attempted. Systematic examination of more than 50 chagasic hearts in my Department (Oliveira, 1967) revealed a discrepancy between the EKG findings and the histological alterations in several cases. Cases with complete A-V block did not show morphological alterations in the bundle of His which could explain the interrupted conduction. These negative results were not surprising since we know that mutability of the EKG changes (Brasil, 1956) is so characteristic of the chagasic cardiopathy. Therefore, the various blocks which appear and disappear at short intervals could not be caused by a definite morphological interruption of the conduction system. The mechanism of the EKG changes needs further investigation, mainly with regard to the influences of the intrinsic nervous system upon the conduction of stimuli.

Summarizing, we can say that the neurogenic chagasic cardiopathy is caused by more or less marked parasympathetic and sympathetic denervation, and as I indicated throughout this exposition, there are many obscure points in the

complex pathogenesis of this disease, which still represents a "new chapter of human pathology".

C. EXOCRINOPATHIES

It is obvious that a disease which alters such different territories of the nervous system can also provoke disturbances in the function of the glands which are under the control of the autonomic nervous system. There are only a few studies about clinical and morphological manifestations of the exocrine glands, some of them originally described by Chagas.

1. *Sialoadenopathy*

Chagas and Villela (1922) observed a symmetrical enlargement of the parotid gland which gives the patient's face a very typical configuration ("cat's face"). This hypertrophy is very frequent in cases of megaesophagus (33% of 1514 cases reported by Mineiro, 1958). The enlargement of the parotid gland with sialorrhea was considered a result of the difficulty in swallowing. Nowadays we know that this hypertrophy of the parotid has the same pathogenetic mechanism as the other Chagas' syndromes, i.e. a parasympathetic denervation. The hypertrophy was verified histologically by Vieira and Hadler (1961). Hyperamylasemia is a common finding in these patients (Vieira, 1961), and pharmacological studies (Vieira, 1964) showed that the salivary glands respond excessively to the application of pilocarpine, according to Cannon's law.

An interesting investigation on patients with megaesophagus and hypertrophy of the salivary glands was performed by Vieira *et al.* (1962). In these patients a subtotal esophagectomy (after Câmara Lopes) was performed and they were fed through a gastric tube. In the first post-operative period a marked decrease in volume of the previous enlarged parotid gland and a fall of serum amylase were observed. When in a second operation a cervical esophagogastric anastomosis was performed and the patient was able to eat by mouth, the parotid glands enlarged again and the serum amylase increased. These observations demonstrate clearly that the salivary glands react to oral stimuli and not to reflexes originating from the esophagus. All salivary glands may show enlargement but this manifestation is more pronounced in the parotids.

2. *Hydroadenopathy*

A typical complaint of chagasic patients is an increased sudoresis and, sometimes, sweating episodes without any apparent cause. Vieira (1963) analyzed sweating from the quantitative point of view, using pilocarpine subcutaneously as the activating drug. He found a marked increase of sweating in chronic chagasic patients when compared with a group of normal individuals.

3. *Gastropathy*

A gastropathy in chagasic patients with a vague symptomatology, i.e. difficult digestion, sensation of plenitude, was described by Porto (1955). These complaints are partly caused by denervation of the gastric musculature which leads to difficulty in emptying, megagastria or "stomach in cascade" (Rezende,

1959); and partly by denervation of the gastric glands responsible for the associated hypochlorhydria (Vieira *et al.*, 1964a).

It is interesting that the denervated gastric glands do not show the expected supersensitivity of the denervated structures which could result in hyperchlorhydria. It seems that this fact is apparently an exception of Cannon's law of denervation. However, studying the gastric mucosa in chronic chagasic patients one finds a dedifferentiation of the epithelium and the fundic glands of the stomach, i.e. intestinalization of the gastric mucosa. The same phenomenon occurs in the high differentiated muscle fibers which fibrillate after denervation until their dedifferentiation, further behaving like smooth muscle fibers. The parietal cells of the fundic glands are highly differentiated, and it is probable that they become dedifferentiated like the striated muscle fibers.

D. ENDOCRINOPATHIES

Manifestations of the endocrine system were described by Chagas (1911), but they were not recognized for many years. Only recent systematic investigations on chagasic patients demonstrated alterations of glucose tolerance test (Reis, 1965) and variations in iodoproteinemia (Lomonaco, 1962). Histopathological studies have not yet been performed. It is known that abortion and premature birth are frequent in pregnant chagasic patients (Oliveira, 1958; Freitas, 1966), but we do not know the mechanism of such disturbances. Concerning the gonads an interesting study in patients with megacolon was made by Haddad *et al.* (1959). After rectosigmoidectomy many patients complained of impotency (males) and frigidity (females). The authors were able to verify that in most such cases these disorders were present long before surgical intervention and in only a few cases was there aggravation of the disturbance. Thus the symptomatology has to be ascribed to Chagas' disease itself. Studies in infected male animals showed diminution in the volume of semen (Ferreira and Oliveira, 1965) and severe alterations of the innervation of the testicles with reduction or inhibition of spermatogenesis (Ferreira, 1967).

E. ENCEPHALOPATHIES

Chagas (1913) described more than 200 cases of neurological manifestations of central origin and called this special chronic form of the disease "Forma Nervosa". Chagas referred to spastic diplegia, dysbasia, athetosis, aphasia, cerebellar ataxia, pseudobulbar paralysis, idiocy, infantilism, etc. and wrote:

> Concerning the frequency of the nervous forms of the trypanosomiasis we have made numerous observations which authorize us to affirm that this disease might be the one that, in human pathology, provokes the largest number of organic changes in the central nervous system.

Very few observations were reported afterwards (Moreira, 1925; Austregésilo, 1927; Borges-Fortes, 1942; Käfer *et al.*, 1961). During the last decade this aspect of the *T. cruzi* infection was studied in animals infected experimentally. Depopulation in nerve cells of the diencephalon and cerebellum was noted by Jardim (1962). Brandão and Zulian (1966) verified a diminution of the number

of Purkinje cells in the cerebellum in chronic chagasic patients. In one case with cerebellar symptomatology the diminution was 95 % of the average number found in normal cases. Alencar (1964) observed a reduction in the number of ganglion cells in the cerebral cortex in chronic chagasic cardiopaths, attributing his findings to a chronic hypoxemia of the brain. I cannot agree with such an interpretation, because ganglion cell destruction during acute Chagas' disease is nowadays sufficiently proven. Recently Armbrust-Figueiredo (1967) called attention to the difficulties of diagnosis of the central nervous system form and to the necessity of performing systematic studies in this forgotten field.

F. MYELOPATHIES

Observations on Chagas' myelopathy were done only in experimentally infected rats by Schwartzburd and Köberle (1959), and they found a marked reduction of the ganglion cells, principally in the lumbar segments of the spinal cord. Pains and some weakness of the musculature of the legs are frequently observed in chronic chagasic patients and are probably related to nerve-cell lesions in the spinal cord. Systematic studies are needed to evaluate the importance of the involvement in this part of the nervous system.

With the enumeration of these syndromes, I have not exhausted the symptomatology of American Trypanosomiasis. Various metabolic alterations and disturbances of intestinal absorption have been studied (Vieira *et al.*, 1966; Campos and Cançado, 1962; Reis and Vichi, 1965; Vieira and Meneghelli, 1966; Meneghelli and Reis, 1967), indicating that practically all homeostatic mechanisms of the organism may be altered to some degree by the *T. cruzi* infection.

VII. Conclusions

The discovery of American trypanosomiasis by Carlos Chagas represents one of the most fascinating events in the history of medicine.

As a rule, the etiological agent sought and demonstrated after a disease is already well known. Peculiarly enough, the reverse path was followed by Chagas! He discovered a parasite in the intestine of a blood-sucking bug, which suggested to him, through an unexplained lucubration, the possible existence of a new disease of animals and human beings sucked by the supposed transmitter. It is almost incredible that such severe pathological manifestations, which represent the "causa mortis" of one third of our autopsy material, would have been overlooked or unknown before Chagas' unique discovery. In fact, many of these manifestations were well known long before the discovery of American trypanosomiasis and not etiologically correlated with it after its discovery. It is interesting that one of these manifestations, which in chagasic etiology was most obstinately denied and is disputed even today—namely the megaesophagus—had already been described (250 years ago) as a widespread plague in the State of Pernambuco (Brazil).

We owe our first knowledge about megaesophagus to the famous English scientist Sir Thomas Willis (1670), who described in his "Pharmaceutice Rationalis" a single case of this syndrome. A second isolated case was published by Blasius (1677), the translator of Willis' work. Whereas in the following two centuries cases of megaesophagus appeared now and then as rarities in the European medical literature, the Brazilian quack Miguel Dias Pimenta (1707) gave a minutiose description of this frequent disease in Pernambuco, recommending its prevention and treatment, considering it to be of parasitic etiology. References on dysphagia as endemic disease in various parts of Brazil are frequent in medical and lay literature, under several peculiar denominations.

A report on Chagas' disease will be incomplete without reference to the most illustrious victim of this illness. Even knowing that it is impossible to prove, we are convinced that Charles Darwin acquired Chagas' disease in Argentina or Chile during his scientific expedition and suffered from Chagas' syndrome until his death. This opinion was suggested first by Adler (1959) and reinforced by Kohn (1963). Those who are acquainted with the biography of Darwin on the one hand and with the various complaints of chagasic patients on the other, accept without hesitation the "posthumous" diagnosis for Darwin's chronic ill-health. The history of Darwin's disease is impressively similar to many histories of chagasic patients; it happens even today that their vague, singular and intense complaints are not properly evaluated and are simply taken to be psychoneurotic symptoms. However, physicians working in endemic areas and familiarized with the plague, consider the chagasic patient as a neurotic. Some skilful physicians are even able to diagnose the disease according to the patient's behavior when entering their office. In fact, the chagasic patient presents a pleiomorphic symptomatology from subtle to severe homeostatic disturbances, turning him into an apparently "neurotic" individual. However, through our investigations we have demonstrated that all these alterations have morphological bases characterized by nerve cell damage of varying degrees. Thus the chagasic patient is really a neuropath, i.e. an individual with well defined organic lesions in different territories of his nervous system.

Finally, American trypanosomiasis shows us a very peculiar pathology of homeostasis of the human organism, and represents even today a new realm in pathology, which becomes understandable through Cannon's law of denervation.

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Trichomonas vaginalis and Trichomoniasis

OTTO JÍROVEC and MIROSLAV PETRŮ

Zoologický Ústav Přírod Fakulty Úniversity Karlovy, Prague, Č.S.S.R.

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I. INTRODUCTION

Trichomonas vaginalis was described by Donné in 1836 and has long been regarded as a harmless commensal of the human vagina. In 1916 Höhne described as a clinical unit the so-called "Trichomonadenkolpitis = trichomoniasis" of women whose vaginae contained many of these flagellates. The

concept of *T. vaginalis* as a primary pathogenic parasite was only gradually accepted. In central Europe credit is due to Rodecurt, although various ideas of his on the resistance of *T. vaginalis* were not substantiated. Without a doubt, *T. vaginalis* is the commonest of the human trichomonads and "Trichomonadenkolpitis" does exist despite the sceptical adverse views of some prominent older gynaecologists. The literature on *T. vaginalis* and trichomoniasis is vast. The first monograph (Trussell, 1947) lists 1586 references. At present, the number of publications on *T. vaginalis* far surpasses 3000. The three symposia dedicated to this subject (Monaco, 1954, "Symposium sur les uréthritides non gonococciques"; Reims, 1957, "Symposium international sur les infestations à trichomonas"; Montreal, 1959, "First Canadian symposium on non-gonococcal urethritis and human trichomoniasis" published 1960) confirm the world-wide interest taken in this human infection. In recent years, three symposia were also arranged in Poland by the Polish Parasitological Society (Olsztyn, 1961; Lublin, 1963; Bialystok, 1965). Additional monographs on this subject are by R. Peter, "Nákaza bičíkovcem poševním u dětí, panen a mladistvých", Prague 1945; J. Okla, "Rzesistkowe zapalenie pochvy i jego leczenie", Warszawa 1954; J. M. Bedoya, "Tricomonas sexual humana", Valencia 1959; N. E. Sidorov, A. M. Korchemkin and A. P. Kolesov, "Trichomonaz močepolovych organov čeloveku", Moscow 1959. Many comprehensive articles have been published in various medical journals, but in most textbooks on parasitology and gynaecology, little attention has been given to the problems of *Trichomonas vaginalis*. An exception is the textbook by Jírovec, "Parasitologie für Ärzte" (Jena, 1960), in which a chapter (26 pp.) is devoted to trichomoniasis. On *T. vaginalis* in the human male there is a monograph by Veynerov and Rozhinski, "Trichomonadny uretrit muzhchin", Kiev, 1956; and a comprehensive study by J. Jíra (1961), "Studie o mužské trichomoniase", with a complete bibliography. An abstract of this paper was published earlier (1958) in *Zentbl. Bakt. Parasitk.* I. *Abt. Orig.* **172**, 310–329.

The present review deals with more recent knowledge of the morphology and biology, pathology and laboratory diagnosis of *T. vaginalis*, with clinical aspects of trichomoniasis in women, men and children, and with the epidemiology and treatment of this infection. The older literature (up to 1947) can be found in the monograph by Trussell (1947), more recent literature in the Proceedings of the two Symposia (Reims, Montreal). We have generally considered more recent papers, although some of them were inaccessible and in others we found a considerable overlap. Nevertheless, 30 years of personal experience of research on *T. vaginalis* and trichomoniasis provide justification for critical appraisal of the various problems discussed.

II. Taxonomic Position of *Trichomonas vaginalis*

The flagellate *T. vaginalis* belongs to the superclass Mastigophora Diesing, 1866, class Zoomastigophorea Calkins, 1909, order Trichomonadina Kirby, 1947, family Trichomonadidae Chalmers and Pekola, 1918, emend. Kirby, 1946. Honigberg (1963) divided this family into two subfamilies: Trichomonadinae, receiving the genera *Trichomonas* and *Pentatrichomonas*, and the

subfamily Tritrichomonadinae with the single genus *Tritrichomonas*. The characteristics of these three genera are given below.

1. Genus *Trichomonas* Donné, 1836
   Four anterior flagella, the fifth (posterior) flagellum terminating with the undulating membrane. No trailing flagellum. Undulating membrane shorter than the body. Capitulum of axostyle only moderately extended, terminating anteriorly in thin pelta, axostyle thin. Parabasal body rod-shaped or with lateral bifurcation. *T. vaginalis* (Donné, 1836) from the human urogenital tract. *T. tenax* (Müller, 1773, emend. Ehrenberg, 1838) Dobell, 1939 (syn.: *T. buccalis*, *T. elongata*) from the oral cavity of man. *T. gallinae* (Rivolda, 1878) Stabler, 1938 (syn.: *T. columbae*) from fowl and pigeon.
2. Genus *Pentatrichomonas* Mesnil, 1914
   Five anterior flagella—four in one group and one solitary flagellum beating in independent rhythm. Posterior flagellum passing into long trailing flagellum. Well-developed undulating membrane extending to the termination of the body. Costa well developed. Capitulum of the axostyle widened by lateral membranes, terminating in a large pelta. Axostyle of medium width. Parabasal body composed of one to several granules, surrounded by an elliptical or spherical zone. *P. hominis* (Davaine, 1860) Wenrich, 1931 from the intestines of man.
3. Genus *Tritrichomonas* Kofoid, 1920
   Three anterior flagella, one posterior flagellum passing into a long trailing flagellum. Well-developed undulating membrane extended to body termination. Axostyle thick, sharply pointed at posterior end, surrounded by one to several periaxostylic rings at the site where it projects from the body. Parabasal body rod-shaped, often very elongated. *T. suis* Gruby, Delafond, 1943 (syn.: *T. foetus*) from cattle and swine.

For concise morphological and physiological data on four species of this subfamily, see Table I.

III. Morphology of *T. vaginalis*

A. Light Microscopy

Investigations by Honigberg and King (1964), using phase contrast, clarified some morphological details of *T. vaginalis*. The shape of the body is variable in both living and preserved forms. Actively swimming forms are ellipsoidal or ovoidal, sometimes spherical. The flagellates are very plastic and may pass through narrow spaces. All strains have the capacity to form pseudopodia-like extensions, which are used in feeding, for attachment to stationary objects, but not for amoeboid movement. All non-dividing flagellate forms have four anterior flagella, somewhat unequal in length; these originate in an anterior basal granule complex. After protargol-staining they are seen to end in small rods or knobs. The undulating membrane and the costa arise in the basal granule complex postero-dorsal to the anterior flagella. The free margin of the

TABLE I

Morphological and physiological data on the four species Trichomonas (*comb. after Kulda*, 1965)

	Trichomonas vaginalis	*Trichomonas tenax*	*Trichomonas gallinae*	*Pentatrichomonas hominis*
Body measurements (average in brackets)	4–32 × 2·4–14·4 μ (10 × 7 μ)	4·2–12·8 × 2·1–14·7 μ (7·4 × 5·3 μ)	6·2–18·9 × 2·3–8·5 μ (10·5 × 5·2 μ)	7–15 × 4–10 μ
Shape of body	oval	piriform	spherical	piriform
Ratio of body length to width	1·4:1	1·4:1	1·8:1	—
Nucleus after Honigberg,	2·4–6·4 × 1·2–3·2 μ (4·2 × 2 μ) oval	1·5–3·3 × 1–2·5 μ (2·5 × 1·7 μ) spherical without nucleoli	1·8–3·1 × 1–2·1 μ (2·4 × 1·3 μ)	spherical with central nucleoli
Ratio of nucleus length/width	2·1:1	1·5:1	1·8:1	—
Chromosomes	5 (Hawes) (Powell)	3 (Hinshaw)	?	5 (Bishop)
Flagella	short, approx. 1/1 of body length	approx. 2/3 of body length	—	long, terminating freely at level of 6th flagellum
Number of anterior flagella	4	4	4	5
Paraxostylar granules	present	absent	present	—
Shape of parabasal body	mostly V-shaped	exclusively rod-shaped	mostly hook-shaped	—
Optimal pH	5·8–6	7·0–7·5	7·0–7·5	7
Optimal temperature	37°C	31–32°C	37°C	30–37°C
Isolation in axenic culture	easy in standard media	not possible in standard media	easy in standard media	easy in standard media
Pathogenicity for mice	pathogenic	non-pathogenic	pathogenic	?
Cytopathogenic effect on tissue culture	strong	none	strong	?

membrane consists of the accessory filament and the recurrent 5th flagellum, which are about equal in length and diameter. The posterior end of the costa is usually obscured by the terminal segment of the undulating membrane. The two rows of paracostal granules are visible quite clearly in hematoxylin-stained preparations and in living organisms viewed in a phase contrast system.

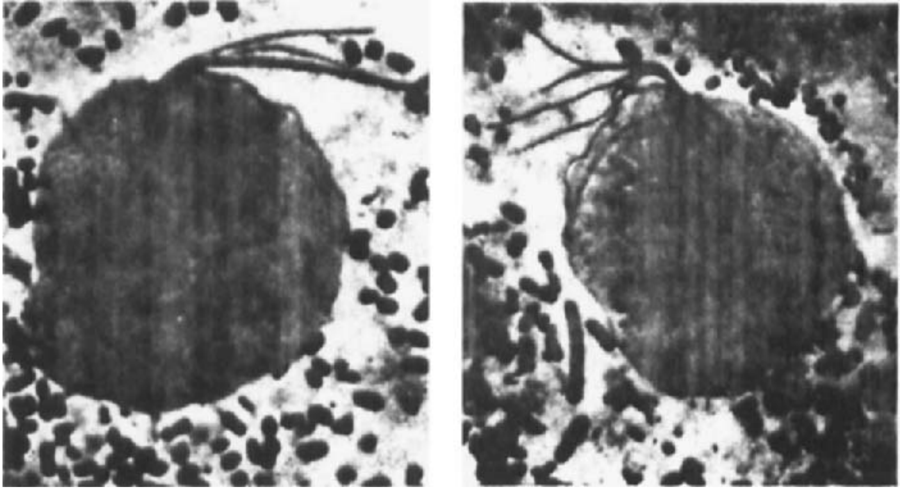


FIG. 1. *Trichomonas vaginalis*. (Giemsa staining. Photo Professor J. Fiala.) ×3000.

The axostyle has a spatulate capitulum about one-third of its total length and this extends anteriorly into a small, crescent-shaped pelta, seen most clearly after protargol staining. The trunk of the axostyle is a thin hyaline rod passing through the centre of the organism and projecting slightly from the posterior surface. Swellings or ridges are noted along the axostylar projection part and may be mistaken for periaxostylar rings, which do not exist in *T. vaginalis*. Constantly present are three rows of paraxostylar granules, a typical arrangement for *T. vaginalis*. The parabasal apparatus consists of the parabasal body associated with one or more filaments. In most strains forms with a V-shaped parabasal body are the more common. The ellipsoidal or ovoidal nucleus is situated near the anterior end of the body, often containing a small spherical nucleolus in a chromatin-free area. In fixed and stained preparations the nuclei appear rather elongate. In the cytoplasm there are chromatophilic inclusions of varying size.

A cytostome seems not to exist and food particles are ingested in the posterior region by the fine, pseudopodia-like processes.

The biometric characteristics of *T. vaginalis* were worked out by Kurnatowska (1964, 1966) as parameters such as: length, breadth, surface of projection volume, shape index (length/breadth ratio) and the plasmonuclear surface and volumetric indices. Statistically significant differences exist in the biometric

characteristics of flagellates from women with asymptomatic, acute and chronic infection, the smallest in acute cases, the largest in chronic infection. *T. vaginalis* from women treated with arsenical drugs are much under average size, though the nucleus retains its original size. Differences in length, breadth and shape of *T. vaginalis* obtained from women at various stages of infection, disappeared when strains were cultured for one month in Pavlova's medium.

Schmidt-Gross (1958) described changes in the shape of the body during metabolic activity.

B. ELECTRON MICROSCOPY

The first studies on the ultrastructure of *T. vaginalis* were made by Shimada (1959) and Inoki *et al.* (1960), the latter writers describing a double-layered nuclear membrane with pores, a lamellar Golgi apparatus, and flagella with 9 peripheral double fibrils and one central fibril.

Ludvík *et al.* (1961) studied ultra-thin sections and total preparations shadowed with beryllium and chrome, and gave this description of the ultrastructure of *T. vaginalis*: four anterior flagella of equal length (15–22 μ), not exceeding the length of the cell body, their ends sharply pointed or terminating in a hook. They consist of 10–11 fine fibrils forming a bundle, which is surrounded by a plasmatic sheath. Nine fibrils are arranged in a circle, one or two stronger fibrils being central. The undulating membrane is 1–1·6 μ thick, its exterior border formed by a fine marginal fibril. In the middle of the undulating membrane, the centrally situated short flagellum also has 10–11 fibrils; it ends in the first half of the cell body along with the undulating membrane, but is never free and trailing. Flagella and undulating membrane arise in the group of the five basal granules situated in the terminal part of the axostylar capitulum. The four costal fibrils also arise in this group, all of them directed towards the centre of the cell. Two are thicker and longer, two thinner and shorter; they are composed of several disks 55 mμ thick.

Anteriorly the axostyle forms a thick capitulum, thin in its median part (0·5–0·7 μ in diameter); its termination outside the cell body is like a delicate thorn 5–7 μ long. The parabasal body (5–6 μ long, 1 μ wide) lies in the anterior part of the cell body, mostly dorsal to the nucleus. Most cytoplasmic, osmiophilic granules (average size 0·4 μ) are crowded round the spindle or drop-shaped nucleus and along the axostyle; finer granules occur along the costal fibrils, especially along the costa beneath the undulating membrane. Nielsen *et al.* (1966) completed these studies on the ultrastructure of *T. vaginalis*. The nuclear membrane is an ordinary, three-layered membrane, about 7 mμ wide. Most nuclei contain several large electron-dense granules, which either represent nucleoli or are simply clusters of chromatic material (chromosomes ?). The parabasal body is morphologically a Golgi zone located near the anterior nuclear pole opposite the axostyle, measuring about 1·5 × 0·5 μ and composed of more or less flattened cisternae with a layering almost parallel to the nuclear surface. Vesicles seem to have arisen from the individual cisternae by a process of constriction or budding. All of them are limited by a triple-layered membrane 7–10 mμ wide. The sheath of the axostyle consists of a monolayer of

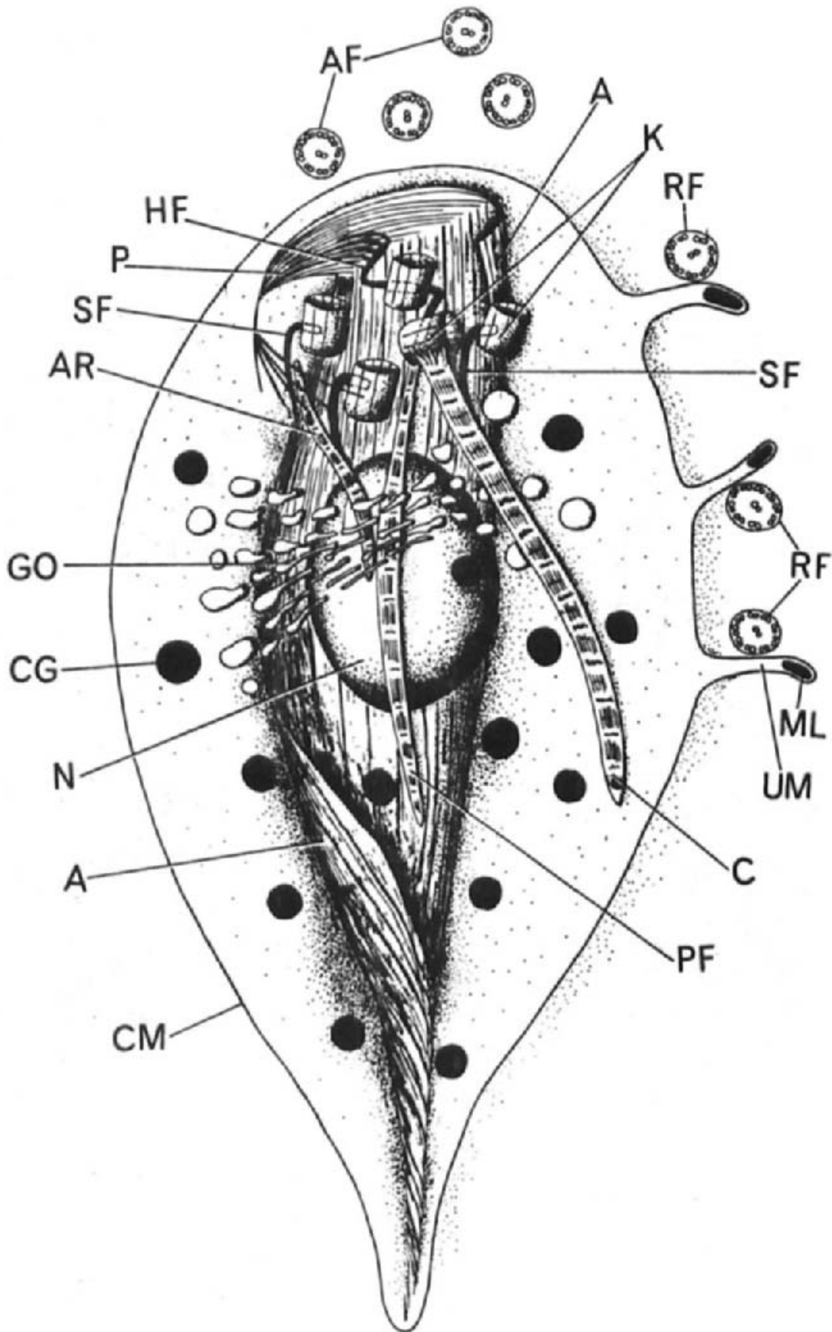


FIG. 2. *T. vaginalis*—semiperspective diagram based on electronmicrographs. *CM*—cell membrane; *A*—axostyle; *P*—pelta; *N*—nucleus; *GO*—Golgi or parabasal body; *C*—Costa; *PF*—Parabasal filament; *AR*—accessoric rootlet fibre; *SF*—sickle-shaped fibres; *K*—Kinetosomes; *AF*—anterior flagella (in T.S.); *RF*—recurrent flagellum (in T.S.); *UM*—undulating membrane (in T.S.); *ML*—marginal lamella (in T.S.); *CG*—paracostal and paraxostylar granules. (After Nielsen *et al.*, 1966.)

about 50–55 parallel, tubular fibres, the external tubules measuring 20 mμ, the internal ones 7 mμ in diameter. Four of the basal granules (kinetosomes) have parallel long axes, distributed radially around the fifth granule, to which the recurrent flagellum is attached. Each individual *T. vaginalis* has at least two rootless flagellar fibres (costae) and a parabasal filament attached to the kinetosomes by sickle-shaped fibres without cross striations. The costa is longer and wider than the filament, its body being characteristically flattened near the kinetosome. It is located near the periphery close to the attachment

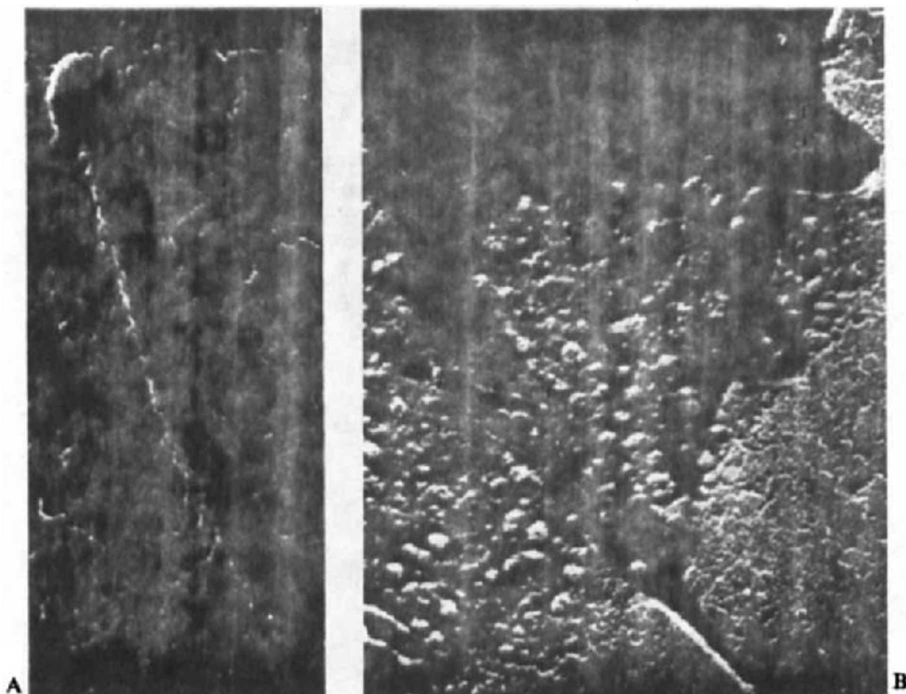


FIG. 3. *T. vaginalis* electronmicrographs. A—End of a flagellum. B—End of the axostyle and undulating membrane. (Photographed by J. Ludvik.)

of the undulating membrane. The parabasal filament lies nearer the centre of the plasma. In rare cases the costa is 13 μ long and 1·5 μ broad. Each of the periods of cross striation in the costa and the filament are subdivided by a less dense cross line, the subfibril inside the costa being arranged longitudinally. The recurrent flagellum is along its entire length attached to the undulating membrane. The endoplasmatic reticulum is found frequently as a corona around the nucleus and is always abundant in the cytoplasm inside the capitulum of the axostyle. Free ribosomes are distributed all over the cytoplasm. Vesicles of different size and small tubules limited by a three-layered membrane are frequently observed at the cell periphery. Larger vesicles and vacuoles, some containing electron-dense material, are situated in the caudal end of the

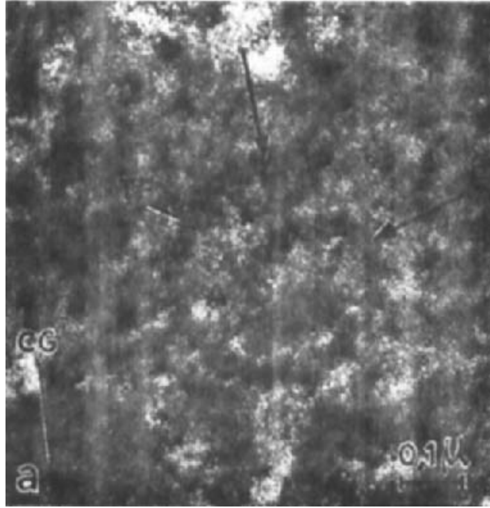


FIG. 4. E.M. section of *T. vaginalis* showing individual fibres (*TF*) of caudal part of axostyle (*A*). Tubules connected side by side by a delicate membrane (arrows). × 90000. (After Nielsen *et al.*, 1966.)

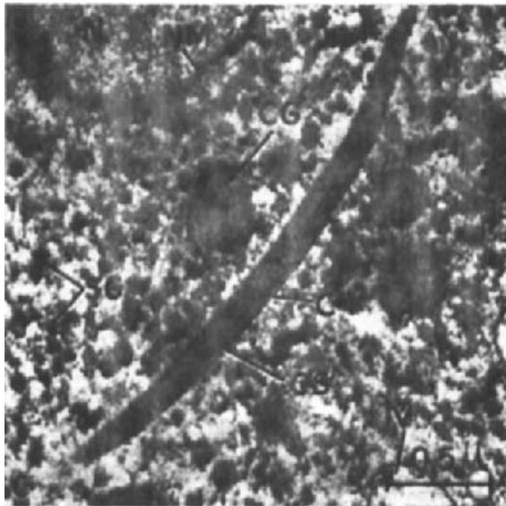


FIG. 5. E.M. section of *T. vaginalis*. Costa (*C*) shown in L.S. Subfibrils (*CS*) within costa arranged almost in parallel to long axis of organelle and at right angles to cross striation. Fibrils approximately 10mμ apart. Segmentation of costa indicated by a single major cross line, but a less dense intermediate line can also be distinguished. × 90000. (After Nielsen *et al.*, 1966.)

body. Most of them seem to be food vacuoles. The paraxostylar granules, measuring about 0·5 μ, are limited by a triple membrane and consist of a coarse, granular, electron-dense matrix. There are also small granules (0·05–0·1 μ) with a dense globular matrix but no limiting membrane.

Smith and Stewart (1966) confirmed Inoki's original observation that there are no mitochondria in *T. vaginalis*. The axostyle appears to be a cup-shaped structure comprised of a single row of 35 fibrils, each about 200 Å in diameter and a constant distance apart. A dense-cross-banded costa with a regular

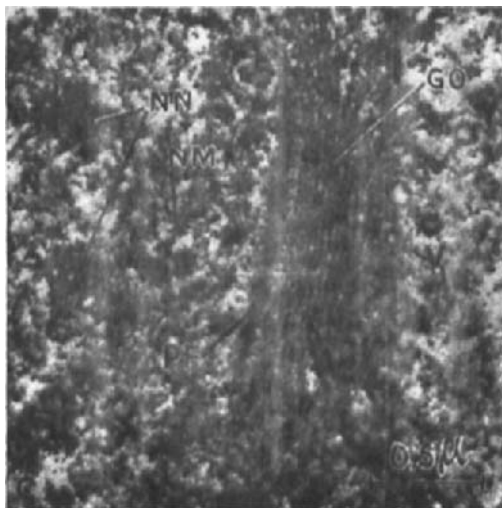


FIG. 6. E.M. section of *T. vaginalis* showing location of parabasal filament between nucleus and parabasal body (Golgi zone). The nucleus (*N*) with nuclear membrane (*NM*) contains nucleoli or clusters of chromatin (*NN*) in the nucleoplasm. *PF* denotes parabasal filament and *GO* parabasal body. Note vesicles (*V*) with parabasal body are increasing in size towards cell periphery. × 31 000. (After Nielsen *et al.*, 1966.)

periodicity of about 400 Å is closely associated with a kinetosome of an anterior flagellum. By differential staining, glycogen deposits were demonstrated. The lack of mitochondria is in keeping with the normally anaerobic life cycle of *T. vaginalis*.

Samuels (1961) found two general types of variation in *T. vaginalis*: (a) drug resistant mutants, (b) clonal strains with hereditable differences of morphology. Some clones had almost exclusively small cells, in others the cells were larger; many giant cells were found in these cultures and, in addition, these organisms contained many granules and vacuoles. The possible sources of these variations may be either nutritional or metabolic differences or an infection with some agent, possibly a virus.

C. REPRODUCTION

T. vaginalis multiplies by bipartition, and the nuclear division is mitotic. According to Hawes (1947), five chromosomes are formed outside the nucleoli.

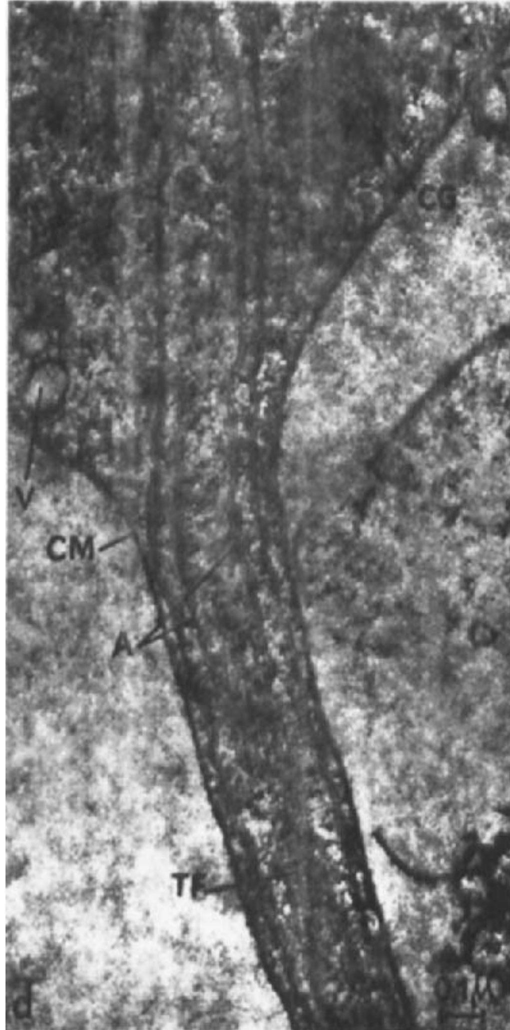


FIG. 7. L.S. through caudal part of axostyle of *T. vaginalis.* A chromatic granule with a paraxostylar location (*CG*) and vesicles (*V*) are found close to the cytoplasmic membrane (*CM*). Axostyle (*A*) consists of several layers spirally wound (cf. Fig. 1). Individual tubular fibres (*TF*) of axostyle tangentially cut, their helical course is clear. × 52000. (After Nielsen *et al.*, 1966.)

The basal corpuscles pass to the opposite poles of the mitotic spindle, but no details are yet available on this process. After division of the nucleus, the locomotory apparatus is completed and finally the plasma divides. The fact that nuclear division is by mitosis is well known to protozoologists, but mitoses are difficult to find and may become indistinct or appear to be atypical after fixation and staining—and dry smears are useless.

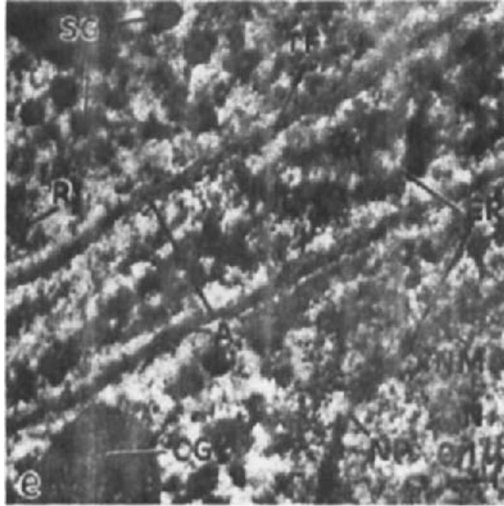


FIG. 8. Section of *T. vaginalis*, axostyle (*A*) in region of lower nuclear pole. *N* denotes nucleus with nuclear membrane (*NM*) and nuclear pore (*NP*). At nuclear pore there seems to be continuity from nuclear membrane to innermost layer of rough endoplasmic reticulum (*ER*). Between *NM* and *ER* a narrow perinuclear space (arrow). *TF* indicates obliquely cut tubular fibres of axostyle and *R* a cluster of free ribosomes in cytoplasm. Part of a chromatic granule lined by triple-layered membrane at *CG* and small cytoplasmic granules intensively stained with lead salts at *SG*. × 54000. (After Nielsen *et al.*, 1966.)

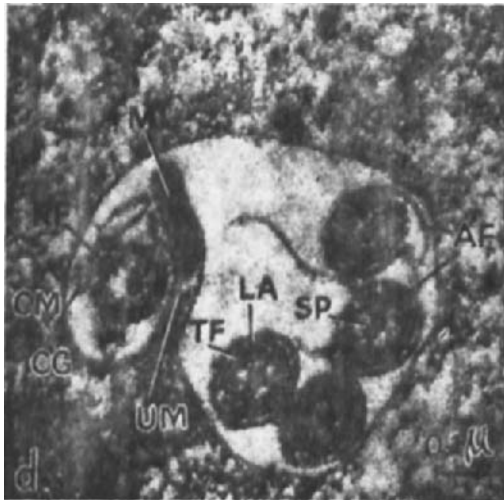


FIG. 9. Section of *T. vaginalis* showing flagella (*AF*) and (*RF*), undulating membrane (*UM*); cytoplasmic membrane (*CM*), lining recurrent flagellum (*RF*) is torn. Marginal lamella (*ML*) of undulating membrane (*UM*) is visible, also nine pairs of peripheral fibres (*TF*) and (*LA*) and two single central fibres (*SP*). *CG* denotes chromatic granulum. × 54000. (After Nielsen *et al.*, 1966.)

Multiple division in *T. vaginalis*, as described by Dyroff and Michalzik (1954), is quite exceptional. The liberation of young forms from the "cysts", as observed by both authors, may be ascribed to erroneous observation. Malyszko (1964) found multiple division of *T. vaginalis*, grown on Roiron medium and also in samples collected directly from patients. Also Hoffman and Malyszko (1966) described multiple division and polynuclear *T. vaginalis* in one out of the 22 women examined before treatment and in 6 women examined on the third day of treatment with flagyl, aminitrozol or nitrofuranon. In the urethral discharge of males, polynuclear *T. vaginalis* were found in 10 patients before treatment and in 12 on the third day of treatment. Unfavourable conditions of the environment may explain increase in numbers of polynuclear forms.

There is no exact confirmation of sexual reproduction of trichomonads including *T. vaginalis*. The drawings by Grimmer (1950) depict either an accidental adherence of two completely independent flagellates or the mentioned constriction of protoplasmic particles which, under certain circumstances, may again become regressive. Dyroff and Michalzik (1954) consider the possible existence of permanent forms without confirming this assumption.

D. THE CYST PROBLEM

The problem of cyst formation by trichomonads has been studied by many protozoologists but the opinion prevails that cysts are not formed by *T. vaginalis*. Some spherical, motionless trichomonads can be found in vaginal discharges and especially in the urethral secretion of the male, recognizable by their sky-blue plasma and the moderately spindle-shaped, reddish-violet, finely granulated nucleus (Giemsa-stained), but these never have any flagella or a distinguishable cyst membrane, well known characteristics of *Amoeba* or *Lamblia* cysts. These formations seem to be degenerate trichomonads destined to die unless they are in time transferred into a culture medium or even into the genital ducts of the opposite sex. Such motionless, unflagellated trichomonads, covered with a mucous layer, can be found in the intestine of various rodents. These also are not cysts but are trichomonads dying on clusters of excrement. They are also transmissible, to a certain degree, perorally to other hosts or can grow in appropriate culture media, but otherwise are also condemned to death. This is never the case with true cysts. Also in the culture cysts are never formed, and the spherical trichomonads are degenerate forms. In some instances it could be proved that the formations, previously considered to be trichomonad cysts, belonged either to other flagellates (*Retortomonas dobelli* a.o.) or were the cells of *Blastocystis*. The stage drawn by Grimmer (1950) is certainly not a cyst but is a decomposed host cell. The cysts observed by Holz (1953) may be either degenerate host cells or yeast-like organisms covered with a thick membrane and bearing large vacuoles. The data by Holz could not be confirmed by Reusse (1955). Studies on *T. foetus* and *T. muris* have shown that these parasites never reach a strictly cystic stage. In old cultures, however, and under adverse conditions they may take on a rounded form, and by the loss of their flagella and undulating membrane they become entirely motionless. As observed in cultures of single trichomonads, rounded-off forms are labile organisms with

very little motility of the endoplasm and with hardly any sign of life, yet still able to regain their former vitality and to reproduce. In such cases amoeboid movement sets in with the protrusion of pseudopodia, soon to be followed by transverse division. Revitalization is also possible without the amoeboid stage, but then the tendency for an early bipartition is missing (Reusse, 1955).

Although Mandoul *et al.* (1946) do not consider the rounded forms of *T. vaginalis* to be true cysts, they ascribe to them an analogous role without exactly proving this assumption.

In Giemsa-stained smears it is sometimes possible to observe rounded, blue-staining formations without nucleus, flagella or axostyle, which may originate from torn-off pieces of flagellates trying to pass through crevices. Hereby, the anterior end with the nucleus, the flagella complex and the axostyle are torn off and the posterior portion, consisting of almost homogenous plasma, may give the erroneous impression of being a cyst. To prove trichomonad cysts, observations of native preparations under the light field, dark field and phase contrast microscope have to be completed with examinations of the conditions of the nucleus, the flagella and the axostyle by cytological methods on wet smear preparations and on protargol-stained trichomonads. Such cysts would also have to be regularly present in the vagina, like the cysts of intestinal *Lamblia*, *Amoeba*, *Chilomastix* and other Protozoa. This may be especially misleading for beginners who are trying to solve the cyst problem without having yet acquired sufficient knowledge of protozoan morphology.

E. SPECIFICITY OF HUMAN TRICHOMONAD SPECIES

The taxonomic position of *T. vaginalis* as an independent species will be discussed in this Section. The conception that *T. vaginalis* is only a certain aspect of *Pentatrichomonas hominis* or of *T. tenax* has been suggested by various authors (e.g. Grollet and Montaugé, 1957), but not agreed by protozoologists who have studied the morphology of *T. vaginalis* for the past 20 years or by the two symposia (Reims and Montreal). The differences in the three *Trichomonas* species are as follows (Table I):

(1) Morphologically, *T. vaginalis* is the largest of the three species. The undulating membrane extends along one half of the body, to which both ends are connected by a fibril. The nucleus is spindle-like or oval. The four free flagella terminate in a hook. *T. tenax* is much smaller, its nucleus is rounded and the ends of the short undulating membrane are connected by an indistinct fibril. The flagella terminate also in a small hook. *Pentatrichomonas hominis* is smaller, its undulating membrane is long, and there is a sixth free trailing flagellum. All five flagella terminate in a sharp point without a hook. These differentiating features are constant and are retained for several years even in cultures or after inoculation into various experimental animals by different methods (unpublished experimental results by Jírovcová, 1963). Cysts are not formed by any of these three species.

(2) Biological differences concern the location in the human body: *T. vaginalis* occurs only in the genito-urinary system. *T. tenax* is found, as a rule, only in the human buccal cavity, but occasionally in the stomach

during achlorhydria; *P. hominis* only in the large intestine. The least resistant of the three *Trichomonas* species to lower temperatures, desiccation, hypo- or hypertonic media and other influences of the external environment is *T. vaginalis*, most resistant is *P. hominis*; the resistance of *T. tenax* lies in between that of the other two species.

(3) Experimental transmissions of the individual species to unnatural environments were performed by Westphal (1936) and Bauer (1943), with negative results.

(4) The epidemiology of the three *Trichomonas* species also favours the view that they are independent species. Long ago, Jírovec *et al.* (1942) emphasized the fact that *T. tenax* does not coincide with *T. vaginalis* in *T. vaginalis* infected women. In fact, *T. tenax* is practically absent in young women with healthy teeth and becomes more common past the age of 40, when teeth generally start to decay; while *T. vaginalis* reaches its peak between the age of 20–40.

Červa and Červová (1961), examining 609 adult women in Prague coprologically and vaginally by cultivation and microscopical methods, found 25% of them to be infected with *T. vaginalis*. *P. hominis* was not observed in any of the examined women. Only in one instance was the flagellate *Enteromonas hominis* and, in one other, *T. vaginalis* isolated from the stool. In central Bohemia, *P. hominis* has been recorded only from children's homes (age limit 6 years). In central Europe, the incidence of *P. hominis* infection in adults is so low that it may easily be disregarded in view of the high hygienic standards of the present. The findings of *T. vaginalis* and *P. hominis* are not explicitly connected. De Carneri and Giannone (1964) statistically studied the eight possible combinations of the three infections (*T. vaginalis* 30·2%; *T. tenax* 43·3%; *Entamoeba gingivalis* 37·6%) of 367 North Italian women, but did not reveal any relationship of association or exclusion among the three parasitoses.

IV. CULTIVATION OF *T. vaginalis*

After it became possible to cultivate bacteria-free strains of *T. vaginalis* by adding penicillin and streptomycin to the media (Adler and Pulvertaft, 1944; Johnson and Trussell, 1944; Jírovec and Peter, 1948; Magara *et al.*, 1953; etc.), cultivation has become easy and cultures can be used standardly even when diagnosing latent trichomoniasis and controlling therapeutic results. *T. vaginalis* grows under anaerobic conditions when a native serum is present. The redox potential can be decreased by adding L-cysteine-chloride or Na-thiogluconate; the pH of the medium is adjusted to 5–6. Glucose or maltose are used for supplying the source of energy. An additional 0·1% of agar stabilizes the cholesterol suspension and slows down oxygen diffusion. Bacteria-free cultures are obtained by adding mostly 400–1 000 units of penicillin and 100–1 000 μg of streptomycin per 1 ml of culture fluid. Any yeasts occurring in the original material can be removed within 24–72 h by adding 300γ mycostatin/ml (see Honigberg, 1957). The most frequent media used are Johnson's CPLM, and the Vf bouillon after Magara *et al.* (1953), after Diamond (1957), after Feinberg (1953) and after Roiron-Rattner (1957, 1958), either in the

original formula or in various modifications. Another improvement is the use of Hall's test tube with an attenuation in the posterior one-third of its length (Fig. 10b); a glass ball, stoppering this attenuated portion, to some extent prevents the diffusion of oxygen from above, providing conditions for better and prolonged growth of the trichomonads under anaerobic conditions. The number of *T. vaginalis* is 10 000–1 000 000 per ml of culture fluid. However, it is not always possible to obtain a free isolate (Holečková-Červová, 1960, records positive results in only about 60–70%), and also pure strains have

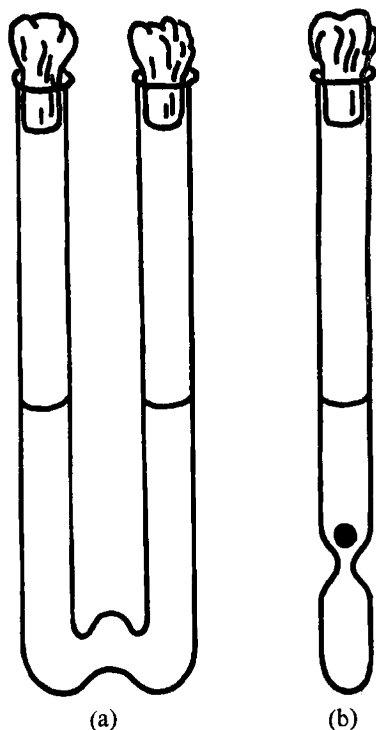


FIG. 10. Culture tubes: W-shaped tube (after de Carneri) and single tube (after Hall).

been found to die for unknown reasons after several months. The same author observed differences in the growth of various strains of *T. vaginalis*, when cultured e.g. in the Vf-bouillon with 10% rabbit serum; some of the strains grow constantly in coarse flakes along the posterior half of the fluid column, others in vertical or diagonal lines.

When used for diagnosis the culture must be observed for 10–12 days every 24 h (Teras *et al.*, 1963). For further breeding the culture must be reinoculated after 3–5 days at a temperature of 35–37°C. Sometimes it is difficult to remove the bacteria. De Carneri (1956) used for this purpose a W-shaped tube (20 × 1 cm) (Fig. 10a)filled under sterile conditions with 15 ml of the CPLM medium,

10% human serum and 1 000 units of penicillin and 1 000 μg streptomycin per ml. The contaminated material, containing trichomonads, fungi and bacteria, is seeded into one arm of the "W" tube. The agar in the medium prevents conversion streams and the fungi are confined to develop in the upper part of the other arm, while the trichomonads penetrate the whole medium. After a 2-day incubation period at 37°C, some drops are removed with a pipette from the second arm and transferred to the same medium in a normal tube.

By increasing oxygen tension it has been possible to induce the formation of multinucleated cells in a very high proportion of 50–80% "giant somatella" (Wirtschafter, 1954). Inoculation into a flask containing 100 ml of medium at 37°C shows after 48 h an oxidized state with reference to methylene blue indicator in the CPLM medium. Such culture incubated under reduced oxygen tension produces, in 48 h, a completely normal culture without giant cells.

Many investigators have cultivated *T. vaginalis* on solid media (Magara *et al.*, 1953; Wirtschafter, 1954; Asami and Nakamura, 1955; Filadoro and Orsi, 1958; Ivey, 1961; Samuels, 1962). The solidity of the culture (CPLM, Diamond etc.) is obtained by an addition of 1–2% of agar; anaerobiosis can be achieved either by cultivation on Fortner plates with *Serratia marcescens*, by feeding the medium with N_2 or CO_2, or by the absorption of oxygen through a mixture of pyrogallol–NaOH. The colonies attain a size of 0·5–2 mm and remain viable in e.g. a N_2 atmosphere for about 11 days. Colonies of 1 mm diameter are composed of about 100000 trichomonads. The advantage of cultures on solid media is the longer viability of the flagellates, the possibility of better isolation from the clones and an easier observation of the inhibition by various *Trichomonas* controlling preparations.

Schoenherr (1958) developed a method for quick identification of the number of cells in *T. vaginalis* and *T. foetus* cultures. He determined the amount of sediment after centrifugation in graduated pipettes under constant g-figure and standard times. Lash's modified nutritive medium with addition of liver proved to be significantly superior. The vaginal secretion with trichomonads contains a "growth factor" for *T. vaginalis*, which is favourable for cell reproduction also in more diluted and normally unsuitable nutritive media. The generation time of *T. vaginalis* was found to be approximately 3·88 h, for *T. foetus* 3·47 h. Axenic culture of *T. vaginalis* in hens' eggs incubated for 11 days was achieved by Müller (1967).

The resistance of *T. vaginalis* to changes in temperature was studied by different authors. Whittington (1951b) found that in temperatures fluctuating between 5·9–15·5°C two strains survived for 3 days but no longer. Another strain resisted 9·2–10·6°C for 2 days and another 2·2–8·9°C only 1 day. In vaginal exudate diluted with water 1:1 one strain survived 4·4–6·7°C only 2 days, another strain in undiluted vaginal exudate −4–0°C for 2 days. MacEntegart (1954, 1959) conserved *T. vaginalis* in culture with 5% glycerol for 26 months at −79°C, and with 5–15% dimethylsulphoxide for 35 months at −170°C.

Stabler *et al.* (1964) found that *T. vaginalis* and *T. gallinae* kept their original pathogenicity for natural and experimental hosts and for tissue cultures for many months in glycerol-containing media at below −70°C in dry ice. Typi-

cally only a small percentage of the flagellates survived. Honigberg *et al.* (1965) conserved both species in the Diamond medium in the presence of dimethylsulphoxide in liquid nitrogen.

Diamond *et al.* (1965) conserved axenically cultivated *T. vaginalis* in the presence of 5% dimethylsulphoxide by freezing and then stored at −170°C in vapour above liquid nitrogen. After 2 years, thawed and subcultured samples of trichomonads showed no significant differences between these organisms and the ones tested before freezing, in view of the virulence for mice after i.p. inoculation.

Pray (1952) divided the bacteria influencing the growth of *T. vaginalis* into three groups:

(1) Those prolonging the life of the culture beyond that of bacteria-free controls (*Staphylococcus aureus* and *S. albus*).

(2) Those having a moderately inhibiting effect (*Brucella suis, Streptococcus lactis, Pseudomonas fluorescens, Alcaligenes faecalis, Sarcina lutea, Bacillus subtilis*).

(3) Those which greatly curtail multiplication of the flagellate and the life of the culture (*Escherichia coli, Aerobacter aerogenes, Pseudomonas aeruginosa, Salmonella schottmuelleri, Proteus mirabilis, Salmonella paratyphi*).

Filtrates of bacterial cultures gave no indication that specific metabolites or antagonistic substances were responsible for the effects observed. Maltose and glucose were found equally effective for prolonging the life of *T. vaginalis* cultures in the presence of bacteria which had an inhibiting effect. Changes in the pH and in the oxidation-reduction potentials gave no indication that the effects of bacteria were due to such factors. Other studies on this point were made by Sorel (1954), de Carneri (1956), Feo (1958) and others.

A 2–3-day old *T. vaginalis* culture grown in the CPLM medium after Hitchcock (1948) produced no inhibition of different *Salmonella* sp., *Aerobacter aerogenes, Pseudomonas aeruginosa, Escherichia coli, Shigella* sp. and *Staphylococcus aureus. T. foetus*, however, produced inhibition of *Salmonella pullorum, S. schottmuelleri* and *Corynebacterium renale.*

V. Biochemistry of *T. vaginalis*

Exact biochemical investigation of *T. vaginalis* becomes possible only after the development of a method for cultivating this species in bacteria-free cultures. The first experiments were performed by Trussell and Johnson; later the problems were pursued by Kupferberg, Baernstein, Asami and many other investigators. Yet our present knowledge of biochemistry is still fragmentary.

T. vaginalis is generally considered to be an anaerobic protozoan parasite; it contains no cytochrome c, no mitochondria and is adversely affected by a high oxygen tension; yet under certain conditions it does take up oxygen. It is capable of utilizing a number of polysaccharides, but only those which contain an α-1·4-glycosidic linkage provide those properties essential for good growth (Wellerson and Kupferberg, 1962).

T. vaginalis uses glucose as one of the essential sources for the production of energy-rich compounds necessary for living and multiplication. Growth in the culture is markedly stimulated by an addition of glucose, fructose, maltose, glycogen and dextrin, and considerably stimulated by sucrose and soluble starch; lactose and galactose did not stimulate multiplication (Asami, 1956). The amount of glucose consumed by the multiplying organisms (from 9000 to 3000000 individuals) was 4·5 mg; lactic acid was produced in an amount of about 20 mg/100 ml during 96 h incubation. In *T. vaginalis*, succinic-, malic- and citric-dehydrogenases have been identified, indicating the probable existence of a TCA cycle during metabolism. Dehydrogenase activities are strictly inhibited by monoiodoacetate; cyanide and malonate had no effect upon the activities. While in *T. vaginalis* catalase activity was found to be low, it was high in *T. foetus* and *T. gallinae* (Asami, 1956). Washed intact cell preparations of *T. vaginalis* oxidize pyruvate and malate. They are unable to utilize other intermediates of the Krebs cycle. Attempts to inhibit pyruvate oxidation with such Krebs cycle blocking agents as malonate, arsenite, parapyruvate and fluoroacetate, were unsuccessful. The Krebs cycle is not the pathway for the oxidation of pyruvate in *T. vaginalis* (Wirtschafter *et al.*, 1956). The principal product of anaerobic glycolysis—lactic acid—was produced in high concentration (about 40% of the total amount of acids). *T. vaginalis* had a high glycogen content—17% of the dry weight of the cell. *T. vaginalis* as an anaerobic protozoan parasite is one of the few nonchlorophyll-containing Protozoa capable of fixing CO_2. The entire amount of radioactive CO_2 supplied during the course of growth was found to be fixed in lactic acid. No significant portion of radioactivity remained within the cell. Labelled CO_2 appeared only in the carboxyl group of lactate and none was found in malate or succinate (Wellerson *et al.*, 1959). This seems to exclude the possibility of CO_2 being incorporated by pyruvate carboxylation. In the degradation of glucose, through the various phosphorylated intermediates ending with pyruvate, which is subsequently converted to lactic acid, the classical Embden–Meyerhof scheme seems to be followed. No cytochrome C is present in *T. vaginalis*. Cyanide resistance of the respiration does not suggest the participation of cytochrome oxidase.

Glycolytic enzymes were most studied in *T. vaginalis* (Baernstein, 1955; Wirtschafter, 1954; Wirtschafter and Jahn, 1956; Kupferberg, 1960; Wellerson and Kupferberg, 1962). In cell-free extracts prepared from mass cultures of *T. vaginalis* were found aldolase, lactic acid dehydrogenase, trioso-phosphate dehydrogenase, trioso-phosphate isomerase, phosphorglucoisomerase, phosphorfructokinase, phosphorglucomutase, pyruvate kinase system, and hexokinase. Phosphorylase and alcohol-dehydrogenase were not demonstrated. Functional sulphydryl groups remain active in KCl-solution for a month or more when stored at 2°C (Baernstein, 1955).

T. vaginalis grown aerobically after Kunitake *et al.* (1962) slowly metabolizes uniformly labelled glucose-U-C^{14} and succinate-2,3-C^{14} to CO_2 and to amino-acids which are then incorporated into protein. Analysis of protein hydrolysates from cells grown on glucose-U-C^{14} reveals radioactivity in 15 amino-acids. A tricarboxylic acid cycle seems to be operating.

The consumption of oxygen is 162 mm² for 100 millions flagellates per hour

—the same number of *T. foetus* consume 215 mm^3 per hour and *T. gallinae* as much as 600 mm^3.

T. vaginalis is not only capable of metabolizing amino-acids but appears to be able to achieve the *de novo* synthesis of nearly all of its amino-acids. The purified DNA from *T. vaginalis* reveals an adenine-thymine type DNA, one which is different from that of most other flagellates (Wellerson and Kupferberg, 1962).

Native serum seems to be indispensable for *T. vaginalis*. Sprince and Kupferberg (1947) obtained from human serum two fractions, both of which are necessary—one fraction soluble in ether, the other soluble in water. It seems that linoleic acid is the active substance necessary for growth. Pantothenic acid is also necessary (Kupferberg *et al.*, 1948).

Cystein and Na-thioglycolate reduce the redox-potential of the medium and are added to the used media. Ascorbic acid, glutamic acid and choline caused a stimulation of the cell multiplications, the first probably by lowering the redox potential of the medium. DL-N-⟨gamma-glutamyl⟩-ethyl-amine acts as a comparative antagonist, probably of glutamine rather than of glutamic acid, and it seems that glutamine is obligatory for the development of the culture of *T. vaginalis* (Back *et al.*, 1950). Cortisone and hydrocortisone inhibit the endogenous respiration and simultaneously the oxidation of Na-succinate and fructose. Oestrogen hormones do not influence the growth in culture (Kupferberg and Johnson, 1941).

Analysis of the purified DNA of *T. vaginalis* by thermal denaturation and density gradient centrifugation shows both to be quite rich in adenine and thyamine. *T. vaginalis* and *T. gallinae* are found to have the AT-type of DNA and to display considerable compositional heterogeneity.

Iyori (1959) follows the protein-N, rest-N, amino-N and ammonia-N in the cultures of *T. vaginalis*. The change in the amount of various nitrogen fractions in the culture media during the growth of *T. vaginalis* was very small compared with that of carbohydrates. The decomposition of protein seems to be more active in the period of cultivation when the number of organisms decreases, than in the early period when they actively multiply. This is probably due to the enzymes liberated from the destroyed organisms.

Ninomiya and Suzuoki (1952) demonstrated on washed suspension cells of *T. vaginalis*, by manometric methods, that glucose and maltose are rapidly oxidized whereas pyruvate and lactate are metabolized at half the rate of the sugars. Succinate, citrate, fumarate, acetate, butyrate, allanine, glutamate and gluconate were not suitable substrates. According to Magara *et al.* (1953) their strains of *T. vaginalis* were not capable of utilizing inuline, glycerine, mannite, dulcite and saracine, and utilized saccharose and rhamnose only in a small degree, whereas good utilization of glucose, galactose, levulose, maltose and glycogene was observed. *T. vaginalis* cannot produce haemolysis, indol, or H_2S, nor liquefy gelatine, produce catalase or decomposed urea. Kupferberg *et al.* (1953) were unable to demonstrate the production of any gas other than CO_2 in any appreciable amount.

T. vaginalis has a limited capacity for oxygen utilization, which is sensitive to cyanide and results in hydrogen peroxide accumulation except when catalase

is also present. A flavoprotein terminal oxidase is indicated, but the importance of the oxygen utilization is not known.

Riboflavin and flavin mononucleotide were isolated from *T. vaginalis*, the first in a concentration of 75 mg/g of cell dry weight (Kupferberg, 1960). Some strains produced hydrogen gas which is probably linked to electron transport. The organisms are essentially anaerobic and therefore depend upon coupled reactions mediated by pyridino- and flavoproteins resulting in the production of reduced compounds (Baernstein, 1963).

T. vaginalis, *T. foetus* and *T. gallinae* show cyanide and azide insensitive respiration. Wellerson *et al.* (1959) isolated considerable amounts of riboflavin from *T. vaginalis*. *T. vaginalis* cannot oxidize all intermediates of the citric cycle, though *T. gallinae* is able to do so. Oxygen uptake in these three species of trichomonads is probably mediated by flavoproteins. There is no doubt that conventional glycolysis is an important system in their metabolism supplemented with other systems linked to NADP and to succinate to furnish electron donors. Electron transport in anaerobic metabolism is limited to dehydrogenase coupling and to the excretion of the reduced compounds (Baernstein, 1963).

Sonic homogenates of *T. vaginalis* contain a metal activating aldolase. Cobaltous and ferrous ions are effective—their action is greatly enhanced by cysteine or thioglycolate. Ethylene-diaminotetracetate is more effective as inhibitor than dipyridyl at pH7. The inhibition may be completely reversed with cobaltous or ferrous salts. The optimum pH for aldolase activity is about 7 and the Michaelis constant is $0{\cdot}25 \times 10^{-3}$ M hexose-diphosphate. Aldolase in *T. vaginalis* is a soluble enzyme. Glucose seems in some degree to have restrained the decomposition of proteins which is used as a source of energy. By paper chromatography 11 amino acids were detected in culture media: aspartic acid, glutamic acid, taurine, glycine, threonine, tyrosine, alanine, arginine, valine, leucine and proline. The same applied to material obtained from the control from uninoculated culture media. *T. vaginalis* showed the ability of deamination, though quite small in amount with cysteine, histidine, arginine and methionine.

Kojima has demonstrated the presence of histamine in the vaginal contents of patients infected with *T. vaginalis*. The experiments of Iyori (1959) showed that in culture *T. vaginalis* did not produce histamine and that it had no decarboxylase to produce histamine from histidine.

Ludvík *et al.* (1961) investigated *T. vaginalis* with cytochemical methods (Fig. 11). The DNA is present only in the nucleus as fine granulations at the periphery of the membrane (detected by the Feulgen nucleal reaction and the staining with methyl green). The endosome contains no DNA and is always Feulgen-negative. RNA was detected with pyronine and toluidin blue (after Brachet) as small granules near the nucleus and the axostyle. It is possible to dissolve all RNA with ribonuclease. The silver-impregnation with protargol (after Bodian) coloured the whole locomotion apparatus, the undulating membrane, the costae, the endpoint of the axostyle and also the nucleus. PAS positive granules can be found in the anterior half of the body and along the axostyle, some are also distributed in the plasma. Best's carmine-stained

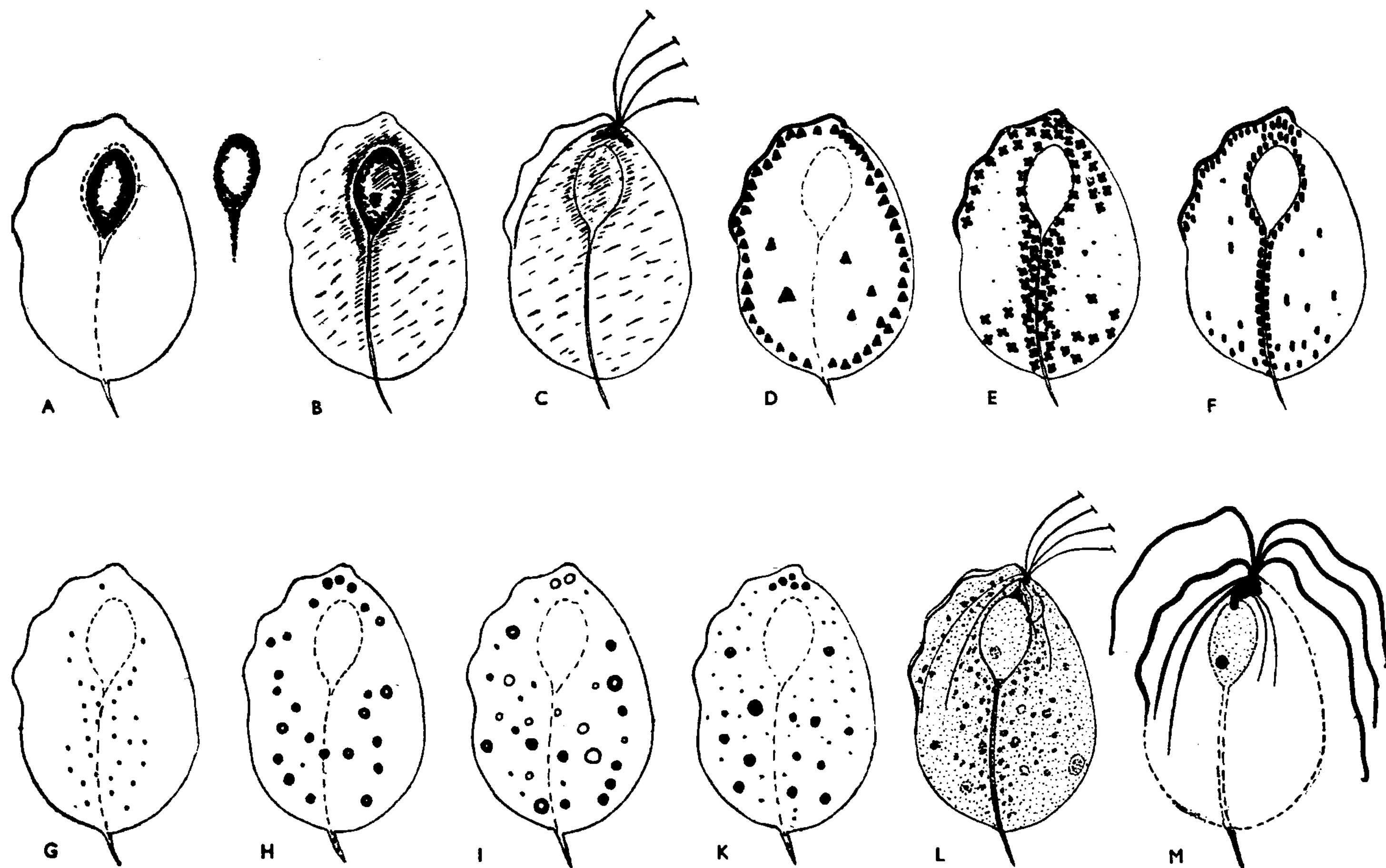


FIG. 11. Some cytochemical reactions on *T. vaginalis*. A: Feulgen nucleal reaction (DNA). B: Brachet staining (RNA–DNA). C: Gram staining. D: Best staining (glycogen). E: PAS staining (MacManus–Hotchkiss). F: Halle staining. G: Ebel staining (volutine). H: Oil red. I: Nile blue sulphate. K: Baker staining. L: Giemsa–Romanowski method. M: Bodian protargol impregnation. (After Ludvík *et al.*, 1961.)

glycogen concentrated mainly under the cell membrane. Solitary glycogene granules are distributed irregularly throughout the plasma. Acid mucopolysaccharides (after Hall) found at these sites are not so plentiful as the PAS positive substances. Lipids are present as small droplets in the plasma; they can be stained with Oil-red O, Sudan III, Sudan IV and Nile blue sulphate.

VI. Serology and Immunobiology of *T. vaginalis*

Serological and immunobiological investigations have been only of theoretical interest until the present, because the finding of *T. vaginalis* by cultivation or direct microscopical examination is easier and more reliable. In mostly symptomless trichomoniasis of man, however, such investigations could be important practically speaking. It seems that the divergences reported by different authors, may be due to the antigenic structure of the individual *T. vaginalis* strains. The height of titres also seems to have no connection with the clinical symptomatology (Korte, 1958).

A. The Complement Fixation Reaction (CFR)

Riedmüller (1932) was the first to develop weak titres in the CFR in guinea-pigs by repeated i.p. injections of vaginal discharges containing *T. vaginalis*. Wendelberger (1936) used an alcoholic extract from *T. vaginalis* suspension as antigen for the CFR. 68% of the 32 examined women with clinical trichomoniasis were positive, 16% without symptoms negative. Trussell *et al.* (1942), using an aqueous antigen, found a positivity of CFR in 42·3% of 110 women with *T. vaginalis* and in 16·5% of 290 women without *T. vaginalis*. Trussell (1947) wrote: "... no practical importance is now attached to the demonstration of CFR. Certainly the test is of no diagnostic value and it remains to be seen whether there is any correlation with spontaneous or therapeutic cure".

New, more promising investigations were performed by Hoffman *et al.* (1966). Using the CFR in the quantitative technique of Kolmer, he found a positivity in 80% of adult female and in 40% of adult male patients, both infected with *T. vaginalis*, the control groups of both sexes showing positive reactions only in 9–10% of the cases. Patients with chronic trichomoniasis showed a higher positivity (95%) than that found in persons at the acute stage (53·8%). The same could be observed in the males—in chronic stages the positivity was 57%, in acute stages 28·5%. Jaakmees *et al.* (1966) used 8 antigens of a serotype and found no positive CFR in the control group. One hundred and seventy patients with *T. vaginalis* showed complete or strong positive CFR with the antigen of at least one serotype. The authors claimed the necessity of using simultaneously antigens from different serotypes. The only serum in which a partial CFR was observed had been obtained from a woman infected with *T. vaginalis* only 2 weeks before the examination. The presence of CF-antibody as well in the blood of *T. vaginalis*-negative sexual partners of patients infected with trichomoniasis could be due to the fact that regardless of repeated negative examinations, these people might still have been infected with *T. vaginalis*. After treatment with metranidazol and the disappearance of

T. vaginalis no later than the fourth day of treatment, followed by a rapid regress of clinical symptoms, the CFR in 24 women showed a continuous decrease in the titre beginning at the third month after treatment; in most cases antibodies disappeared completely from the blood within a year at the most. Similar results were also obtained by studying the dynamics of CFR in 16 male patients treated with metranidazol. There is no explanation why CF-antibodies disappear from the blood of some people considerably sooner than from others.

B. AGGLUTINATION AND AGGLOMERATION REACTIONS (AR)

Tokura (1935) probably first indicated the formation of agglomerating and killing antibodies in rabbits inoculated with repeated i.v. injections of *T. vaginalis* mixed cultures killed with formalin. Trussell (1946), studying the development of agglutinins in rabbits injected with axenic cultures of *T. vaginalis*, found macroagglutination less satisfactory than microagglutination. The highest titres obtained were 1:5000 to 1:10000. MacDonald and Tatum (1948) obtained specific antisera against *P. hominis*, *T. vaginalis* and *T. foetus* by injecting formalinized whole cells for demonstration of agglutination and agglomeration of these trichomonads. *T. vaginalis* and *P. hominis* were antigenically identical also in cross-reaction at the same titre. Specific adsorption of antisera by either *T. vaginalis* or *T. hominis* removes the antibody for both. *T. foetus* was antigenically different. Tatsuki (1957), using the agglutination test, obtained positive reactions in 84·4% of infected women, the mean agglutination titre being 1:274. In the control group without *T. vaginalis* the positivity was also high, 52·9%, but the titre relatively low (1:30). The lysis of *T. vaginalis* in sera from non-infected women was positive in 60% of the cases, from infected women in 57%. The author states that trichomonas cannot be diagnosed by the agglutination test because *T. vaginalis* is also agglutinated by blood sera of uninfected persons. Also Lanceley (1958) demonstrated the presence of an agglutinating body against *T. vaginalis*. From 20 strains of *T. vaginalis*, 18 were agglutinated in an immune serum and 2 were not. From 10 strains tested against 2 antisera prepared from different specimens, 6 were agglutinated by the one and 4 by the other, but no strain by both antisera. The titre in the immune serum was 1:320, in the control 1:40. Stepkowski and Bartoszewski (1959) obtained positive results using the agglutinin test in 60% of positive females, using the CFR, in only 30%.

Very important investigations were conducted by Teras and his collaborators. Teras (1961) reported that normal human serum contains agglutinins for *T. vaginalis* in titres 1:40–1:80, also normal rabbit serum in titres 1:20–1:80. Therefore, the titre up to 1:80 cannot be regarded as specific; on the other hand the CFR may be regarded as specific. Teras (1959, 1961, 1966) succeeded in finding a reliable explanation: the agglutinin titre of the sera depends largely on the strains of *T. vaginalis* and of its serotype; one serotype gave 3–4 times higher agglutinin titres than the other. In people without contact with *T. vaginalis*-infected patients the agglutinin titre with an antigen never exceeded the titre 1:160, which is regarded as the limit titre of normal agglutinins. In

TABLE II

Review of the results of agglutination reaction in females and males suffering from trichomoniasis

(*After Teras* et al., 1966)

Clinical form of tricho-moniasis	Female				Male			
	Number of patients	Agglutinin titre			Number of patients	Agglutinin titre		
		1:480 and higher	1:200–1:400	up to 1:160		1:480 and higher	1:200–1:400	up to 1:160
Acute	44	31	13	—	—	—	—	—
Subacute	46	34	12	—	12	10	2	—
Chronic	70	55	15	—	39	22	17	—
Latent	11	11	—	—	32	15	17	—
Total	171	131	40	—	83	47	36	—

patients suffering from trichomoniasis the titre was 1:200–480 and higher (Table II). Specific agglutinins were demonstrable in nearly all men and women suffering from trichomoniasis. This was only due to the fact that the AR was used simultaneously with all serotypes (Table III). Within a year after recovery from trichomoniasis in the male and within 16 months in the female, the agglutinin titre decreased in nearly all cases to its limit in normal blood (1:160). The rate of decrease seems to depend neither on the clinical form of trichomoniasis nor on the initial titre. This supposition was confirmed by Nigesen (1963). There seems to be a temporary infection immunity in the case of genito-urinary trichomoniasis (Teras *et al.*, 1965).

TABLE III

Dependence of results of agglutination reaction on the serotype of the antigen of T. vaginalis *in cases of genito-urinary trichomoniasis* (*After Teras* et al, 1966)

Clinical form of tricho-moniasis	Female					Male				
	Number of patients	Serotypes				Number of patients	Serotypes			
		TN	TLR	TRT	TR		TN	TLR	TRT	TR
Acute	44	26	22	30	26	—	—	—	—	—
Subacute	46	28	23	32	29	12	5	5	8	9
Chronic	70	50	39	53	42	39	17	12	21	18
Latent	11	10	8	6	8	32	16	12	16	16
Total	171	114	92	121	15	83	38	29	45	43

In further experiments, Teras (1961) vaccinated 16 rabbits i.v. with living cultures of *T. vaginalis* 3–4 times in 10-day intervals, and i.v., i.m. or s.c. with cultures killed by heating. The highest agglutinin titre (up to 10 000) was found in rabbits vaccinated with living cultures. After injection of killed trichomonads the titre was up to 1:5 000. It seems most probable that *T. vaginalis* causes only a non-sterile immunity in rabbits, which vanishes soon after the disappearance of the infective agent from the organisms.

Summarizing the investigations by Teras *et al.* (1961–1966), the results obtained in the diagnosis of genito-urinary trichomoniasis by means of CFR and AR can be considered reliable only in cases in which serotypes most common in the given area are used as antigens in both reactions.

C. THE INTRADERMAL TEST (IDT)

The results of the IDT with specific antigen prepared from pure *T. vaginalis* were unsatisfactory in cases of genito-urinary trichomoniasis (Adler and Sadowsky, 1947; Lanceley, 1958), because positive reactions were obtained not only from patients suffering from trichomoniasis, but also quite often from persons with no detectable *T. vaginalis* infection. Adler and Sadowsky (1947) found positive reactions in 81 % of the Trichomonas-group and in 23 % of the control group, without correlation between the severity of the symptoms and the intensity of the skin test). Better results were obtained by Kawai *et al.* (1961), who noted that the IDT becomes positive about 2 weeks after the onset of infection. Two extracts were obtained from *T. vaginalis*, one without specific characteristics, the other, identified by 3 spots on 2-dimensional paper chromatography as being composed of some polysaccharides, causing a specific IDT in women with *T. vaginalis* infection. The IDT was performed on the forearms of 530 out-patients and was considered positive if the area of erythema attained more than 10 mm diameter. An erythema of more than 18 mm always indicated the presence of *T. vaginalis*. The area of erythema decreased with treatment and the test became negative when the trichomonas disappeared from the smears. Therefore this skin test has a diagnostic value for *T. vaginalis* infection.

Jaakmees and Teras (1966) obtained better results with corpuscular antigen than with antigen-lysates, confirming the conclusions drawn by Anita-Radtchenko (1959) and Sinelnikova (1961). The corpuscular antigen prepared from the serotypes TLR, TN, TRT and TR caused either reddening or swelling in every person in the control group (20 men and 20 women), but those symptoms were demonstrable in all women (41) suffering from trichomoniasis. Therefore the IDT can be recommended for the diagnosis of genito-urinary trichomoniasis, although the specific allergy does not depend on the content of CF- and AR-bodies in the blood serum. It is evident that the CFR and AR can be substituted by the IDT and vice versa in the diagnosis of trichomoniasis. The best IDT is obtained with the serotypes TR and TLR. Higher allergic reaction occurred more frequently in women with acute or subacute trichomoniasis, less often in women with chronic and latent forms (Table IV). A positive reaction could also be obtained from persons negative for *T. vaginalis*,

TABLE IV

Dependence of results of the intradermal test on the clinical form of trichomoniasis in the female and male

(*After Jaakmees and Teras*, 1966)

	Clinical form of trichomoniasis	Number of patients	Intensity of reaction		
			++ or +++ for females, only ++ for males	+	0
Female	acute and subacute	75	21	38	16
	chronic and latent	51	6	26	19
	Total	126	27	64	35
Male	acute and subacute	8	3	2	3
	chronic	26	7	10	9
	latent	28	1	13	14
	Total	62	11	25	26

but this occurred only in cases where the sexual partners of the patients were ill with *T. vaginalis* (latent stages or infections of some time ago).

D. OTHER SEROLOGICAL REACTIONS

Haemagglutination test (HT): Lanceley and MacEntegart (1953) were not able to find a positive HT in experimentally infected women.

Immunofluorescent reaction was employed by MacEntegart (1958) and later by Kučera and Kramář (1965). In the indirect Coons method sera positive results were obtained from 17 women and 2 men suffering from trichomoniasis at various stages, while sera of the 6 negative persons were also negative.

Protection test (PT): in the experiments by Teras (1961) the PT of 19 blood sera, after injections of mice with a pure culture of *T. vaginalis* s-o. from patients with vaginal trichomoniasis, shows two distinct groups: the first comprises 11 sera with a high protective effect (in the injected mice in general *T. vaginalis* did not cause any pathological changes, or they were very slight). In the second group of 8 sera a weaker protective effect was observed, but compared with the unprotected controls the pathological changes in the protected mice were slighter. There was no link between the protective effect of the investigated sera and the results of CFR, between the degree of the protective power and the clinical form of trichomoniasis, the duration of the infection or the age of the patients. In his following work Teras (1963) found

no differences in the PT in mice with antisera from strong virulent and low virulent strains of *T. vaginalis*. Testing the pathogenicity of different serotypes of *T. vaginalis* on monocellular cultures, Teras and Tompel (1963) could find no differences. Only immediately after the isolation from patients was the effect considerably higher.

Vava (1958) immunized mice with three injections at 4-day intervals of heat-killed *T. vaginalis* cultures, followed by an injection of living *T. vaginalis* cultures into the hindleg: only in 24% of the immunized animals were *Trichomonas* abscesses formed, in contrast to 100% in the unimmunized animals. After an immunization with *T. gallinae* and *T. vaginalis*, abscesses occurred in 43% of mice in the first case and in 47% in the second case. This shows that a partial immunity to *T. vaginalis* develops even after an immunization with *T. gallinae* and *T. foetus*.

The *mucous-agglutination reaction* (MAR), after Rom and de Thiery (1958), with the mucous content of vagina from infected women, gives no applicable results. This is an important difference between *T. foetus* and *T. vaginalis* (Florent, 1938).

Weld and Kean (1958) described in the human serum a *cytolytic factor*, destroying *T. vaginalis* in a few minutes. This factor is inactivated by heating for 30 minutes at 56°C. In the experiments of Reisenhofer (1963) all of the examined human sera as well as the sera from horses, cows, dogs, sheep and pigs showed the property of damaging *T. vaginalis*. Their cytolytic powers are reduced by storage and inactivation. While most of the human sera will not agglutinate *T. vaginalis*, the latter will be agglutinated by all the animal sera examined, mostly in the dilution of 1:32 (1:16:164). Samuels and Chun-Hoon (1964) showed that active normal sera or other body fluids from amphibians, reptiles, birds and mammals (e.g. chicken egg yolk, yolk-sac contents, human ascitic fluid and cow milk) cytolyse *T. angusta*. Heat-inactivated sera only agglutinated this species.

The *Sabin-Feldman reaction* (SFR) on toxoplasmosis is highly specific for toxoplasmosis and there is no cross-reaction in women infected with *T. vaginalis* (Piekarski *et al.*, 1957). The opinion of Michalzyk (1953) that the SFR is unspecifically positive in such cases has not been confirmed. There is no relationship between toxoplasma and trichomoniasis. Fuchs *et al.* (1964) studied the possible influence of trichomoniasis on the results of toxoplasmin tests in 722 women. No statistically significant difference was found in the incidence of positive tests in infected and non-infected groups of women. CFR on toxoplasmosis in 294 women had the same negative results.

E. DIFFERENT SEROTYPES OF *T. vaginalis* IN LABORATORY ANIMALS

Schoenherr (1956), using AR, CFR and precipitation, distinguished not only *T. vaginalis* from *T. hominis*, *T. gallinae* and *T. foetus*, but also some serotypes in *T. vaginalis*. Kott and Adler (1961) proved by simple cross-agglutination and cross-adsorption tests that *T. vaginalis* is not a serological homogenous species, but consists of a number of distinct serotypes, though all

strains have agglutinogens in common. Eight distinct serotypes were found among 19 strains of *T. vaginalis*. The results of both reactions were constant during an observation period of 2½ years. Two strains seem to have changed their antigenic structure after being contaminated with bacteria. Two distinct serotypes could easily be distinguished among 5 strains of *P. hominis*—the serotype I had some agglutinogens in common with *T. vaginalis*, but not the serotype II. Sera prepared against 3 strains of *T. tenax* did not agglutinate any of the strains of *T. vaginalis* and *P. hominis* examined. Specific sera against *T. tenax* contained two types of antibodies: one agglutinating the flagellates and one paralysing their flagella.

Hoffman and Gorczynski (1964), using AR, HR and CFR, could distinguish in the 23 strains of *T. vaginalis* the existence of several serological types. Thirteen strains had the same antigenic structure as that of the three strains used for preparations of the antigens; 5 strains were comparable to that of another TA strain; and the remaining 5 strains were serologically different from all used strains.

TABLE V

Distribution of typified strains of T. vaginalis *according to clinical forms of trichomoniasis*

(*After Teras* et al., 1966)

Clinical form of trichomoniasis	Total strains	Serotype of *T. vaginalis*			
		TN	TLR	TRT	RT
Acute	9	1	—	2	6
Subacute	25	3	5	8	9
Chronic	45	4	4	16	21
Latent	20	6	4	3	7
Total	99	14	13	29	43

Teras (1959, 1962, 1963, 1965), Nigesen (1963) and Jaakmees (1965) found four additional different serotypes, designated TR, TN, TRT and TLR (this designation is not a good one). The distribution of these serotypes in 99 patients is shown in Table V. Of 100 isolated *T. vaginalis* strains, 43 % belonged to the serotype TR, 29 % to TRT, 14 % to TN and 13 % to TLR. It is interesting that in 32 cases of the 34 married couples, the strains isolated from both spouses belonged to the same serotype. In the two not corresponding cases the husbands had extramarital intercourse. Table VI shows that none of the four serotypes was found to be associated with only one particular clinical form, neither could any correlation between the antigenic properties and the virulence be observed. Only in the AR and CFR could the authors find differences in the intensity of these reactions. All four serotypes have an antigenic component in common.

TABLE VI

Dependence of complement fixation and agglutinin reaction on the serotypes in patients infected with the serotypes TR, TRT, TN, or TLR of Trichomonas vaginalis

(*After Teras* et al., 1966)

	Serotypes of *T. vaginalis*	Total strains	With serotype TR	TRT	TN	TRL	With homologous serotypes	With all serotypes
Complement fixation	TR	43	43	32	33	33	43	24
	TRT	29	24	29	23	24	29	29
	TN	14	12	12	13	13	13	11
	TLR	13	9	12	11	13	13	9
	Total	99	88	85	90	93	98	64
Agglutination 1:320 and higher	TR	43	43	30	27	24	43	15
	TRT	29	17	28	19	11	28	6
	TN	14	7	8	13	4	13	2
	TLR	13	7	8	9	13	13	3
	Total	99	74	84	68	52	97	26

The use of a polyvalent antigen, prepared from all known serotypes of *T. vaginalis*, is necessary to estimate correctly the value of all serological reactions for the diagnosis of trichomoniasis, as well as to check the results of treatment and to investigate different questions concerning immunity (Teras *et al.*, 1965).

VII. EXPERIMENTAL INFECTION IN LABORATORY ANIMALS

Earlier studies performed with bacteria-infected cultures of *T. vaginalis* or directly with *Trichomonas* containing vaginal secretions are, at present, only of historical interest. As soon as bacteria-free cultures became available, interesting results were achieved in renewed experiments.

A. INTRAVAGINAL TRANSMISSION

Schnitzer and Kelly (1954) and Uhlenhuth and Schoenherr (1955) transferred a culture of *T. vaginalis* to the vagina of the Golden Hamster, infecting 20–30% of experimental animals after one inoculation, 80–90% after repeated inoculation. The infection persisted for 1 year and longer, and also a transmission from one hamster to another was possible. Cavier and Mossion (1957) describe a successful intravaginal transmission to rats in permanent oestrus. Castrated female rats were injected either daily with 1·2–5 μg oestradiol-benzoate in oil; or every 8th day 5 mg of crystal suspension of this substance was implanted subcutaneously, or 10 mg every fortnight. The consequence was a permanent oestrus. *T. vaginalis* multiplied in the vagina of the rats and

persisted for about 40–76 days after the discontinuation of the hormonal treatment. The same results in non-castrated rats were obtained by Combescot *et al.* (1957b). In this case a permanent oestrus of the rats was induced by a subcutaneous implantation of 20 mg oestradiol. Successful infection was achieved in 80–100% of the cases. Daily injections of 2·5–5 mg progesteron caused the disappearance of *T. vaginalis*. Combescot *et al.* (1957c) observed in castrated rats that the pH in the vagina remained almost neutral (6·6–7·5). In artificially induced hibernation (e.g. with chlorpromazine) the temperature in the vagina was reduced to 20°C. This had no effect on *T. vaginalis*, which has been found capable of surviving even in dead animals at a temperature of 6°C (Combescot *et al.*, 1957a). Mandoul *et al.* (1957) tried unsuccessfully to obtain a lasting infection with *T. tenax* in the vagina of young castrated rats, which had been brought artificially into a state of permanent oestrus; the presence of the parasite in the vagina could be confirmed only 48–96 h after inoculation. This also proves that both parasites are independent species. Vershinskii (1957) intravaginally infected hamsters, this infection lasting from 2 weeks to several months. In two animals, triflagellate trichomonads, measuring only 6–7 μ, could be seen in the vagina.

Soszka *et al.* (1962) and Ginel (1962) inoculated guinea-pigs with a pure culture of *T. vaginalis*. This successful inoculation was used by these authors in later work (1962) for biochemical studies of *T. vaginalis* infection. The *Trichomonas* inflammation in the vagina of guinea-pigs was expressed by an accumulation of acid or neutral mucopolysaccharides on the surface of the epithelium. In the germinative layer of the epithelium a decrease of the DNA and an increase of the RNA were registered. Following treatment, the RNA slowly receded and the DNA increased significantly in the nuclei in this layer. According to many investigators, long-lasting elevations of the RNA and DNA are significant for neoplastic states. The long-lasting process of infection with *T. vaginalis*, as well as uncompleted treatment, changes the cells and the intercellular substance metabolism in the epithelium of the vagina, which may be the starting point for a pre- and carcinomatous state. Similar observations were also made by Kazanowska (1962). Ginel (1962), inoculating the vagina of guinea-pigs with pure cultures of *T. vaginalis*, found on the third day distinct symptoms of inflammation of the reproductive tracts, expressed by a swelling and reddening of the vulva and by the presence of an abundant, yellow, foamy and offensively smelling secretion. Great masses of leucocytes, *T. vaginalis*, and shedded and changed epithelial cells could be demonstrated on the slides. There were also numerous mononuclear macrophages present among the leucocytes. The epithelial cells were characterized by the presence of relatively large hyperchromatic nuclei of two types: one with a coarsely grained chromatin, the second without granular structure. The guinea-pigs aborted usually single foetuses, which were macerated. The entire mucous membrane of the vagina exhibited all the morphological characteristics of inflammation: swelling of the substratum, dilatation and hyperemia of the blood vessels particularly under the epithelium, often giving the impression of endothelial penetration, and the presence of numerous white cells migrating in the connective tissue and epithelium.

B. PERITONEAL TRANSMISSION

Peritoneal transmission of axenic cultures of *T. vaginalis* in mice produces a purulent fibrinous peritonitis with necrotic foci in the liver, pancreas, spleen, lymphatic nodules and other abdominal organs, with the formation of ascitic fluid (Schnitzer *et al.*, 1950; Hamada, 1953; Yamagata, 1954; Teras, 1954–1966; Iwai, 1957; Vershinskii, 1958; Paronikjan, 1958; Reardon *et al.*, 1961; Honigberg, 1961–1966; and some other recent investigators). The infective dose is 1·5–4 millions (Teras, 1954; Vershinskii, 1958), the LD_{50} is 1·75 millions of flagellates (Nigesen, 1961). Cultivation on media containing agar, methyl-cellulose or gelatine stimulates the pathogenicity of *T. vaginalis* and *T. gallinae* (Honigberg, 1959). Many mice died during the first week p.i. Also in guinea-pigs, chronic inflammation and formation of granulation tissue could be observed. Iwai (1957, 1959) detected differences in pathogenicity in three strains of *T. vaginalis* with the aid of i.p. inoculation. Reardon and Jacobs (1958) isolated a highly virulent strain C and a low virulent strain R from mice. Strains of *T. vaginalis* isolated from patients with acute and subacute colpitis proved to be much more pathogenic than those isolated from patients with chronic colpitis (Bogowsky and Teras, 1958; Reardon and Jacobs, 1958; Reardon *et al.*, 1961; and others).

Teras and Roigas (1966) published studies on 171 freshly isolated strains. Seventy-four of them (43%) were found to be of high pathogenicity, 76 of medium (44%) and only 21 of low pathogenicity (12%). A non-pathogenic strain has not yet been found.

The earliest and most frequent pathological change is fibrinous purulent peritonitis, located particularly in the region of the liver and spleen. One of the most characteristic phenomena, observed especially in mice killed at the end of the observation period, was a conglomerate consisting of liver, stomach, spleen, pancreas and the lymph nodes of the upper part of the abdominal cavity, all these organs being covered with a fibrinously purulent exudate containing many leucocytes and trichomonads. Often foci resembling abscesses and containing an enormous number of leucocytes and *T. vaginalis*, localized on the mesenterium and on the peritoneum covering the intestine, were detected. Strains of high pathogenicity invaded the liver, causing typical necrotic foci. The macroscopic aspect showed grey foci, of a slightly yellowish shade and of cheese-like density. Their size varied greatly—in some cases they were hardly as big as a pin, in others they occupied almost a whole lobe. Trichomonads were detected also in the blood vessels passing through the necrotic foci of the liver. Ordinarily there was a thickening and an inflammation of the intima of the veins. Pathological change, detectable as a rule only in histological investigations, could be observed most frequently also in the pancreas, in the gastric wall and in the lymph nodes of the abdominal cavity. More uncommon was the invasion of trichomonads into the spleen, this being observed generally only in lethal cases. Only in a few cases was an invasion into the suprarenal glands observed. A high death rate (5–8 days at an early stage) was characteristic for these highly pathogenic strains.

Inoculation of strains with a medium pathogenicity was at first also followed

by fibrinously purulent peritonitis, sometimes in the form of circumscribed foci. However, these changes were detectable later and to a smaller extent, the amount of exudate in the peritoneal cavity being smaller in such instances. *T. vaginalis* also invades the liver, but these cases are rare and occur at a later period; in some cases it penetrates the pancreas (atrophy of glandular follicles), and rarely the gastric wall and the lymph nodes. The death rate of infected mice was markedly lower (up to 21 days). Inoculation of strains with low pathogenicity showed, in animals killed during the first days after inoculation, only a weak exudation and simultaneously some proliferative changes. Very rarely trichomonads were found to invade the abdominal organs. The mice died within 4–8 weeks. A comparatively simple method for testing the pathogenicity of strains of *T. vaginalis* has been worked out by the authors: 10–11 mice are inoculated i.p. and killed after 10 days. The results are tabulated as an index with 10 points. The most important and severest injury is the necrosis in the liver. Index O-mice die spontaneously, index 10-mice are without infection and without any pathological changes. The more invasive strains are more resistant to osarzol, sanazine and uropine (Teras, 1958). Neither the pathogenicity of *T. vaginalis* nor the clinical forms of the genito-urinary infection have any correlation with the serotype of the trichomonads. On the other hand the clinical forms of trichomoniasis depend on the degree of pathogenicity of *T. vaginalis* (Teras, 1965).

From 48 *T. vaginalis* strains isolated by Laan (1966), 26 strains were highly pathogenic for mice (i.p.), 18 were of medium and 4 were of low pathogenicity. The pathogenicity of cultivation in TN-1 medium (up to 4 months) did not change essentially, but decreased constantly in prolonged passage up to 32 months. The pathogenicity of the serotypes TLR and TN disappeared in prolonged passage in medium *T. vaginalis*-1. The virulence of *T. vaginalis* is changeable; it is reduced by stovarsol. On the other hand, the antigenic properties among the strains of *T. vaginalis* are stable *in vitro* as well as *in vivo*. The fermentative activity in the production of acid from maltose and glucose was less intensive in strains with high virulence, than that in the strains of medium and low virulence, but no changes could be observed in the fermentation of lactose, sucrose and manitol. Only the agglutination proved to be stable in prolonged passages *in vitro* and repeated passages *in vivo*. The strains of *T. vaginalis* could be identified only by their antigenic differences.

In sections of different organs of mice infected with *T. vaginalis* cultures, a specific immunofluorescence was observed, which was particularly strong in kidney and lung sections, but less intensive in liver and spleen sections (Karbowski, 1966). These results indicate that the immunofluorescent method is suitable for revealing the distribution of trichomonads in the tissues of experimentally infected mice and probably also in man.

C. SUBCUTANEOUS INOCULATION

Subcutaneous inoculation of *T. vaginalis* in mice produced subcutaneous lesions with a great number of flagellates (Schnitzer *et al.*, 1950; Paronikjan, 1958; Honigberg, 1961; and others). The acute stage developed during the

first 8 h, later changing into reparative processes with histiocytic infiltration and giant cell formation. After 1–2 weeks all pathological changes disappeared. *T. vaginalis* were distributed in the edematic tissue and could also be found in more distant macrophages.

Honigberg (1961) developed a very simple method for the determination of the pathogenicity of different *T. vaginalis* strains: subcutaneous inoculation of mice with fresh axenic cultures and subsequent measurements of the volume of lesions caused by the flagellates. The mean volume of 6-day-old lesions served as the basis for evaluating the relative pathogenicity level of a given strain. A statistical comparison (Honigberg, 1961) of subcutaneous lesions produced in mice by the several strains of *T. vaginalis* reveals that these volumes faithfully respect their relative virulence to the natural host. The least virulent strains of *T. vaginalis* are still more harmful to mice than the least pathogenic strains of *T. gallinae*. Maintained in culture the strains of *T. vaginalis* become attenuated in their virulence. The presence of agar must have some influence upon the physiology of the parasites, rendering them more pathogenic, and the presence of methyl-cellulose was found similarly influential upon lesion-production. The lesions were smaller when mucin was substituted for agar. Abscesses produced by the parasite in the presence of gelatin were not enlarged.

In subcutaneous lesions produced by *T. vaginalis* (after Frost and Honigberg, 1962), the mechanisms of progression in all strains were studied orderly and found to involve: influx of polymorphonuclear leucocytes, multiplication of parasites, death of leucocytes, destruction of the host tissues with the lysis of the abscess wall, edema of the surrounding tissues and spreading of the flagellates, influx of leucocytes, remultiplication of the parasites and continuation of the cycle, the progression of which results in pure mantles of either leucocytes or trichomonads formed against the inner wall of the injection pocket. Both the precise picture and time sequence vary with the strain of *T. vaginalis* and are related to its pathogenicity. An excellent correlation was found between the experimental laboratory data evaluated by the mean volumes of subcutaneous lesions produced by the parasites in C-57/B1 mice 6 days after inoculation, and the severity of vaginal and cervical diseases of the female patients (Honigberg *et al.*, 1966). Ivey and Hall (1964) found no relationship between the human host status and the virulence of the strain for mice infected by the intraperitoneal route. Only after subcutaneous inoculation most strains from symptomatic patients showed more marked virulence for mice than did any of the strains isolated from normal persons.

With great probability the results of infestation experiments also depend on the strains of mice used. Kulda (1965), in applying Honigberg's methods, proved that minimal development was noted in the strain C-57/B1 (132 mm^3) and maximal in the mouse strain A (309 mm^3). He recommends the strain Balb/c as a standard for testing virulence. The strains DBA/2 and DBA seem unsuitable for these purposes.

Subcutaneous inoculation of germ-free guinea-pigs with *T. vaginalis* cultures shows important differences between the strain C with a strong virulence and the R-strain with a low one. The same was observed when using mice for

i.p. inoculation. The strain C killed the mice in a few days, while strain R did not (Newton *et al.*, 1960).

D. OTHER PATHWAYS OF INFECTION

Weld and Kean (1956) succeeded in inoculating *T. vaginalis* into the anterior chamber of the eye and into the vitreous body of rabbits. In 22 out of the 28 inoculated eyes flagellates were found in both sites. The best method is the inoculation into the vitreous body. Even after intramuscular inoculation deep abscesses containing numerous trichomonads are formed after 2–3 weeks. Infection could not be obtained in mice infected intranasally, orally and intra-intestinally (Paronikjan, 1958).

Inoki and Hamada (1954) describe changing experiments with *T. vaginalis*. When inoculating mice i.v. with 0·1–0·5 ml of washed chicken erythrocytes, and additionally with pure *T. vaginalis* culture, 70% of the mice died within 14–40 days. The recultivated trichomonads were found to grow in media which had been considered unsuitable and are described as having 3 flagella and a long undulating membrane. This change from *T. vaginalis* to *T. foetus* has not been confirmed by other authors and there are certain doubts as to its correctness.

E. PATHOGENICITY OF *T. vaginalis* FOR CELL CULTURES

This pathogenicity was shown by Hogue, and Honigberg *et al.* (1961–1966) continued the studies with different cell cultures (HeLa cells, chick-liver cells, fibroblasts, epithelial cells, and others) and *T. vaginalis* strains of different virulence. *T. vaginalis* caused degenerative changes in all three cell types and, to a lesser or greater extent, also in the filtrates of rich cultures (Honigberg and Ewalt, 1961). All the effects are more pronounced in infection with more pathogenic strains. The original pathogenicity could be maintained for two years from the time of isolation in axenic cultures by keeping them at about −72°C in the presence of glycerol (Honigberg and King, 1962). Healthy trichomonads of the pathogenic strain are often found within fibroblasts and especially epithelial cells, which then undergo many abnormal changes. Pathological changes occur also in cells which are neither in close contact with nor contain any trichomonads. In fibroblasts the division is stopped, commonly in the prophase, and these cells later degenerate. The introduction of cell-free filtrates of active *Trichomonas* cultures results in the appearance of many abnormal changes in the cell cultures, and these are similar to but typically less extensive than those observed in the presence of the parasites. The effects of filtrates from cultures of pathogenic strains are more pronounced than those of the mild ones. In chick-liver cultures, within 2 h over 70% of macrophages contain one or more parasite. In most instances the intracellular trichomonads degenerate. Occasionally the flagellates can multiply and may ultimately destroy the phagocytes. Only 1% of the epithelial cells in the avian tissue cultures are invaded after 2 h, 6% after 8–12 h. The epithelial cells appear somewhat more resistant than fibroblast-like cells. HeLa cells are rarely invaded by the flagellates. The cytoplast of uninfected cells is retracted and the

nuclei show signs of degeneration. It is likely that some at least of the substances produced by the parasite either alone or in combination with the degenerating tissue are lytic in nature.

Christian *et al.* (1963) used relatively small inocula (5000 organisms) for infection of HeLa cell cultures. They observed no intracellular parasites, but reported the appearance of lesions that were lined with trichomonads. After massive inocula (5×10^5) of a relatively pathogenic strain, Honigberg and Ewalt (1961) observed parasites within some cells, especially in advanced infections. The parasites must have exerted both mechanical and chemical effects.

Trypsin-dispersed chick-liver cultures, after infection with a relatively pathogenic strain, show the following changes (Sharma and Honigberg, 1966). Fibroblasts and epithelial cells gradually lose their cytoplasmic RNA. Nuclear DNA levels of all cell types appear to show no significant changes. Glycogen is not stored in the fibroblasts, but epithelial cells show much of this polysaccharide and macrophages show moderate amounts. Acid mucopolysaccharides are not demonstrable by this method. Progression of the infection leads to a large accumulation of lipids in fibroblasts and epithelial cells. Significant loss of lipids is seen in phagocytes which contain *T. vaginalis*. Phospholipids were not demonstrable.

VIII. Trichomoniasis as a Clinical Entity

Genito-urinary trichomoniasis has been acknowledged as a clinical entity since the description by Höhne (1916). Perju (1957) gave the following definition:

> La trichomoniase urogénitale est une entité morbide parasitaire produit par le flagellé *Trichomonas vaginalis* chez l'homme et chez la femme evoluant sous diverses formes uniques ou associées et présentant un marqué caractère vénérien.

A. *Trichomonas* in Adult Women

The clinical symptoms are very well known and nothing of importance has been published in recent years. Only some observations concerning the pathobiology must be mentioned.

1. *Pathobiology of* T. vaginalis

The greatest number of trichomonads appear in vaginal secretion in the late luteal phase and in the early oestrogen phase (VII, I) and then in phases II of the cycle according to De Allende, the lowest number appearing in phases IV, V and VI (Kurnatowska, 1958). In climacteric women the population density of *T. vaginalis* varies irregularly. Experiments *in vitro*, using cervical mucus of 30 women and *T. vaginalis* strains from 24 patients, indicate that the cervical mucus may serve as a barrier preventing the trichomonads from entering. In 91 % of pregnant women infected with *T. vaginalis* inflammatory manifestations are present in the vagina.

After abortion, during labour and puerperium, complications occurred twice as often in women with *T. vaginalis* as in the control groups. The most common complications in puerperium were: a higher temperature, foetid excretions and puerperal inflammation of the uterine mucous membrane (Jedrzejczak, 1966). In view of the evidence that 90% of the 975 cases of trichomoniasis had erosions, it can be assumed that the inflammatory conditions induced by *T. vaginalis* may have an effect on the occurrence and development of cervical erosion. For that reason antitrichomonial measures should be considered in the prophylaxis of precancerous states (Zawadzki, 1966).

The penetration of other organs by *T. vaginalis*, very common in laboratory mammals inoculated with pure culture, seems to be very rare in women. A case is reported by Hoffman *et al.* (1966):

> from a patient suffering from generalized carcinoma with concomitant trichomonads in the vagina, the authors could cultivate *T. vaginalis* from the cancerous foci in the lungs and liver and from section of the spleen and vaginal mucous membrane.

Two isolations of *T. vaginalis* from the oviduct are reported by Zwierz and Klyszejko (1964).

Moore and Simpson (1954) and McEwen (1960) point out psychosomatic symptoms during trichomoniasis, such as sexual disturbances, dispareunia, frigidity, moral laxity, emotional instability, religious obstacles. Certainly every physician must take into account these psychic complications, but we must refuse their postulate: "*T. vaginalis* is a normal contaminator of the vulva and vagina, waiting only for favourable conditions for reproduction and, therefore, not a venereal disease".

Contrary to that, many new investigations proved trichomoniasis to be a true venereal infection and venereal disease.

2. T. vaginalis *in the urinary tract*

T. vaginalis survives in the female urethra, in the paraurethral glands, in mucous crypts etc., causing apparently less damage than in the vagina, although adequate histological studies are not yet available. Evidence of trichomonads in urine was provided by many authors in the last decade. It is possible that bacteria are transported to the bladder by *T. vaginalis* (Kean, 1955). Some relapses can have their origin in urethral infection. Grys (1966) found *T. vaginalis* in the bladder, accompanied by an increased number of inflammatory elements in the sediment. Catheterization can introduce *T. vaginalis* into the bladder from infected urethra (Grys, 1966). Glebski (1966) could not find any differences in the incidence of *T. vaginalis* in the urine from diabetic patients (4·5% positive) and the controls (4%). In 76% of trichomoniasis cases Durel and Roiron-Rattner (1957) also found trichomonads causing urethritis in women. Glebski (1966) assumed that certain features of the urine such as specific weight, pH, albumin content, presence of cylinders, erythrocytes and leucocytes in the sediment, and presence of bacteria occur in statistically frequent cases in connection with the presence of trichomonads, *T. vaginalis* has no effect on the morphological composition of the blood.

Candiani has found no differences in the content of pregnandiol, estrogen and 17-keto-steroids in the urine during *Trichomonas* infections.

Grys (1964), investigating by cultivation samples collected from the genital organs of 387 women, found *T. vaginalis* in urethra alone in 1·7% of the cases, in the paraurethral glands in 3·2%, in the external orifice of the vagina in 3·2%, in the urethra, paraurethral glands and vagina in 68·8%, in the urethra, paraurethral glands, vagina, cervical canal in 13·1%, in the urethra and paraurethral glands in 3·2%, in the urethra and external orifice of vagina in 0·6% and in paraurethral glands and external orifice of vagina in 12·7%. Out of 32 patients operated who had been infected with *T. vaginalis* before the operation, the presence of trichomonads in the corpus and in the cervical canal was observed in one case only.

Littlewood and Kohler (1966) noted *T. vaginalis* in a female infant of approximately 28 weeks gestation with abdominal distension and readily palpable kidney on the 19th day of life. The flagellates were present in the urine and were associated with significant pyuria. On the 64th day oral treatment with metronidazol (60 mg t.d.s. for 7 days) was started and this rapidly eradicated the parasite and the pyuria.

3. *The influence of* T. vaginalis *on the vaginal epithelium*

Bechtold and Reicher (1952), Řeřábek *et al.* (1953) and Teter and Polachowski (1954) showed that in vaginal smears containing *T. vaginalis* the epithelial cells acquire a malignant aspect as in anisonucleosis, poikilonucleosis. large granulation of the chromatin etc. Pundel (1957), Charvet *et al.* (1957), and others confirmed these findings. Papanicolau and Wolinska (1955) summarize this problem as follows:

> In cases of *T. vaginalis* infestation vaginal smears exhibit a characteristic pattern: a marked increase in the number of the cornified cells of the superficial squamous type. Some of the cells have irregular outlines and are covered with smudges and grayish mucus. The cytoplasm is often dense and intensively acidophilic. The nuclei are deeply stained in well preserved cells, while in degenerating cells they are faintly stained and have a pinkish appearance. Marked keratinization of the cells is occasionally noted. The parabasal cells are often prominent and appear singly or in small clusters. They show some variation in size and a more pronounced vacuolization of the cytoplasm than seen in normal cells and, sometimes, an increased affinity to acidophilic stains. All these changes may in some cases be the result of a concomitant secondary inflammatory condition. In a number of *T. vaginalis* positive smears exfoliated cells may exhibit marked nuclear atypia. This consists chiefly in an enlargement, irregularity in form and hyperchromatia of the nuclei. These changes when pronounced may lead to a false interpretation of the cell as malignant. It is thus important to explore thoroughly every vaginal and cervical smear showing nuclear atypia to rule out the presence of trichomonads. This does not imply that every smear with abnormal nuclear forms, in which trichomonads have been found, should be considered to be negative for malignancy. On the other hand, more conclusive evidence should be sought before such a smear is reported as definitely positive. Nuclear atypia in association with trichomonads may be observed not only in the superficial, intermediate and parabasal squamous cells, but also in cells of

endocervical origin. Extreme cytologic abbreviations are seen in a relatively small number of trichomoniases. In most instances the smear shows only minor morphological changes and in many cases there may be a complete absence of clinical symptoms.

The identification of *T. vaginalis* in smears stained after Papanicolau is more difficult than by the Giemsa method, because the flagella, the undulating membrane and the axostyle are not stained.

Kazanowska (1962) observed inflammatory changes resembling in appearance mucous membranes observed in precancerous states. Occasionally a coëxistence of cancer and trichomonads is observed.

After Frost (1962), the cytology of the *T. vaginalis* infestations can be divided, in view of its cellular reaction, into three parts: the general background, the regressive cellular changes of degeneration due to tissue injury and destruction, and the progressive cellular changes of regeneration in reaction to injury of dysplasia and of neoplasia. All three components of the cellular response pattern may be found. In such cases a clear differentiation is most important.

Holtorff and Krimmenau (1960), studying colposcopy of trichomoniasis, made the following observation: in 4·1 % of the patients the vagina was not inflamed in spite of the presence of trichomonads; in 10·5 % the irritation was not visible to the eye, but colposcopy revealed a diffuse, flat papillary formation. In 18·1 %, grade I of the inflammation was a diffuse vaginitis with formation of papillae spreading over the cervical and vaginal mucous membrane. This became clearly visible after the iodine test. In 23·7 % grade II of inflammation was a diffuse reddening or reddish spots on the macroscopic picture. Colposcopy showed very well developed slightly elevated edematous papillae of connective tissue with marked hyperemia of the vessels. Often, the papillae coalesce in the presence of the accompanying edema, appearing as small irregular spots. Inflammation grade III was most frequent (30·9 %). Macroscopic symptoms are red spots, which start bleeding at the slightest touch. Colposcopy showed numerous foci of varying appearance—fine, washed out, edematous, highly elevated above the general surface. These round foci are marked by hyperemia, dark-red dense, capillary loops, their margins being moistened with a yellowish secretion. Less frequent are fine fibrinous membranes giving the appearance of fibrinous colpitis. The mucous membrane is highly edematic, most sensitive and bleeds easily (colpitis granularis).

4. *Sterility and* T. vaginalis

Long-lasting infection of *T. vaginalis* is followed by sterility (Gautier and Biguet, 1957; and others). On the other hand, after successful treatment, which has cured the patient or at least has caused the disease to become latent, many can conceive and give birth to a healthy, normal child. Phagocytosis of the sperms by trichomonads, as emphasized by Kolesov (1950), is of no importance; in fact we have never observed it during all these years; but it is possible that toxic metabolites of *T. vaginalis* may reduce the motility of the sperms. According to Hynie *et al.* (1960) this temporary sterility depends on the following factors:

A. In *Trichomonas-colpitis* the mucus of the vagina and cervix is covered with purulent discharge (pseudocervical blockade) so that even short therapeutic measure can cure this sterility. The situation is worse when endocervical inflammation is present and the spermatozoids are caught up in the cervical canal (cervical blockade).

B. In the case of less intensively motile spermatozoids in a more diluted ejaculate it was possible to observe that the spermatozoids were stopping in a medium with more plentiful and active trichomonads. The stopping of the spermatozoids gave the impression that they had come into an area contaminated by toxic substances from the trichomonads. More flagellates had a greater destructive influence on the spermatozoids. In a supernatant from a *T. vaginalis* culture these spermatozoids showed very little motion after 2 h. The crushed trichomonads considerably restrict the motility of the spermatozoids after 1–1½ h. After 3 h there was no movement. Crushed cultures obtained by autolysis for 1–2 days strongly reduced the motility of the spermatozoids within a few minutes, while a 24-hour-old crushed discharge containing trichomonads stopped the intensely motile spermatozoids within 2 h. *T. vaginalis* may be the cause of sterility especially in the culminating stage of very heavy infection. This may apply to cases in which the male's semen is relatively weak and the ejaculate is not sucked into the uterus intensively enough. Thus the spermatozoids remain longer in contact with the vaginal secretion containing trichomonads and find it difficult to penetrate the cervix by their own propelling force.

C. Through inflammatory processes caused by the pathogenic influence of *T. vaginalis* and by pyogenic bacteria, the introitus vaginae often becomes very painful, making cohabitation very difficult and leading to dispareunia.

In the practice every woman suspected of sterility should be examined for the presence of trichomonads and, in positive cases, treatment of both husband and wife should be started before taking other therapeutic measures to cure sterility.

5. *Microbic vaginal pictures and the dynamic aspect of* Trichomonas *infestation*

Mere bacteriological classification of the cleanliness of the vagina (Manu af Heurlin, 1914) does not satisfy clinical needs, for it omits gonococci, *T. vaginalis* and pathogenic yeast organisms. With regard to the entire microfauna and microflora and to the histological elements, Jírovec *et al.* (1947–1948) proposed a new classification of the vaginal biocenosis in adult women. Their six basic groups (called microbic vaginal pictures) were numbered I–VI. The technique is very simple: 2 smears from vaginal contents are dryed in the air, fixed with methyl alcohol, then one is stained with Giemsa 1:10 for 1 h, the other by the Gram method, and both are examined under a microscope (×1000). The basic microbic vaginal pictures are:

I. Normal physiological picture of a healthy adult woman with many epithelial cells, many *Lactobacillus vaginalis* and few leucocytes.

II. Non-purulent bacterial discharge with many epithelial cells, few leucocytes but many different bacteria—cocci, rods, sometimes *Vibrio vaginalis* but no *Lactobacillus*.

III. Purulent bacterial discharge with few epithelial cells, many leucocytes and a mixed flora of bacteria and cocci.

IV. Gonorrheal discharge—in the acute phase many gonococci, Gram-negative of typical form, are found inside the leucocytes; in the chronic form the gonococci could be detected mostly by cultivation.

V. *Trichomonas* discharge.

VI. Vaginal mycosis with *Candida* div. sp., *Torulopsis* sp., *Saccharomyces* div. sp., and others.

The period of sexual rest (1–10 years approximately)—an unoestrogenized vagina—is characterized by the physiological picture "O" expressed cytologically only by basal and parabasal cells; microbes of all kinds are absent, or present only in very small numbers, and in particular there are no *Döderlein* bacilli. No other microbes, e.g. *Vibrio*, *Trichomonas* or yeast-like organisms, occur in this period.

This classification has been introduced to Czechoslovakia, Poland, G.D.R., Rumania and Yugoslavia as a simple routine diagnostic method (Table VII).

In microscopical examination of trichomonal discharge the flagellates are frequently accompanied by bacteria and purulent cells. Sometimes solitary trichomonads in the otherwise normal picture of the vaginal biocenosis indicate a latent trichomoniasis (I/V). Some outstanding gynaecologists have been led to believe that trichomonads in the vagina are quite harmless. The dynamic conception of Peter and Jírovec (1950) shows a logical connection of all stages of the infection encountered in the practice, and at the same time explains the incongruities in the literature. In the course of *Trichomonas* infection they distinguish three or four types concerning the V microbic vaginal picture.

V/A-Trichomoniasis acuta (Fig. 12)

A few days after infection, mostly transmitted by sexual routes, quickly reproducing trichomonads appear in the vaginal secretion, the numbers of epithelial cells decrease, Döderlein's *Lactobacillus* subsides and the inflammation is unfailingly announced by a considerable number of leucocytes. Other bacteria which adjoin later are entirely missing at this stage or can be found only in insignificant numbers. The clinical manifestation, however, does not differ from the subsequent stage. The discharge is strikingly thin, whitish, yellowish or greenish, sometimes dropping from the genitals and often mixed with gas bubbles. This acute trichomoniasis, generally of short duration, is seldom observed in time by the physician as women delay their visit and come for examination when the second stage has set in.

V/B—Culminating trichomoniasis (Fig. 13)

The smears contain numerous trichomonads, many leucocytes and many different bacteria. There is less epithelium and Döderlein's *Lactobacillus* has disappeared. Clinically, there is a thin discharge as described above, lasting for several weeks, months or even years, of the same or fluctuating intensity. This stage, most frequently encountered by the physician, is followed by a chronic stage.

TABLE VII

Frequency of microbic vaginal pictures in women visiting gynaecological ambulatories etc. (sick population)

Microbic vaginal picture	Dáňa, Prague (1949) (1944–1947) %	Žižková (1956) Prague		Kostič, Beograd (1955)		Holtorff, Dresden (1961)	Engelbrecht and Müller, Berlin (1962) %	Lipenský and Viehweg, Demmin, GDR (1963) %
		(1951) %	(1952) %	women (1949) %	men (1958) %			
I	6·0	23·6	24·9	6·0	3·3	4·0	10·1	8·66
II	22·0	21·4	25·1	23·0	18·4	24·0	51·0	15·33
III	24·0	23·2	20·0	22·0	26·1	7·4	24·0	38·0
IV	1·4	0·1	—	3·8	0·56	—	—	0·33
V	38·0	25·0	23·1	38·0	39·0	62·5	14·9	33·0
VI	8·6	6·7	6·7	6·35	12·6	1·9	—	1·0
Number of examinations	3000 = 100%	3255 = 100%		1827=100%		625 = 100%	820 = 100%	300 = 100%

TABLE VIII

Dynamic aspects of Trichomonas *infections*

Microbic vaginal picture	Dáňa, Prague (1949) %	Holtorff, Dresden (1961) %	Engelbrecht and Müller, Berlin (1962) %
V/A	7·6	0	6·1
V/B	68·0	33·0	59·5
V/C	24·4	37·1	22·3
Transitory	—	11/V 29·9	V/B–C 12·1

V/C—Chronic trichomoniasis (Fig. 14)

Trichomonads can be seen in varying numbers, many epithelial cells, few leucocytes and a mixture of rods, cocci and other bacteria, but no Döderlein bacilli. Women complain of an atypical discharge. This chronic stage is sometimes transformed by a keen reduction of the trichomonads into a latent stage I/V with the normal numbers of Döderlein's *Lactobacilli* and epithelial cells, only very few leucocytes and few flagellates, detected in most cases only by culture (Fig. 15). This stage can be artificially brought about by treatment. Table VIII shows the findings by three investigators from different localities.

T. vaginalis is also often associated with different species of yeast-like organisms and yeasts (Microbic picture V/VI). Feo (1953) found in a group of

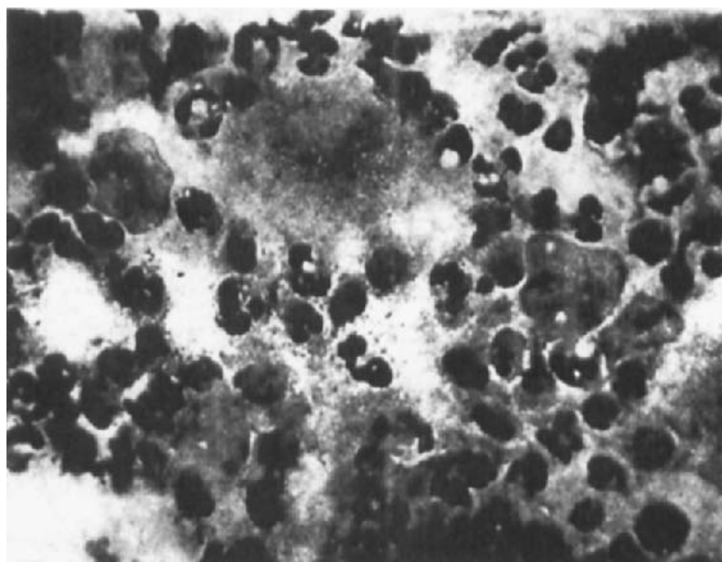


FIG. 12. Microbic vaginal pictures: acute stage of trichomoniasis (V/A). Many trichomonads and leucocytes, few epithelial cells, no bacteria. ×800. (Giemsa staining—photo O. Jírovec.)

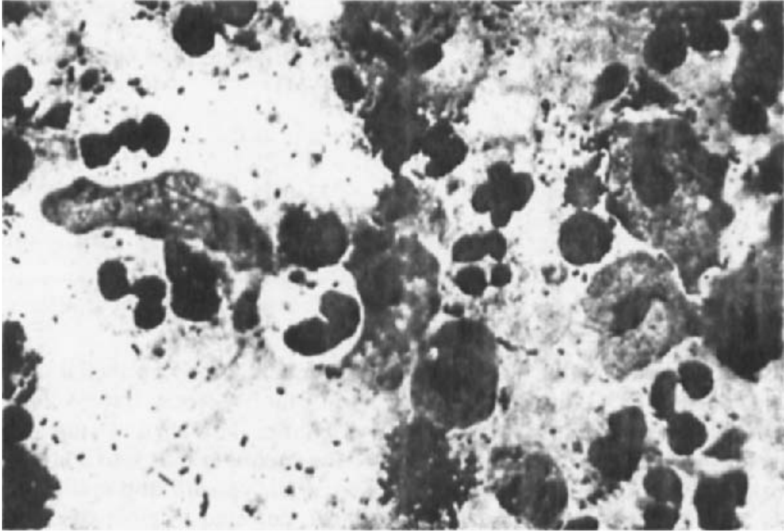


FIG. 13. Microbic vaginal picture: culminating trichomoniasis (V/B). Many trichomonads, leucocytes, bacteria and few epithelial cells. Without Döderlein *Lactobacilli*. × 2000. (Giemsa staining—photo Professor J. Fiala.)

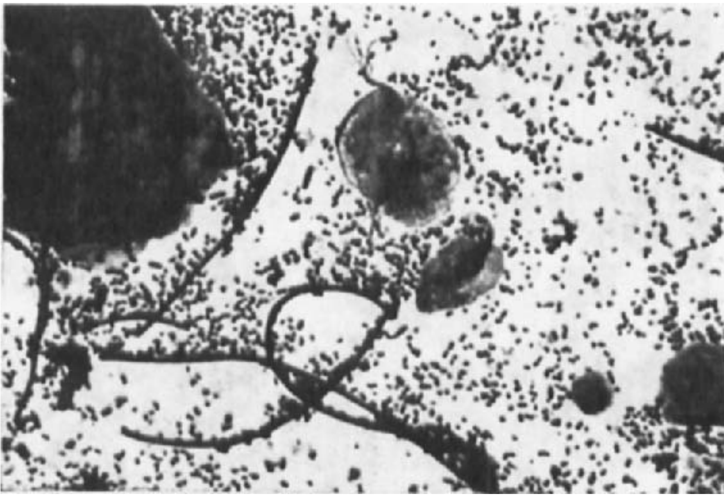


FIG. 14. Microbic vaginal picture: chronic trichomoniasis (V/C). × 1 500. (Giemsa staining —photo Professor J. Fiala.)

200 white women trichomonads in 16·5% and *Candida* in 12%; in a group of 500 negroes the percentages were 43·6% and 15·2% respectively. Capriora *et al.* (1957) detected 40·8% of trichomonads infection and 11·3% of vaginal mycosis in 1204 women; in 3% both infections were present. Lauras and Garin (1958) found, in 36 women with trichomonads, *Candida albicans* in 12% and *Aspergillus fumigatus* in 6%. Kurnatowska (1958) gave an account of such associated infections (*Candida* sp., *Torulopsis* sp., *Aspergillus* sp., *Trichothecium* sp.) which are particularly resistant to treatment with metronidazol.

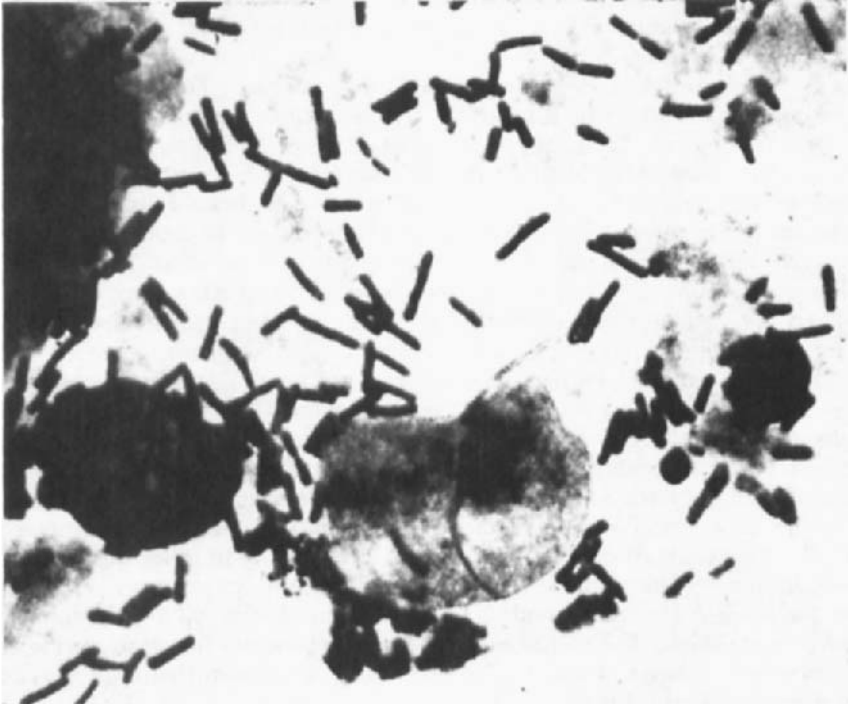


FIG. 15. Microbic vaginal picture: latent trichomoniasis (I/V). Many Döderlein lactobacilli and epithelial cells, few leucocytes. No inflammatory reaction. ×2000. (Giemsa staining - photo Dr. J. Lom.)

Lang *et al.* (1960) record the combination of *T. vaginalis* and *Candida* in 31% of cases, of trichomonas alone in 43% and of *Candida*-vaginitis in 21·3%. Peoples *et al.* (1957) isolated in 14·7% of 34 persons a peripneumonia-like organism, but attributed no significance to this association. Petrů (1966) subjected the vaginal secretions of 6258 women to mycological examination and isolated yeast-like organisms in 16·3% of a total of 1020 mycologically positive cases; vaginal trichomoniasis was also found in 162 patients (15%). These numbers indicate the low coincidence of vaginal mycoses with vaginal trichomoniases; most of the mycologically negative cases of this group (30%) were found *Trichomonas*-positive. At present nothing definitive can be said

about the antagonistic relations: these may be due to the influence of highly increased acid conditions in the vagina caused by yeasts, to a certain competition for glycogen, or to other and unknown mechanisms.

B. *Trichomonas* IN GIRLS

Older and recent investigations have emphasized the fact that *T. vaginalis* infection is very rare in girls and virgins but that the incidence of infection is rapidly increasing after defloration. Peter (1945, 1957, *et seq.*) found, in child records (Prague), that out of a total of 11 500 girls aged 0–14 years there were only 109 recordings of *T. vaginalis*; of these positive cases, 13 were sucklings from mothers infected with trichomonads, 5 were girls aged 4–7 years, 20 girls aged 9–11 years and 71 girls aged 12–14 years. These findings are in accord with the observations by Peter that *T. vaginalis* can settle only in an oestrogenized environment. The suckling is under the influence of its mother's oestrogen for several weeks after birth, Döderlein's *Lactobacillus* is present in the vagina of newborn girls and glycogen is abundant in the epithelial cells, representing the same environment as in a healthy adult woman. Trichomonads are acquired during birth if the mother is infected with flagellates. Another possibility of transmission to newborn babies is due to highly unhygienic conditions. There is no evidence of the duration of infection because the infected sucklings are treated immediately and can be cured relatively soon, even by older therapeutic methods. During the rest period of the child's genitalia, conditions in the vagina are unsuitable for the development of trichomonads, being practically due to the absence of glycogen in the vaginal mucous membrane. Should trichomonads penetrate into such a vagina, they disappear after several days or do not settle at all. Two out of the five cases of *Trichomonas* infection in the girls observed by Peter occurred during the pubertas praecox. Only at the onset of puberty, when the ovaries start to produce hormones, do conditions become suitable for *T. vaginalis*. At this stage also *Lactobacillus vaginalis* infects for the second time, now definitively the vagina of prepubertal or pubertal girls. The higher incidence of *Trichomonas* infection during this period has its own epidemiological reasons, such as masturbation, coitus *ante portas*, etc. (Peter, 1945).

Lang (1959) investigated 110 cases of vaginitis in girls who had not yet started to menstruate and found only four infected with *T. vaginalis*. Crowther (1962) observed two well-documented cases in infants: both children were of low birth-weight and in one there was a congenital abnormality of the urinary tract associated with meningomyelocele. Using the oral route both were cured by giving 50 mg metronidazol three times daily for 7 days. Komorowska *et al.* (1962, 1964) confirmed the opinion of Peter: of 35 newborn babies up to 3 weeks old, 17·2 % were *T. vaginalis*-positive, of 1 101 girls up to the age of 10 years 0·8 % were positive, of 870 girls past the age of 10, 10·4 %. Girls without oestrogenic reactions of the vaginal epithelium do not show *T. vaginalis*. The ovulary cycles occurred in 27 % of girls infected with *T. vaginalis* and only in 3 % in non-infected ones (Komorowska and Kurnatowska, 1964). Girls sharing a bed or using the same bath and lavatory as adult infected persons very seldom

become infected. *T. vaginalis* occurs in girls who are under the influence of genital hormones; very rarely, the infection occurs during the resting period of the genital organs. Urinary infection by *T. vaginalis* appears to be also very rare in infancy and childhood.

C. *Trichomonas* IN MEN

At first, *T. vaginalis* in the human male was considered a rarity and each case was published as an interesting report. Later recordings became more numerous, at first in connection with the so-called unspecific urethrites, later as some kind of latent infection without clinical symptoms. Today, trichomoniasis in the male is about as frequent as in the female, differing only in its course; in the male the infection is mostly latent or persisting in a subclinical form, while in the female the infection shows external symptoms—a discharge—in its acute and culminating stage. Since syphilis and gonorrhoea have been treated successfully with antibiotics, the subject of trichomoniasis in the male should be given more attention by venereologists and urologists. The number of papers published on this subject was about 5490 in the years 1894–1956 (Jíra, 1958), but these are only of historical value in view of the fact that trichomoniasis as a typical venereal infection is cosmopolitan in its distribution in the male and the female. In Germany trichomoniasis in the male was studied by Rodecurt (1929–1957), Bauer (1942–1966), Keutel (1955–1958) and others, who published numerous papers on this subject; in England by MacEntegart (1952–1959), Harkness (1933–1953), King (1959–1960), Lanceley (1953–1958), Whittington (1951–1957) and others; in Spain mainly by Bedoya (1957–1960); in Rumania by Perju (1951–1964); in Yugoslavia by Kostič (1954–1960); in Poland by Kozlowski (1951–1954); in Czechoslovakia by Kučera (1940–1957), Jíra (1954–1958) and others; in the USSR by Teocharov (1957–1958), Teras (1954–1966), Rožinski (1948) and others; in Italy by Nazzaro and Valenti (1953); in the USA by Coutts (1955–1958) and Feo (1944–1956).

Kostič for trichomoniasis in the male, has used the classification of the microbic pictures specified by Jírovec *et al.* (1947–1948). Jíra (1958) distinguished the following stages of these pictures: (*a*) primary acute stage, immediately following infection by coitus and mostly accompanied by strong urethral discharge; (*b*), primary subchronic stage with a slow onset and meagre discharge; (*c*), primary latent stage with a symptomless course throughout turning the infected male into a vector. All three stages change into the chronic stage especially if the infection has not been diagnosed correctly and treated accordingly. This stage may last for years; the discharge may disappear for some time only to exacerbate after being provoked by e.g. a cold, some wrong food, excessive sexual intercourse or alcohol. The standard treatment with antibiotics, sulphonamides and urinary antiseptics may have some effects on the accompanying bacteria but not on the trichomonads. According to Jíra (1958) the incubation period is 1–5 days in 20% of the cases, 6–10 days in about 40%, 11–15 days in 10%, 16–30 days in 20%, and 1–3 months in about 9%. Site of infection: *T. vaginalis* was recovered not only from the urethra, but also from

the preputial sac, the para-urethral glands, the seminal vesicle, Morgagni's lacunae and the prostate, less frequently from the epididymis or the testicles. Krupicz (1964) and others report on the occurrence of a purulent ulcerous or excoriative balanitis; there are also recordings of litreitis, cowperitis, vesiculitis, epididymitis (8–30% of cases), deferentitis, cystitis (Piringer and Piringer, 1957) and especially prostatitis (40–70%). Keutel (1955) found solitary trichomonads in an epididymis after epididectomy (in a frozen section and in phase contrast). In his opinion the prostate is the most common site for *T. vaginalis* in the male. In keeping with Holz (1953), Keutel considers the frequently occurring non-flagellate rounded *Trichomonas* in the male to be cysts in cultures of *T. vaginalis*, which had been kept under unsuitable conditions, e.g. changing temperature and pH, and considered them to be true cysts, even making a drawing of cyst partition! The author confessed to difficulties in confirming the cysts, respectively the round forms in the native preparation because of their similarity to inflammatory cells.

In the urethrogenital organs of the male *T. vaginalis* can either live as a commensal or change into a true pathogen, influenced by various factors. It is difficult to know whether this change is greater in younger men or whether more frequent exposure to infection through coitus is the decisive factor. According to Jíra (1958), the highest incidence of infection occurs at the age of 21–25 years (48%) and of 26–30 years (26%), decreasing with increasing age. Association with bacterial infection is of some importance, but Lanceley and MacEntegart (1953) confirmed in experiments with pure cultures of *T. vaginalis* on 5 volunteers that the ciliate has a primary pathogenicity. It was possible to produce urethritis five times and prostatitis twice; in three of the inoculated cases trichomonads were found in urethral discharge on the 6th–9th day and persisted for 44–94 days. According to Bauer (1960), 16% of urethral discharge in trichomoniasis in the male is microscopically sterile, 33% shows a moderate and 51% a high incidence of bacteria. Naturally, there are also mixed infections with gonococci. The bacteria in urethritis with or without trichomonads are practically the same not only in view of the species but also in their incidence (Feo *et al.*, 1956). Mostly they multiply secondarily in an environment inflamed by the trichomonads. Information on the change of resistance of the host's organism by an eventual activation of a simultaneous latent infection with certain bacteria or viruses, and also on the different pathogenicity of *trichomonas* strains, is very scanty. Hypospady, diverticle strictures and urethral stenosis seem to facilitate the infestations by *T. vaginalis* (Hancock, 1959; Shepard, 1959). Of 364 males whose wives or sexual partners had *T. vaginalis* infection Kostič has demonstrated the organisms in 39% in their urogenital tracts. The urethra was involved in all cases (39%), the prepuce in 1·38%, the prostate in 6·6%, the bladder in 25·4%, the renal pelvis in 1·7%.

The coital origin of male *Trichomonas* infection is generally recognized. Extragenital acquisition of the infection in the male—if this indeed exists—is extremely rare. According to various statistics, the percentage of *Trichomonas*-carriers by partners of infected females is estimated to range from 20–100% (e.g. after Bedoya *et al.*, 1958, it is at least 76%). These numbers are, in fact, greatly dependent on the methods of examination, their repetition, and especi-

ally on the experience of the investigators. A latent infection in both sexes may lead to a latent or evident disease in the partner (Keutel, 1958). The percentage of participations of trichomoniasis in the male infected with non-specific urethritis ranges, according to the various authors, from 3–68%; according to Jíra the average is about 30%. Keutel (1957) recorded a decrease of fructose content in the sperms, which in view of the shortened activity of the sperms may be considered one of the reasons for sterility. There is still some uncertainty about the length of time for which the trichomonads can persist in the uro-genital organs of the male. Some consider the possibility of their spontaneous disappearance after 2–3 months, others assume the infection to persist for many years (Feo, 1956; Siboulet, 1957; Jíra, 1958; and others). Bedoya believes in a lifelong infection. During sexual intercourse the trichomonads from the prostates and from the walls of the urethra are carried with the ejaculate into the vagina.

IX. Diagnosis of *T. vaginalis* Infection in the Human Female and Male

Because the diagnosis of *T. vaginalis* infection cannot be based on clinical symptoms alone, especially if the infection is in its chronic or latent stage, microscopical examination has to be carried out. When this is negative, it must be completed by cultivation. Trichomonads are most abundant during the first 2 days following menstruation. Should no flagellates be found in the vagina it is necessary to examine the urethra, the paraurethral ducts and the greater vestibular gland. It is most difficult to demonstrate trichomonads in the cervix because even after a careful removal of the vaginal secretion with tampons, some trichomonads may still remain and simulate their presence in the cervix. There it can practically be proved only in operational material. For the practice it is sufficient if trichomonads are demonstrated in the vagina, the urethra and in the urinary sediment. Careful records are made of the clinical findings in the vagina, the cervix and the external genitals of the amount, colour, smell and appearance of the vaginal secretion.

In native preparations the flagellates retain their motility for several hours when kept at a temperature of 20°C under a coverslip sealed with paraffin. Temperatures exceeding 44°C kill the trichomonads in a short time and therefore the preparation should never be heated over a flame. The pressure of the coverslip sometimes causes the trichomonads to change their shape, and eventually to develop pseudopodia-like processes which serve neither for motion nor for the intake of food. Various mistakes may occur in a diagnosis made from a native preparation. In a weak infection the incidence of flagellates is so low that the preparation may be considered negative. On the other hand, leucocytes may be set into motion by viable spirochetes, flagellated bacteria, sperms or ciliated cells adhering to them (operation materia), thus simulating trichomonads. Coutts *et al.* (1959) recommend unstained dry smears observed in a dark field—the flagella are very clearly visible. Addition of 0·1% safranin after Starzyk *et al.* (1958) or brilliant cresyl blue after Holtorff (1957) facilitate

the finding of *T. vaginalis* in native preparations. Only dead flagellates are stained intensively red, resp. blue; the living remain unstained—in opposition to the stained leucocytes, epithelial cells etc., in the preparation.

The diagnosis from a native preparation should always be completed by a Giemsa-stained preparation. The original solution is diluted by 1:10 with freshly boiled or buffered distilled water (pH 7·2–7·6) and stained for one hour. The trichomonads can be easily recognized by the sky-blue colour of their plasma and the reddish-violet, oval to slightly pointed granulated nucleus. In a well stained preparation the locomotion apparatus with the axostyle stain carmine red; but frequently remain unstained. In such preparations it is even possible to determine the microbic vaginal picture given in the classification by Jírovec *et al.* (1947, 1948). Bacteria stain violet (including the genera *Vibrio*, *Leptotrichia*, *Fusiformis*, *Spirochaeta*), gonococci blueish, yeast cells blue with reddish granules in the plasma (Jírovec, 1960).

A Gram-preparation serves for completing the diagnosis: the gonococci are Gram-negative; the pyogenic staphylococci and streptococci, all yeast-like organisms and *Lactobacillus vaginalis* are Gram-positive. Trichomonads are Gram-negative but are difficult to recognize. Other staining methods (gentian, methylgreen-pyronin, methylene-blue, hematoxylin, Papanicolau's method) are unsuitable for an exact proof and although they may demonstrate a massive infection, a few disappeared trichomonads are always overlooked. The media used for cultivation are Johnson's CPLM medium, Vf-bouillon after Magara, Feinberg, etc. (see addendum). They increase the number of positive cases by about 10%, but there are instances where negative results are obtained in the culture even if the microscopical finding was positive. Therefore the use of both methods is recommended. Perju (1964) emphasized the need for examining also the mothers or all female persons who had been in close contact with *Trichomonas*-positive young girls or sucklings.

Also every male suffering from urethritis or other urological infection should be thoroughly examined for *T. vaginalis*, similarly his wife or female partner. Microscopical native preparation and Giemsa- and Gram-stained preparations have to be made from the urethral and prostatic secretion from the urinary sediment, and carefully examined for the presence of trichomonads. At the same time cultures should be made from the same material (Jíra, 1958; Kurnatowska, 1958; Hoffman *et al.*, 1961; Roigas and Rubanovitch, 1963; Těmín, 1965, personal communication; and others).

X. Chemotherapy of Vaginal Trichomoniasis

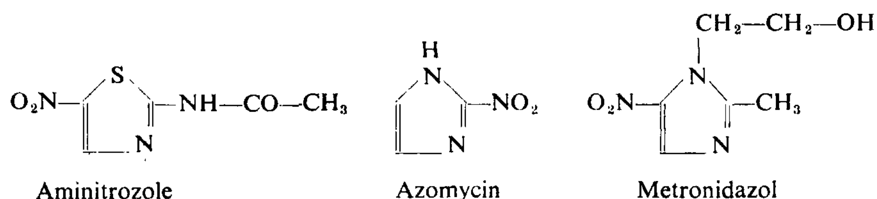
Chemotherapy of vaginal trichomoniasis is still a most difficult problem in spite of the use of various drugs in different combinations. Although drugs were found to remove *T. vaginalis* from the vagina, it was not possible to remove them from the urethra, the Skene's ducts and the greater vestibular gland, which caused relapses especially in patients with lowered defence mechanisms (through another infection, colds, etc.). Another drawback was the length of time which most of these therapeutic methods required. An even more difficult problem was the treatment of the male prostate; when this organ had been

infected with trichomonads, there was almost no hope for successful treatment. In addition to these difficulties it was almost impossible to persuade apparently healthy latent *Trichomonas*-carriers to undergo such long-lasting and personally most disagreeable treatment, and these remain a source of re-infection. The combination of trichomonadocidal, bacteriostatic and mycocidal substances after Peter and Jírovec (1946, 1947), effective on three pathogenic groups of organisms and serving as a basis for the production of effective preparations such as "Triflocid" and "Fluocid" (Spofa), "Anafluose" (Guillaumin), "Tricolpon" (Organon), "Viozol" (Ciba), and others, could in view of only local application in both sexes, not fully prevent the occurrence of relapses and re-infection. Also after the introduction of antibiotics given either by mouth or applied locally no permanent cure could be achieved. Treatment with e.g. tyrothrycine (Ibañez, 1951; Iger and Kupperman, 1955; Šubert, 1957), terramycin (Greene, 1952; Kostič, 1954), aureomycin (Kostič, 1954; Bradburn, 1952) and others proved to be unsuccessful, although they were found to be highly inhibitive to growth or directly toxic in experiments *in vitro*; e.g. anisomycin 3–12 μg (Lynch *et al.*, 1955), actinomycin 200 γ/ml, chloramphenicol 125 γ/ml, oleandomycin 1 000 γ/ml, iturin 250 γ/ml (Reginster *et al.*, 1958), tyrothricin and aureomycin 1:3000–30000, teramycine 1:10000–30000, chloramphenicol 1:25000–50000, tyrothricin 1:10000 (Lützenkirchen, 1952), thiolutin 0·2 μg/ml, neomycin 125–250 μg/ml (Seneca and Ides, 1953), sanazin 1:3500 to 10000 (Teras, 1959). No inhibitive effect was observed with penicillin and streptomycin (although both are used even for preparing bacteria-free cultures of *T. vaginalis*), bacitracin, griseofulvin, nystatin and triacetin (Barr and Brent, 1960; and others). The medium used in inhibition experiments should not contain too much serum, which greatly lowers inhibition. This fact was demonstrated in experiments by Körber and Fleckenstein (1954), who could not obtain inhibition with chloramphenicol 1:1 000 after adding 50% of serum.

The findings by Magara *et al.* (1954) on the effect of oral application of the isolated antibiotic trichomycin, suggested the advantage of investigating further trichomonadocid substances which could be given by mouth. The first report by Magara *et al.* seemed most impressive: trichomycin 1:200000 completely inhibited *T. vaginalis* in the culture; 60000 IU of trichomycin given in daily doses to patients infected with trichomonads made the parasites disappear within 1–6 weeks, while after daily doses of 240000 IU the parasites disappeared after an average of 4 days. A total dose of 1 000000 IU (100000 IU daily) was supposed to cure trichomoniasis permanently. These experiments, repeated in Europe, did not confirm the successes achieved in oral treatment with trichomycin (Bauer, 1960; Bedoya and Fernandez Ortega, 1957; Caterall and Nicol, 1957; Durel, 1958; and others).

Recently reports became available on the highly trichomonadocidal effect of the antibiotic hamycin. According to some investigators the motility of trichomonads becomes arrested 10–30 min. after the application of a solution of 1–1·5 γ/ml; other writers report inhibition of growth in the Vf-bouillon with a dose of 0·5 γ/ml; a dose of 0·66 γ/ml kills the flagellates after 48 h. Hamycin seems to be five times as effective as trichomycin and Metronidazol.

The first systemic drug to be used in oral therapy was Aminitrozole-(Tritheon = 2-acetylamino-5-nitrothiazol; Cuckler *et al.*, 1955), but in re-examinations by Caterall and Nicol (1957), Dunlop *et al.* (1958) and Thiery *et al.* (1960), disappointing results were obtained (toxic symptoms, successful cure in only 8–16 %). This problem became definitively solved by the synthesis of Metronidazol (Cosar and Julou, 1959), developed from azomycin.



Aminitrozole Azomycin Metronidazol

The first reports on a surprisingly successful oral therapy with Metronidazol-designated Flagyl were recorded by Durel *et al.* (1959) from France, and Sylvestre *et al.* (1959) from Canada. These results were confirmed by various authors (successful therapy in 90–100 %). Today, Metronidazol is the best drug against *T. vaginalis*; its secondary effects are minimal (rarely vomiting, nausea, gastric complaints, sensation of dryness in the mouth, lingua pillosa, passing exanthema, foul taste) and never strong enough to discontinue the cure.

Metronidazol is a chemical drug (hydroxy-2′-ethyl-1-methyl-2-nitro-5-imidazol) produced by "Specia" under the name Flagyl, and also produced and sold by various firms under different names. Solubility in water is 1 %, in ethanol, ether, chloroform 0·5 %, pH of the saturated aqueous solution at 20°C is 5·8. Toxicity is very low: LD_{50} for mice by mouth after a single dose is 4·3 g/kg (in comparison, Tritheon 0·63 g/kg). The maximum tolerable dose for mice (5 times daily) is 2·5 g/kg (compared with a daily oral dose of 0·2 g/kg Aminitrozole). Cultures of *T. vaginalis* are still killed with 1:400000 within 24 h (Bock, 1961). Tritheon becomes effective in doses of 1:75000, azomycin 1:25000. Inhibition of growth still occurs at a dilution of 1:1–13000000 (Durel and Roiron-Rattner, 1960; Watt and Jennison, 1960; and others). Daily treatment with 12·5 mg/kg by mouth prevents the formation of trichomonad abscesses after inoculation of *T. vaginalis* culture under the skin of mice, corresponding to about 1/200 of the LD_{50} of the 5-daily doses. The urine remains trichomonadocid during the first 4 h after treatment even when diluted to 1:100–1000, the same applies for the blood serum 1:10. The urine contains 50–390 μg/ml, the blood 6–13 μg/ml, the saliva only 0–1·4 μg/ml.

Ten to 15 % of the Metronidazol is eliminated by the urine in 48 h (Durel *et al.*, 1959). The dark urine of some patients receiving Flagyl is due to the presence of an azo-dye resulting from the condensation of two partially reduced Metronidazol molecules, probably following renal excretion of the metabolites (Manthei and Feo, 1964). Metronidazol has no bacterial activity and does not interfere with normal vaginal flora—*Lactobacillus vaginalis* develop also in the presence of 0·3 % Metronidazol. It also has no antifungal activity, in contrast to trichomycin. Metronidazol also killed *Giardia lamblia*, *Trichomonas muris*, *Chilomastix mesnili*, *Entamoeba muris* and *Entamoeba histolytica*

(Bock, 1961; Schneider, 1961; Mandoul *et al.*, 1961). Its action on *T. vaginalis* cultures is inhibited by purines (adenin, guanine, hypoxanthin, inosin, xanthin: Samuels, 1962).

Perju *et al.* (1963) studied the effect of Metronidazol on the ultrastructure of *T. vaginalis*. After 40 minutes of incubation in a 1:80000 solution the trichomonads showed the first alterations. Metronidazol penetrating the cell acts as a chemotoxic substance, the metabolic processes are paralysed, the plasma is lysed and disorganized, and the canalic membranes are altered. Perju and Strimbeanu (1964) showed that *in vivo* 99% of trichomonads are immobilized in 9–12 h, total destruction occurring in 13 h, while *in vitro* it occurs in 9 h without exception. Eight to 9 h after administration of Metronidazol round forms of *T. vaginalis*, condensed and contracted, appear; after 10–11 h important alterations occur on the cytoplasm, there is a loss of granules, an appearance of vacuoles, total cytolysis at the periphery, pycnosis and caryorhexy of the nucleus; the end is a complete lysis of the parasite.

The number of leucocytes and of the pyogenic bacteria decreases rapidly within 24–48 h. After 4–5 days *Lactobacillus vaginalis* starts to multiply anew; 8–14 days later the leucocytes disappear almost completely and *Lactobacillus vaginalis* starts to reproduce heavily. After 6–10 days the colposcopic finding is normal. Only in about 22·6% of cases is there a partial reduction of leucocytes and a persistence of the mixed bacterial flora.

No essential changes either in the female or in the male blood proteins are brought about. Only in a few cases could a temporary decrease in the number of leucocytes be observed. Therefore it is necessary to examine the white blood count before Metronidazol treatment, and also in the oourse of it (Teras *et al.*, 1963b, c).

In treated patients pregnancy is normal and, up to present, no damaging effect of Metronidazol has been observed in newborn babies, although Metronidazol passes into the foetal circulation and is also present in relatively high doses in the milk of the mother (Bertrand, 1964; and others).

Rats treated with Metronidazol revealed no modification in fecundity, in duration of gestation, in number of rats per litter, in proportion of stillborn rats, in mortality during the first week of life, in malformations or changes of spermatogenesis. Only after 2 months and a daily dose of 1 g/kg did about half of the male rats show some lesions of limited extension, not inducing a decrease in fecundity (Gautier *et al.*, 1960).

The great and singular advantage of Metronidazol is its ability to kill *T. vaginalis* not only in the vagina but also in all extravaginal foci; the treatment is of short duration (250 mg 3 times daily for 7 days), highly effective, and the male partner can simultaneously be treated by mouth to forego possible reinfection right from the outset. There is some uncertainty about the possible resistance of *T. vaginalis* to Metronidazol. Jennison *et al.* (1961) could not observe any resistance *in vitro*, but de Carneri *et al.* (1963) described two therapeutic failures in 14 patients suffering from trichomonal vaginitis and treated both orally and locally with Metronidazol; this failure was attributed to natural resistance of the 2 strains of *T. vaginalis* to the drug. In experiments with mice a dose of 20 mg/kg, resp. 50 mg/kg was needed for both strains, in

comparison with only 5 mg/kg necessary for the other strains to avoid the formation of subcutaneous abscesses.

It is still uncertain whether Metronidazol may provoke vaginal mycosis (Rom *et al.*, 1961). In numerous cases treated with Flagyl, the occurrence of yeast-like organisms has been observed. Metronidazol is known not to effect pyogenic bacteria, and therefore the use of some bacteriostatic drug (e.g. sulphonamide intravaginally) is recommended. In keeping with the findings of Teras *et al.* (1963c) an intravaginal treatment with Metronidazol seems superfluous. The healing process is accelerated by an intravaginal application of combined drugs (e.g. Triflocid or Fluocid), at the same time preventing the occurrence of Candidae by the contents of boracic acid in the drugs. No definite decision has been made as yet on the necessity of implanting Döderlein *Lactobacilli* during Metronidazol treatment. In practice it would be difficult to ascertain that the "living" cultures are still viable at the time of implantation.

The oral therapy with Metronidazol has greatly eased treatment of trichomoniasis in the male and has therefore become most successful. There is no need to make urethro-vesicle irrigations using a variety of disinfectant on therapeutic agents. Metronidazol effects the urethra, the prostate and all other sites in the urino-genital system. We confirmed the 100% success of Metronidazol treatment on our own material. In every case it is necessary to treat the other sexual partner to prevent re-infection ("ping-pong infection"). Since the introduction of Metronidazol the use of all other trichomonadocidal agents has become superfluous. Further research may develop even more suitable oral substances which are less toxic.

XI. Epidemiology of Trichomoniasis

The epidemiology of trichomoniasis is the object of two extensive reports by Kučera at Reims (1957) and Chappaz at Montreal (1960b), both convinced about the venereal transmission of *Trichomonas* infection. Concerning some publications from recent years we can summarize our knowledge as follows: The average frequency of *T. vaginalis* infection is about 10% in normal population (2–15% after Kučera, 1957). The percentage depends on the age—the maximum being achieved during the highest sexual activity (Mascall, 1954; and others); on the methods employed for detection; and on the experience of the authors. As an example of *T. vaginalis* distribution in central Europe we give the results of Petrů (1964): of 123000 gynaecologically examined women in Bohemia and Moravia, 27·3% were found to be infected (material from gynaecological and obstetric departments). In the districts of Prague the percentage of the infected women was, in the years 1954–1956, 24·2%, and 10 years later 25·8%; the maximum number of cases occurring in 30–40 year-old patients. The transmission by sexual intercourse becomes evident when estimating the social situation. In married women coming to the gynaecological clinics the percentage of positive cases was 23·7%, in divorced women 48·2%! The authors assumed more sexual promiscuity in the divorced women. In female clerks the positivity was 28·1%, in female factory workers 28·9%, in unemployed women only 20·9%. The normal infection rate was demonstrated

TABLE IX

Distribution of Trichomonas vaginalis *in healthy female population*

	England		USA				Poland		Czechoslovakia			
	Whittington (1951a)		Feo (1956)				Stroczyñska *et al.* (1961)		Jírovec *et al.* (1942)		Vojtěchovská and Petrů (1968)	
			white		negroes							
Age groups	Number positive	%	Number	%	Number	%	Number	%	Number	%	Number	%
15–19	13	7·7	—	—	—	—	1	—	93	5·3	44	11·4
20–29	321	3·1	38	2·6	38	10·5	52	22·6	69	17·3	107	16·9
30–39	1981	8·6	54	3·7	33	9·1	65	15·4	16	37·5	185	13·0
40–49	26	7·7	52	3·8	78	19·2	47	21·3	3	—	193	14·1
50 and over	5	0	295	2·8	152	10	34	17·7	—	—	147	7·5
Total	562		169		301		199		181		676	

in examinations of about 700 female workers of a factory in Central Bohemia —the positivity was 12·6% (Vojtěchovská and Petrů, in press). Petrů *et al.* (1956) performed *Trichomonas* examinations on 116 female patients suffering from psychosis. The positivity of 25% is in approximate keeping with the general gynaecological praxis. There was no striking coincidence between a positive toxoplasmine test and the incidence of *Trichomonas* infection.

TABLE X

Distribution of Trichomonas vaginalis *in women visiting gynaecological ambulatories, etc. (sick population)*

Age groups	Jírovec *et al.* (1942) ČSSR		Kučera and Král (1944) ČSSR		Ašmera and Linhart (1962) ČSSR		Engelbrecht and Müller (1962) DDR	
	Number positive	%	Number	%	Number	%	Number	%
15–19	15	20	33	42	144	25	—	13·1
20–29	18	35·5	336	31·8	278	47·2	—	16·5
30–39	72	41·6	185	42·8	273	34·4	—	19
40–49	54	22·2	35	68·6	122	36·8	—	21·8
Over 50	31	13	99	44·0	43	30	148	13·3
Total	252		498		860		820	

Coloured women showed an increase in positivity (Feo, 1953), due to bad hygienic conditions, more sexual promiscuity and the lack of contraceptives (Gray, 1961). After the menopause in negroes over 60 years about 10% were infected, in white people only 1%. Burch *et al.* (1959) showed in negroes a frequency nearly eight times higher than in white people, with highest prevalence in women of both races between the age of 30–50 years. Lambillon *et al.* (1954) found highly virulent infection in 20% of the white female population. In negro women the positivity was 40–50%, mostly without clinical symptoms. Mandoul and Fleurette (1950) noted the same infection as in Europe or the USA in Mohammedan women in North Africa (24%); here too the maximum incidence occurred during the sexual period of life.

The frequency of infection does not seem to change with the various seasons —in spite of the persistence of infections for many years. Only Glebski (1964), observing the frequency of *T. vaginalis* in the urine sediment of 40000 males and females (1:1) for 5 consecutive years, found a positivity in 7–14% with a minimum infection rate from December to February and a maximum from August to November. His results should be confirmed by other methods, because the observation of urine sediment alone is not sufficient for such research.

The recent study of Gaudefroy and Vernier concerns 19 680 cases detected; and in the light of statistical determinations and a security limit of 95%, the

frequency diagrams between 1959 and 1961 have evidenced a maximal frequency in March, in July and in October–November. On the other hand, from 1962 to 1965 the seasonal variations disappear in a progressive manner. No plausible explanation may be advanced; however, if the venereal nature of this infestation is admitted, it may be noted that there exists a relation between the seasonal variations of Trichomoniasis and the frequency of marriages; this relationship having been encountered on the national scale and even abroad. Furthermore, these variations appear to have been influenced by the systemic trichomonicides, the use of which dates from 1960. In all cases, the frequency diagrams passed from 25–29 % in 1960 to 11–13 % in 1965.

Buxton (1958) found 221 female prisoners 70 % positive; from 715 patients from a psychotherapeutic clinic only 15 % were positive. Of 157 subordinated members of the staff of a girls' college none were positive. Herbst *et al.* (1960) examined 450 female prison inmates and found *T. vaginalis* in 62 %. In older women and in those condemned many years earlier the infection was very rare; women older than 60 years were all negative.

Bogusz-Rożkowska and Zablotniak (1966) investigated prisoners (478 men and 111 women) confined for 1–24 months. Of the men 8·8 % were infected with *T. vaginalis*, of the women 47·7 %. In persons deprived of sexual intercourse the trichomonads may persist in their genito-urinary tract for at least 2 years.

Trichomoniasis genito-urinaria is a typical venereal infection, as almost unanimously accepted at the conferences at Reims (1957) and Montreal (1959) and also earlier (Bauer, 1943; Bedoya, 1957; Caterall and Nicol, 1960; Jírovec, 1957, 1960; Jíra, 1958; Dellepiane, 1957; Kean, 1955, Keutel, 1955–1957; Kostič, 1954; Kučera, 1957; Ottolenghi-Preti, 1957; Peter, 1945, 1957b; Teras *et al.*, 1961–1966; and others). Teras *et al.* (1961–1966), tracing the sexual contacts of numerous females and males, proved 61 infestation chains transmitting the infection by sexual intercourse. Of the 1135 examined women about 70 % were positive, of the 998 men 36·6 %. In the opinion of these authors the same measures as for any other venereal disease should be applied in the control of trichomoniasis. The further spread of *T. vaginalis* can only be prevented by regarding it as a venereal disease falling under the act of compulsory registration. Kaarma and Koplus (1963) determined that "conflicts in family are often caused by the fact that the wife does not recover from trichomoniasis in the course of several years, the consequences being dispareunia and other psychic disorders which often make family life impossible".

Extragenital contamination has not yet been proved in any of the cases, although it is possible. Experiments of Peter (1945) and Jírovec and Peter (1948a) showed, contrary to the claims of others (Rodecurt, etc.), that *T. vaginalis* is not resistant to freezing, temperatures above 44 °C, drying and direct sunlight. Tests with *T. vaginalis* revealed that in urine at 20 °C, inoculated with vaginal secretion, the majority of trichomonads remained viable after 9 h, whereas after 20 h only very few were still alive. In vaginal secretion applied to wood, brass, toilet paper, towels or bathing sponges the flagellates lived for 1–2 h, rarely 5–6 h. Contact with water killed them within 35–40 min (Fig. 16). Transmission of *T. vaginalis* takes place mainly through sexual intercourse,

exceptionally by indirect contact at toilets with towels etc. These findings were confirmed by many authors: Kessel and Thompson (1950) found that *T. vaginalis* persisted only a few hours, when droplets of the discharge were placed on the enamel surface of wooden blocks and tested under natural conditions of drying at room temperature. No survival was demonstrated after 7 h. Whittington (1958) demonstrated that *T. vaginalis* in vaginal discharge remained alive for only up to 45 min on polished surfaces of lavatory seats. Only 4 out of 30 patients with *T. vaginalis* left infective material on a toilet seat after they had used it. Burch *et al.* (1959) showed the possibility of transmission by means of communal use of fomites, by culturing *T. vaginalis* on pieces of wash cloth keeping them at room temperature for up to 23 h after being used for cleansing the external genitalia of infected women. Chappaz *et al.* (1961)

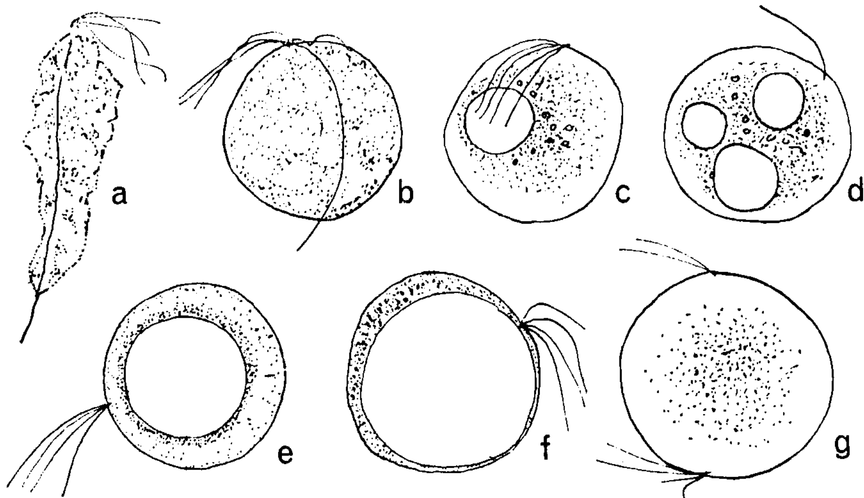


FIG. 16. *T. vaginalis*. *a*: in hypertonic solution, *b–g*: in hypotonic solution—vacuolization and total degeneration. (After Jírovec and Peter, 1948a.)

showed in 30 observations, that the survival time of *T. vaginalis* on clothes does not exceed 4 h providing there is desiccation. The authors have been brought almost to the point of denying the possibility of transmission of *T. vaginalis* from one woman to another by clothes, underclothes or sanitary towels. In another paper Chappaz *et al.* (1961) emphasize that extragenital infection has not yet been proved in any case. Paralleling the opinion of Whittington, the authors denied emphatically any possibility of contamination by toilet seats.

Also the short survival time of *T. vaginalis* in fresh water, described by Weiler (1938), Wu (1938), Morenas (1945), Peter (1945) and Jírovec and Peter (1948a), was confirmed in new publications by Teras *et al.* (1961). The rapidity of the destruction of *T. vaginalis* depends on the concentration of mineral salts in the

water. In the water supply in Tallinn the lysis of *T. vaginalis* occurred within 10 min, in water of swimming pools the trichomonads perished in 10–30 min, in soap-solution of 0·15–0·3% they die immediately. Infection by *T. vaginalis* in water is impossible and the authors see no reason to have any doubts on the venereal origin of this infection. Also Sarosiek (1967) could not prove the presence of *T. vaginalis* in culturing water from artificial and natural swimming-pools in Szcezecin—such water cannot be regarded as harbouring trichomonads. The failure to obtain *T. vaginalis* cultures may be caused by its rapid spread in water, its short life and by chlorine disinfection of artificial water-ponds.

Čatár *et al.* (1967) admitted the possibility of transmission by the thermal waters of a spa used for the treatment of gynaecological affections (Lúčky, near Ruožmberok). On admission to the spa there were 15·6% *trichomonas*-positive women. After the treatment for the entire period investigated, a total of 22·7% of females were positive. Three of the 16 examined strains of *T. vaginalis* survived in mineral waters for as long as 22–72 h with well preserved motility and multiplication capacity. Four of the examined strains lived for only 35–55 min in the thermal water. Recovery isolation of *T. vaginalis* was successful at the end of 15 and 45 min from the beginning of thermal water treatment. (One factor was forgotten by the authors—the possibility of sexual promiscuity in the spa and fresh infection, or reactivation of an old, until now latent, infection with *T. vaginalis.*)

The opinion of Depoorter (1959) that the toxicity of the urine for *T. vaginalis* explains the spontaneous cure of urethritis, is certainly wrong. Not only is diluted urine an excellent medium for maintaining *T. vaginalis* for some time, but also the infection of the urinary tract persists for many years. In diluted urine the viability of *T. vaginalis* is retained for a longer time than in water (Jírovec and Peter, 1948a).

In spite of the low resistance of *T. vaginalis* to external factors (low osmotic pressure, temperature up to 25°C, sunshine, drying etc.) and the absence of true cysts, the transmission by other routes than sexual intercourse must be extremely rare and the principal pathway of the infection in both sexes is the sexual one.

APPENDIX

MEDIA FOR CULTIVATION OF *T. Vaginalis*

A. CONTAINING SERUM

1. *Johnson CPLM medium*

Bactopeptone 32 g, bactoagar 1·6 g, L-cysteinchloride 2·4 g, maltose 1·6 g, liver infusion (Difco, made as directed on bottle) 320 ml, Ringer solution (NaCl 0·6%, $NaHCO_3$, KCl, $CaCl_2$ each 0·01%) 960 ml, NaOH N/1 10–13 ml are boiled 10 min to melt agar; filter through coarse paper. Add 0·7 ml of 0·5% aqueous methylene blue. Adjust to pH 5·8–6. Tube in 8 ml amounts in medium size tubes, plug tubes and autoclave 15 min. After cooling add 1 ml

of sterile (filtered) human serum. Incubate at least 4 days. This medium is stored at room temperature and discharged when the blue zone of unreduced methylene blue extends past the middle of the column of fluid, usually after 2–3 weeks.

Petrů (1961) developed a method for storing this medium to be used in routine diagnosis of *T. vaginalis* infection: instead of tubes, transfusion flasks of 250–500 ml are used for storage of the ready-made medium CPLM. The rubber stoppers are pierced with a thick injection needle and the flasks are steam-sterilized at 100°C. Then the injection needle is removed from the hot flasks to prevent non-sterile air from entering the flasks during cooling. Sterilization in steam (1 h) is repeated for 2 consecutive days without replacing the injection needles. After cooling, 10% of human or sheep serum is added (by injection needle) to the sterile medium, and the flasks can then be kept at +2°C for at least 3 months. Shortly before use, 1 000 IU of crystallized penicillin and 1 mg streptomycin are added to 1 ml of medium, and Hall tubes are filled with this mixture to about 6 cm height. Fresh media are yellowish-green in colour; when the colour of the medium turns bluish-green, they are no longer fit for use. No differences have been found between the use of a fresh and a 3-month-old medium.

2. *Trypticase medium after Sprince and Kupferberg* (1947)

Dissolve 1 g maltose and 1 g bactoagar by boiling in 400 ml distilled water and filter through filter paper. Then dissolve 20 g trypticase BBL, 1·5 g cysteinchloride and 0·48 ml of 0·5% methylene blue and 600 ml of Ringer solution. After correcting the pH to 6 with N/1 NaOH, dispense the medium (9·5 ml) into sterile tubes and autoclave. Before use add 0·5 ml sterile serum to each tube.

3. *Medium after Feinberg* (1953), *sterilized by filtration*

Proteolysed liver Panmede 25 g, NaCl 6·5 g, dextrose 5 g, inactivated horse serum 80 ml, dist. water 1 000 ml, penicillin 1 million IU, streptomycin 0·5 g, pH adjusted to 6·4 by adding approximately 9 ml N/1 NaOH per litre. The mixture is sterilized by Seitz-filter and stored in screw-capped bottles in a refrigerator. The medium can also be dried or freeze-dried. After 3 months' storage at +4 to 5°C no deterioration of the antibacterial power is evident. For diagnosis 7 ml of this medium are filled in glass tubes and examined 4–5 days after inoculation. If only few trichomonads are present, it is desirable to centrifuge the cultures before examination.

4. *Senton's modification of the Feinberg's medium*

2 g ascorbic acid, 1·2 g L-cysteinchloride, 5 g glucose, 0·5 g Oxoid-liver dry extract are dissolved in 920 ml Hartley digest broth, pH corrected to 6. The mixture is autoclaved. Before use add 80 ml horse serum, 0·5 g streptomycin and 500000 units penicillin-G and pour into tubes.

5. *Vf-bouillon after Magara* et al. (1953)

Hacked beef liver 50 g, 10% HCl 10 ml, bactopepsin 0·5 g, dist. water 1 000 ml are heated after mixing for 24 h at 48°C, stirring occasionally. Next day the solution is heated for 10 min at 80°C, filtered through filter paper and heated once more for 15 min at 100°C. After correcting the pH to 5·4 with 10% NaOH, the solution is heated for 25 min to 100°C, and after filtering it is poured into Hall's culture tubes, sterilized by heating for 30 min at 100°C for 3 consecutive days and then stored at +2°C. Glucose as a 3% sterile solution is added to make the glucose content 0·5%. Sterile serum is added to the extent of 10%. Penicillin (1 000 IU) and streptomycin (1 mg/ml) could be added together with the glucose.

6. *Medium after Diamond* (1957)

20 g trypticase BBl, 10 g yeast-extract, 5 g maltose, 1 g L-cysteinhydrochloride, 0·2 g ascorbic acid, 0·8 g K_2HPO_4 are dissolved in 900 ml dist. water, pH corrected to 6, 0·5 g bactoagar added and the mixture autoclaved. After cooling to 48°C add 100 ml serum, 1 million IU penicillin-G and 1 g streptomycin-sulphate, fill in sterile tubes (5 ml). Can be stored for 3–4 weeks in refrigerator. Before use heat tubes in water-bath to 35–37°C.

7. *Medium after Roiron-Rattner* (1957/8)

20 g pepton, 1 g asparagin, 5 g glucose, 10 ml liver extract, 490 ml meat extract, 2 g cryst. Na_2HPO_4, 2·5 g NaCl, 1 g ascorbic acid, 500 ml dist. water are poured into tubes (5 ml), autoclaved and before use mixed with 1 ml inactivated horse serum and the standard dose of penicillin and streptomycin. pH = 6. Supposed to be most suitable for cultivation of *T. vaginalis* from the genitals of the male.

8. *Medium after Pavlova* (1938)

Is used in some East-European countries: 8·5 g NaCl, 1·469 g Na_2HPO_4. 12 H_2O, 0·45 g KH_2PO_4, 1 000 ml dist. water are dissolved and filled in tubes (5 ml) and autoclaved. 1 ml horse serum and 2 loops of sterile rice starch are added to each tube. Suitable especially for primary culture. Trichomonads grow together with bacteria.

B. WITHOUT SERUM

9. *Medium after Jírovec and Peter* (1948)

Used Johnson CPLM-medium without serum but added a small piece of cooked rabbit or guinea-pig liver (2 × 0·5 × 0·3 cm) to each tube. pH = 6–6·2. Sterilization 3 times for 1 h at 100°C. Penicillin and streptomycin are added before use.

10. *Medium after Sorel* (1954)

Used a pepton-bouillon (pH = 6·2) with a small piece of meat (1 × 0·5 × 0·5 cm) in each tube, sterilized by autoclaving 20 min at 120:C. Antibiotics added before use. Microscopical examination 3 days after inoculation, transmission after 7 days.

11. *Medium after Samuels and Bell* (1962)

They devised a new serum-free medium C-6 containing cream: 28 g trypticase BBL, 3 g dry yeast extract, 0·075 g KH_2PO_4, 1·5 g glucose, 0·08 g citric acid, 0·4 g ascorbic acid, 0·2 g thiomalic acid, 25 ml 10% liver-powder infusion, 0·1 ml light cream (table or coffee cream), 0·08 g cholesterol, dissolved in 4 ml ethylalcohol. The dry ingredients are added to 800 ml dist. water, then the liver extract and cream, and finally cholesterol are added to the medium heated at 50°C. N/1 KOH is added to reach pH 6·8 and the volume brought up to 1000 ml. After standard autoclaving this medium retains nutritional value for at least 3 weeks, when stored at room temperature in the dark. Approximately 5×10^4 organisms are transferred weekly to 10 ml of medium in screw-capped tubes. *T. vaginalis* attains a population density of 10×10^4 organisms/ml and above.

12. *Medium after Barbarowski* (1966)

Used for primary cultures of *T. vaginalis*: 3·5 ml Ringer solution (1000 ml dist. water, 8·6 g NaCl, 0·3 g KCl, 1 g $CaCl_2$), 1 ml 10% glucose, 0·5 ml 10% ascorbic acid. The growth is sometimes very rich, subcultures are usually not possible.

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* All *Canadian Symposium* references refer to the First Canadian Symposium, held in Montreal in 1959, on non-gonococcal urethritis and human trichomoniasis.

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Liver Involvement in Acute Mammalian Malaria with Special Reference to *Plasmodium knowlesi* Malaria

BRIAN MAEGRAITH

Department of Tropical Medicine, Liverpool School of Tropical Medicine, Liverpool, England

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I. Introduction

Maegraith (1948) introduced a concentrated physiological approach to the study of pathological processes in malaria, which has revealed that many of the disturbances that arise in the host during infection are basically non-specific to malaria and occur in other acute medical states. This has been demonstrated most clearly in regard to the liver and the kidneys, but it is equally true of other organs and tissues of the host. This is not surprising when it is considered that the reactions of a given organ to any form of stress are limited by its structure and function and by the reactions of its blood vessels and other auxiliary tissues.

Changes in tissues resulting from infection may arise locally from direct histotoxic effects, which may be specific, or from dynamic circulatory disturbances or changes in vascular permeability. They may also arise from general

effects, e.g. poisoning from circulating toxins, which again may be specific, or from generalized phenomena such as circulatory failure in shock (basically non-specific).

Sporozoites and merozoites both have the power to penetrate liver tissue cells and multiply therein, and merozoites initiate the erythrocytic phase.

Garnham (1966) has described the chain of events following the rupture of the exoerythrocytic schizonts of *Plasmodium cynomolgi*. There is an immediate phagocytic response, first polymorphonuclear then lymphoid macrophages and plasma cells become involved. In the course of a few days fibroblasts appear and the infiltrating cells retire. "No permanent damage of the organ is apparently produced, either immediately or as a remote cirrhotic effect."

Most authors would accept the last statement (Garnham, 1966) as true for other forms of mammalian malaria. There is thus general agreement that the erythrocytic (E) phase of the parasite life cycle is the more important, and possibly the only important pathogenic stage in the mammalian host (Maegraith, 1966a).

In this case "pathogenesis" refers to the pathophysiological changes induced in the host as a result of the infection (Maegraith, 1955). The influence of the changing physiological and biochemical environment of the infected host on the *Plasmodium* are discussed only briefly in the relevant context since very little is known about this point, except in bacterial infections (Smith, 1964).

Discussion of the pathogenesis of the liver lesions in malaria must take into account all mechanisms which are responsible for functional and structural disturbances in the organ arising from non-specific reactions, and those which can be attributable more directly to the infection itself. The effects of the latter are frequently also non-specific because a given tissue can react in only a limited number of ways.

An example of the first type of reaction in the liver in malaria is the response to the hyperactivity of the sympathetic nervous system initiated by the infection (Skirrow *et al.*, 1964). The second type of reaction is exemplified by the histotoxic activity of certain factors, to be discussed later, circulating in the blood of the infected host which cause fatty degeneration of parenchymal cells and inhibit mitochondrial oxidative phosphorylation (Ray, 1958; Ray and Sharma, 1958; Riley and Maegraith, 1961, 1962).

This review is concerned mainly with these reactions in simian and to some extent in human and rodent malarias. Reactions in avian malaria are not discussed, since, at the cellular level, these infections present completely different problems from mammalian malaria, in that avian parasites, unlike mammalian, inhabit nucleated erthyrocytes, possess mitochondria and metabolize carbohydrate by means of the Krebs cycle.

The pathological and physiological disturbances in malaria vary considerably in severity and duration according to the infecting *Plasmodium* and the host. The processes involved have so far been most carefully studied in *P. knowlesi* infections in rhesus monkeys (*Macaca mulatta*). The work on this infection in so far as it concerns the liver is the main subject of this review.

It will be noted that throughout this text the structure of the liver is discussed in terms of the hepatic lobule. This is because the work on the liver circulation

clearly implies that, in its broadest three-dimensional sense, a lobular arrangement is the basic intrahepatic vascular pattern (Andrews, 1957). Acceptance of this fact in no way prejudices the views of Elias (1949) and others on the syncytial cellular architecture.

II. Biochemical Evidence of Hepatic Dysfunction in Malaria

The so-called liver function tests are often deviated from normal in malaria in man, especially in severe acute infections, in which there is hepatic enlargement and the bilirubin content of the plasma rises and jaundice may develop (Maegraith, 1948); in such cases, as in *P. falciparum* infection in man, the histological picture is one of congestion and centrilobular degeneration and necrosis.

Signs and symptoms of disturbance of liver function may appear in the course of all forms of malaria, both naturally acquired and artificially induced. The most serious disturbances occur in acute *P. falciparum* infections and its complications, especially blackwater fever and bilious remittent fever, the latter being characterized by acute hepatic failure (Maegraith, 1948, 1959, 1966a, b).

In severe infections, there is a fall of albumin, provided the disease continues long enough; there is a more consistent reduction in chronic infections. In human malaria the serum content of α_1-globulin may rise and that of α_2-globulin may fall (Dole and Emerson, 1945; Tella and Maegraith, 1965). γ-Globulins increase; β-globulins show variable changes. Serum concentration of fibrinogen increases in many cases, but may fall in the late stages of severe infection (Maegraith, 1948; Devakul *et al.*, 1966).

The fall of albumin has been regarded as primarily due to failure of its synthesis in the liver, although there is some evidence of leakage from the albumin pool (Maegraith, 1948, 1966a). Fibrinogen changes may be related either to liver synthesis, failure of usage, or the reverse. This is considered elsewhere in the discussion on disturbances of coagulation in malaria.

Changes in blood sugar are variable (Sinton and Kehar, 1931; Fulton and Maegraith, 1948; Maegraith, 1948). A rise of serum glucose content commonly occurs in *P. vivax* and *P. falciparum* paroxysms, but in the terminal stages of the latter the levels fall and may become very low. Devakul (1960) has recorded a blood sugar of only 4 mg% in the late stages of a fatal infection. This is in keeping with the observations of Fulton (1939), Mercado and von Brand (1957), and Devakul and Maegraith (1958) on the fall of blood glucose concentrations in *P. knowlesi* and *P. berghei* infections and the concurrent loss of glycogen from the liver cells, particularly those from the central lobular regions. This represents a true disturbance in liver function (Maegraith, 1954a).

It is interesting to note that in *Babesia canis* infections in puppies there is a similar terminal depletion of liver glycogen; this is not, however, reflected in the blood sugar, which remains within normal limits (Gilles *et al.*, 1953; Maegraith *et al.*, 1957).

Deviations in liver function tests and sometimes overt signs of liver failure including jaundice have frequently been reported in *P. knowlesi* infections in

M. mulatta and in other simian malarias (Devakul, 1958; Maegraith, 1959; Desowitz and Pavanand, 1967). Similar deviations have been reported in rodent malaria, especially *P. berghei* infections (Maegraith, 1954a).

Much of the experimental work in man has been done in blood-induced infections in syphilitics. Very little has been done to study such liver functional changes serially in otherwise healthy individuals or to follow up the subsequent recovery patterns. Sadun *et al.* (1966) have now provided some information in this respect in *P. falciparum* infections in man (American military personnel infected with drug resistant parasites, and some healthy volunteers) and in experimentally infected splenectomized chimpanzees and in *P. berghei* infections in mice.

In all infections except those in the volunteers they found increases in the transaminases and in the SGPT:SGOT ratio, positive cephalin flocculation tests, lowered fasting serum glucose and alkaline phosphatase, a fall in total protein and in albumin and increase in γ-globulin. The volunteers were prisoners in an American jail who were inoculated with a million parasites of a Uganda strain of *P. falciparum* and were allowed to remain infected for as long as 32 days following infection. Parasitaemias of 24000–88000 parasites per mm^3 blood were reached, after which the parasites were controlled by quinine. It is interesting to note that deviations in liver function were not detected in these subjects, although in the individuals naturally infected in Vietnam deviations occurred even in the absence of clinical evidence of hepatic insufficiency, and even with moderate parasitaemia. The authors conclude that the malarial infections disturbed certain liver functions and noted that these recovered slowly after the termination of the infection.

These findings are characteristic of *P. falciparum* infections elsewhere in the world (Maegraith, 1948, 1966a).

III. Pathological Lesions in the Liver in Malaria

Lesions developing in *P. knowlesi* malaria are discussed separately (see Section IV).

There are many detailed descriptions of the liver lesions which develop in man in acute *P. falciparum* infection. The basic picture is similar to that seen in *P. knowlesi* infections. The tissues are macroscopically coloured by the malaria pigment held in the Kupffer cells lining the sinusoids. Microscopically these cells are hypertrophied and hyperplastic and contain, in addition to the granular pigment and haemosiderin granules, phagocytosed parasitized and non-parasitized erythrocytes and free parasites. The liver cells may show granules of haemosiderin and bilirubin; they do not contain malaria pigment. Cells in the centrilobular zone show some fatty infiltration and may be degenerate and necrotic; the peripherally placed cells are not usually involved in these processes. The sinusoids are dilated and, in the absence of severe haemolysis, are congested. All vessels may be involved or only the centrally placed ones. The central veins are dilated and congested. There are no characteristic changes in the portal tracts, although in some cases there is some lymphoid cellular infiltration and vascular congestion (Maegraith, 1948).

All stages and variations of this centrilobular lesion may be present. The development is dependent on the time the infection has been active. In persons dying rapidly after infection there may be only sinusoidal dilatation and congestion; in others in whom the infection has continued for some weeks, the full picture may be present. The lesion is not *post mortem*; it can be identified in liver biopsy (Maegraith, 1948, 1966a).

The liver lesions occurring in acute *P. berghei* malaria in mice and rats have been described by many authors (Maegraith, 1954a; Garnham, 1966). They closely resemble those seen in other plasmodial and protozoal infections. In early lesions the sinusoids are dilated and congested, especially in the central region of the lobule, and the central veins are dilated and congested. In later lesions the characteristic centrilobular degeneration and necrosis with fatty changes develop and may involve the midzonal cells. The affected tissues often have the appearance of having "collapsed" and the structures are almost unidentifiable, except for large numbers of Kupffer cells, some in milder stages of degeneration, and all filled with masses and granules of malarial pigment; the peripheral structures are usually relatively intact, but the Kupffer cells contain considerably less pigment than those in the central necrotic areas. One interesting difference from the lesions in *P. knowlesi* infection is the relative absence of haemosiderin from the hepatic cells, even the necrotic cells and in the Kupffer cells. It is not known whether this reflects a relatively less important role of haemolysis in the rodent than in the simian malaria. It has been noted that in chloroquine-resistant strains of *P. berghei* very little malarial pigment is found in the livers, reflecting the general failure of the parasite to synthesize haemozoin (Peters *et al.*, 1965).

Similar lesions have been described in other acute rodent malarias (Garnham, 1966).

The electron microscopic appearance of the lesions in acute *P. berghei* malaria are very similar to those described below in *P. knowlesi* infection.

IV. Liver Lesions in *P. knowlesi* Infection and Other Simian Malarias

A. *P. knowlesi* Malarias

The recorded work has been carried out with two strains of *P. knowlesi*. Up to 1950 the parasites were obtained from the original strain discovered by Napier and Campbell (1932) and isolated by Sinton and Mulligan (1933). This parasite gradually lost its virulence and all recent studies have been made with a second strain (the Nuri strain) described by Edeson and Davey (1953) and isolated by Jaswant Singh *et al.* (1953). The parasite is usually blood passaged through the sensitive rhesus monkey host, *Macaca mulatta* (which is not the natural host), but it can be transmitted by various species of *Anopheles* mosquitoes (Garnham, 1966). The infection is commonly regarded as a useful model for comparison with human infections, but has certain defects in this respect, one of which is a very sharp 24-h schizogony cycle. Recently studies have been carried out with *P. coatneyi* which also causes an acute and commonly fatal infection in *M. mulatta*, but which has a 48-h cycle and thus more

closely resembles *P. falciparum* (Garnham, 1966; Desowitz and Pavanand, 1967). There is some evidence that *P. coatneyi* is antigenically related to *P. falciparum* (Stein and Desowitz, 1964; Desowitz *et al.*, 1966).

In *P. knowlesi* malaria in the rhesus monkey *M. mulatta*, illness does not develop until the parasitaemia in the peripheral blood exceeds 1–2% schizonts or 10–20% ring forms. Sporulation is not associated with paroxysm and there is frequently no fever. Three clinical types of infection are recognized: the "lytic", in which haemolysis predominates, with haemoglobinuria in the last 48 h of the infection; the "acute shock", in which the final episode occurs in 4–5 days, with the rapid onset of shock, cyanosis, cold extremities and low blood pressures and death; and "prolonged shock", in which the animals survive in shock for up to 24 h. The latter types are not usually associated with severe haemolysis (Chongsuphajaisiddhi, 1966).

The general histopathology and the pathophysiological progress of malaria induced by *P. knowlesi* has been described elsewhere by many authors and need not be considered here (Taliaferro and Mulligan, 1937; Menon, 1939; Maegraith, 1948, 1966a).

As will be explained later, the processes involved in the evolution of the acute liver lesion (which disappears after successful treatment) include acute dynamic intrahepatic circulatory disturbances and a serum factor which inhibits cellular respiration and oxidative phosphorylation. The lesions are basically non-specific to malaria. Similar centrilobular damage occurs in many other acute medical conditions (Maegraith *et al.*, 1947).

The liver lesions seen at autopsy or in biopsy differ in detail according to the type of clinical pattern developing in the individual monkey, but are essentially similar.

B. LESIONS IN LYTIC INFECTIONS

Lesions are seen earlier and in more advanced forms in lytic infections in which the animal survives for a longer period than in the infections characterized by the appearance of shock. In the lytic infections the processes involved are (1) those initiated by the infection *per se*, which will be described later and which are common to all forms of the malaria, and (2) the modifications instituted by the severe haemolysis. As in all pathological developments, the final picture depends very much on the length of time the processes are able to operate. This is a basic principle.

The liver is commonly enlarged and congested, blood flowing freely from the cut surface and vessels. The portal veins are dilated and distended with fluid blood containing parasitized and non-parasitized erythrocytes, macrophages which have taken up these cells and both haemosiderin and malaria pigment, polymorphs containing the latter, and the usual leucocytes of which lymphocytes predominate.

Histological examination of biopsy and autopsy material in fatal *P. knowlesi* infection has not revealed any vascular changes in the portal vessels and tracts. A considerable infiltration of the tracts with round cells is, however, common in human and simian malaria (Maegraith, 1948).

The sinusoids in early lesions are dilated throughout the lobule and are filled with fluid and erythrocytes, many of them parasitized. The dilatation and congestion are more pronounced in the sinusoids in the midzonal and central regions of the lobule. In more advanced lesions, in which parenchymal cellular changes have become obvious, there may be little evident dilation of the peripheral sinusoids, and on occasion, the maximum dilatation is seen in the midzonal area. The central veins are dilated and contain many parasitized erythrocytes and macrophages.

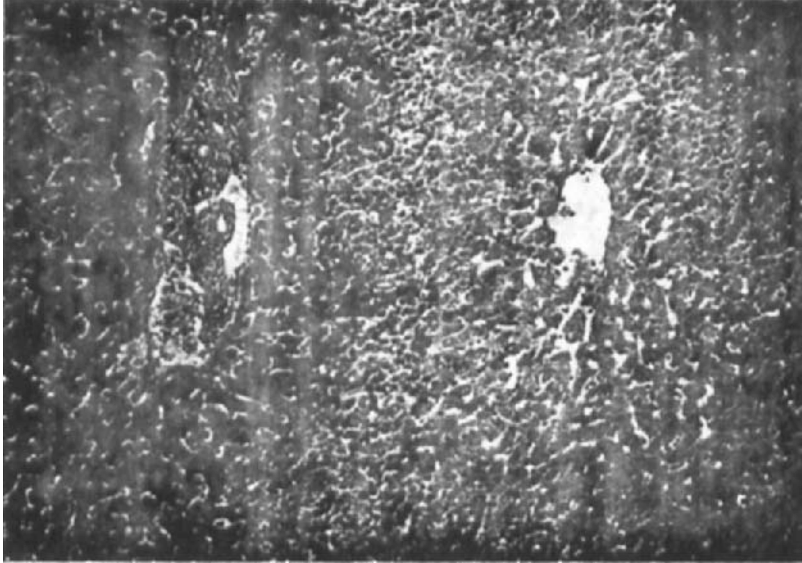


FIG. 1. Liver in "lytic" *P. knowlesi* malaria (early stages of infection). H. and E. Note dilatation of central vein and sinusoids. There is loss of staining of cells in centrilobular area. Glycogen is absent from these cells but present in peripheral cells.

The Kupffer cells are swollen and laden with malaria pigment, haemosiderin and parasitized and unparasitized erythrocytes, sometimes free parasites. Free Kupffer cells may be present in the sinusoids and central veins.

In the early lesions the parenchymal cells show little change, beyond some fatty degeneration commonly most developed in the cells in the peripheral zone. As the process develops, changes become visible in the cells in the central zone. The first sign is often some failure to take up stains, the central cells being much paler than the peripheral cells. It is at this stage that glycogen disappears from the centrally placed cells, in contrast to those on the periphery (Mercado and von Brand, 1957; Devakul and Maegraith, 1958).

In severe lesions, degeneration of the cytoplasm becomes obvious, with deposition of fat and breaking up of the substance, which may now stain deeper pink with eosin. Fine and then coarse granulation appears and changes in the nuclei develop. Some of the latter first stain deeper, others take stains poorly. Karyorrhexis and pyknosis are evident in the most advanced lesions,

when the whole central area may present an appearance of having "collapsed" or become infarcted, and the tissue elements are often difficult to identify (Maegraith, 1954a). In such lesions there is usually a great deal of malarial pigment, mostly in the Kupffer cells, which are less affected by the degenerative processes than the polygonal cells.

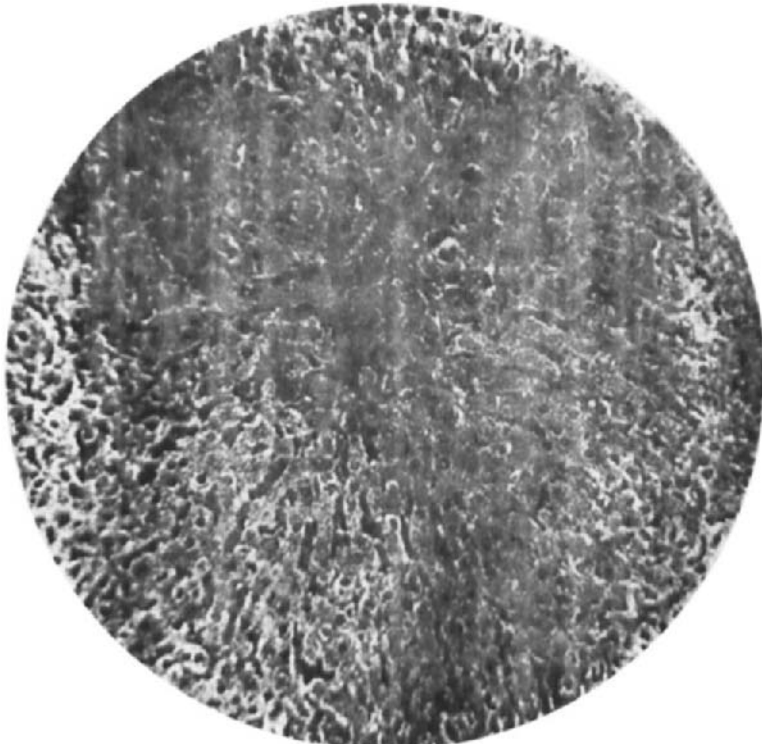


FIG. 2. *P. berghei* infection. Early changes in liver circulation. Intense dilatation and congestion of central vein and centrally placed sinusoids.

In animals which survived for a long period with developing centrilobular liver lesions, infiltration of the necrotic areas with polymorphonuclear leucocytes and macrophages was sometimes present.

The distribution of centrilobular damage through the liver is not uniform; some parts of the organ are affected more than others in a completely irregular fashion.

The damaged parenchymal cells often contain bile thrombi. Considerable deposits of haemosiderin are common, usually scattered as small brown-green granules in the cytoplasm, staining blue with Perl's reagent. Malaria pigment is never seen in the epithelial cells.

Skirrow (1962) has pointed out the very sharp demarcation between the degenerate and necrotic tissue in the central region of the lobules and the

relatively undamaged contiguous cells. In some cases necrotic cells abut immediately against cells showing little or no evidence of damage. Sharp demarcation between the central and peripherally placed cells is often visible at an early stage, before severe degeneration or necrosis develop; it is notable, for instance, at the point where the cells of the affected zone take stains badly and first lose their glycogen (Fig. 1).

This would appear to indicate that the differentiation between the zones of living and necrotic cells is primarily decided by factors other than those finally responsible for the cellular death (such as the soluble cytotoxic factor to which

FIG. 3. Liver in late stages of "lytic" *P. knowlesi* infection in *M. mulatta*. Note dilatation of centrilobular sinusoids and central vein with disintegration of centrilobular cells; dilated sinusoids lined with pigment filled Kupffer cells and fatty degeneration of cells in midzone; relatively little change in periphery of lobule. (Courtesy Dr. T. Chongsuphajaisiddhi.)

reference is made later). The most probable explanation is the circulatory reorganization which takes place following the intrahepatic vasoconstriction induced by the intense sympathetic hyperactivity which, as pointed out below develops before the structural changes in the affected cells become extensive (see p. 208).

The disappearance of glycogen from the centrilobular region occurs early and persists with the development of the lesion. It appears to be due to mobilization and subsequent failure of glyconeogenesis, since in both *P. berghei* infections in mice and in *P. knowlesi* infection in *M. mulatta* the glycogen can be partly restored to the cells by intravenous administration of fructose or glucose, the process being enhanced by hydrocortisone (Mercado and von Brand, 1957; Devakul and Maegraith, 1958). The mobilization of the glucose from the cells

is probably a manifestation of the hyperactivity of the sympathetic nervous system (see later).

That there are changes in mitochondria in the centrally placed cells can be seen in histological preparations stained by Heidenhain's specific stain. The cytoplasm of the cells at the periphery of the lobule contains normal numbers of particles; that of the cells involved in the central degenerative lesion contains

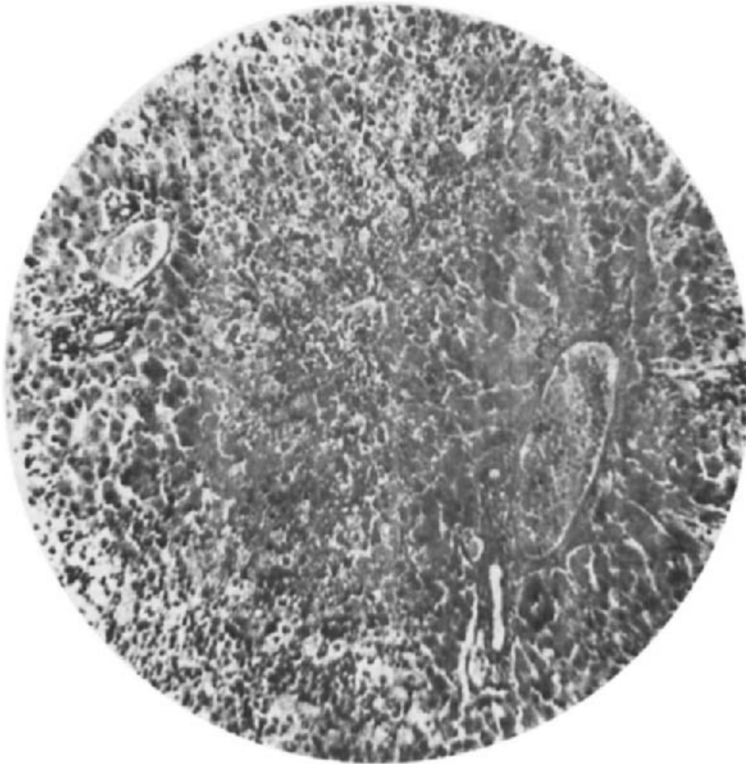


FIG. 4. Centrilobular damage in acute *P. berghei* infection (at death 7 days after overt infection). Note "collapse" and intense pigmentation of central area. Pigment is haemozoin, which is relatively scanty at periphery of lobule.

fewer well stained particles and variable numbers of minute vacuoles which are increased in number in the obviously necrotic cells. These patterns are clearly dependent on mitochondrial changes and are mirrored by the electron microscopic picture already described (Skirrow, 1962).

Chongsuphajaisiddhi (1966) and his colleagues have recently followed the liver cell changes in detail under the electron microscope in serial biopsies taken during the course of acute *P. knowlesi* malaria in individual *M. mulatta* monkeys which developed lytic infections and died on the 8th day after infection. They first observed changes in parenchymal cell structure on the 7th day.

The mitochondria of some cells were swollen and irregular in outline; the organelles in other cells appeared normal; except that they were no longer electron dense and displayed many cristae. By the following day changes were very marked. Mitochondria were swollen, distorted, electron transparent and contained few cristae. The endoplasmic reticulum was increased in amount and

FIG. 5. Electron micrograph of part of a normal liver parenchymal cell from a control biopsy specimen (*M. mulatta*) showing nucleus (*N*) with nuclear membrane (*nm*), elements and vesicles of the endoplasmic reticulum (er_1 and er_2), mitochondria (*mi*) and a "lysosome" (*l*). Some cristae mitochondriales (*c*) are visible and numerous glycogen granules (*g*) are present in the cytoplasm. × 20000. Cf. Fig. 6.

was frequently arranged closely about the damaged mitochondria. Lysosomes containing cellular debris were visible in many cells. The cytoplasm was vacuolated, probably as a result of lipid deposition. Nuclear changes included clumping of granular nucleoplasm and undulation of the containing membrane. Some cells, presumably from the central region (at this stage centrilobular changes were obvious histologically), showed extreme mitochondrial damage and widespread vacuolation; these cells did not contain glycogen granules.

In the biopsy taken immediately before death most cells examined were disorganized and necrotic. Few recognizable mitochondria were visible. Vacuolation was extensive. The microvilli of the sinusoidal margins and bile canaliculi were lost. The endoplasmic reticulum was completely fragmented, with small vesicles. The nucleoplasm was electron transparent and the membrane was

FIG. 6. Electron micrograph of part of a biopsy specimen taken on the 7th morning of infection of *M. mulatta* with *P. knowlesi*. Changes in the nucleoplasm and in the nuclear membrane (*nm*) are evident. Mitochondrial swelling and distortion are marked and proliferation of the endoplasmic reticulum elements (er_1) is apparent around these particles. Their cristae (*c*) are less numerous and distinct than normal. Large vacuoles (*va*) probably represent simple lipid deposition. Disorganization of the vesicles of the E.R. (er_2) is evident. Note the presence in the cytoplasm of several abnormal particles (*pa*). × 20000.

incomplete and dilated. A few cells, presumably from the peripheral zone, contained swollen mitochondria which appeared to be undergoing degeneration; some contained a few normal mitochondria, lysosomes and glycogen granules.

It is interesting to note that the plasma inhibitory factor can first be detected in the course of this infection at about the same time as the mitochondrial

changes appear under the electron microscope. As will be seen later, it is not likely that this factor is solely responsible for the cellular lesions; the circulatory disturbances exert a synergistic effect.

Changes can also be observed in the endoplasmic reticulum, which is commonly redistributed at an early stage and sometimes comes to lie around the affected mitochondria. There is also evidence that there may be lysosomal changes, leading to their disruption and contributing to the eventual death of the cell. Maegraith *et al.* (1963) and Fletcher (1964) have suggested that anoxia, arising partly (as anoxic anoxia) from the circulatory disturbances described below and partly (as histotoxic anoxia) from the mitochondrial damage, might stimulate lysosomal degeneration which would in turn further damage the cell by the action of liberated hydrolytic enzymes. Histochemical evidence of this has been obtained by examining the distribution of acid phosphatase in the central zonal liver cells in malaria infected monkeys (Popper and Schaffner, 1961).

C. LESIONS IN MALARIAL SHOCK

In the infected animals which die soon after the onset of shock, the cellular lesions are not well developed, and the major changes are extreme dilatation of the central veins and sinusoids with pronounced congestion, the vessels being packed with erythrocytes, both infected and non-infected. The dilatation is often more pronounced in the peripheral zone. The Kupffer cells exhibit changes similar to those described above except that they contain considerably less haemosiderin than in the lytic cases in which haemolysis is intense.

In cases where shock persists for 12–24 h before death, sinusoidal dilatation is again pronounced, most often in the central zone near the central veins; sometimes dilatation occurs only in this area. Cellular damage is sometimes marked, with degenerative and necrotic changes similar to those described above in the lytic infections, except for the minimal amounts of haemosiderin present.

It will be noted that the cellular changes are more pronounced in the infections which last longer. Thus they reach full development in the lytic cases, which die within 7–8 days after the overt infection, and are least obvious in the animals which die shortly after the appearance of acute shock, usually on the 4th to 5th day.

The importance of time in the development of centrilobular lesions has also been demonstrated in perfusion experiments in dogs. Thus Delorme (1951) demonstrated that centrilobular changes in the perfused dog liver developed only where perfusion with deoxygenated blood was continued for a long time. This was later confirmed by Maegraith *et al.* (1951).

D. SUMMARY

The principal lesions in the liver in acute *P. knowlesi* infection thus present in the central zone in the form of sinusoidal dilatation and congestion followed by cellular degeneration and necrosis, affecting primarily the parenchymal cells and later the contiguous Kupffer cells and other tissues. These lesions are

characteristically unevenly scattered through the organ; not uncommonly they are especially pronounced in the tissue near the surface, but they may appear anywhere. As will be seen, this uneven distribution is probably determined to some extent by the equally irregular vasoconstriction which occurs, involving particularly the smaller vessels of the portal vein, and giving rise to circulatory stagnation more pronounced in the central regions because of the alternative sinusoidal pathways which exist at the periphery of the lobules.

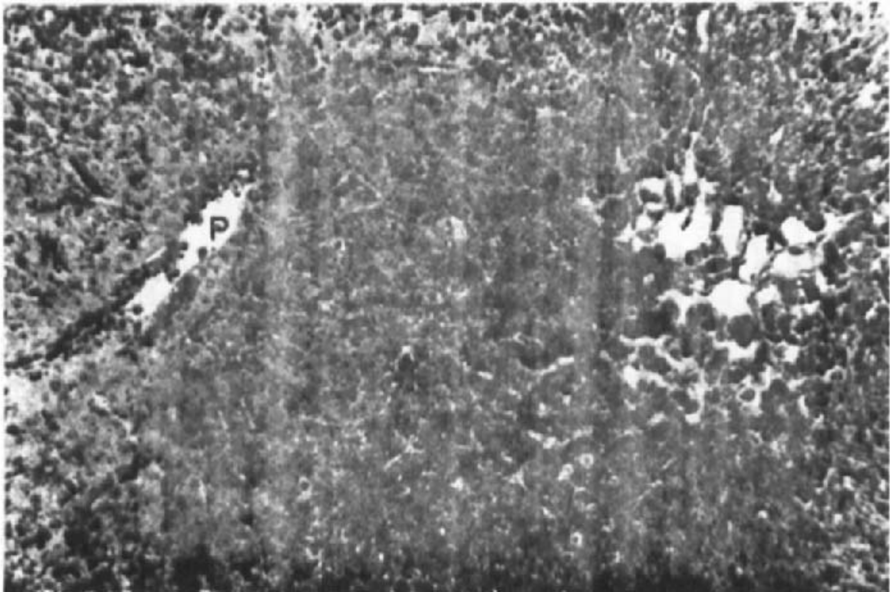


FIG. 7. Frozen section of liver of *M. mulatta* infected with *P. knowlesi* stained with oil red O showing centrilobular distribution of fat (stained red); cells adjacent to the portal tract (*P*) are unaffected. The mechanical trauma involved in preparing the section has caused some of the necrotic centrilobular cells to be lost. × 150. (Courtesy Dr. M. Skirrow.)

The fatty changes which occur in the central zones of the lobules are related to the more extensive cellular changes which prevail there. Thus fat globules are frequently seen in degenerating cells in which mitochondrial damage has become evident (Fletcher and Maegraith, 1962a; see later). Fatty infiltration in the peripheral cells appears independently of the centrilobular degeneration and may often be seen before the latter can be detected. Ray and Sharma (1958) have shown that fatty changes can be produced in these cells by intravenous inoculation into rhesus monkeys of serum from infected animals. The process, which can be inhibited by choline, appears to be different from that initiating the centrilobular fatty changes and cellular necrosis.

The circulatory changes mentioned above are probably related to sympathetic nervous system stimulation, which leads to the demonstrable portal venous constriction, with consequent retardation of blood flow through the liver. This point will be discussed in detail later.

Studies made under the electron microscope on the cellular changes in the liver (Fletcher and Maegraith, 1962a; Fletcher, 1964; Maegraith, 1966a; Chongsuphajaisiddhi, 1966) have thrown some light on the mechanisms involved and, as will be seen later, support the thesis that one of the factors involved is the soluble substance detectable in the serum of the infected animal which inhibits the respiratory processes of the cell (Maegraith *et al.*, 1962; Riley and Maegraith, 1962).

The most notable features in the degenerating liver cell in the central zone of the lobule are swelling and eventual destruction of the mitochondria. In the early stages the pattern resembles that seen in carbon tetrachloride poisoning where the permeability of the particles is increased, allowing the escape of coenzymes needed for oxidative metabolism. This also appears to be the action of the serum factor which inhibits cellular respiration in normal mitochondria. If such mitochondrial damage were allowed to continue, degeneration and necrosis of the cell might be expected leading to its death.

E. OTHER SIMIAN MALARIAS

Desowitz and Pavanand (1967) and colleagues (Eyles *et al.*, 1962) have described liver lesions in acute infections with *P. coatneyi* in three splenectomized *M. mulatta.* In one animal early centrilobular degeneration and necrosis were noted in a biopsy taken on the 8th day. Thereafter haemolysis and haemoglobinuria developed and centrilobular necrosis was massive at autopsy. Similar lesions were observed in one other fatal haemolytic infection and in one acute infection, also haemolytic, which ultimately resolved. In two animals the transaminases rose above normal, fell after the peak parasitaemia was passed and rose again during the subsequent haemolysis.

P. fragile, which is a highly fatal infection in *M. radiata*, also produces severe centrilobular necrosis, with massive deposition of pigment in the Kupffer cells (Garnham, 1966).

V. Pathogenesis of Liver Lesions

A. INTRAHEPATIC CIRCULATORY DISTURBANCES

The hepatic centrilobular degeneration and necrosis found in malaria in man, monkeys and rodents also occur in many other conditions, including parasitic infections such as *Trypanosoma congolense* infection in cattle (Unsworth, 1956, personal communication), *Babesia canis* infections (Gilles *et al.*, 1953) in puppies, many acute generalized bacterial infections and acute medical states associated with shock (Maegraith *et al.*, 1947).

The common appearance of this lesion in such a wide range of conditions indicates that it is non-specific in origin. Maegraith (1944) suggested that one factor involved was obstruction to blood flow through the liver, leading to stagnant circulation in the lobules, especially in the central zone. Maegraith *et al.* (1947) posited a constriction of the hepatic venous tree as the mechanism responsible for the vascular changes, and subsequent work by Andrews (1948),

Andrews *et al.* (1949), Andrews (1951) and Hecker (1954) supported this view, in so far as the perfused organ is concerned.

More recent work in the intact animal by means of X-ray opaque angiography (Franklin and Janker, 1936; Daniel and Prichard, 1951a, b; Daniel *et al.*, 1951, 1952a, b) has, however, demonstrated that in *P. knowlesi* infection in *M. mulatta* the major dynamic change in the vascular tree is an intense constriction of the portal vein and its smaller intrahepatic branches (Skirrow *et al.*, 1964). Although there is some indication that the hepatic veins, are also constricted, the principal areas involved are the portal veins, and hence the obstruction to hepatic blood flow is primarily on the afferent side of the sinusoidal bed.

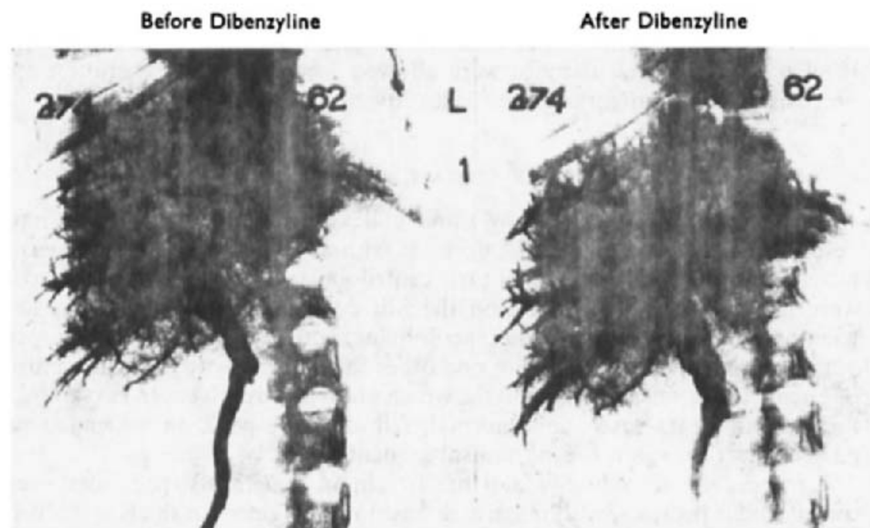
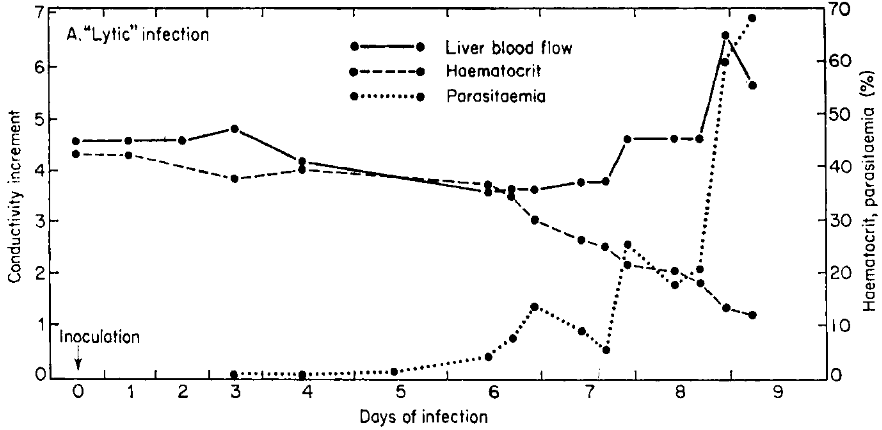


FIG. 8. Relief of extrahepatic and intrahepatic portal constriction during acute malaria (*P. knowlesi* infection in *M. mulatta*) following adrenergic blockade with Dibenzyline. (Courtesy Dr. M. Skirrow.)

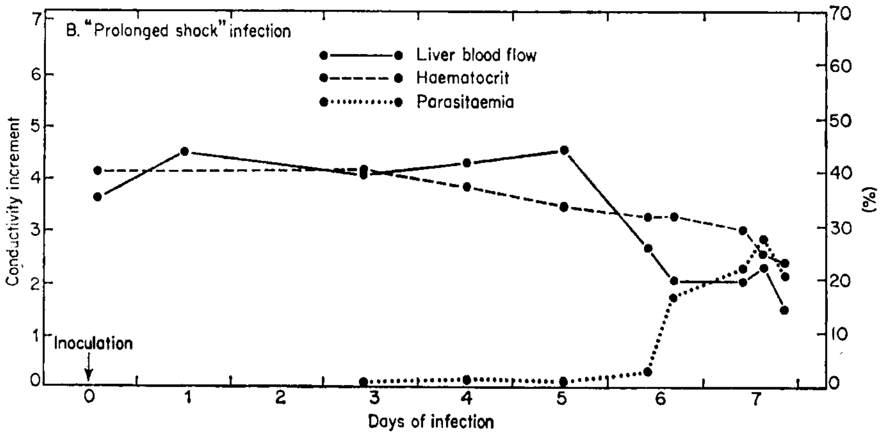
The vasoconstriction is evident both in advanced simian malaria and in various forms of shock, including oligaemic and that resulting from gut manipulation. That it can be relieved by ganglionic or adrenergic blockade indicates that it originates from hyperaction of the sympathetic nervous system (Skirrow *et al.*, 1964).

Chongsuphajaisiddhi *et al.* (1966) have now examined the intrahepatic vasoconstriction in malaria and in shock and its effects on the liver blood flow and portal venous pressure by more elegant angiographic techniques and by the application of internal calorimetry for the estimation of liver blood flow (Grayson, 1952; Dosekun *et al.*, 1960; Chongsuphajaisiddhi, 1966).

In infected animals studied in Liverpool in the late days of infection the arterial blood pressures were below normal, most notably in the clinical types of infection characterized by shock. The venous pressures were slightly raised, except in prolonged shock, in which there was a consistent fall. The most striking change in all types of infection at the stage at which intrahepatic portal



(A)



(B)

FIG. 9. Total liver blood flow in *P. knowlesi* malaria in *M. mulatta*. A. "Lytic" infection. Haemolysis began on days 7–8. Note considerable rise of liver blood flow and fall of haematocrit. B. "Prolonged shock" infection. Note rapid fall of hepatic blood flow and relatively high level of haematocrit. (After Chongsuphajaisiddhi, 1966.)

vasoconstriction was demonstrable was increase in the portal pressure, the mean rise being least in the animals in prolonged shock.

This rise in portal venous pressure was observed in all monkeys examined after the 5th day of the infection and was usually most pronounced in shocked animals. Its cause was primarily the concurrent intrahepatic portal vasoconstriction described above. That this was the case was demonstrated by the reduction in portal pressure which occurred after administration of adrenergic blocking agents, simultaneously with the relaxation of the vasoconstriction (Skirrow *et al.*, 1964).

TABLE I

Mean values in M. mulatta *normal and infected with* P. knowlesi
(After Chongsuphajaisiddhi, 1966.)

Type of infection	Parasitaemia (%)	Haematocrit (%)	Pressures		
			Arterial (mm Hg)	Venous (cm saline)	Portal (cm saline)
Lytic	61	11	70	4·3	15·5
Acute shock	29	36	41	4·0	13·9
Prolonged shock	21	26	41	2·6	11·1
Normal monkey	—	40–45	97	3·4	8·8

It will be noted from Table I that the lytic type of infection was characterized by much lower haematocrit values and higher erythrocyte parasite infection rates than those recorded in shock types. The latter can be explained on the grounds that the animals with lytic infections commonly survived beyond the 7th day after infection, sometimes to the 9th, whereas the shock types of infection terminated earlier. The low haematocrit in the lytic animals was, of course, primarily caused by the very acute haemolysis. Changes in blood volume in these infections are discussed on p. 214.

B. LIVER BLOOD FLOW

Evaluation of the haemodynamics of the liver is rendered very difficult by the complexity of the intrahepatic vascular bed and its complicated connexions with the splanchnic and somatic circulations. The frequent necessity to examine the circulation in an animal which has undergone laparotomy, the inadequacies of the available methods and the difficulties in the intact animal of interpreting the behaviour of the hepatic vascular beds in terms of the total visceral and body circulation, have all combined to make the study of the normal liver circulation difficult and that of the liver in the sick host the more so. The problem is aggravated by the differences which occur in the responses of various vertebrate hosts to the same stimuli, because of differences in effective drug levels or stimuli (Bradley, 1963).

Extensive studies on hepatic circulation by modern perfusion techniques have shown that stimulation of pre- and postganglionic fibres of the splanchnic nerves causes intense intrahepatic vasoconstriction equivalent to that produced by adrenaline (Andrews, 1951; Andrews *et al.*, 1954, 1955; Hecker, 1954). Stimulation of the vagi has no effect. On the other hand, Ginsberg and Grayson (1954) noted in rats that a fall in arterial pressure caused reflex vasodilatation in the liver, mediated through the vagus. They advanced the view that there may exist an intrinsic auto-regulating mechanism for the blood flow through the liver, which is not necessarily neurogenic but which may be governed by direct vascular reaction to changes in intraluminar pressure.

The response of the liver vessels to acetylcholine varies considerably from species to species and is dependent to some extent on the dosage and the state of the liver at the time of administration. Notable effects on flow are produced only with very large doses of acetylcholine (Andrews *et al.*, 1956). Skirrow and Maegraith (1964), using angiographic methods, noted that in rabbits intraportal injection of acetylcholine caused constriction of the portal venous tree, apart from the main trunk. Chongsuphajaisiddhi (1966) obtained similar results in rhesus monkeys and considered that the vasoconstriction was reflex, following the fall in systemic blood pressure (Ginsberg and Grayson, 1954). A secondary factor was possibly the release of noradrenaline from tissue by the acetylcholine, as postulated by Brandon and Rand (1961).

It has been concluded from the somewhat conflicting evidence that the normal control of the liver blood flow is operated through the sympathetic nervous system and not the parasympathetic. As will be seen, this thesis is important in interpreting the circulatory phenomena occurring in the liver in acute malaria which are such significant factors in the development of the characteristic centrilobular lesions.

The hepatic sympathetic nerve supply is vasoconstrictive, acting largely on the portal venous tree; on the whole, the parasympathetic has little effect on the vessels (Bauer *et al.*, 1932; Wakim, 1944; Daniel and Prichard, 1951b; Green and Kepchar, 1959; Shoemaker, 1964; Andrews, 1957). Adrenaline and noradrenaline have vasoconstrictor effects on hepatic artery and portal vein in dogs and monkeys (Andrews *et al.*, 1956). Intraportal injection of adrenaline leads to constriction of the intrahepatic portal tree in the dog and the monkey, accompanied by a rise in portal venous pressure and an increase in transhepatic circulation (Daniel and Prichard, 1951b).

Skirrow and Maegraith (1964) confirmed these angiographic findings and noted that adrenaline and shock caused a reduction in the density of the sinusoidal shadow and relatively early appearance of the contrast medium in the hepatic veins. They deduced that there was evidence of a functional intralobular shunt, possibly produced by sinusoidal constriction, causing the blood to take a more direct course through the lobule without passing through interconnecting sinusoidal channels. The effects of sympathetic stimulation and of adrenaline on the total liver blood flow are very variable and depend to some extent on the constrictor effect on the hepatic vessels of reflex vasodilatation of the hepatic artery in the presence of a rise in systemic blood pressure and vasoconstriction of the splanchnic bed (Ginsberg and Grayson, 1954; Andrews *et al.*, 1955; Ueda *et al.*, 1962; Chongsuphajaisiddhi, 1966).

Wiggers *et al.* (1946, 1950) demonstrated that hepatic vascular resistance and portal pressure were greatly increased in dogs in oligaemic hypotension. Skirrow *et al.* (1964) and Chongsuphajaisiddhi (1966) obtained similar results in rhesus monkeys in oligaemic shock, traumatic shock and in the late stages of *P. knowlesi* malaria. They found that the circulatory changes in these conditions were identical, indicating non-specific origin. In the liver in late *P. knowlesi* malaria there is intense portal venous vasoconstriction, which is relieved by the administration of adrenergic blocking drugs, and thus seems to be mediated through the sympathetic nervous system. Further support for the latter view

and for the concept that such circulatory changes are concerned in the development of the centrilobular lesions in malaria is given by the results of experiments of Ray and Sharma (1958), who prevented the development of centrilobular lesions in *M. mulatta* infected with *P. knowlesi* by previously subjecting the animals to bilateral sympathectomy.

It should be noted here that the centrilobular damage which occurs as a response to administration of carbon tetrachloride to rats can also be delayed or prevented by blockading drugs or transection of the spinal cord (Calvert and Brody, 1960). This again suggests that an important causative factor in these lesions is sustained hyperactivity of the sympathetic nervous system, although other factors are involved, such as the direct action of the drug on the mitochondrial membranes and the endoplasmic reticulum. The hydropic cellular degeneration appears in the midzonal rather than the central zonal cells (Andrews, 1948; Maegraith, 1948). At a later stage the latter become involved.

There seems to be a close relationship between vasoconstriction of sympathetic origin and the ultimate development of cellular degeneration and necrosis in the centrilobular zone. The latter might arise from stagnation of local blood flow, as posited above (Maegraith, 1944, 1948), but it is not at first easy to see how such stagnation can arise from vasoconstriction which primarily involves the portal venous tree.

Angiographic and calorimetric studies of hepatic blood flow in *P. knowlesi* infections in *M. mulatta* have thrown considerable light on this complex matter, which is further complicated by the fact that centrilobular lesions are most highly developed in the lytic forms of the disease, in which it has been demonstrated that the total liver blood flow is actually increased, despite the portal constriction, as a result of decreased blood viscosity following the massive haemolysis (Chongsuphajaisiddhi, 1966).

In infected animals which died quickly after the early onset of shock and in those which survived shock for 12–25 h, the total hepatic flow considerably decreased with the development of circulatory failure.

In this respect it should be remembered that the flow across the lobule is dependent on the pressure gradient from the periphery to the centre and on the resistance offered by the sinusoids. In the liver this gradient is extremely labile and is on average roughly equivalent to only 6 cm of water, so that the balance can be easily disturbed. Using micropuncture techniques in rats, Nakata *et al.* (1960) found that about 70% of the total portal venous pressure head was dissipated between the portal vein at its point of entry and the pre-sinusoidal venules; they found that adrenaline produced an increase in this pressure drop, so that the net result of the increase in vascular resistance was reduced pressure at the periphery of the lobule (Chongsuphajaisiddhi, 1966).

Maegraith (1944) and Maegraith *et al.* (1947) posited that the centrilobular lesion was partly the result of stagnant anoxia and suggested that the most likely explanation was constriction of the hepatic veins leading to reduced intrahepatic blood flow. Such constriction can easily be demonstrated in the dog but is more difficult to show in other mammals (Andrews, 1951). However, recent work in Liverpool has demonstrated in the late stages of *P. knowlesi*

malaria that there is some degree of constriction in the hepatic veins which can be released by adrenergic blocking agents. Earlier work had not demonstrated this (Skirrow *et al.*, 1964) but Skirrow (1962) had pointed out, in line with Maegraith and Findlay (1944), that sinusoidal drainage would be affected considerably by even very small changes in the hepatic veins. This had been shown by Brauer *et al.* (1959) in the perfused rat liver, in which they found that a rise in hepatic venous pressure above the hydrostatic pressure in the portal vein progressively decreased the perfusion rate. Similar results were obtained by Andrews and Maegraith (1951). This factor clearly obtains in the rhesus monkey both in malaria and in shock, in which the average venous pressure in the inferior vena cava at the level of the hepatic venous orifices is raised above normal.

Skirrow and Maegraith (1964) reported that in the monkey adrenaline and noradrenaline caused reduction in the density of the sinusoidal shadow and posited the existence of some form of intralobular shunting arising in the presence of the drug which would result in resistance to flow. Similar effects to those caused by adrenaline might be expected in the monkeys infected with *P. knowlesi* as a result of the current sympathetic activity.

Other mechanical obstructive factors may influence the sinusoidal flow in malaria. The swollen Kupffer cells could act in this way, as would the haemoconcentration seen in the acute shock which sometimes appears immediately preceding sporulation of the parasites and may lead rapidly to death (Maegraith, 1948, 1966; Skirrow, 1962). The effect of haemoconcentration has been studied in blood at low rates of flow by Wells (1965), who found that at stagnant rates of flow the viscosity rose to ten times that of blood in normal flow rates; increased concentrations of heavy molecules, especially fibrinogen and the globulins, also greatly augmented the viscosity. These factors may all affect the sinusoidal flow in malaria, in which fibrinogen and γ-globulin concentrations increase both in human *P. falciparum* and simian *P. knowlesi* infections (Dole and Emerson, 1945; Devakul, 1958). In the latter, Skirrow (1962) demonstrated a rise of fibrinogen plasma content from 2·6 mg per ml to 6·1 mg per ml at the end of the acute infection.

Skirrow and Maegraith (1964) interpreted their angiographic findings in the normal monkey following administration of adrenaline as indicating that some form of intersinusoidal shunting of blood may have taken place. This in itself would affect the total movement of blood through the sinusoids, causing some slowing of circulation in the vessels not involved in the shunt.

Richards and Brearley (1962) found that in the dog portal injection of adrenaline and noradrenaline produced a fall in the rate of BSP extraction which was greater than could be explained in terms of the observed fall in portal flow. They suggested that this resulted from some kind of circulatory shunt. In view of the rich arteriovenous plexus along the bile ducts described by Andrews *et al.* (1949) and the excretion of BSP into the bile (Andrews *et al.* 1956), there is some possibility that the shunt may occur on the arterial side of the circulation. Skirrow and Maegraith (1964) obtained evidence in support of the existence of circulatory shunts in monkeys and, from the reduction in density of sinusoidal shadows and early appearance of contrast medium in the

hepatic veins occurring after the administration of adrenaline, concluded that the shunt was largely intrasinusoidal. The overall effect was that sinusoidal flow was redistributed so that some blood took a more direct route through the lobule from periphery to central vein, by-passing the interconnecting channels, the "ladder-rung" sinusoids of Brauer (1963). In support of the view that there may be alternative sinusoidal routes for blood through the liver are the findings of Hollenberg and Dougherty (1966), using $Krypton^{85}$ to examine liver flow via portal venous and hepatic arterial pathways. These authors have suggested that there are certain portal vessels which do not share sinusoidal pathways with the hepatic arterial flow and which may thus serve as preferential high flow channels. The results of the administration of adrenaline could be interpreted in terms of a shunt of portal flow through such low resistance vessels.

Daniel and Prichard (1951a, b) demonstrated by angiography that redistribution of blood flow in the rat liver occurred on administration of adrenaline. In normal conditions the contrast medium after injection into the portal vein spread evenly through the sinusoidal beds. Under adrenaline the medium was confined to the portions of the liver lobes near the hilar region, the peripheral parts receiving very little. This form of restricted flow was occasionally seen in monkeys in late *P. knowlesi* infection, but was not a constant feature and therefore cannot be regarded as a regular contributor to the interference in central sinusoidal flow (Skirrow, 1962; Skirrow *et al.*, 1964). It nevertheless offers further evidence of differential functional vascular pathways in the organ, despite the absence of any firm anatomical evidence of specific translobular vessels.

The development of shunt pathways in the monkey liver in malaria and shock may be effected by the processes noted above, but there are other factors to consider, notably dynamic or passive restriction of flow in the sinusoids themselves.

There is some evidence that the sinusoids in some animals, including rats, can be actively constricted by adrenaline or by sympathetic stimulation (Wakim, 1944; Seneviratne, 1949). Hecker (1954) and Maegraith (1958) observed constriction of the sinusoids after injection of adrenaline into rats but failed to detect the existence of the postulated inlet and outlet sinusoidal "sphincters", the evidence for which was later summarized by Knisely *et al.* (1957). Gibson (1959) and Nakata *et al.* (1960) also failed to detect such sphincters. Nevertheless, Andrews *et al.* (1955) in liver perfusion experiments in a number of mammals produced clear evidence of the existence of outflow control mechanisms, which they thought were located in the small lobular and sublobular hepatic veins, and possibly at the distal termination of the sinusoids, as in the "small sluice" channels of Deysach (1941).

On balance, the evidence is in favour of the existence of some form of reorientation of sinusoidal flow in the normal monkey challenged with adrenaline and in animals in late *P. knowlesi* malaria and in shock (Skirrow *et al.*, 1964). The redistribution of blood flow is such that the blood takes a more direct route from the periphery of the lobule to the central vein, missing the interconnecting sinusoidal channels (Brauer, 1963). Thus, the parenchymal cells lining the latter may suffer from stagnant anoxia while others on the more direct routes

do not. This state of affairs probably exists for some time before there is any detectable reduction in total hepatic flow.

Such a fall in total flow occurred in *P. knowlesi* malaria in shocked animals but not in the lytic forms of the disease in which the viscosity of the blood was reduced by the removal of erythrocytes subsequent to haemolysis, and the consequent fall in haematocrit. In the latter animals the total hepatic flow actually increased during the late stages although it was reduced to some extent by the vasoconstriction, since an increased flow was recorded when blocking agents were introduced. Nevertheless, the centrilobular lesions were most pronounced in the lytic animals, indicating that the cells lining the interconnecting sinusoids must have suffered from the effects of a stagnating circulation despite the high rate of total liver flow.

The severity of the lesions in the lytic infections was partly due also to the relatively long survival of the animals, compared with those which died in shock and the consequent persistent exposure to increasing stagnation of the relevant parts of the microcirculation, plus the increasing anoxaemia arising from this and the profound reduction in numbers of circulating erythrocytes.

As will be seen later, a synergistic factor of great significance in all forms of liver cell damage noted in the *P. knowlesi* infections is, however, believed to be a soluble substance which circulates in the plasma and has been found to inhibit cellular respiration and oxidative phosphorylation (see p. 217).

C. SYMPATHETIC HYPERACTIVITY

Hyperactivity of the sympathetic nervous system (with elevation of concentrations of circulating catecholamines) in *P. knowlesi* infection is evidently an important phenomenon in the evolution of the pathophysiology of the host body as a whole. It is not confined to the liver.

Since first postulated by Maegraith (1944), Maegraith and Findlay (1944) and Maegraith *et al.* (1945) as the rational explanation of renal failure and anuria in blackwater fever, intrarenal vasoconstriction causing reduction of total renal flow and corresponding failure of glomerular filtration and tubular function has been demonstrated in malaria in man, in *P. knowlesi* infection and in *P. berghei* infections. There is evidence of some degree of redistribution of the intrarenal circulation in the infected monkey whereby the cortical flow is restricted more than the medullary, and in *P. knowlesi* infected animals the vasoconstriction can be released, as in the liver, by adrenergic blockading drugs (Skirrow *et al.*, 1964; Maegraith, 1966). As in the hepatic vasoconstriction, the changes in renal blood flow in the monkey malaria occur in various forms of shock and may be regarded in such circumstances as part of the general reaction of the body to circulatory failure.

In malarial shock in *P. knowlesi* infections the mesenteric vessels, both arteries and veins, undergo constriction. The constriction of the mesenteric veins is real, since the pressure in them is raised, which would not be the case in collapse (Chongsuphajaisiddhi, 1966). The functional significance of this constriction is a notable reduction in intestinal absorption of xylose and of

amino acid (α-amino-isobutyric), which can be reversed by adrenergic drugs (Migasena *et al.*, 1967).

It is interesting to note here that in the lytic type of infection, the disturbances of intestinal absorption are measurable by the 4th day, some 2–3 days before the hepatic or renal vasoconstriction can be demonstrated by angiography. It would appear, therefore, that the hyperactivity of the sympathetic nervous system develops for some time before the latter changes can be identified, and is an earlier phenomenon than might have been expected.

Vessels in other parts of the body are also constricted, including the superficial veins. Moreover, the blood-oxygen saturation is lower in superficial than in the deep veins, suggesting a redistribution of the circulating blood centrally into the great vessels. This again indicates pronounced sympathetic activity (Wang *et al.*, 1947; Skirrow, 1962).

The general picture is that of the stress response in which the sympathetic nervous system evokes visceral and cutaneous vasoconstriction, as it does in the early stages of shock (Franklin, 1951; Green, 1961; Johnson, 1964).

D. OTHER CIRCULATORY FACTORS

Certain other points should be noted. Continuous adrenaline administration or sympathetic hyperactivity *per se* may cause a decrease in the circulating blood volume; under these circumstances an accumulation of fluid occurs in the tissues. At the same time, aggregation of erythrocytes may sometimes be seen in the sinusoids of the liver and in the small blood vessels. In the latter case the aggregation is enhanced in some areas, such as the brain, by the local escape of heavy molecules and water, following changes in endothelial permeability. Vasoconstriction of the vessels of the hind limbs in dogs leads to apparent increase in blood viscosity which, as pointed out above, increases rapidly as the rate of flow diminishes (Pappenheimer and Maes, 1942; Wells, 1965).

It is reasonable to infer that phenomena such as these may also play a role in the retardation of the hepatic microcirculation, at any rate in the interconnected sinusoids.

The blood flow through vessels is usually streamlined, with the erythrocytes centrally placed and plasma in contact with the vessel wall. This is not the case in sinusoids, in which the flow tends to be mixed, with frequent impacts between erythrocytes and between erythrocytes and the vessel walls; in some instances, under trans-illumination, sinusoids may be seen filled with plasma only, whereas elsewhere in the same preparation they may be filled with a turbulent suspension of erythrocytes (Hecker, 1954; Maegraith, 1958). Similar patterns may sometimes be seen in peripheral capillaries of other tissues (Billings and Maegraith, 1938). Conditions leading to lessening of the pressure gradient across the sinusoids would aggravate this picture and so reduce effective flow. This may well be important in the impedance of flow in the malarial liver.

Other factors which would increase frictional resistance are probably also involved. These include increases in blood viscosity, such as occur in the haemoconcentration which occurs in shocked animals following loss of

circulating plasma volume, and from local loss of fluid resulting from vasoconstriction of the small portal vessels (Pappenheimer and Maes, 1942; see above).

Other factors involved here would include the so-called "permeability factors" damaging the endothelial function probably at pre-sinusoidal level, including ferritin, histamine, lactate, kinins and toxic factors induced by the host–parasite reactions.

These are discussed elsewhere. Their importance will certainly vary from organ to organ. Where the vascular endothelium is highly impermeable, as in the brain, they probably exert considerable influence (Migasena, 1967; Maegraith, 1966). Where the membrane is largely permeable, as in the liver (the lymph from which has practically the same constituent structure as plasma), their effects would be minimal.

This applies also to the possible activity of the serum factor, which damages the mitochondria of the liver cells and also those of the endothelial cells and so disturbs their functions. Again, the physiological effects of such functional failure would probably show in the escape of heavy molecules and water, as occurs in inflammation, but this would be apparent only where the endothelial membrane was relatively impermeable, not in the liver.

Migasena (1967) and Migasena and Maegraith (1967) have recently shown that in the last 24–48 h of *P. knowlesi* infection ^{131}I-tagged albumin passes freely into the cerebrospinal fluid and brain substance and equally back into the blood from the CSF via the arachnoid villi. The increased net movement is slightly slower into the blood; thus there is a small increase in CSF protein content. This indicates some dysfunction of both membranes. Increased escape of protein has also been shown by fluorescence techniques and autoradiography to occur across the small vessels in the brain substance in mice in the late stages of *P. berghei* infection, and the use of dyes has confirmed the equivalent passage of water. This escape of protein and water across a previously impermeable membrane is the situation in which inflammatory stasis occurs, arising from local loss of water from the plasma, increased viscosity of the blood, with relevant decrease in flow rate and ultimately aggregation of the erythrocytes and even complete cessation of flow. The result is "stasis" similar to that seen in acute inflammation. This phenomenon is reversible (if not continued to the irreversible stage) by anti-inflammatory agents. In this respect it is interesting to note that the above changes in the blood-brain barrier in malaria can be reversed by cortisone and by chloroquine, which is a powerful anti-inflammatory drug (Migasena and Maegraith, 1967).

This process is unlikely to be important in the progress of lesions in the liver cells associated with sinusoids, at any rate until stagnation develops, but it may well be a factor in the reduction of flow in the constricted small portal vessels, which lowers the pressure gradients at the periphery of the affected lobules.

Support for the view that changes in vascular permeability occur in acute malaria infections has recently been offered by Desowitz and Pavanand (1967), who have demonstrated a permeability increasing factor in serum from monkeys infected with *P. inui* and *P. coatneyi*. The responsible agent was not

characterized but its activity was blocked by the antihistamine drug promethazine.

Other pharmacologically active substances are released in malaria which may decrease the impermeability of endothelial membranes. Amongst these are the peptides, especially the kinins. Tella (1962) and Tella and Maegraith (1966a, b) have examined this problem in *P. knowlesi* malaria. They find that the plasma concentration of bradykininogen fell rapidly to very low levels on the 3rd to 4th day of the severe infection in *M. mulatta* (which terminated fatally by the 8th day). At the same time kinin output in the urine considerably increased. This suggested that bradykinin and presumably other kinins were being released in the plasma in relatively large quantities, and were so able to exert their physiological effects. This release has now been confirmed (Bhabani and Maegraith, 1966).

It should be noted that the possibility of circulating peptides being active pathogenic agents is by no means new. It was first suggested by Menkin (1936), and a polypeptide held to be responsible for some of the phenomena of acute inflammation, including increased capillary permeability, was detected by Chain and Duthie (1939). Maegraith (1948) posited similar agents in *P. falciparum* malaria, and more recently Goodwin *et al.* (1960) have identified pharmacologically active peptides in the blood and urine of animals infected with *Babesia rhodaini.*

Extreme anoxic anoxia also grossly increases the permeability of isolated capillaries to protein and fluid, as Landis (1927) and Florey (1926) showed in the frog and mammalian omentum. Florey described the vessels as appearing as though the fluid content had been filtered off, leaving a "solid mass" of corpuscles within the lumen. The stasis, if not too long persisting, could be resolved by reoxygenation of the tissues.

It is possible that a process of this sort ultimately develops in the stagnant environment of the sinusoids affected by the redistribution of the intralobular circulation referred to above. There is, as yet, no direct evidence of this.

Once a state of tissue hypoxia is created by such factors as we have considered above, metabolic processes will be modified accordingly. Tissue oxygen uptake will be limited by the deficient oxygen supply (Selkurt and Brecker, 1956). Partial anaerobiosis results in the impairment of metabolism of pyruvate into carbon dioxide, with a consequent rise in lactate in both blood and tissue. As the process extends, there may be decrease in the pH, CO_2 and bicarbonate in the local blood, and increase in pyruvate and inorganic phosphate. These phenomena in turn, whether intracellular or extracellular, will lead to other metabolic failures and disturbances in the body economy. There is some evidence that lactate itself may be involved in precipitating shock; it also releases adrenaline from the adrenal medulla (Woods *et al.*, 1956). Lactate and other agents, including histamine, serotonin and the kinins (see above), may effect the permeability of the endothelium and allow the escape of heavy molecules and accompanying water (Fine, 1965a, b; Miles, 1961; Maegraith, 1948, 1966a).

Devakul (1958) recorded a small but progressive rise in plasma volume during *P. knowlesi* infection in *M. mulatta* associated with small increases over

the period of schizogony. This continued until the terminal stages, when there was a sharp fall. Her findings corresponded closely with those of Overman and Feldman (1947), using the original strain of *P. knowlesi*. The pattern was most obvious in lytic cases in which the loss of erythrocytes was very pronounced in the terminal stages. The slight increase in plasma volume was unable to compensate for this, hence the fall in total blood volume. Skirrow (1962) re-examined the changes in blood volume in shocked infected monkeys in terms of circulating erythrocyte mass and reported similar findings. He concluded that the sharp final fall in total blood volume was not the primary cause of the shock in the lytic case, although it may be in the animals which die early in shock. Using a double isotope technique, Miller *et al.* (1967) have observed a fall in total blood volume after the third sporulation in *P. coatneyi* infection in rhesus monkeys.

Gibson *et al.* (1947), using radioactive isotopes and dyes, showed that trapping of blood occurred, as was suspected, in the minute vessels of the tissues in haemorrhagic shock in dogs. Maegraith *et al.* (1959) suggested that the release of such trapping and the return of the blood to the circulation was the explanation of the dramatic recovery from malarial shock which occurred after the administration of noradrenaline to *P. knowlesi* infected monkeys.

Skirrow re-examined this problem using ^{59}Fe- and ^{51}Cr-tagged erythrocytes in dogs in "normovolaemic" shock (Wiggers, 1950). He found that trapping occurred in the spleen, the liver and the lungs. The liver was always affected and exhibited extensive centrilobular necrosis; the trapped erythrocytes could not be returned to the circulation by transfusion or noradrenaline.

The use of tagged erythrocytes for determining whether sequestration of cells was occurring, was not possible in malaria because of the erythrocyte loss from the infection. As expected, tagged cells introduced into the circulation during *P. knowlesi* infection were rapidly diluted. However, mixing time was usually normal or nearly normal. This does not exclude trapping, since even in the presence of extensive sequestration in shock in the dog, mixing times were nearly normal. Noradrenaline produced very little increase in the circulating erythrocyte mass. Thus, if trapping in the liver were present in the infected animals, the drug did not release it.

Changes in blood volume may be significant in alterations of the intrahepatic blood flow in *P. knowlesi* malaria in the period following the penultimate schizogony, when the high parasitaemia is associated with an increase in haematocrit.

The swelling and escape of the actively phagocytosing Kupffer cells may also offer some obstruction to the sinusoidal flow.

The same would apply to deposit of fibrin either in the circulating blood itself or in the spaces of Disse. In the Liverpool experiments with *P. knowlesi* infection no histological evidence of fibrin was obtained, either with Weigert's or Lendrum's modification of picro-Mallory's stain.

A possible source of obstruction to flow might also be "sludging", as described by Knisely *et al.* (1945), in which erythrocytes circulate as small clumps, apparently as a result of fibrin deposits on their surfaces. Sludging can be observed in the peripheral blood very late in *P. knowlesi* malaria, but there is

still considerable doubt as to whether its formation is in itself *post hoc* or *propter hoc* (Maegraith, 1966a).

Stickiness of erythrocytes, both parasitized and unparasitized, and of leucocytes such as is seen in acute inflammation may also be important in certain anatomical regions, but this does not appear to be the case in the liver (Maegraith, 1948, 1966a).

On the whole, the consensus of opinion is that the circulatory obstruction to liver flow is dynamic rather than mechanical, and that the essential elements are those described above, which appear to be mediated by sympathetic hyperactivity.

Anoxaemia and associated anoxic anoxia resulting from erythrocyte loss during haemolysis is not a major factor in either *P. falciparum* malaria or *P. knowlesi* infections, except in extreme blackwater fever in the former and in the terminal stages of a lytic infection in the latter (Devakul and Maegraith, 1958; Maegraith, 1966). The same is true for phagocytosis (Zuckerman, 1964). Other factors concerned with the development of anaemia in malaria, such as bone marrow depression in *P. falciparum* infection (Srichaikul *et al.*, 1967), the unavailability of malaria pigment for re-synthesis of haemoglobin (Deegan and Maegraith, 1958) and the withholding in the bone marrow of young erythrocytes from the peripheral circulation (Thonnard-Neumann, 1944), are also of minor importance.

The failure to demonstrate any consistent or extensive degree of intravascular thrombosis at autopsy in *P. falciparum* and *P. knowlesi* infections remains somewhat of a puzzle (Maegraith, 1948, 1959, 1966).

It is possible to some extent that fibrinolysis may be involved, despite the fact that the fibrinogen content of plasma may be increased during the infection. Devakul *et al.* (1966) have studied the rate of disappearance of ^{125}I-labelled human fibrinogen in blood-induced *P. falciparum* infections in Thailand and have reported that in two patients who were clinically severely ill, with high parasitaemia and raised bilirubin, the radioactive fibrinogen was very rapidly removed from the circulation. They consider that this may well indicate intravascular coagulation; in life this may temporarily exceed fibrinolysis and help aggravate vascular obstruction, the balance becoming reversed after death. As pointed out above, this could be a subsidiary factor in slowing the intrahepatic blood flow.

It is now clear that coagulation defects do occur in malaria. Dennis *et al.* (1966a, b) have recorded in *P. knowlesi* infections prolonged one-stage prothrombin times, abnormal serial thrombin times, decreased fibrinogen concentration and thrombocytopenia. They report similar changes in chloroquine-resistant *P. falciparum* in Vietnam. Similar findings have been reported from Thailand (Parttraporn *et al.*, 1967) and thrombocytopenia has also been recorded by Sodeman and Jeffery on antimalarial therapy. They suggest the strong possibility of accelerated intravascular coagulation in the acute infection. However, hypercoagulability as such has not yet been demonstrated in malaria.

The possibility has been discounted that the degenerative processes involved in the evolution of the liver lesions in malaria might be aggravated by anoxic

anoxia arising from deviation of the oxyhaemoglobin dissociation curve to the left so that oxygen could not be discharged at the partial pressures obtaining at the tissue face.

In all forms of malaria in which this point has been studied, it has been found that the physico-chemical properties of the pigment are normal. In the late stages of infection when acidosis develops, the curve is deviated to the right, allowing for easier escape of oxygen to the tissues (Rigdon, 1945; Jones *et al.*, 1951; Maegraith, 1959).

Malaria pigment is not involved. It is a metabolic modification of the haemoglobin molecule in which the iron is oxidized and the globulin denatured and partly proteolysed (Deegan and Maegraith, 1956; Sherman *et al.*, 1965). It is inert and has no effect on the tissues either when free or after phagocytosis. The electron microscope shows the pigment particles within the cytoplasm of the parasite surrounded by a membrane presumably derived from the original vacuole containing the haemoglobin ingested by phagotrophy (Fletcher and Maegraith, 1962b).

E. HUMORAL FACTORS RESPONSIBLE FOR BIOCHEMICAL LESIONS

The circulatory phenomena referred to may be enough in themselves to provoke cellular damage in the affected lobules, provided they continue long enough.

There is another factor, however, which acts synergistically and enhances the effects of the developing anoxic anoxia. This is cytotoxic anoxia, which inhibits the usage by the cell of the oxygen available to it.

Evidence of such biochemical damage has been found in both *P. knowlesi* and *P. berghei* infections in which the respiration and oxidative phosphorylation of the hepatic parenchymal cells are both inhibited, as can be demonstrated in isolated mitochondria. Equivalent structural changes can be demonstrated in the mitochondria of affected cells. The organelles are swollen and irregular, with an intact but convoluted double membrane, electron transparent substance and disappearance of cristae. Changes also occur in the endoplasmic reticulum. As explained elsewhere, the damaged mitochondria ultimately become vacuolated and disintegrate.

Similar changes in mitochondrial enzyme activity occur when the serum from infected animals (*P. knowlesi* and *P. berghei* infections) is added to ageing normal monkey or mouse mitochondria. Fractionation of the serum has shown that the active agent is a substance with a molecular weight of less than 1000, which has not yet been separated from the lactic acid fraction of the ultrafiltrate (Thurnham, 1967).

This substance apparently acts by disturbing the mitochondrial membrane, leading to the loss of co-factors. The resultant inhibition of respiration and oxidative phosphorylation may be considerably reduced *in vitro* by the addition of the coenzymes (Riley and Maegraith, 1961, 1962; Maegraith, 1966; Thurnham, 1967).

The raised levels of inorganic phosphate which occur in the plasma in *P. knowlesi* infections and in *P. falciparum* infection in man, may also be concerned in the process, since phosphate obtained in the ultrafiltrate shows some inhibitory activity.

In the concentration found in the filtrate lactic acid will also inhibit mitochondrial respiration *in vitro*, but only in the acid form; salts are inert. The major inhibitory factor, on the other hand, is active at neutral pH. It is possible that the factor may work synergistically with the lactic acid.

It is interesting to note at this point that lactic acid, which accumulates in the plasma and in the damaged cells themselves, may be concerned in the genesis of shock, since it can apparently initiate intense sympathetic activity and give rise to the extreme vasoconstriction which accompanies it (Schumer, 1966).

The present Liverpool thesis is that the cellular damage seen in malaria, in this instance in the liver, is explicable partly in terms of cytotoxic anoxia, arising perhaps primarily from the activity of the soluble factor referred to above, with synergistic effects, perhaps in the way of "preparing" the mitochondria, from lactic acid and other substances, including long chain unesterified fatty acids, which have been shown to accumulate both in the blood and in the liver substance.

The ultimate effect is the inability to use oxygen, with resultant accumulation in the cell of hydrogen ions and fall in cell pH; if this situation continues for long enough the cell will die.

The mitochondrial inhibiting factor in the plasma can be detected only in the late stages of an infection. As noted above, the circulatory changes can be demonstrated earlier. It seems likely that the latter may operate in some way similar to *in vitro* ageing of the organelles, which has been shown to increase their sensitivity to the action of the factor.

Thus the anoxic and cytotoxic elements may act synergistically ultimately producing cellular degeneration and death. The curious distribution of the liver lesions in the centrilobular region is probably best explained initially in terms of the redistribution of blood flow, as described above. Once initiated, the processes are accelerated by a summation effect, which can be demonstrated *in vitro* by the intensification of the inhibitory action of the serum factor on ageing, as compared with fresh, mitochondrial suspensions, and by the similar intensification of its effect on the presence of C_{18}-unsaturated fatty acid, such as *cis*-vaccenic acid, in concentrations of the latter which, by themselves, are ineffective.

In areas of the liver where the circulation is not so restricted, as at the periphery of the lobule, the action of the factor alone appears to be inadequate to induce severe cell degeneration or necrosis.

The serum factor may well be a direct link between the parasite living in its erythrocyte environment and the infected host. Its origin has not yet been determined, but its activity is non-specific and is shared by many other substances, including the fatty acids mentioned and to some extent carbon tetrachloride. For the reasons stated, it may be regarded as one of the active agents involved in the liver cell damage.

F. FATTY DEGENERATION

Other factors also seem to be present, since fatty degeneration is seen sometimes in malaria not only in the centrilobular lesions but also in the cells lying in the periphery adjacent to the portal tracts. In the cells in the central zone the fatty changes occur at about the same time or even later than the mitochondrial damage and the fatty droplets may often be seen in intimate contact with organelles in the first stages of degeneration. In the peripheral areas, where fatty changes are by no means always seen, they are independent of mitochondrial changes, which often cannot be detected in the fat-containing cells. Ray and Sharma (1958) have reported that injection of serum from a monkey infected with *P. knowlesi* results in peripheral cellular fat deposits. These can be prevented by choline, which has no effect on the fatty changes in the central degenerating cells. What this agent is in the serum is unknown, but it is clearly not the respiration inhibitor discussed above.

The appearance of fatty changes in the degenerating cells suggests some link with lipid metabolism in general. This possibility has recently been examined in detail in *P. knowlesi* infection in *M. mulatta.* Angus (1967) has studied the lipids in erythrocytes (infected and non-infected), the plasma and the liver in acute *P. knowlesi* infection in *M. mulatta.* In the late stages of this infection there is a post-hepatic hyperlipaemia, a considerable increase in lipids in parasitized erythrocytes and cell-free parasites, and a variable increase in liver lipids.

The latter can be demonstrated histologically, especially in the areas of centrilobular degeneration and necrosis in *P. falciparum*, *P. knowlesi* and *P. berghei* infections (Maegraith, 1948, 1954; Mercado and von Brand, 1957; Skirrow, 1962).

As pointed out elsewhere the centrilobular degenerative changes and to some extent the mitochondrial damage in the cells involved resemble the changes induced by carbon tetrachloride. The processes by which the latter develop have been reviewed by Lombardi (1965). It has been found that the fatty changes precede the mitochondrial and that the lesions in the latter are not directly concerned in the deposition of fat in the necrosed cells. Electron microscopic studies have indicated that the endoplasmic reticulum is concerned in the early stages of the poisoning with carbon tetrachloride, rather than the mitochondria, and that the early changes in the reticulum are about contemporaneous with the visible increase in triglycerides.

Since the latter are transported in the plasma in association with lipoproteins which are believed to be synthesized by the endoplasmic reticulum, it has been suggested that the accumulation of triglycerides in the liver in carbon tetrachloride poisoning may arise from blockage of their release into the plasma, or from failure of protein synthesis. This would explain the fall of plasma lipid, which is a feature of the poisoning.

The process in malaria seems somewhat different, in that accumulation of triglycerides is not always evident in the liver in *P. knowlesi* infections at death, although in the series of monkeys Angus found a mean level greater than normal. In animals with heavy parasitaemia, however, over the last 24 h of the infection high levels were found in the liver (as can be seen histologically).

Again, in the late stages of the infection the plasma content of lipids is high, largely due to triglycerides (as the lipoprotein faction) together with an increase in free fatty acids, and there is also some increase in phospholipids. This is in direct contrast to the findings in carbon tetrachloride poisoning.

Moreover, as pointed out, in malaria (*P. berghei* and *P. knowlesi* infections) the rise in liver lipid occurs simultaneously with or later than the early mitochondrial damage and there is no rise in calcium ion content.

The rise in plasma lipids may be related to failure of acceptance by the tissues. On the other hand, the high plasma content of free fatty acids may change the balance of handling and oxidation in the liver and directly induce triglyceride accumulation.

The demonstrable hyperactivity of the sympathetic nervous system may contribute to the increased concentration of free fatty acid in the plasma (Cardon and Gordon, 1959), particularly in animals which survive for long periods in shock. So may the anorexia which develops (Goodman and Knobil, 1959).

There is a large increase in lipids in parasitized erythrocytes, including free fatty acid, but largely phospholipid, reaching a maximum at schizogony (Ball *et al.*, 1948). Most of this lipid is probably discharged into the plasma at schizogony; an increase in free fatty acid at this stage can sometimes be demonstrated in *P. knowlesi* infection. Although there may be an element of the increased plasma lipid acquired in this way, the evidence indicates that most of it arises from non-specific host response to the infection. It is unlikely that chylomicrons are involved, since there is invariably anorexia and absorption of fat from the intestine is reduced in the late stages of the infection (Migasena *et al.*, 1967).

On balance, it seems probable that the lipaemia is related to increase in serum lipoprotein, which has recently been demonstrated in the late stages of *P. knowlesi* infection in the Liverpool series of monkeys (Angus, 1967; Migasena *et al.*, 1967a).

The total cholesterol plasma level in *P. knowlesi* infection varies considerably but is higher than normal in the late stages, especially in shocked animals (Kehar, 1937; Angus, 1967). In the late stages of the infection there is a marked fall in esterified and a corresponding rise in unesterified cholesterol. This reversal of the ratio of cholesterol to cholesterol esters was also observed by Kay (1951) in human malaria. This reflects on hepatic synthesis, which may be influenced by the striking anorexia which develops in the late stages of the infection, but hepatic dysfunction is indicated, as it is in the changes of circulating cholesterol and raised levels of esters found in viral hepatitis and obstructive jaundice, possibly arising from inability of the liver cells to esterify cholesterol. An additional quota of cholesterol may come from the concomitant erythrocyte destruction as suggested by Kehar (1937) in lytic cases of *P. knowlesi infection*. However, this is probably minimal, since acute induced anaemia *per se* had little effect on the free fatty acid levels. The anaemia which develops in the long-term *P. falciparum* infections in splenectomized gibbons in which there is no histopathological involvement of the liver is not accompanied by changes in fatty acid levels.

The significance of the rise of fatty acid concentration in *P. knowlesi* lies in the fact that these acids inhibit oxidative processes of mitochondria (Pressman and Lardy, 1956). Moreover, certain long chain unsaturated acids are haemolytic (Laser, 1948; Morton and Todd, 1950), and some have pharmacological activities resembling those of kinins (the so-called slow reacting substances; Jaques, 1959; Vogt, 1958). Riley (1961) found an increase of $C_{18:1}$ acids in the liver in *P. knowlesi* and *P. berghei* infection, and Angus *et al.* (1967) have reported increased content of similar acids (oleic and possibly *cis*-vaccenic acids) in the serum and parasitized erythrocytes in *P. knowlesi* infection. Thurnham confirmed that these acids, in the concentration found in plasma, slightly inhibited oxygen uptake by mitochondria and had a synergistic effect when added to small amounts of inhibitory serum or ultrafiltrate, which contains no lipid (Thurnham, 1967). They may therefore be involved in some way in the genesis of the liver damage.

Wallace *et al.* (1967) have examined the lipid content of the liver in mice infected with *P. berghei* and have noted that late in the disease the lipid composition of the liver and of the parasites are similar. They suggest that the observed liver lipids may thus to some extent be influenced by the massed parasites present in the organ. There was no change in lipase activity as compared with uninfected animals. The authors make the interesting point that the changes in free fatty acids were mostly the reverse of those in other lipid fractions, suggesting that the pool of fatty acids derived from depot lipids may be used for biosynthesis by the parasite.

G. IMMUNE AND SENSITIVITY REACTIONS

The possibility that immune and immuno-sensitivity reactions may play some part in the pathogenesis of the tissue lesions in malaria was first suggested by Gear (1946) and Maegraith (1948). This is of particular interest in view of the recent demonstration of their importance in certain circumstances and infections in increasing vascular permeability and causing acute vascular inflammation and anophylaxis (Dixon, 1962; Weigle, 1964; World Health Organization, 1965). The possibility of local immune reaction arising from polygonal cells acting as antigens reacting to homologous cell antibody developed as a result of cellular chemical changes initiated by the infection, must also be considered, since comparable reactions are known to cause lesions in the kidneys (Schwentker and Comploier, 1939). Ward and Conran (1966) have studied the fluorescent antibody responses in splenectomized monkeys infected with *P. cynomolgi*. In the 2nd and 3rd weeks of the infection, they noted that the endothelial surfaces of the hepatic sinusoids were lined with beaded fluorescence, indicating antigen. The Kupffer cells were laden with fine and coarse granular material containing antigen. These deposits and granules of antigen were not associated with γ-globulin or β_1C-globulin (as they were in the renal, capillary loops). The significance of these apparent antigen–antibody complexes is not yet clear. Dixon (1966) has reported deposits of host γ- and β_1C-globulin along the glomerular capillary basement membrane in renal biopsies of Nigerian children with the nephrotic syndrome believed to be

derived from *P. malariae* infection (Gilles and Hendrickse, 1963; Edington and Mainwaring, 1966).

Recent studies of complement component β_1C in these children have indicated that the disease has an immunological basis in which soluble antigen–excess antigen–antibody complex is involved (Soothill and Hendrickse, 1967).

Anophylaxis resulting from antibody–antigen reactions in horse-serum sensitized animals causes intense intrahepatic vasoconstriction of the same type as seen in *P. knowlesi* malaria (Andrews *et al.*, 1949; Skirrow *et al.*, 1964).

In dogs this reaction has been found to occur locally following intrahepatic injection of the antigen (Maegraith *et al.*, 1949). Such local reactions may be important in the establishment of certain hepatic lesions, since Harinasuta and Maegraith (1954) have shown that amoebic liver abscesses following intraportal infection of *Entamoeba histolytica* can be induced in guinea-pigs only if the animals are "prepared" by chronic gut infection or previous injection of amoebic antigen. Maegraith (1954b) has suggested that the injected amoebae act as antigen in the small vessels of the liver, reacting with antibody to fire off a local vascular constriction similar to that seen in the dog after local injection of antigen into the liver substance in a serum sensitized host. He proposed that the environmental changes so elicited would allow the parasite to survive and multiply. Support is given for this view in the centrilobular sinusoidal congestion and dilatation seen not uncommonly at the periphery of the developing amoebic liver abscess (Maegraith, 1954b).

It is possible that somewhat similar mechanisms are involved in the genesis of the liver lesions in malaria.

VI. Comment

Hepatic dysfunction is common in mammalian malaria.

In acute infections characteristic lesions develop in the central region of the hepatic lobule. In this area and sometimes extending to the midzonal region, the polygonal cells may present all stages of degeneration and necrosis. In a given case the extent and degree of the lesion depends on the severity of the infection and the time it has acted.

Very similar centrilobular lesions occur in many other acute infections and medical states including shock. It has been suggested, therefore, that the basic pathogenic processes involved in producing the lesions in malaria may be non-specific.

The pathogenesis of the lesions in malaria has been studied most in *P. knowlesi* infections in rhesus monkeys (*M. mulatta*).

It has been shown that in this infection at least two major pathogenic factors are involved.

(1) Portal venous constriction leads to redistribution of intralobular blood flow whereby some blood may be routed direct through the lobule to the central vein so that other sinusoidal pathways are by-passed, leading to stagnation and anoxic anoxia.

The intrahepatic vascular disturbances are associated with pronounced and continuous hyperactivity of the sympathetic nervous system.

(2) A cytotoxic substance, not yet categorized, but with a molecular weight of less than 1 000, has been demonstrated in the blood in *P. knowlesi* and *P. berghei* infections. This inhibits the respiration and oxidative phosphorylation of the mitochondria of the polygonal cells. Mitochondrial structural damage can be demonstrated in these cells in the late stages of infection.

The experimental evidence for the existence of these two factors is given above in some detail, with emphasis on the most recent work.

It is considered that the circulatory redistribution and the cytotoxic factor act synergistically and are important agents in the production of the centrilobular lesion.

Other factors which may influence the genesis of the hepatic lesions are discussed.

The processes described above are examples in a specific organ of the pathophysiological responses of the host to the parasite infection. They must clearly be related to the whole picture of the development of malaria in the host, much of which is basically non-specific to the plasmodial infection.

To see them in correct perspective it will help to quote from a recent review of the pathogenesis of malaria (Maegraith, 1966a).

> At some stage of its development, the malaria parasite in the erythrocyte must initiate the whole process. The early or initiating mechanisms must influence the metabolizing tissues of the host, and we have evidence that this is executed at any rate in the late stages by diffusable factors arising from somewhere in the parasite-erythrocyte complex.
>
> One of several factors may be involved in this initial stage, acting on various points in the host and influencing the physiological and biochemical balance of the latter.
>
> For instance, endocrine balance may be disturbed; the autonomic nervous system becomes involved; the dynamics of the circulation of blood through the organs is changed, with physiological effects which may involve other organs and the metabolism and physiology of the whole body. Pharmacologically active substances, such as kinins, are at some stage produced and exert their effects.
>
> In this way a physiological chain reaction is set up, with effects that vary in the individual host, leading to local and general disturbances which are at first reversible but, with time, may become irreversible and lead to tissue death, evidenced as pathological changes.
>
> If unchecked, the chain reaction goes on expanding; more and more features become irreversible and eventually general circulatory failure or some other calamity results in death of the host.
>
> This simple concept of initiating factors starting off a chain of pathogenic physiological effects, with inter-related processes involving such things as hormone balance, membrane permeability, local changes in organ circulation with or without general vascular collapse, is useful in framing the broad picture of a developing infection. The essential early reversibility of the phenomena in general and later irreversibility help also to demonstrate the vast importance of time as a factor in such reactions.
>
> This pattern is a good basis for promoting research.

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Experimental Chemotherapy of Schistosomiasis mansoni*

J. PELLEGRINO AND NAFTALE KATZ

Instituto de Biologia, Faculdade de Filosofia, Universidade Federal de Minas Gerais and Instituto Nacional de Endemias Rurias, Centro de Pesquisas René Rachou, Belo Horizonte, Minas Gerais, Brazil

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I. Introduction

Since the employment of tartar emetic (Christopherson, 1918) as the first effective antischistosomal agent, half a century ago, only a limited progress in the chemotherapy of schistosomiasis has been achieved in comparison to that observed for other helminthic diseases. In fact, despite the great effort expended in the search for active compounds, only a few have reached the clinical stage and emerged as antischistosomal agents of proved value. The development of schistosomicides more effective and with wider range of action than those at present available is still a great necessity for meeting the world problem of schistosomiasis (Standen, 1967).

The difficulties and expenses involved in the laboratory maintenance of the life cycle of schistosomes on a scale required for the routine screening of a large number of compounds is probably the chief reason why the chemotherapy of experimental schistosomiasis has been limited to relatively few workers. The first systematic laboratory experiments on the chemotherapy of schistosomiasis were undertaken by Kikuth and Gönnert (1948) at the Bayer Laboratories, Elberfeld, Germany, beginning in 1932. In August 1938, after screening

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about 4000 substances, including those synthesized during the mid-to-late 1930's by Mauss (1948), a new active antischistosomal agent was found and subsequently named Miracil A. Further developments on the "miracil" series of compounds led to the discovery of Miracil D, the first metal-free compound which proved to be of therapeutic value in human schistosomiasis (Kikuth *et al.*, 1946; Kikuth and Gönnert, 1948, 1949). However, since the pioneer work carried out by Oesterlin (1934) and Bayer investigators, a progressive interest of many pharmaceutical industries towards chemotherapy of schistosomiasis has been observed. According to Standen (1967), it may be that as many as 250000 chemical substances have now been tested against schistosome infections in laboratory animals.

For many practical and economical reasons, the major portion of experimental work with schistosomes has been carried out with *Schistosoma mansoni*. Although the life cycle of *S. haematobium* has been successfully maintained under laboratory conditions (Capron *et al.*, 1965), this species is not very suitable for experimental chemotherapy (difficulty of infecting the snail vectors; loss of the predilection of this species for the urogenital tract in small laboratory animals, etc.). *S. japonicum* life cycle is difficult to perpetuate in the laboratory and this species is known to be the most resistant to treatment with all available antischistosomal agents. Therefore, *S. mansoni* is the fluke of choice for experimental work. On the other hand, should any promising drug be found active against *S. mansoni*, it would subsequently be tested in animals or in patients infected either with *S. haematobium* or with *S. japonicum*.

In connection with the search for an ideal antischistosomal agent, the speculations of Fairley (1951), Newsome (1962b) and Friedheim (1967) deserve a mention. A schistosomicide does not merely have to be active against the worm and inactive against the host; it needs more qualities. First of all, any new treatment will have to have practical advantages over antimonials and lucanthone. The great drawbacks to the chemotherapy of schistosomiasis have been the side effects and insufficient activity. The first properties of the perfect drug are therefore: (1) absence of side effects and toxicity in man, and (2) very high activity against the three main human schistosomes. From a practical point of view, long courses are useless because they are so often not completed. Another difficulty is that injections are convenient or preferred in one area and tablets in another. The next three properties are that the drug should be: (3) efficient when given in a course of four days or less; (4) equally effective by injection or by mouth; (5) inexpensive to market. Antimonials and lucanthone are known to have no effect on the early stages of schistosome infection before the worms have matured and begun to lay eggs. If the ideal drug killed immature schistosomes it would be valuable for treatment of distressing symptoms sometimes seen in the first 6–12 weeks and for mass treatment and prophylaxis during eradication or control schemes. The next two properties, therefore, are that it should be: (6) active against all stages of the schistosome in the mammalian host, and (7) prophylactic, curative and safe when given in single oral doses at intervals of one week or more. A final and very important prerequisite would be (8) chemical stability in solution or in tablets under all storage conditions. However, as has been pointed out by Newsome (1962b),

this ideal drug is only a dream, but it is an aim and a yardstick for measuring the small gains that have been made and are yet to come. A less ambitious and probably a wiser position is to admit that the problem of chemotherapy of schistosomiasis demands three types of drugs (Friedheim and De Jongh, 1959; Bruce and Sadun, 1963; Pellegrino, 1967; Standen, 1967): (a) a *prophylactic* drug which will prevent infection; (b) a *suppressant* which may not necessarily impose irreversible harm upon the schistosomes but will prevent egg laying and thus interrupt transmission; (c) a *curative* drug which will destroy most, or all, of the adult worms. Actually, as far as the experimental *S. mansoni* infection in the mouse is concerned, there are many unrelated chemical substances displaying variable degrees of curative properties (Standen, 1963; Pellegrino and Faria, 1965); and at least one potent suppressant (Campbell and Cuckler, 1967) and one highly prophylactic agent (Mors *et al.*, 1967) are known. These different antischistosomal properties are not necessarily shared by the same compound.

Since the development of the oogram method as an experimental screening model (Pellegrino *et al.*, 1962), we have been engaged in an extensive program on chemotherapy of schistosomiasis mansoni. In the present review the chief contributions within the last two decades concerning the maintenance of the life cycle of *S. mansoni* in the laboratory, the different approaches for the assessment of antischistosomal activity, and the experimental development of some newly emerged drugs will be outlined and discussed, with emphasis on the routine methods employed in our laboratories for screening and preclinical trials in schistosomiasis.

II. General Considerations

The development of new drugs for the chemotherapy of schistosomiasis has followed three major lines: the empirical, the selective, and the biochemical.

The empirical approach is a blind screening where large numbers of compounds are tested with a view to finding a chemical lead. This is the most usual and rewarding method in large-scale experimental chemotherapy and will remain so until sufficient knowledge of schistosome biochemistry and physiology can be amassed and thereby provide the basis for a more rational approach. Therefore, until this relatively neglected field receives serious attention, the highly speculative process of looking for the needle in the haystack will continue (Standen, 1963).

Empirical screening of compounds *in vitro* is greatly handicapped by the fact that biochemical degradation in vertebrate hosts—necessary to form active metabolites—is known to occur with several groups of antischistosomal agents (miracils, *p*-aminodiphenoxy-alkanes, etc.). Furthermore, there is not a good correlation between *in vitro* and *in vivo* tests. For instance, diaminodiphenoxyalkanes, highly active against *S. mansoni in vivo* (Raison and Standen, 1955; Hill, 1956) are of low activity against the same species *in vitro*, whereas alkylenebisbenzylamines, of high activity *in vitro* (Bueding and Penedo, 1957), are known to be of little interest *in vivo* (Standen, 1963). The

same lack of correlation is known to occur with glucosamine and naphthoquinones (Bueding *et al.*, 1947, 1954; Brener, 1960c; Pellegrino *et al.*, 1962; Standen, 1963). Possibly, a more rational approach aiming to minimize the drawbacks of the *in vitro* screening might be the exposure of drugs to liver microsomes (Rosi *et al.*, 1967) prior to drug testing.

For routine *in vivo* screening, the working model comprising the white mouse as the host and *S. mansoni* as the infecting agent is almost invariably employed. However, Okpala (1959) and Berberian and Freele (1964) prefer to use the hamster as a primary host. Both suppressive and curative effects can be used as basic criteria for the search of new chemical leads. Although the former seem to be more reliable and sensitive (Pellegrino *et al.*, 1962; Pellegrino and Faria, 1965), the latter approach to drug development is generally preferred (Standen, 1967). It must be remarked, however, that (a) all curative agents definitively suppress egg laying, (b) the process of oviposition is probably the most sensitive target for demonstrating drug action and (c) suppressants may not necessarily act as schistosomicidal agents (for instance, nicarbazin). On the other hand, should any drug be found that interferes with egg laying (i.e. induces oogram changes), it would certainly come to laboratory evaluation as a curative agent. It is agreed that the search of prophylactic compounds demands a different screening approach (Pellegrino, 1967; Standen, 1967).

The selective approach is the biological investigation of compounds chemically related to those already known to display some antischistosomal activity. The principal aim is to increase activity and/or decrease toxicity through chemical modifications of the parent compound. Bioassays can be conducted by *in vivo*, *in vitro* and/or *in vivo–in vitro* tests. Furthermore, a proper knowledge of the factors governing the structure–activity relationships plays an important role in the experimental development of new antischistosomal agents. In this connection, a remarkable achievement has been accomplished recently by the Sterling–Winthrop's group (Rensselaer, New York). It was found that a microbial transformation product of lucanthone, the 4-hydroxymethyl derivative, is indeed the therapeutically active metabolite of lucanthone (Rosi *et al.*, 1965). Starting from this point it was further demonstrated that the antischistosomal activity of related thiochromones and xanthen-9-ones is greatly enhanced when the 4-methyl group is replaced by the 4-hydroxymethyl group. Therefore, for this class of compounds, the metabolic hydroxylation of the 4-methyl group represents a necessary step before the drugs acquire antischistosomal activity (Rosi *et al.*, 1967). As a practical consequence of this finding, the activity of the newly emerged Abbott's piperazine derivative (A-16612) could be greatly enhanced by hydroxylation of the 4-methyl group (Rosi *et al.*, 1967).

A thorough perusal of the literature concerned with experimental chemotherapy of schistosomiasis reveals that an enormous volume of research has been undertaken in many pharmaceutical industries on the selective approach, exploring the leads offered by the empirical screening (Kikuth and Gönnert, 1949; Raison and Standen, 1955; Caldwell and Standen, 1956; Hill, 1956; Standen and Walls, 1956; Gorvin *et al.*, 1957; Ashley *et al.*, 1958; Collins *et al.*,

1958; Lämmler, 1958; Elslager *et al.*, 1961; Gönnert, 1961; Thompson *et al.*, 1962; Strufe, 1963; Stohler and Frey, 1964b; Wilhelm and Schmidt, 1966; Korolkovas, 1967, etc.). This work resulted in the development of at least two acceptable schistosomicides for clinical use, namely, niridazole and hycanthone (Pellegrino and Katz, 1968a). However, as was pointed out by Standen (1967), it is clear that investment by the pharmaceutical industry in research into schistosomiasis has been large, yet the return has been small.

During the past two decades, basic studies on the biochemistry and physiology of schistosomes have been carried out chiefly by Bueding and co-workers (Bueding *et al.*, 1947, 1953, 1967; Bueding, 1949, 1950, 1952, 1959; Bueding and Koletsky, 1950; Bueding and Charms, 1951; Bueding and Peters, 1951; Mansour and Bueding, 1953, 1954; Mansour *et al.*, 1954; Bueding and MacKinnon, 1955a, b; Bueding and Mansour, 1957; Timms and Bueding, 1959; Barker *et al.*, 1966). These studies have contributed much to elucidate the mode of drug action of already known antischistosomal agents, but up to now not a single compound has emerged as an effective agent of practical value through the biochemical approach. Some aspects of the mechanism of antischistosomal activity will be considered later in connection with the experimental development of novel schistosomicides.

There has been a need for more fundamental knowledge concerning the exact metabolic pathways used by adult schistosomes. Growing out of these studies has been the hope that such knowledge would lead to more rational and probably more effective therapy (Senft, 1965). In assessing the amino acid and protein metabolism of *S. mansoni*, *in vitro*, and using a chemically defined medium (Senft, 1963), the following working hypotheses were advanced by Senft (1965):

1. The schistosomes depend heavily on carbohydrates both as a source of energy and as a pool from which to manufacture amino acids, particularly alanine.
2. Of the physiological amino acids, histidine, tryptophan, and arginine are most readily utilized under experimental conditions. Aspartic acid is possibly used. Since urea and ornithine are produced *in vitro* and only when arginine is present in a defined medium, it is likely that an arginase enzyme system is an important part of the schistosome nutritional economy.
3. The production of proline may indicate a proline–urea cycle.
4. Schistosomes can digest globin by releasing both free amino acids and peptides. The peptides appear to be fairly large; they have a positive charge, partially diffuse through a cellophane dialysate bag, and upon hydrolysis yield a variety of amino acids. Arginine, histidine and tryptophan are present in the peptides.

Although schistosomes live in an environment at considerable oxygen tension, their existence apparently does not depend on respiratory metabolism, and they can be cultured for a period of some days anaerobically, during which time carbohydrate is utilized at a considerable rate. The chief product is lactic acid, formed by the Embden–Meyerhof–Parnas' path of phosphorylating glycolysis (Bueding, 1959). Under aerobic or anaerobic conditions, glucose is

rapidly metabolized and as much as 15–26% of the weight of parasites disappears from the culture medium per hour but is not converted to glycogen. Some of the sugar is oxidized to CO_2 and water. After a period of anaerobiosis the rate of oxygen consumption does not increase under aerobic conditions, as usually happens with other helminths. Cyanine dyes are strongly absorbed by *S. mansoni* worms and depress their respiratory but not their glycolytic activity. This fact shows that glycolytic processes in schistosomes are of more importance than the oxydative ones (Bueding, 1950).

It was shown that some enzymes of schistosomes are not identical with those of mammalian tissues although these enzymes catalize the same reactions in the parasite and in the host (Mansour and Bueding, 1953; Mansour *et al.*, 1954; Bueding and MacKinnon, 1955a, b; Bueding, 1959). Species differences in the nature of enzymes catalyzing the same reaction might be of interest for the development of chemotherapeutic agents because they may afford opportunities to select specific inhibitors against the enzyme of the parasite without affecting that of the host (Mansour *et al.*, 1954). Although some of the metabolic pathways and enzymatic systems of schistosomes are already known, there is still a need of more fundamental knowledge before the biochemical approach could effectively aid in designing new antischistosomal agents.

III. Culture of Snail Vectors

The existence of strains of parasite and intermediate host species, differing in infectivity and susceptibility, has been demonstrated for several parasitic diseases. The studies of Vogel (1942), Stunkard (1946), Cram *et al.* (1947), Cowper (1947), Files and Cram (1949), Abdel-Malek (1950), Files (1951), Newton (1953, 1955), Paraense and Corrêa (1963), Kagan and Geiger (1965) and Saoud (1965a, b) have clearly shown that a snail serving as an intermediate host for a schistosome in one geographical area may be poorly susceptible or even refractory to infection with the same parasite from a different area. Of particular interest, in this connection, is the report of Files (1951) that whereas *B. glabrata* from Puerto Rico is susceptible to a local *S. mansoni* strain, a Salvador (Bahia) strain was shown to be completely refractory. A great majority of 23 populations of *B. glabrata* (including snails from Puerto Rico, St. Kitts Islands, Venezuela and Brazil) were found by Paraense and Corrêa (1963) to be highly susceptible to a Belo Horizonte strain of *S. mansoni*, showing infection rates above 50%. Six populations, however, proved less susceptible or even highly resistant to infection. The degree of populational susceptibility of *B. glabrata* must be considered as an aspect of genetic intraspecific variation. According to Kagan and Geiger (1965), schistosomes can become adapted to different strains of snail hosts and they consider that the genetic constitution of the miracidium may be an important factor in determining its invasiveness. Therefore, for establishing the life cycle of schistosomes in the laboratory it is wiser that both infected definitive hosts and the snail vector should be brought together from the same endemic area.

Biomphalaria glabrata is the most suitable snail for mass culture. It is known that this snail, maintained in aquaria, has a distinct preference for certain sites

for egg-laying and that egg output is lowered in the absence of favourable conditions. When sprigs of *Ludwigia palustris* are floated on the water surface, a burst of egg-laying usually occurs within 24 h (Standen, 1951a). *B. glabrata* also readily deposit their eggs on polyethylene plastic sheeting floated on the water and usually prefer it to glass or vegetation (Olivier *et al.*, 1962). In our laboratories, egg clutches are obtained by introducing 3–6 half-grown but sexually mature *B. glabrata* into polyethylene bags (Fig. 1), containing about

FIG. 1. Egg clutches of *Biomphalaria glabrata* deposited on the sides of plastic bags.

500 ml of dechlorinated water, and maintained in a well-illuminated room at 25–26°C (Pellegrino and Gonçalves, 1965). Water is changed every week and fresh lettuce is used as food for the adult snails. Egg clutches are deposited on the sides of the bag. They can be easily handled by everting the sac after removing the snails and draining the water. Adult snails are removed from the bags when eggs are close to hatching. Newly hatched snails feed on the algal film that usually grows on the plastic. A fine powdered food consisting of a mixture of Cerophyl[R], conventional pellet diet for guinea pig, wheat germ, and Pablum[R] (4:2:2:1) is also dusted on the water surface. It is known that the critical stage in development occurs during the first 2 weeks or so after the young snails have emerged from the eggs (Standen, 1951a). With our technique, the mortality among young snails is negligible. After 2 weeks the snails are transferred to plastic containers filled with dechlorinated water and artificially aerated, where they are kept until they reach the size of about 0·5 cm. Figure 2 shows a general view of the "nursery" room, which is maintained at 25–27°C.

Half-grown snails are finally transferred to biologically-balanced glass aquaria (Fig. 3). The bottom of each aquarium is covered with a layer of sand and sterilized earth which supports a variety of aquatic weeds (*Elodea*, *Valisneria*, *Cabomba*, etc.). The water is pumped from a well to a cement–asbestos

reservoir from which it is distributed to the aquaria at the rate of 10 liters/day/aquarium. The excess of water is automatically drained through a syphon. Artificial aeration and illumination are provided for 8 h a day. Each aquarium contains 30 liters of water which is enough to keep about 200 snails under

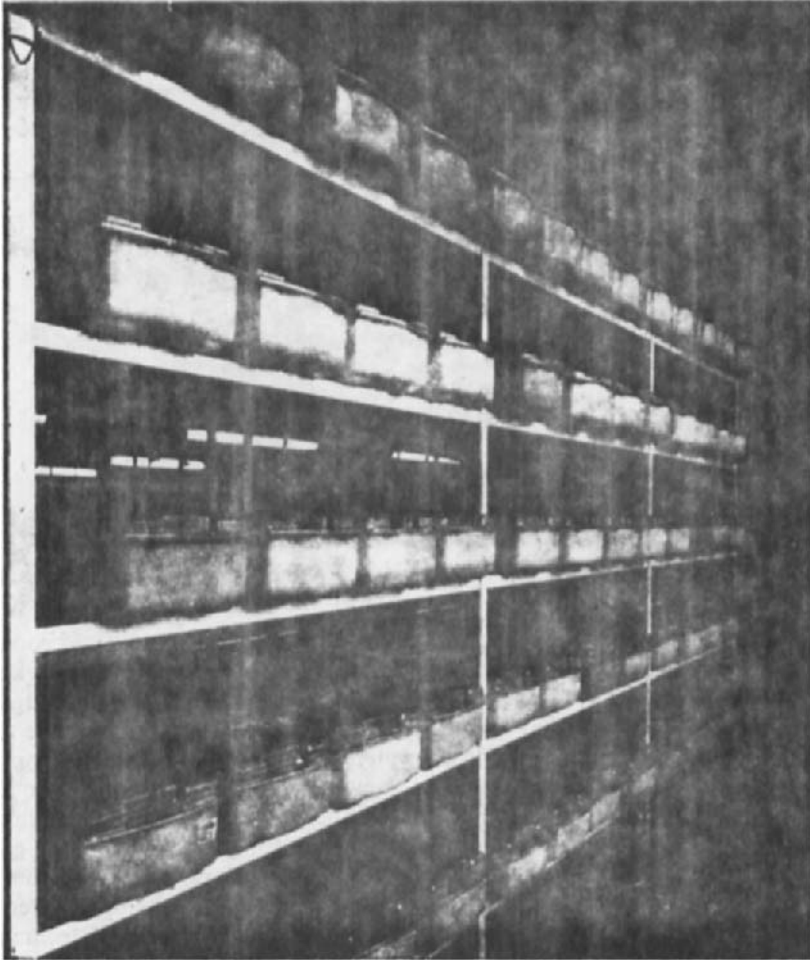

FIG. 2. Young snails are reared in plastic aquaria with artificial aeration until they reach the size of about 0·5 cm in diameter. General view of the "nursery" room.

favourable environmental conditions. The snail room is maintained at 25–27°C. Fresh lettuce and the diet recommended by Etges and Ritchie (1966) are used for feeding the snails. With this procedure, maintenance is minimal and the aquaria can be kept for several months without special care. Microflora and fauna, when in excess, are controlled by *Daphnia* (Berberian and Freele,

1964), while snail feces and decaying vegetable or food materials are removed by oligochaete worms such as *Tubifex* (Standen, 1963).

Breeding experiments conducted by Ritchie *et al.* (1963a) under various conditions have demonstrated that the physical environment has a marked effect on the growth-rate and maturation time of *B. glabrata.* Small numbers of snails maintained on an adequate diet in gently flowing water began egg-laying 21 days after hatching and shell diameters of 20 mm were attained in 2 months. These results are believed to approach the maximum biological potential of *B. glabrata.* Actually, when 8–9 days are allowed for incubation of the eggs, the egg-to-egg cycle could be completed in one month (Ritchie *et al.*, 1963a).

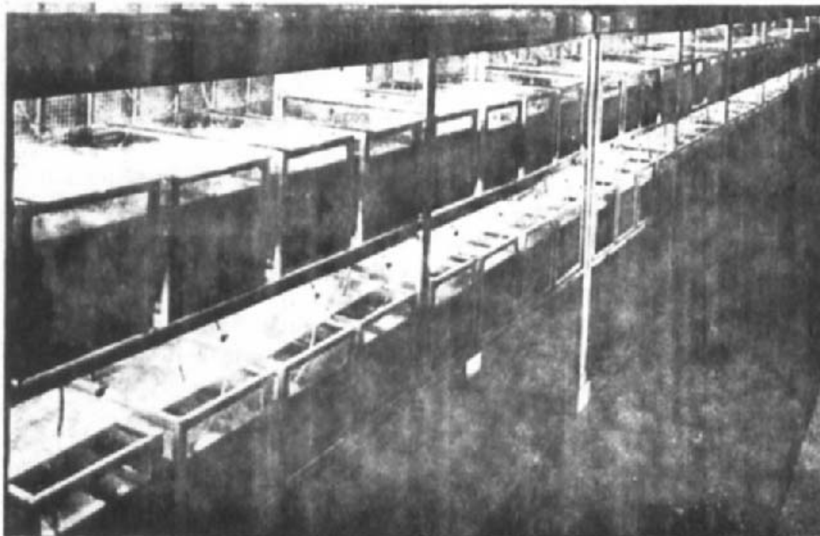

FIG. 3. Snail room showing the glass aquaria used for rearing juvenile and adult *Biomphalaria glabrata.* The room is kept at 25–27°C. The biologically balanced aquaria are artificially aerated. Natural water, pumped from a well, is allowed to flow through the aquaria.

Optimal physical conditions for the culture of *B. glabrata* are pH-range from 7·2 to 7·8, high oxygen tension, absence of chlorine, copper, and zinc from the water, calcium carbonate concentration of approximately 18 ppm., sodium–calcium ratio of 1·0, and temperature of 23–28°C (Cowper, 1946; Michelson, 1961; Frank, 1963; Standen, 1963). In studies of the crowding effect on populations of *B. glabrata* it was found that in more dense populations there was a reduced rate of growth of the individuals as well as a reduction in fecundity (Chernin and Michelson, 1957a, b). When *B. glabrata* snails were reared singly from hatching in water–glass aquaria the life span was observed to be about 18 months and the reproductive span was 15–16 months. When 2 or 5 snails were cultured in battery jars with 4 liters of continuously changing water, the life and reproductive spans were, respectively, 6 and 5 months for

2 snails per jar and 8 and 7 months for 5 snails per jar. The reproductive potential of *B. glabrata* was estimated to be 14000 eggs (Ritchie *et al.*, 1966a).

A technique has been described by Rowan (1958) for mass production of *B. glabrata* for experimental work when space for aquarium tanks is limited. Six tanks are used, each of 45 liters capacity. One tank contains a number of adult snails which serve as egg-producers for the colony, the others are fitted with a hammock of fine-mesh nylon net which stands in the water to a depth of about 10 cm. Egg-masses from the stock tank are placed in the hammock and the young snails are thus confined in the upper layer of water. The water in the tank is changed every 11–12 days and the young snails are provided with food in the hammock. As many as 800 snails can be raised to about 5 weeks old in each unit. Two types of food are provided to both young and mature snails. Small pieces of tender head or leaf lettuce are fed in excess so that several pieces are always present in each hammock. In addition, a dry mixture of food (Purina dog chow, 50 parts; dry silt, 50 parts; calcium carbonate, 1 part) is sprinkled on the surface of the water once or twice each day. A system for mass-producing the snail *B. glabrata* and cercariae of *S. mansoni* has been described by Sandt *et al.* (1965).

Although lettuce is generally used in most laboratories for breeding schistosome-bearing snails, other types of food and formulations have been proposed. Calcium alginate preparations introduced by Standen (1951a), and modified by Moore *et al.* (1953) and by Sandground and Moore (1955), present some advantages such as uniformity and easy storage under frozen state (Lee and Lewert, 1956). The use of a diet consisting of a mixture of Cerophyl[R], wheat germ cereal, Glandex fish food, and powdered milk in a ratio of 4:2:2:1, respectively, was favored by Moore *et al.* (1953) and by many workers since (Etges and Ritchie, 1966).

IV. Infection of Snails

In the last section the importance of the schistosome-snail relationship was discussed in connection with the maintenance of the life cycle of *S. mansoni* in the laboratory.

For the infection of *B. glabrata*, miracidia can be easily obtained from eggs excreted with the feces or retained in tissues (liver and intestine preferably) of mice and hamsters experimentally infected with *S. mansoni*. Digestion methods have been proposed for the isolation of schistosome eggs from tissues of infected animals (Bénex, 1960; Smithers, 1960; Browne and Thomas, 1963). According to Smithers (1960), trypsin digestion by itself, although removing most of the unwanted tissue, fails to remove collagen fibers, which cause the eggs and traces of cell debris to clump together. Further treatment with pepsin digests the collagen, leaving the eggs free of foreign material. A final washing by centrifugation can be regulated so that most of the dead eggs are removed in the supernatant. At least 100000 eggs have been obtained after the digestion of liver and gut from 5 infected hamsters, about one-third of them being mature. Digestion does not harm the viability of miracidia.

A simple procedure has been developed by Ritchie and Berrios-Duran (1961) for recovery of *S. mansoni* eggs from livers and intestines of mice and hamsters sacrificed after 7 weeks of exposing the animals to 200 and 500 cercariae, respectively. The primary feature of this technique is linked with the fact that a sharp layering can be obtained between tissue homogenates in 1·7% saline and a 2·0% saline solution. Layering is accomplished by introducing a clear 2·0% saline through an aerating stone at the bottom of a side-arm Erlenmeyer flask containing the homogenate. As the latter is lifted the eggs fall through the interface into the clear solution. The flow (70–100 ml/min) is continued until the homogenate is all discarded through the side-arm overflow. In Standen's technique (1949, 1953a) infected hamsters are killed, the gut is removed and its contents and superficial layers scraped into a mortar, the liver is added, and the whole is ground gently to a smooth paste with 0·9% saline and a little pure fine sand. The paste is then suspended in cold water or saline and allowed to sediment in the dark before decanting the supernatant, leaving the eggs and heavier particles behind. Re-suspension and decanting are repeated until a clear supernatant is obtained. The final sediment is suspended in fresh water at 28–30°C and when placed under bright light the eggs commence to hatch within ½–1 h.

Studies on the factors governing miracidial hatching have demonstrated that the osmotic pressure of the saline, low temperature, and darkness represent inhibitory conditions whereas fresh water, higher temperature, and illumination stimulate the hatching process (Maldonado and Acosta Matienzo, 1948; Maldonado *et al.*, 1950a, b; Standen, 1951b). Several factors determine the longevity of the free-living miracidium. Some of them are directly related to the miracidium itself, such as the time elapsed from egg deposition to its arrival in water. External factors are primarily related to water qualities (pH, oxygen tension, osmotic pressure and temperature). According to Maldonado and Acosta Matienzo (1948), 91% of the miracidia remain active 1 h after hatching but only 25% after 8 h. The average life span is from 5 to 6 h.

Several techniques for the concentration of miracidia, making use of their positive phototropism and negative geotropism, have been developed (Stunkard, 1946; Ingalls *et al.*, 1949; Chaia, 1956).

Two types of method are used for the infection of *B. glabrata*: individual and mass exposure. In the first one, snails of approximately 1·0 cm diameter (10–14 weeks old) are exposed to about 10 miracidia each in small glass containers with the minimum volume of water required for snail movement (Cram, 1947; Standen, 1952). Successful infection rates are dependent upon the number of miracidia per snail (Schreiber and Schubert, 1949) and the temperature of the water (De Witt, 1955).

For mass infection, batches of 300 *B. glabrata* in 30-liter glass aquaria are exposed to about 5000 miracidia and maintained at an average temperature of 27°C. In this connection, it is important to remark that newly emerged miracidia can swim at 690–750 cm/h and can locate and infect snails situated 86 cm horizontally or 33 cm downwards and probably at greater distances (Chernin and Dunavan, 1962).

The relative merits of individual and mass exposure of *B. glabrata* to miracidia of *S. mansoni* were evaluated by Moore (1964). A larger number of individually exposed snails survived the incubation period, but this was more than offset by the fact that the proportion of infected snails from mass exposure was double that from individual handling, and the development time was significantly less. Mass exposure methods require a minimum of space, labor and time for the exposure of large numbers of snails and must be preferred (De Carneri, 1957; Ritchie *et al.*, 1963b; Standen, 1963; Moore, 1964).

B. glabrata snails may be simultaneously parasitized by numerous miracidia. However, during the first days of infection, many will fail to develop, so that the number of mother sporocysts in multiple infections is smaller than the number of miracidia that actually penetrate (Maldonado and Acosta Matienzo, 1947). Extensive migration of large numbers of cercariae, and the intense tissue reactions associated with trapped degenerating cercariae, are important factors in causing death of the snails (Pan, 1965). High mortality of infected snails appears 6–7 weeks after infection and coincides with heavy emergence of cercariae. In order to lower the mortality rate, Ritchie *et al.* (1963b) recommend keeping positive snails at a density of 10–20/6 liters of water contained in refrigerator pans at depths of about 7 cm. The water must be changed once or twice a week and the snails kept without aeration. The results obtained showed an overall survival rate of 48% after 18 weeks from the time of exposure to miracidia. According to Barbosa *et al.* (1954) infected *B. glabrata* live an average of about 40 days but some specimens can live more than 5 months liberating cercariae every day. Infected snails shed an average of 4598 cercariae daily during the whole period the infection lasted. Some specimens that maintained the infection for more than 4 months liberated a total of more than half a million cercariae.

The temperature of the aquaria where infected snails are maintained during the prepatent period should not fall below 25–26°C (Standen, 1952; Stirewalt, 1954). Many snails can lose their infection within several months after exposure when they are kept at lower temperatures.

The prepatent period is lengthened as the snail maintenance temperatures are lowered. According to Stirewalt (1954), the emergence of cercariae by *B. glabrata* begins at 18 days (31 to 33°C), 22–23 days (26–28°C) and 35–56 days (23–25°C).

For infecting laboratory animals, cercariae are obtained by exposing positive snails (30–50) to artificial light for about 2 h. The use of a great number of snails is important to balance the sexes of schistosomes in the experimental vertebrate host.

V. Infection of Laboratory Animals

An ideal vertebrate host to be used in experimental chemotherapy of schistosomiasis would have to satisfy the following prerequisites: (a) high susceptibility to schistosome infection—with a normal morphological and physiological development of the worms—and a regular excretion of viable eggs with the feces; (b) availability in large numbers and a low cost maintenance

in the laboratory; (c) facility of handling and close correlation between toxicity and therapeutic activity of drugs in that host and man. Since, amongst known susceptible animals to schistosome infection, there is not a single host which is able to satisfy all these requirements, it is wiser, from a practical point of view, to employ a suitable host for a primary screening and several hosts for pre-clinical trials.

Mouse—The white mouse is considered, by the great majority of research workers, the animal of choice for chemotherapeutic studies in schistosomiasis mansoni. Infection of mice with *S. mansoni* cercariae is easily achieved by intraperitoneal, subcutaneous, and percutaneous routes.

Moore and Meleney (1955) demonstrated that when cercariae of *S. mansoni* are injected into the peritoneal cavity of mice, over 50% of the total worms recovered can be found there for at least 168 days. The worms remaining in the peritoneal cavity continue their growth and development, without benefit of blood, and at least some of the female worms are able to complete their sexual maturation and produce fertile eggs in which miracidia develop. For routine chemotherapeutic work, the intraperitoneal route has been favored by Schubert (1948a), De Carneri (1958), and Berberian and Freele (1964). It must be remarked, however, that the final location of part of schistosomes in the peritoneal cavity may handicap the assessment of antischistosomal drug activity. At Hoffmann-La Roche and Ciba Laboratories the infection of mice with 80–100 *S. mansoni* cercariae by the subcutaneous route is employed (Stohler and Frey, 1963, 1964a; Lambert and Stauffer, 1964).

Infection by the percutaneous route has been preferred by most workers. Two methods are generally used: (a) the wading method of Standen (1949), and (b) the tail-immersion method. The wading method is described by Standen as follows: Mice 3–4 weeks old are allowed to run in 1·25 cm of warm water to stimulate voidance of feces and urine and they are then placed in suitable-sized wide-mouthed glass jars provided with a bung and ventilating shaft. The mouse is able to move but not lie down. The cercarial suspension is introduced through the vent by a spring-loaded syringe, allowing 130–150 cercariae per mouse. The jars are placed in the warmth for 20 min and the mice are then removed to dry off in warm wood-wool. The drying-off process is necessary to avoid death from exposure to water.

The tail-immersion method is described by Olivier and Stirewalt (1952), Luttermoser (1954), Stirewalt and Bronson (1955), Berrios Duran (1955), and Lee and Lewert (1956). In our laboratories, mice of both sexes, weighing about 20 g, are anesthetized by an intraperitoneal injection of Somnifène Roche (0·1 ml of a 10% solution in sterile saline). This dosage immobilizes the mice for 3–4 h, which allows the infection of over 400 animals in one morning by a skilled technician. A group of about 50 laboratory-infected *B. glabrata* is placed in a 250-ml beaker with 200 ml of dechlorinated water and left under an electric lamp for 2 h. After removing the snails the beaker is gently shaken and three 1-ml samples are withdrawn and poured onto 3 × 2 inch microslides fitted with paraffin rings. To each well one drop of staining solution (acetic acid, 2 ml; formalin, 5 ml; methylene blue, 100 mg; tap water q.s. to 100 ml)

is added. The average number of cercariae per ml is then determined with the aid of a dissecting microscope. The cercariae must be pooled from the largest possible number of infected snails in order to balance the sexes. The volume of the suspension containing 100–200 cercariae is transferred to 12 × 75 mm test tubes by means of an automatic pipette. The delivery opening of the pipette must have an aperture of at least 2 mm diameter to avoid damage to the cercariae. Dechlorinated water is added to fill the tubes within 1 cm of the top and they are placed in a row of 11 in a special metal rack. As can be seen in

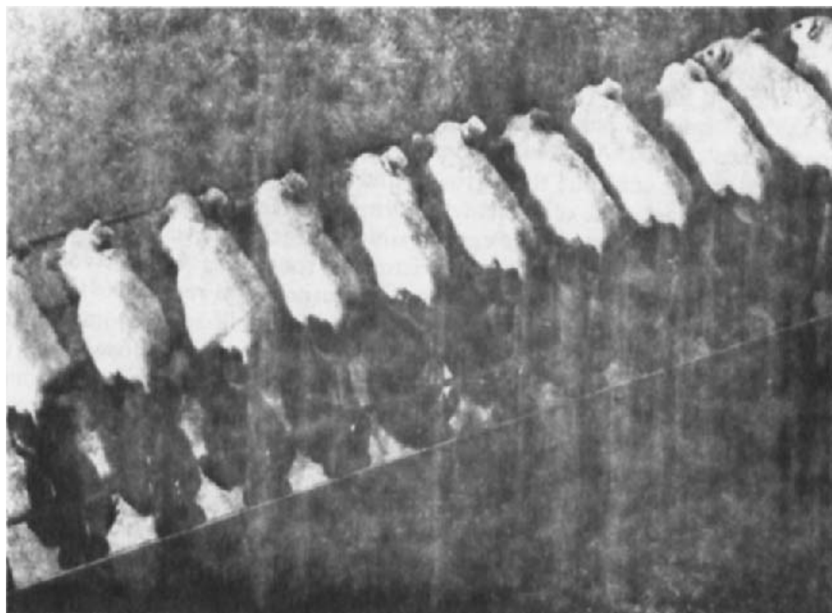

FIG. 4. Infection of mice by the tail-immersion method.

Fig. 4, the animals are infected by immersing their tails into the tubes containing cercariae. The exposure period is 45 min, after which the mice are returned to their cages. The mean percentage development of cercariae to adult schistosomes is about 20% and practically all mice become infected. In the observations of De Witt (1965), the percentage of cercariae that successfully penetrated and matured in mice was found to be strongly influenced by environmental temperature. The optimal temperature for penetration was between 30 and 40°C.

Hamster—The percentage of worm recovery from hamsters exposed to *S. mansoni* cercariae depends on the route of infection. When cercariae are injected intraperitoneally, worm return is less than 20% (Cram and Figgat, 1947; Moore *et al.*, 1949; Yolles *et al.*, 1949; Stirewalt *et al.*, 1951; Berberian and Freele, 1964). In the hamster, the majority of the worms migrate from the peritoneal cavity by 14 days, and none are found in this location after 28 days,

in contrast to what happens in mice (Moore and Meleney, 1955). Exposure of the shaved skin of hamsters to cercariae affords a regular rate of infection and a better yield (30–40 %) of adult schistosomes (Cram and Figgat, 1947; Moore *et al.*, 1949; Faria and Pellegrino, 1963).

In our laboratories, adult *Cricetus auratus* are routinely infected via the cheek pouch (Pellegrino *et al.*, 1965a). *S. mansoni* cercariae are pooled, counted, and the final suspension adjusted to contain 60–80 organisms per ml. The suspension is gently stirred and aliquots of 1 ml are aspirated by an automatic glass pipette and the liquid introduced into the cheek pouch of hamsters previously anesthetized with sodium pentabarbital (3 % solution, i.p., 0·3 ml/10 g). The percentage of cercariae recovered as adult schistosomes is high (30–50 %) and the mortality of infected animals relatively low (about 10 % after 8 weeks).

Monkeys—A comprehensive study on the susceptibility and comparative pathology of ten species of primates exposed to infection with *S. mansoni* has been undertaken by Sadun *et al.* (1966b). Fifty-four primates were exposed to single graded doses of 50–2000 *S. mansoni* cercariae (Puerto Rico strain). They belong to the following 10 species: *Macaca mulatta* (rhesus monkey), *M. cynomolgus* (irus monkey), *M. speciosa* (stump-tail monkey), *Cebus apella* (capuchin monkey), *Ateles geoffroyi* (spider monkey), *Papio anubis* (baboon), *Pan satyrus* (chimpanzee), *Callithrix aurita* (marmoset), *Saimiri sciureus* (squirrel monkey), and *Tupaia* sp. (tree shrew). While all of these species could be experimentally infected with *S. mansoni*, their susceptibility and course of infection varied considerably. Variations in the intensity and type of tissue responses were also observed at various dosage levels. In general, three major reaction patterns could be distinguished: in the rhesus, irus and stump-tail monkeys, there was a high percentage recovery of worms, good worm development, and numerous eggs widely distributed throughout the colon, small intestine and liver. After a few months following infection in these animals, there was a tendency toward gradual self-cure. In the baboon and chimpanzee there was a lower worm recovery but egg excretion was maintained over relatively long periods. Most of the eggs were in the colon. In the marmoset, squirrel monkey and tree-shrew, the infection tended to be aborted from its onset. The worm and egg recoveries were low, the miracidial infectivity for snails was also low or absent and the pathological features were irregular and atypical.

Rhesus monkeys (*Macaca mulatta*) have been widely used in experimental chemotherapy of schistosomiasis mansoni (Kikuth and Gönnert, 1948; Hill, 1956; Bruce and Sadun, 1963, 1966; Thompson *et al.*, 1962, 1965; Campbell and Cuckler, 1963; Sadun *et al.*, 1966a). Other monkeys belonging to the genera *Cercopithecus*, *Papio*, *Macaca*, and *Cebus* have also proved to be useful for preclinical trials (Oesterlin, 1934; Coelho and Magalhães Filho, 1953; Newsome, 1953, 1963, 1964; Luttermoser *et al.*, 1960; Elslager *et al.*, 1961; Gönnert, 1961; Bruce *et al.*, 1962; Meisenhelder and Thompson, 1963; Pellegrino *et al.*, 1965b, 1966, 1967a, b; Katz *et al.*, 1966b, 1967b).

The use of rhesus monkeys in experimental chemotherapy of schistosomiasis

is handicapped by its cost, difficulty in handling, and by the self-limiting nature of the infection (Vogel, 1949, 1958, 1962; Naimark *et al.*, 1960; Lichtenberg and Ritchie, 1961; Jachowski *et al.*, 1963; Smithers and Terry, 1965; Ritchie *et al.*, 1966b; Sadun *et al.*, 1966b). In our laboratories, *Cebus apella macrocephalus*, a very good host for *S. mansoni* (Coelho and Magalhães Filho, 1953; Brener and Alvarenga, 1962), is routinely used for the last stage of preclinical trials. Some advantages for employing *Cebus* monkeys are (a) availability at a relatively low cost, (b) ease of handling and maintenance under common laboratory conditions, (c) high percentage of cercariae maturing to schistosomes (30–50%), (d) large numbers of schistosome eggs excreted with the feces and found in the rectal mucosa, (e) prolonged pattern of the infection without tendency to spontaneous cure, and (f) good correlation of therapeutic response as compared with man (Pellegrino and Katz, 1967, 1968a; Pellegrino *et al.*, 1965b, 1966, 1967a, b; Katz *et al.*, 1966b, 1967b).

Susceptible monkeys are easily infected by percutaneous, subcutaneous, and oral routes. The cutaneous route is preferable because it affords a more regular infection and a better worm return. In our laboratories, *Cebus* monkeys are fastened to a metal holder, abdomen upward, and a cercarial suspension (150–200 cercariae per animal) is pipetted gently onto the surface of the chest and abdomen, applying only sufficient fluid to be retained by the hair. After 30 min the animals are returned to their cages. Not later than 7 weeks, eggs can be regularly found in rectal snips obtained by mucosal curettage.

Other vertebrate hosts such as gerbils, cotton-rats, and mastomys have been occasionally used for chemotherapeutic studies in schistosomiasis mansoni but without advantages over the animals already mentioned (Watson *et al.*, 1948; Lagrange and Scheeqmans, 1951; Ragab, 1962; Lambert and Stauffer, 1964; Abdallah *et al.*, 1965; Lambert, 1966). According to Stirewalt (1963), the monkey, mouse, hamster, cotton-rat, squirrel, and woodchuck are usually accepted as highly susceptible to *S. mansoni* since all exposed animals become infected, 20% or more of the cercariae applied mature, and miracidium-producing eggs are present in the excreta.

VI. Assessment of Antischistosomal Activity

In routine screening programs, where many hundreds of compounds are to be examined each year, the main considerations in selection of a technique are simplicity and maximum economy in expenditure of animals and labor while preserving the essential requirement of activity in compounds of unknown potency (Standen, 1963). Once an active drug has been found through the primary screening, all possible information concerning its antischistosomal activity in different hosts as well as the intimate mechanism of action should be obtained. *In vivo* and *in vitro* bioassays are designed to evaluate the comparative activity of substances related to a chemical lead for establishing the structure–activity–toxicity relationships.

In chemotherapeutic studies of schistosomiasis, emphasis is generally made on the different susceptibilities to chemicals displayed by immature schistosomes and sexually mature forms. Kikuth and Gönnert (1948) demonstrated

that compounds belonging to the miracil series are inactive against *S. mansoni* in mice infected 33 days previously, but active against infection of 48 days' duration. This group of compounds was also inactive in monkeys with infections of 15 and 25 days. The fact that mature schistosomes are more susceptible to the action of antischistosomal agents was demonstrated to occur with antimonials (Schubert, 1948c; Luttermoser, 1952; Standen, 1955b; Brener and Chiari, 1957; Stohler and Frey, 1963), Hoechst compounds S-688 and S-616 (De Meillon *et al.*, 1956; Lämmler, 1958; Luttermoser *et al.*, 1960), *p*-aminodiphenoxyalkanes (Standen, 1955b), tris (*p*-aminophenyl) carbonium salts (Thompson *et al.*, 1962), and niridazole (Lambert and Stauffer, 1964; Lambert, 1966; Sadun *et al.*, 1966a; Yokogawa *et al.*, 1966). According to Campbell and Cuckler (1963), Merck's compound SN 10,275, a 2-phenyl quinoline, behaves differently because it acts better on immature than on sexually mature schistosomes. Therefore, if the relationship of maturity to drug susceptibility is likely to hold with the majority of schistosomicides, it is of the greatest importance that the screening of compounds in experimental chemotherapy should be carried out against schistosomes known to be mature, unless the search is for drugs of a prophylactic activity (Standen, 1955b; Pellegrino, 1967).

VII. Drug Testing *in vitro*

A number of attempts have been made to maintain *S. mansoni* and *S. japonicum* *in vitro* and to culture the stages of development which occur in the vertebrate host (Senft and Weller, 1956; Mao and Lyu, 1957; Robinson, 1957; Cheever and Weller, 1958; Clegg, 1959, 1965).

Simple culture media, consisting basically of serum and balanced physiological solution, are able to allow the schistosomes to survive for periods long enough for drug testing. A completely synthetic medium containing amino acids, nucleic acid derivatives, vitamins, salts, and glucose was described by Senft and Senft (1962), and special apparatus, simulating the natural habitat conditions of the parasite in the mammalian host, were devised by Newsome and Robinson (1954), Senft (1958), and Clegg (1961). Regeneration (Cheever and Weller, 1958), sperm formation (Clegg, 1959), and egg production by *S. mansoni* (Robinson, 1956, 1960; Newsome, 1962a; Senft and Senft, 1962; Lambert and Stauffer, 1964) have been investigated under *in vitro* conditions.

The general procedures involved in drug testing *in vitro* were outlined by Standen (1963) as follows:

The culture medium consists of equal volumes of Tyrode solution and horse or guinea pig serum with streptomycin and penicillin added, adjusted to pH of about 7·4. The serum and Tyrode solution are sterilized by Seitz filtration. The antibiotic solution is made up to contain streptomycin, 5 000 units/ml, and penicillin, 2 500 units/ml. Suitable cleansing of glassware is important. Synthetic detergents should not be used since they appear to adsorb accumulatively on the glassware and ultimately to interfere with the cultures. Glassware is therefore washed in water, soaked overnight in chromate–sulfuric acid,

washed in running tap water for 20 min, rinsed in distilled water, dried, and oven-sterilized at 160°C for 2 h.

Five ml of serum-Tyrode is transferred aseptically to a Carrel flask and to this 0·1 ml of antibiotic solution is added, giving 500 units of streptomycin and 250 units of penicillin per flask. The flask is incubated at 37°C.

Mice infected 10 weeks previously are killed by cervical fracture, the abdomen is shaved and the whole animal swabbed with 70% alcohol. Taking all sterile precautions the abdomen of the mouse is opened and the portal and mesenteric veins are severed immediately behind the paired schistosomes. These are coaxed from the veins by gentle stroking pressure to avoid damage and are transferred to sterile Tyrode solution at 37°C for inspection. Two pairs of undamaged schistosomes are transferred by Pasteur pipette to each warm Carrel flask and incubation is continued at 37°C.

For drug testing, the compound is dissolved in Tyrode, buffered as required, and an aliquot of the solution replaces an equal volume of normal Tyrode in making up the flasks. Drug-free control cultures may continue viable for 2–3 weeks but daily observation of the drug cultures for 4 or 5 days should be sufficient for determination of adverse effects upon the schistosomes: loss of the ventral sucker function, separation of paired worms without recopulation, alterations of the muscular activity-pattern, inhibition of egg laying, immobilization and death of one or both sexes. The nature of these effects may vary according to the type of compounds under test.

It has been demonstrated that trivalent antimonials cause a marked inhibition of the glycolysis of *S. mansoni* (Mansour and Bueding, 1954; Bueding, 1959) by blocking the formation of hexosediphosphate from its precursor, fructose-6-phosphate, through interference with the enzyme phosphofructokinase.

The early evidence that compounds of the "miracil" series are degradated in the vertebrate host and then converted into active metabolites (Halawani *et al.*, 1949; Newsome, 1953; Newsome and Robinson, 1960) have been recently confirmed by Rosi *et al.* (1967) using liver microsomes from different laboratory animals in experiments conducted *in vitro*.

Aerobic glycolysis of *S. mansoni* is markedly inhibited, *in vitro*, by 2-methyl-1,4-naphthoquinone (Bueding *et al.*, 1947; Bueding and Peters, 1951). The antiglycolytic properties of naphthoquinones *in vitro* were found to be less in a medium containing serum, than in one containing salts only, because of the combination of the drugs with proteins. Like naphthoquinones, several compounds such as cyanine dyes (Bueding, 1950), glucosamine (Bueding *et al.*, 1954), alkyldibenzylamines (Bueding, 1959), and tyramine (Dodin and Brygoo, 1964)—active *in vitro* upon adult schistosomes—were found to have little interest when tested *in vivo*.

Experimental *in vivo/in vitro* studies conducted by Standen (1962) with *p*-aminophenoxyalkanes have demonstrated that compounds of this series exert a profound and irreversible effect upon the permeability and physical structure of the cuticle of *S. mansoni* worms, interfering with its transport function.

In worms removed from mice that had been given a stock diet containing

1 % of tris(*p*-aminophenyl) carbonium chloride or pamoate (TAC) for 7–8 days, it was observed that the oral sucker, the pharynx, and the acetabulum are either paralysed or they exhibit only weak uncoordinated movements, whereas the activity of other somatic musculature remains unchanged (Bueding *et al.*, 1967). A causal relation between inhibition of acetylcholinesterase and the paralysis of the adhesive organs was indicated by an immediate reversal of the paralysis upon the *in vitro* exposure of the worms to mecamylamine and atropine, which act as cholinergic blocking agents in *S. mansoni*. The adhesive organs resumed their normal coordinated movements within 1–3 min in the presence of mecamylamine at a concentration of 2×10^{-4} molar or upon exposure to atropine at a concentration of 1×10^{-4} molar. These observations suggest that TAC treatment inhibits acetylcholinesterase activity resulting in an accumulation of acetylcholine in those nervous and neuromuscular systems that regulate the function of the worm's adhesive organs (Bueding *et al.*, 1967).

In vitro tests with niridazole have shown that this compound is responsible itself for schistosomicidal effects and that its metabolites play no part in the action of the drug (Lambert and Stauffer, 1964; Faigle and Keberle, 1966; Lambert, 1966, 1967).

VIII. Drug Testing *in vivo*

Schistosomiasis testing of new compounds in laboratory animals is usually performed in two successive steps. In the first one, drugs of unknown activity are screened and, for this purpose, the working model "*S. mansoni*-mouse" has been widely used. Once a substance is found to display some antischistosomal activity, additional information is obtained through preclinical trials generally conducted in mice, hamsters and monkeys.

The more important criteria for the assessment of antischistosomal activity can be summarized as follows:

Reduction in the number of excreted eggs

Considering the fact that the administration of antischistosomal agents produces a temporary or definitive suppression of egg laying, the decrease or complete cessation of egg passing by treated animals has been used for the evaluation of therapeutic activity.

In the technique of Kikuth and Gönnert (1948), the assessment of activity is mainly based on the decrease or complete cessation of egg passing by treated mice. Dosing is started 48 days after exposure to *S. mansoni* cercariae, when eggs are regularly present in feces. The effectiveness of a drug was classified by these authors as follows:

A. *No action*, which is indicated by the extrusion of eggs being continued for at least 3 weeks (stool examination and hatching test).

B. *Slight action*, which is indicated by interruption or brief cessation of egg extrusion, and at autopsy, by mature worms being accompanied by dead ones or by worm-debris.

C. *Action*, which is indicated by cessation of egg extrusion for at least 3 weeks, followed by relapse, and at autopsy, by most of the worms being dead

and disintegrating, though there might be rare living worms or living eggs, and pigment might be found diffusely in crush preparations.

D. *Cure*, which is indicated by the feces becoming free from eggs, with no relapse, during an observation period of at least 6 weeks, and at autopsy by no trace being found of living worms or living eggs, and by no diffusely distributed pigment being found in crush preparations of the liver. It must be remarked that stools do not become free from eggs until 14 days after the beginning of treatment, even when a cure has been achieved.

According to De Carneri (1958) and Lambert (1967), the most sensitive index of the action of any particular drug on *S. mansoni* is the interruption or cessation of egg passing. However, Schubert (1948a) has pointed out that the presence or absence of viable eggs in the feces is very variable even in untreated mice.

Quantitative estimations of the number of extruded eggs is of particular interest for the assessment of antischistosomal activity (Bell, 1963; Newsome, 1963).

In the experimental chemotherapy of schistosomiasis, the evaluation of activity based on the search for excreted eggs has also been extensively used in hamsters (Bueding *et al.*, 1953; Newsome, 1963) as well as in several species of primates (El Ayadi, 1947; Luttermoser *et al.*, 1960; Elslager *et al.*, 1961; Bruce *et al.*, 1962; Bruce and Sadun, 1966; Sadun *et al.*, 1966a).

Reduction of eggs in tissues

In Schwink's (1955) technique, female white mice are sacrificed 3 weeks after the beginning of treatment. Liver and intestines of treated and control mice are placed intact in 5% NaOH solution and digested for 6–8 h at 55–56°C. Egg counts, made on the residues, showed an inverse correlation between the number of eggs and the size of the dose. Intestines proved more satisfactory than livers. According to Schwink (1955), advantages of this method are (a) its rapidity and (b) the facility of preserving the organs of treated animals by refrigeration, until time is available for processing and egg countings. However, Brener (1959) claims that this technique is of no value for screening purposes.

The relative number of eggs in the liver after treatment, grossly recorded by a score system, was also used in assessing drug activity (Luttermoser, 1954; Thompson *et al.*, 1962; Lambert and Stauffer, 1964).

In the technique of Brener *et al.* (1956) the schistosome granulomas formed around eggs accumulated in the liver are separated, counted, and compared with the number found in untreated mice (Pellegrino and Brener, 1956; Brener, 1957, 1962; Brener and Chiari, 1957).

Increase in the survival time of treated mice

Active drugs may prevent death of infected mice by destroying the schistosomes before they can lay large numbers of eggs and cause extensive damage to the viscera. In Luttermoser's technique (1954), mice which had been exposed to cercarial concentrations (22–25 *S. mansoni* cercariae per g body-weight), lethal to young animals within 7–8 weeks, are given early treatment (maximum

tolerated dose) with the compounds under test. The increase of the survival time of treated animals, compared with that of untreated controls, would indicate antischistosomal activity. This criterion was used by Okpala (1959) for assessing drug activity in experimentally infected hamsters.

Reduction in worm burden

This criterion is of fundamental importance for the assessment of antischistosomal activity and for a sound quantitative evaluation of active compounds belonging to the same or different chemical groups (Watson *et al.*, 1948; Raison and Standen, 1955; Brener and Pellegrino, 1958; Luttermoser, 1959; Luttermoser *et al.*, 1960; Brener, 1962; Thompson *et al.*, 1962, 1965; Campbell and Cuckler, 1963; Stohler and Frey, 1963, 1964a; Lambert and Stauffer, 1964; Bruce and Sadun, 1966; Berberian *et al.*, 1967a, b).

Hepatic shift of schistosomes

Schubert's technique (1948a, b) and its modification by Standen (1953a, b, 1963) are based on the fact that drug activity in mice harboring mature *S. mansoni* infection is accompanied by a change in the distribution of schistosomes within the hepatic portal system. Normally, in mature infections, 60–70% of worms are located in the mesenteric veins, 20–30% in the portal vein, and 0–20% in the liver (Fig. 5). In hamsters, a similar pattern of worm distribution has been reported (Faria and Pellegrino, 1963; Fig. 6). Most antischistosomal agents exert a profound effect upon the distribution pattern at some time

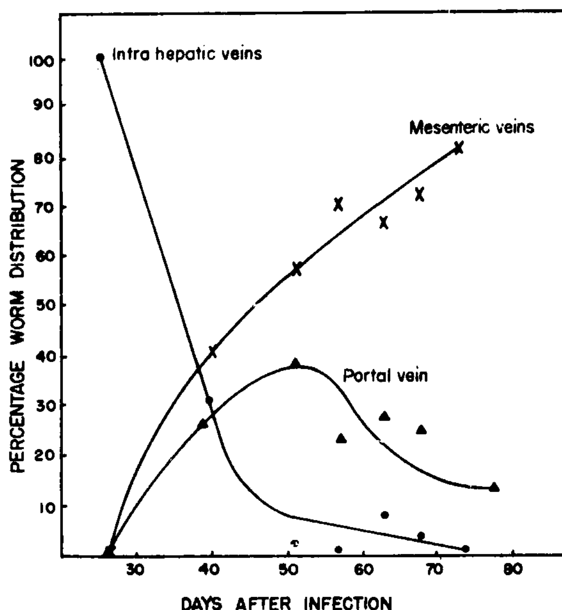

FIG. 5. Distribution of *Schistosoma mansoni* female worms within the hepatic portal system of mice harboring balanced bisexual infection. (Adapted from Standen, 1953, *Ann. trop. Med. Parasit.* 47, 25–43.)

after administration of an effective dose. The schistosomes release their hold upon the walls of the mesenteric veins and are shifted, passively, by the blood stream to the liver. According to the nature of the drug this hepatic shift may occur almost immediately, as with trivalent antimonials (Buttle and Khayyal, 1962; Khayyal, 1964; Brener, 1965), or may be delayed for 1–2 weeks, or may occur at some intermediate interval. The time between dose and shift and the proportion of the worms affected can vary in terms of dose-response. With subsequent drug detoxification in the liver the effect can be temporary and be followed by re-migration of worms to the mesenteric veins, providing a picture

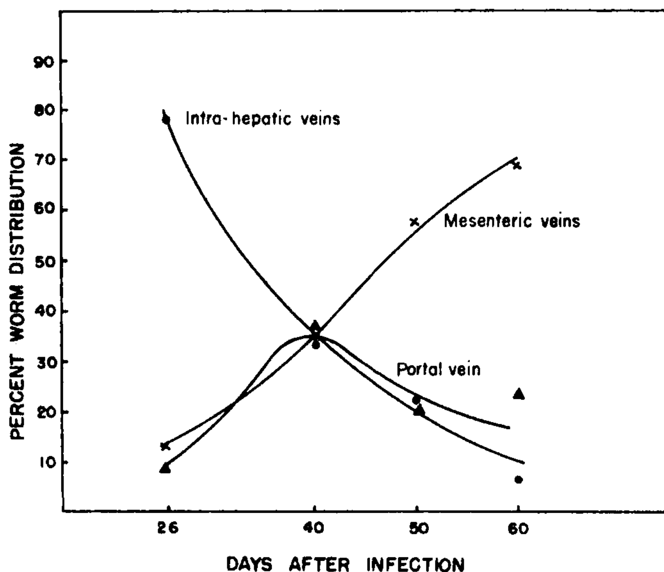

FIG. 6. Distribution of *Schistosoma mansoni* worms within the hepatic portal system of hamsters experimentally infected.

of relapse. Again, some drugs exert an irreversible effect upon the schistosome and those worms shifted to the liver never recover (Standen, 1967). The hepatic shift occurs after the administration of effective drugs to mice, hamsters and monkeys experimentally infected with schistosomes. The recovery of worms is greatly facilitated by the use of perfusion techniques (Yolles *et al.*, 1947; Ruiz, 1952; Pellegrino and Siqueira, 1956; Radke *et al.*, 1961; Duvall and De Witt, 1967).

Hepatic shift of schistosomes is not necessarily correlated with schistosomicidal activity. In fact, mature worms can be swept back to the liver after administration of glucosamine (Abdallah *et al.*, 1959), dimercaprol (Khayyal, 1965b) as well as anesthetics (Dickerson, 1965b; Khayyal, 1965a). Accordingly, in testing compounds for antischistosomal activity, animals must be killed by a physical method and not by an overdose of anesthetic.

A lung shift of schistosomes occurring in mice after chemotherapy has also been reported (Hewitt and Gill, 1960, 1962; Geake, 1962; Warren, 1962; Dickerson, 1965a).

Oogram changes

Studies on the oviposition of *S. mansoni* demonstrated that eggs are laid in the immature state and that a period of about 6 days is needed for the embryo to mature (Gönnert, 1955a; Prata, 1957; Pellegrino *et al.*, 1962; Fig. 7). It is assumed that oviposition constitutes a continuous process at least during a

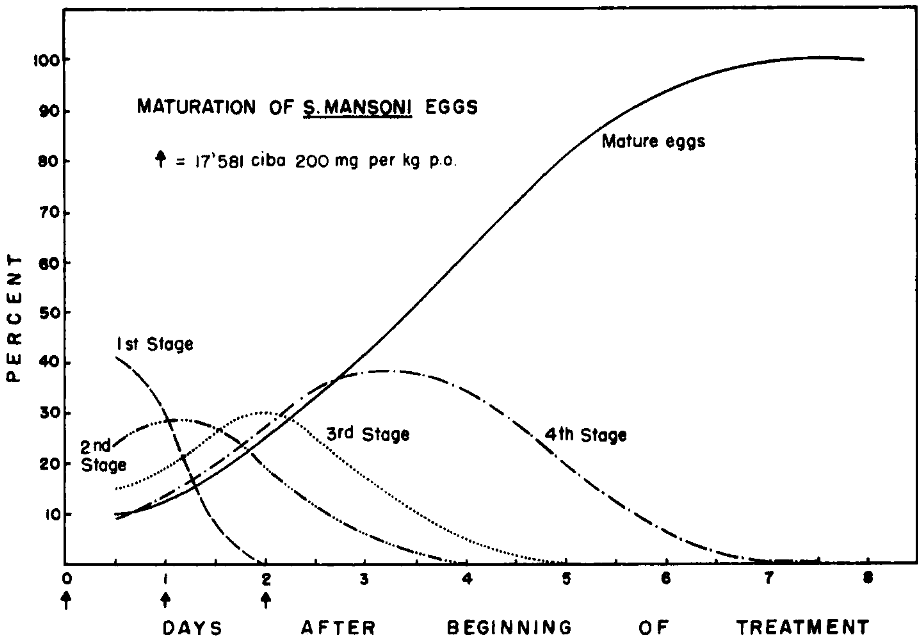

FIG. 7. Schematic representation of the maturation of *Schistosoma mansoni* eggs in the intestinal wall of infected mice. After the administration of 17'581 Ciba's thioxanthone, an early interruption of egg-laying occurred. The disappearance of 1st-stage eggs was followed by the successive disappearance of immature eggs of the following stages. From the 7th day on, all viable eggs found in the intestinal wall were mature. (Adapted from Pellegrino *et al.*, 1962, *Am. J. trop. Med. Hyg.* **11**, 201–15.)

certain period after migration of the worms to the mesenteric veins, and that a female can lay about 300 eggs a day (Moore and Sandground, 1956). Thus, shortly after egg-laying has begun, the intestinal wall may show a large number of eggs (Fig. 8).

By submitting mice infected with *S. mansoni* to treatment with active drugs, there occurs, in the intestinal wall, a progressive change in the number of eggs and in the percentage of viable eggs in the different stages (1st stage, embryo one-third the diameter of the egg; 2nd stage, embryo one-half the diameter of

the egg; 3rd stage, embryo two-thirds the length of the egg; 4th stage, embryo occupying practically the entire eggshell; mature egg, miracidium fully developed; see Fig. 9). This change—whatever the mode of drug action may be (loss of muscle tone and shift of worms to the liver, action on reproductive organs of the parasite, death of worms, etc.)—is brought about by the interruption of oviposition in the intestinal wall and by the maturation of viable eggs already there. Accordingly, the percentage of mature eggs increases progressively after

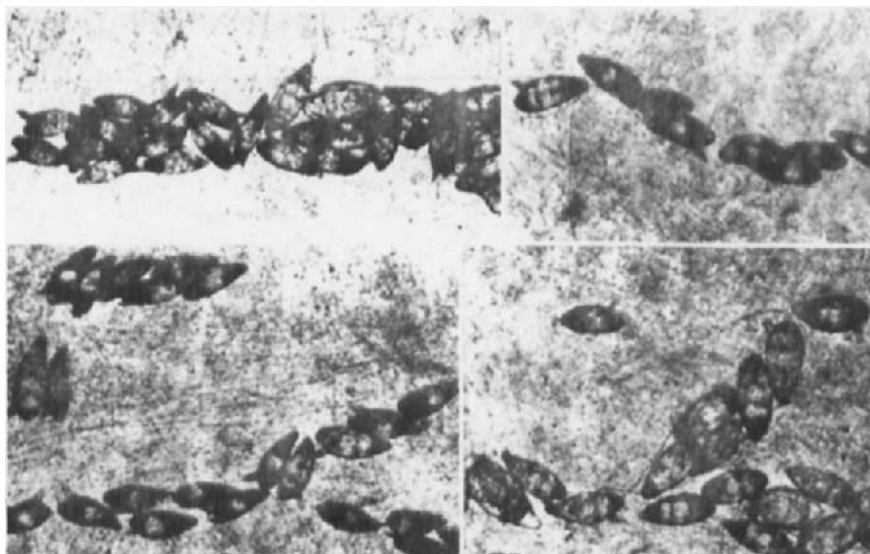

FIG. 8. Press preparations of intestinal fragments from mice infected with *Schistosoma mansoni* showing large numbers of viable eggs in all evolutive stages.

the administration of effective compounds (Fig. 10). Changes in the number and character of eggs (oogram) provide a simple, sensitive and reliable criterion for drug screening (Pellegrino *et al.*, 1962; Pellegrino and Faria, 1964, 1965; Brener, 1965).

The oogram method proved very helpful for the study of relapse (Pellegrino *et al.*, 1963; Figs 11, 12, 13) as well as for the detection of drug activity on schistosome eggs (Hill *et al.*, 1966; Monteiro *et al.*, 1968).

Other criteria for assessing therapeutic activity

Besides the criteria already mentioned, supplementary information may be of great value in connection with the evaluation of drug activity in schistosomiasis: separation of coupled schistosomes, opacity and depigmentation of worms, gross morphological alterations (diminution in size, absence of intra-uterine eggs, atrophy of the ovary, vitellaria, and testes), decrease of pigment deposited in the liver, presence of worms ensheathed in inflammatory tissues, etc. (Bang and Hairston, 1946; Kikuth and Gönnert, 1948; Standen, 1953a, 1955a; Gönnert, 1955b; Hill, 1956; Brener and Pellegrino, 1958; Lambert,

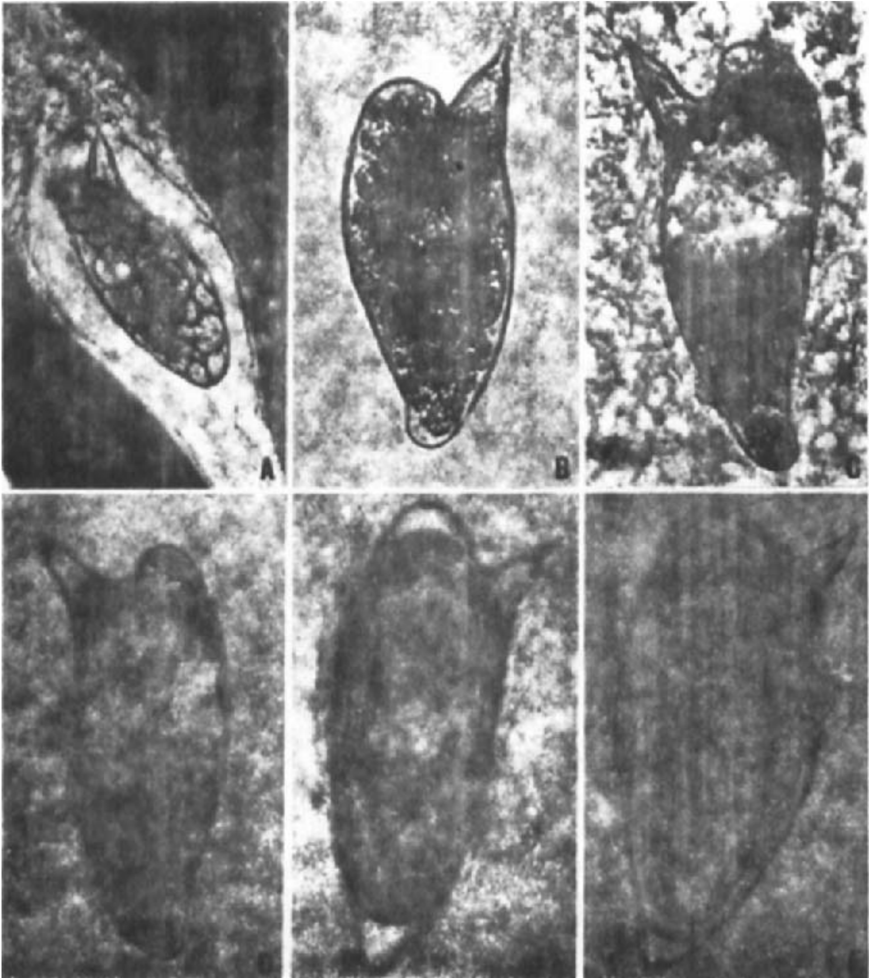

FIG. 9. Viable eggs of *Schistosoma mansoni*. *A*, uterine egg; *B*, immature egg of the 1st stage; *C*, immature egg of the 2nd stage; *D*, immature egg of the 3rd stage; *E*, immature egg of the 4th stage; *F*, mature egg.

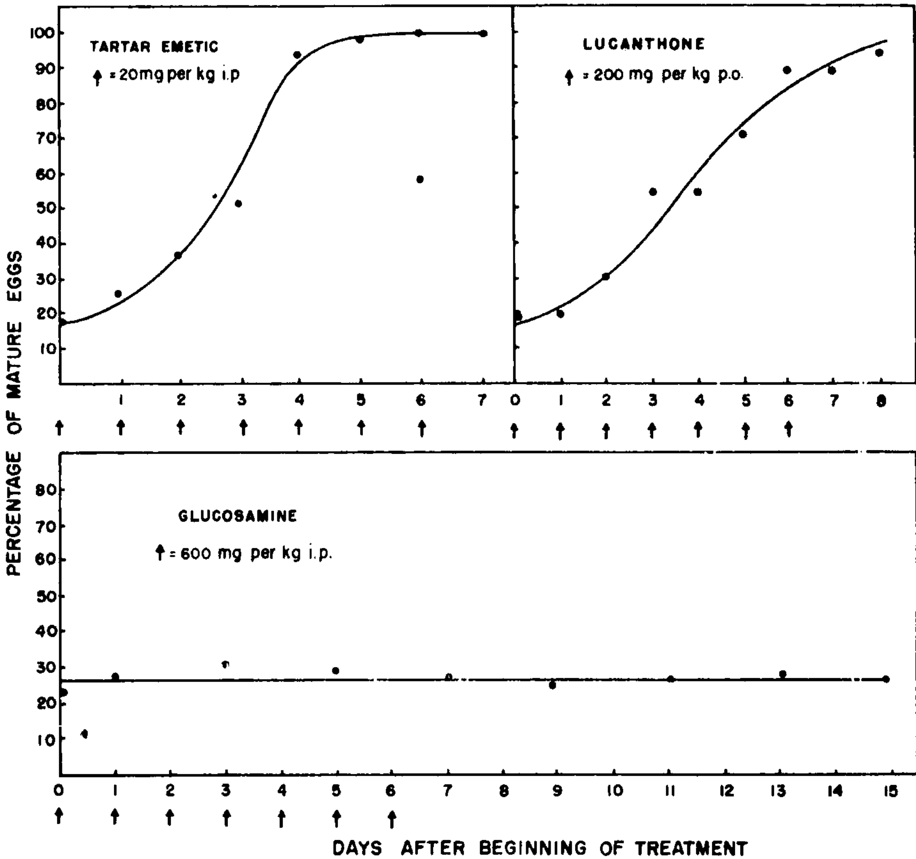

FIG. 10. Curves obtained by plotting the percentage of mature eggs from intestinal fragments of mice treated with tartar emetic (20 mg/kg), lucanthone (200 mg/kg), and glucosamine (600 mg/kg). Note that with tartar emetic and lucanthone the percentage of mature eggs increased progressively, reaching 100% (active compounds). Treatment with glucosamine did not change the percentage of mature eggs (inactive compound). (Adapted from Pellegrino *et al.*, 1962, *Am. J. trop. Med. Hyg.* 11, 201–15.)

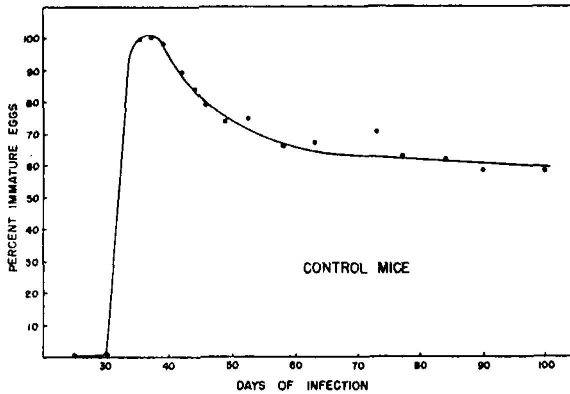

FIG. 11. Curve obtained by plotting the percentage of immature viable eggs from intestinal fragments of mice experimentally infected with *Schistosoma mansoni*. Each point represents the average from two animals. (Pellegrino *et al.*, 1963, *J. Parasit.* **49**, 365–70.)

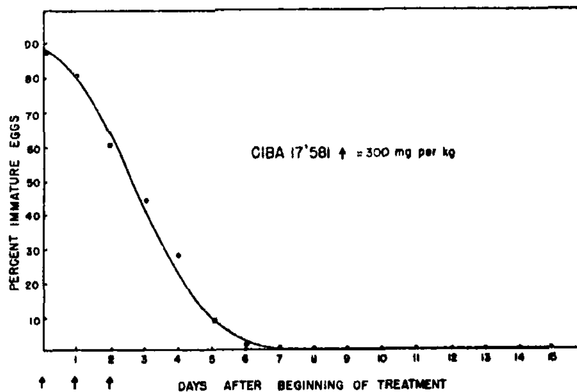

FIG. 12. Curve obtained by plotting the percentage of immature viable eggs from intestinal fragments of mice, treated with Ciba's 17'581, 45 days after exposure to cercariae of *Schistosoma mansoni*. No resumption of egg laying (relapse) was observed during the period of observation. Each point represents the average from two animals (Pellegrino *et al.*, 1963, *J. Parasit.* **49**, 365–70.)

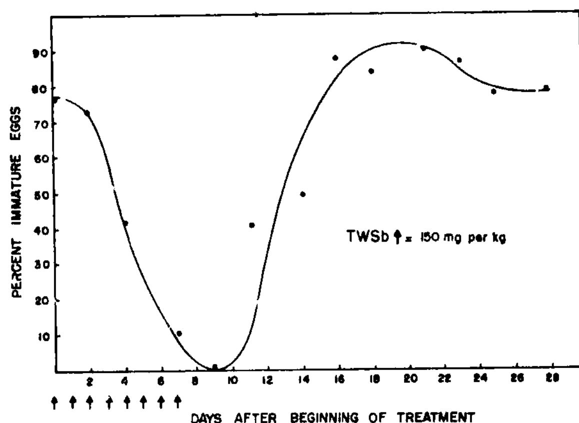

FIG. 13. Curve obtained by plotting the percentage of immature viable eggs from intestinal fragments of mice, treated with TWSb (Astiban[R]), 50 days after exposure to cercariae of *Schistosoma mansoni*. Resumption of egg laying (relapse) took place a few days after treatment had been stopped. Each point represents the average from two animals. (Pellegrino *et al.*, 1963, *J. Parasit.* **49**, 365–70.)

1966; Sadun *et al.*, 1966a; Striebel and Kradolfer, 1966; Bueding *et al.*, 1967).

It must be remarked that although for screening purposes the use of a single host and a single criterion may be adequate, the final evaluation of a promising drug must be based on experimental work carried out in different laboratory animals and using multiple criteria for assessing antischistosomal activities.

IX. QUANTITATIVE APPROACHES FOR THE EVALUATION OF ANTISCHISTOSOMAL ACTIVITY

In order to compare the antischistosomal activity of drugs belonging to the same or to different chemical groups it is imperative to use quantitative criteria of assessment. This aspect is particularly important in connection with the selective approach, when less toxic and more active compounds are to be selected before starting pharmacological clinical trials.

The percentage of reduction of worm burden in treated animals as compared with a control group proved to be a suitable criterion and has been used by many workers (Caldwell and Standen, 1956; Brener, 1960a, 1962; Luttermoser *et al.*, 1960; Stohler and Frey, 1963, 1964a; Stohler *et al.*, 1963; Lambert, 1966).

Therapeutic indexes based on toxicity data (LD_{50}) and figures corresponding to the dose which clears 50% or 90% of infected animals have been widely employed (Schubert *et al.*, 1949; Hill, 1956; Collins *et al.*, 1959; Brener, 1960a, b, 1962; Berberian and Freele, 1964; Rosi *et al.*, 1965, 1967; Berberian *et al.*, 1967a, b).

Quantitative approaches considering the proportion of animals extruding eggs, the reduction of schistosome granulomas in the liver and eggs retained in the tissues of treated animals in comparison with a control group, have also

been proposed (Brener *et al.*, 1956; Brener, 1957, 1959; De Carneri, 1958). The use of the hepatic shift method for a quantitative assessment of anti-schistosomal activity was favored by Standen (1953a).

The administration of decreasing doses of 23 known schistosomicides to groups of 10–20 mice harboring mature *S. mansoni* infection showed that, in general, a very low dosage level is sufficient to produce oogram changes. In fact, alterations of the oogram could be detected even with daily doses (for 7 consecutive days) as low as 1/100 of the LD_{50} (Becanthone, Ciba 17'581,

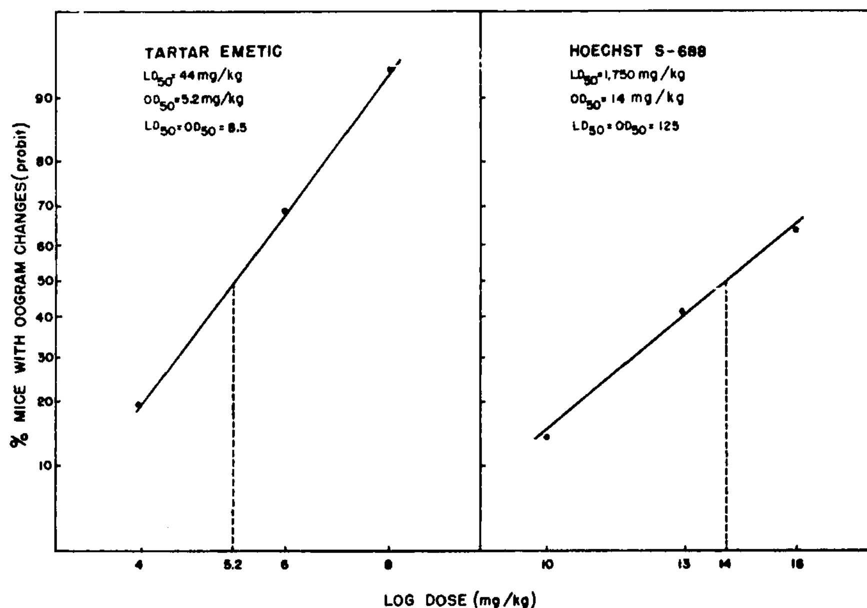

FIG. 14. Quantitative aspects of the oogram method. Relationship between log dose and percentage of mice with oogram changes. Each point represents the average from a group of 20 animals.

Hoechst S-688, Hoechst S-616, Schistocide 1593-A, SN 10,275; Table I). A further evaluation of the oogram method, under a quantitative basis, demonstrated that a linear relationship exists between the log of the dose and the percentage of mice presenting oogram changes (Fig. 14). It was then possible to determine the OD_{50} values (daily dose in mg/kg, administered for 7 consecutive days, which produces oogram changes in 50% of treated mice) and to relate these values with the respective LD_{50}'s. Table I summarizes some of the data obtained in our laboratories. All compounds in this Table and their respective chemical names are listed in the paper by Pellegrino and Faria (1965). It is obvious that these data apply only to mice. Many substances displaying high antischistosomal activity in mice are poorly active or even completely ineffective in humans.

TABLE I

*LD_{50}, OD_{50} and therapeutic index (LD_{50}:OD_{50}) for several antischistosomal agents**

Drugs	Route	LD_{50} (mg/kg)	OD_{50} (mg/kg)	LD_{50}:OD_{50}
Tartar emetic	i.p.	44	5·2	8·5
Fouadin	i.p.	1400	52	26·9
Astiban	i.p.	2500	60	41·7
Triostam	i.p.	175	7·4	23·6
Antiomaline	i.p.	90	13	6·9
Antimonyl EDTA	i.p.	85	5·8	14·7
Lucanthone	p.o.	1250	32	39·1
Becanthone	p.o.	5000	40	125·0
Ciba 17'581	p.o.	3800	16·6	228·9
Hoechst S-688	p.o.	1750	14	125·0
Hoechst S-616	p.o.	520	3·8	136·8
Mirasan	p.o.	500	8·1	61·7
Schistocide 1593-A	p.o.	1500	13·6	110·3
Schistocide T-109	p.o.	500	11·9	42·0
Schistomide	p.o.	>5000	130	>38·5
Emetine	i.p.	40	2	20
Ro 1-9334/5	i.p.	37	~3	~12·3
TAC pamoate	p.o.	>5000	680	>7·4
Tritheon	p.o.	1000	240	4·2
SN 10,275	p.o.	>5000	25	>200·0
Hoechst S-201	i.p.	>5000	480	>10·4

* All drugs in this Table and their respective chemical names are listed in the paper by Pellegrino and Faria (1965, *Am. J. trop. Med. Hyg.* **14**, 363–369).

The quantitative oogram method from rectal snips, as described by Cançado *et al.* (1965) and Cunha and Carvalho (1966), proved useful for the assessment of antischistosomal activity in monkeys (Katz *et al.*, 1966b; Katz and Pellegrino, 1968). Table II shows the results of quantitative oograms performed in 3 *Cebus* monkeys. In the first animal, treated intramuscularly with hycanthone (sulfamate salt) at the dose level of 3 mg/kg/day × 5, a persistent interruption of egg laying was observed. The second monkey, dosed with a single i.m. injection of 10 mg/kg, presented a relapse 8 weeks after treatment. The variation in the number of eggs per gram of rectal tissue, observed in the control monkey, represents a common pattern observed in untreated animals.

Only a few attempts have been made to evaluate the correlation between the activity of antischistosomal agents in laboratory animals and in man. Although the systematic use of mice, hamsters and monkeys in preclinical studies can provide a great deal of informative data for a tentative forecast of a possible efficiency in clinical trials, it must be pointed out that whether an antischistosomal agent will be fully or partially active or whether it will be totally ineffective will depend on the metabolism of that particular compound in a particular host (Berberian *et al.*, 1967a; Katz *et al.*, 1967b; Rosi *et al.*, 1967).

TABLE II

Quantitative oograms from Cebus *monkeys experimentally infected with* Schistosoma mansoni

Monkeys	Duration of infection when egg counts were started	Number of viable eggs per gram of rectal tissue — weeks												Remarks
		1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th	
1	2 months	2418	9029*	348	60	0	0	0	0	0	0	0	0	Interruption of egg laying
2	3 months	3923	1836†	0	125	0	0	0	0	0	0	409	893	Temporary interruption of egg laying. Relapse
Control	42 days	1447	—	3918	—	1697	—	8365	—	5399	—	6438	2465	Common pattern observed in untreated *Cebus* monkeys

* Treated with hycanthone 3 mg/kg/day × 5, i.m.
† Treated with a single dose of hycanthone, 10 mg/kg, i.m.

There is evidence in the literature that different strains of *S. mansoni*, as was demonstrated for *S. japonicum* (Hsü *et al.*, 1963), differ to some degree in their response to treatment with the same drugs. Gönnert and Vogel (1955) have shown that the Egyptian strain of *S. mansoni* is more resistant than the Liberian strain as far as Miracil D treatment is concerned. In fact, 6 doses of 100 mg/kg killed 97% of the Liberian but only 10·6% of the Egyptian parasites. With the Liberian strain the extrusion of eggs ceased permanently or for a long period, while with the Egyptian strain it continued apparently unchanged in many of the mice. According to Thompson *et al.* (1965), the Liberian strain seems to be hypersensitive to TAC pamoate when compared with the Puerto Rico strain. By using the collective evidence of the variations in the morphological and the biological characters of different strains, it has been shown that *S. mansoni* is not a uniform species but that distinct and interstrain variations do occur (Saoud, 1965a, b, 1966).

In the experiments conducted by Stirewalt *et al.* (1965), mice of four different strains (C3H, Swiss, Beige, Hairless) were exposed to standard numbers of *S. mansoni* cercariae under controlled laboratory conditions. Reproducible differences were observed in (a) average numbers of penetrating cercariae, (b) percentage of individual mice penetrated by at least 90% of the available cercariae, (c) worm burdens, (d) maturation of penetrants, and (e) consistency of mice with reference to these characteristics. Therefore, differences in *S. mansoni* strains as well as differences in host strains must be taken into consideration in any comparative or quantitative studies dealing with antischistosomal drug evaluation. In our laboratories, a reference *S. mansoni* strain (LE), isolated from a schistosome patient in Belo Horizonte, Brazil, is routinely used for experimental chemotherapeutic trials.

X. Drug Screening by the Oogram Method

After a preliminary toxicity test, drugs to be screened are administered to groups of 5 mice at a daily dose level corresponding to one-fifth of the LD_{50}, for 5 consecutive days (from Monday to Friday), 45–50 days after exposing the animals to 100–120 *S. mansoni* cercariae by the tail immersion method. Mice of both sexes, weighing about 20 g, are used. When sufficient drug is available, both oral and parenteral routes are employed. Otherwise, drugs with water solubility above 10% are administered intraperitoneally and the poorly soluble compounds are given by gavage, suspended in 5% gum tragacanth or Cremophor EL (25% by volume in normal saline solution). In special cases drugs are mixed in the diet and supplied *ad libitum* to the mice for 5 consecutive days. The use of a Monday–Friday schedule for screening, instead of 7 days of treatment as earlier proposed (Pellegrino *et al.*, 1962), is permissible as comparative results are obtained when the same total dose of active compounds is divided into 5 or 7 daily doses (Pellegrino and Faria, 1965). The practical implications of the use of a short treatment schedule are of great significance when large numbers of compounds are to be tested.

Three days after the end of treatment the animals are sacrificed by a blow on the neck. It is not recommended, for a primary screening, to hold the treated

mice for a longer period because when the activity of drugs is not pronounced or when active drugs are administered in very low dosage schedules, the oogram can return to a normal pattern within a few days. The skin over the abdomen of the dead mouse is grasped with the thumb and forefinger of each hand, pulled until torn, and retracted up over the head and down over the tail. The peritoneal cavity is then opened and 2–3 l-cm fragments of the distal part of the small intestine are cut off. While rinsing in tap water, each fragment is opened lengthwise with a pair of scissors and thereafter transferred and dried over an absor-

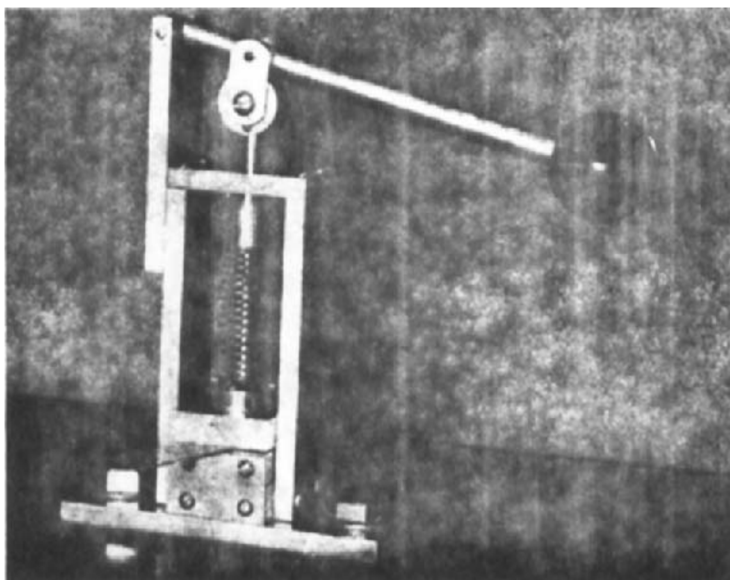

FIG. 15. Apparatus used to make press preparations of intestinal fragments from infected animals.

bent paper. Preparations are obtained by pressing each fragment between a microscopical slide and a plastic cover-slip using a specially designed apparatus (Fig. 15). The use of a projection microscope (Reichert's Visopan Model, Fig. 16) greatly facilitates the examination of intestinal press preparations. About 200 schistosome elements (viable eggs in their different stages of development, dead eggs and shells) are usually counted and properly classified under low magnification. Higher magnification is used whenever any doubt arises in classifying a schistosome element. Table III shows the oogram pattern observed in 200 control mice, 8 weeks after the exposure to *S. mansoni* cercariae. In this group of animals 100 schistosome elements were counted and classified for each mouse. As can be seen from the Table, the range of variation is rather high but in all instances all stages of viable eggs are present. Actual counts are usually unnecessary for screening purposes: when the examination of a few microscopical fields reveals the presence of viable eggs in all stages of development it is concluded that egg-laying was not affected (Fig. 8). A drug is

considered active and worthy of further evaluation when eggs of one or more immature stages are absent. Technical details of the screening procedure by the oogram method are illustrated in our film "Science against Schistosomiasis" (Ciba Film Unit, 1966).

In examining press preparations from intestinal fragments, it is of great importance cautiously to observe the morphological aspects of immature eggs. An unusual oogram pattern has been found in mice infected with *S. mansoni* and treated with a high dose of niridazole (525 mg/kg/day × 2). Although all worms had been shifted to the liver, oograms performed within 5 days after dosing were apparently normal, with eggs in all developmental stages as

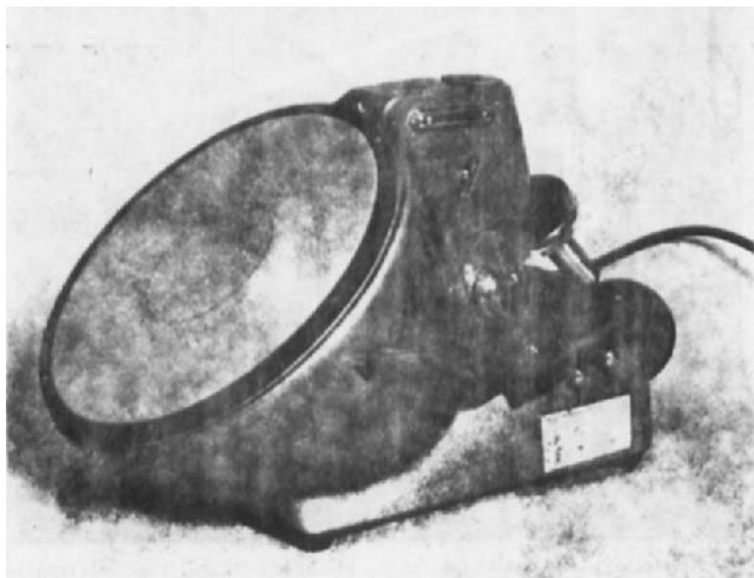

FIG. 16. Projection microscope (Visopan model, Reichert) used for the examination of press preparations (oogram).

happens after the administration of substances not displaying antischistosomal activity. However, immature eggs showed discreet but definite alterations, namely, abnormal shape of the egg shell and/or ooblast, and small amount of vitelline granules scattered over the ooblast (Monteiro *et al.*, 1968; Fig. 17). In this particular case, the oogram revealed a daily increase in the number of dead eggs. At the 6th day they had reached 74% of the total number of counted eggs, in sharp contrast to the normal figure in untreated mice which is rarely above 10%. A direct drug action upon *S. mansoni* eggs has been also reported to occur in mice treated with a nitrofurylacrylamide derivative (Hill *et al.*, 1966).

Table IV shows the results obtained in mice infected with *S. mansoni* and dosed with 18 known antischistosomal agents, as far as oogram changes and distribution of female schistosomes were concerned. As can be seen, there was

TABLE III

Oogram pattern from 200 mice experimentally infected with S. mansoni. *From each animal 100 schistosome elements were counted and classified*

Schistosome elements	Mean values (%)	Range of variation (%)
1st stage	15·0	3–35
2nd stage	14·0	3–51
3rd stage	25·3	7–52
4th stage	11·6	3–31
Mature eggs	29·2	10–57
Dead eggs and shells	4·9	0–31

a good correlation between the hepatic shift and oogram changes. The data reported by Brener (1965) amply support this conclusion.

It is worth stressing again that oogram changes are directly related to disturbances occurring in the egg-laying process of the parasite and also indirectly reflect either a schistosomicidal activity or a mere hepatic shift of worms. Since the principal goal of a screening method is to detect any anti-schistosomal activity, the oogram represents an important tool for the development of new leads in experimental chemotherapy of schistosomiasis. It is

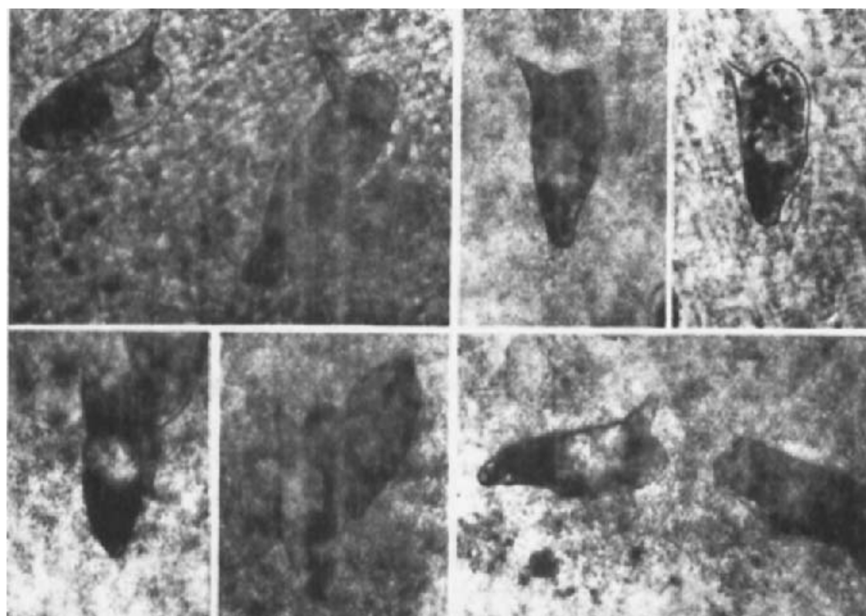

FIG. 17. Altered immature eggs of *Schistosoma mansoni*, in different stages of development, from intestinal fragments of infected mice treated with niridazole (525 mg/kg/day × 2).

TABLE IV

Oogram changes and distribution of female schistosomes in groups of mice treated with 18 *known antischistosomal compounds for* 5 *consecutive days and sacrificed* 3 *days after the end of treatment.* (*Adapted from Pellegrino and Faria,* 1965).

Drugs	Daily dose (mg/kg) for 5 consecutive days and route		Oogram changes/ animals examined	Distribution of female worms (%)		
				Liver	Portal vein	Mesenteric vessels
Tartar emetic	8·8	i.p.	10/10	43·5	13·0	43·5
Fuadin	280	i.p.	10/10	60·0	6·7	33·3
Astiban	500	i.p.	10/10	54·5	9·1	36·4
Triostam	35	i.p.	10/10	70·3	10·8	18·9
Antiomaline	18	i.p.	10/10	23·1	38·4	38·5
Antimonyl EDTA	17	i.p.	10/10	55·6	33·3	11·1
Lucanthone	280	p.o.	9/9	84·3	2·6	13·1
Ciba 17'581	760	p.o.	10/10	94·5	0·0	5·5
Hoechst S-688	350	p.o.	10/10	100·0	0·0	0·0
Hoechst S-616	104	p.o.	6/6	100·0	0·0	0·0
Mirasan	100	p.o.	8/8	100·0	0·0	0·0
Schistocide 1593-A	300	p.o.	10/10	81·3	0·0	18·7
Schistomide	400	p.o.	9/9	100·0	0·0	0·0
Emetine	8	i.p.	4/4	75·0	0·0	25·0
Ro 1-9334/5	7·4	i.p.	10/10	19·5	24·4	56·1
TAC pamoate	1500	p.o.	8/8	27·6	17·2	55·2
Tritheon	200	p.o.	6/10	28·8	22·4	48·8
Tritheon	250	p.o.	11/12	53·1	12·5	34·1
Hoechst S-201	1000	i.p.	5/10	30·8	30·8	38·4
Hoechst S-201	1250	i.p.	9/9	58·0	19·3	22·7
Control	—	—	0/10	6·9	26·7	66·4

obvious that should any drug be found that induces oogram changes it would certainly come to further evaluation with different criteria for assessing antischistosomal activity.

XI. PRECLINICAL TRIALS

All compounds showing antischistosomal activity in the oogram screening test are, in our laboratories, further evaluated on mice, hamsters, and *Cebus* monkeys experimentally infected with *S. mansoni.* Data on worm burden, distribution of schistosomes within the hepatic-portal system, morphological conditions of the worms and quantitative oogram are then obtained and properly analysed. This bulk of information proved to be of fundamental significance for a tentative forecast on the possible activity of a particular compound in clinical trials.

Mouse— Groups of 10–12 mice, 45–50 days after exposure to 100–120 *S. mansoni* cercariae, by the tail immersion method, are treated with decreasing dose levels of the active compound (5–7 consecutive days), taking into consideration the data previously provided by the screening test. The animals are sacrificed by a blow on the neck 3 days after the end of treatment and, besides the oogram from intestinal and liver fragments, the following additional data are obtained: distribution of schistosomes within the hepatic-portal system (intra-hepatic, portal, and mesenteric veins), percentage of dead worms in the liver, and gross morphological structure of female worms.

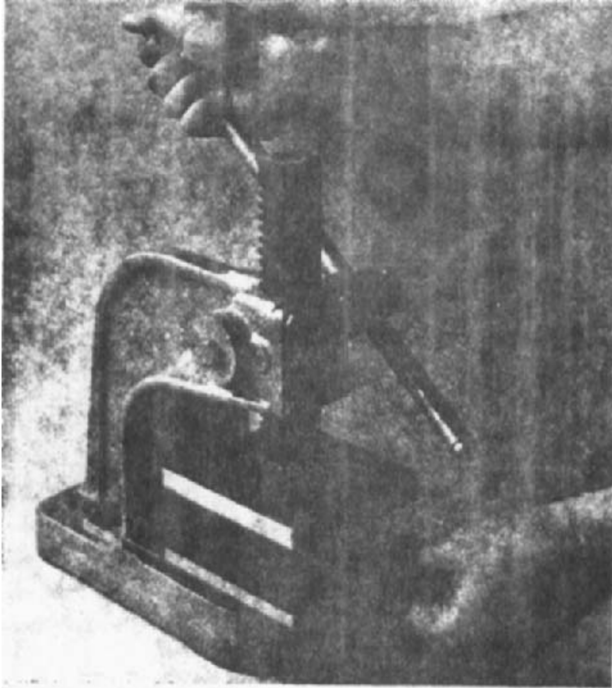

FIG. 18. Press apparatus used for squashing whole livers between two glass plates. (Courtesy of Dr. Paul Thompson, Parke, Davis and Company.)

The schistosomes in the liver, portal vein, and mesenteric vessels are recovered by perfusion using the Pellegrino and Siqueira technique (1956) adapted to mice. The worms are counted (male, female, paired worms) and the female schistosomes are microscopically examined in fresh preparations, attention being paid to the presence and gross morphology of intra-uterine eggs as well as to the internal structure of the reproductive apparatus. The entire liver is thereafter removed, squashed between two glass plates with a special pressing apparatus (Fig. 18), and examined under a dissecting microscope for dead worms.

Hamster—Groups of 5–7 adult hamsters (*Cricetus auratus*), 7–8 weeks after exposure to 60–80 *S. mansoni* cercariae via the cheek pouch (Pellegrino *et al.*, 1965a), are treated with decreasing dose levels of the compound to be tested, for 5–7 consecutive days.

The same procedures that have been outlined for mice are used for the assessment of antischistosomal activity in hamsters.

***Cebus* monkey**—Adult *Cebus apella macrocephalus* Spix, 1823 are exposed by percutaneous route to 150–200 *S. mansoni* cercariae. Treatment is started from 3–7 months after exposure. In general, a 5-day course of treatment is adopted, the dose levels being selected according to the data previously obtained in mice and hamsters.

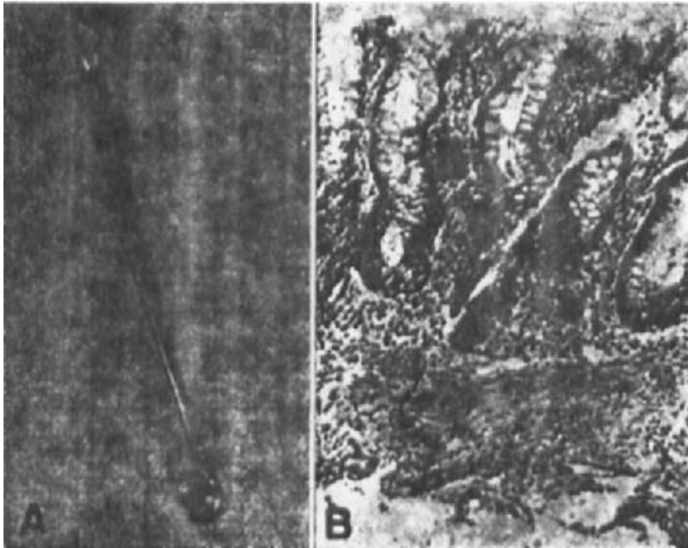

FIG. 19. A. Curette used for the collection of rectal snips from *Cebus* monkeys. B. Histological section of rectal tissue obtained by mucosal curettage.

The assessment of drug activity in *Cebus* monkeys is made through repeated mucosal curettages performed at different periods after treatment. A stainless-steel curette (Fig. 19a) with a 15-cm handle and a spoon of 2 mm height and 4 mm internal diameter is used. The instrument is introduced through the anus and the rectal mucosa is curetted at a distance of about 10 cm (Pellegrino *et al.*, 1965b). Four fragments are normally obtained, weighing about 30 mg, bringing only the mucosa and muscularis mucosae (Fig. 19b). Rectal snips, slightly dried on filter paper, are strongly pressed between slide and cover-slip, and examined with a projection microscope. All schistosome elements are counted and classified (viable eggs: 1st, 2nd, 3rd, 4th stages, and mature eggs; dead eggs and shells). The assessment of drug activity is based on the gradual disappearance of viable eggs in rectal snips. By weighing the rectal fragments obtained by mucosal curettage, and counting all schistosome elements, the

number of eggs per gram of tissue is then calculated. This quantitative approach (Katz *et al.*, 1966b) is of great help for the evaluation of antischistosomal drug activity in monkeys.

When a persistent interruption of egg-laying is observed, further data are obtained by sacrificing the monkeys 4 months after therapy. In such cases, the search for worms is made by perfusing the hepatic–portal system. Oograms are performed in intestinal and liver fragments and samples of organs taken and fixed for pathological studies.

XII. Experimental Development of New Antischistosomal Agents

The development of antischistosomal agents from 1918 up to 1962 was extensively reviewed by Standen (1963). In this comprehensive review, the antischistosomal activity on laboratory animals, the mechanisms of drug action, and the chief aspects related to clinical trials have been discussed. The following groups were considered: antimonials, xanthone derivatives, deoxybenzoins, *p*-aminophenoxyalkane derivatives, tin compounds, glucosamine, cyanine and triphenylmethane dyes, naphthoquinones, rhodamines and hydantoins, and dehydroemetine.

Within the last five years some new compounds displaying antischistosomal activity have been referred to in the literature. These novel drugs will be briefly considered with emphasis on niridazole and hycanthone, which proved to have a remarkable therapeutical activity in clinical schistosomiasis mansoni.

Trichlorphone—(0,0-dimethyl-1-hydroxy-2,2,2-trichloroethylphosphonate, Bayer Laboratories). In 1962, Cerf *et al.* investigated the possibility of using organo-phosphorus compounds less injurious to mammals for the treatment of human parasitic helminths. It was found that trichlorphone gave encouraging results in the treatment of ancyclostomiasis, ascariasis, trichuriasis, creeping eruption, and intestinal schistosomiasis. These findings were subsequently confirmed by Talaat *et al.* (1963).

No antischistosomal activity could be detected by Abdallah *et al.* (1965) on mice, gerbils and *Cercopithecus aetiops* experimentally infected with *S. mansoni* and treated, *per os*, with trichlorphone. The schedules used were 100 kg/mg/day, for 3–6 consecutive days (mice and gerbils), and 5–10 mg/kg/day for 10 days for monkeys. In patients infected with *S. haematobium* and *S. mansoni*, and treated with 5 mg/kg/day for 12 consecutive days, the percentages of cure were 65 and 18 %, respectively.

In our laboratories, the antischistosomal activity of trichlorphone was investigated on mice, hamsters, and *Cebus* monkeys experimentally infected with *S. mansoni*. Oogram changes could be detected in 100 % of mice and hamsters treated, *per os*, at the dose levels of 200, and 100 mg/kg/day for 7 days. No significant activity was found in *Cebus* monkeys dosed up to 30 mg/kg/day × 5. A transient interruption of egg-laying (rectal biopsies) was observed in 6 out of 12 patients, infected with *S. mansoni*, after treatment with trichlorphone at the dose level of 7·5 mg/kg, every fortnight, 5 times (Pellegrino and Katz, 1968b).

SN 10,275—((6,8-dichloro-2-phenyl-4-quinolyl)-α piperidyl carbinol hydrochloride, Merck Laboratories). According to Campbell and Cuckler (1963), mice could be protected against infection with *S. mansoni* cercariae by feeding them on a diet containing 0·1% of SN 10,275 for a week, before or after exposure. When treatment was performed 4 weeks after infection, the dose of 50 mg/kg/day ×3, given orally, cured all mice, and so did a single dose of 100 mg/kg or a diet containing 0·05% drug, given for 2 weeks. In mice harboring mature infections (8 weeks) most of the schistosomes were killed by a diet of 0·05% for 1 week, or a single oral dose of 250 mg/kg. Apparently SN 10,275 acts on immature better than on mature worms. This drug was also reported to be active on rhesus monkeys (Campbell and Cuckler, 1963).

The daily dose of SN 10,275, administered orally for 7 consecutive days, necessary to produce oogram changes in 50% of mice infected with *S. mansoni* was found to be 25 mg/kg (Pellegrino and Faria, 1965; Pellegrino and Katz, 1968a). In hamsters experimentally infected with *S. mansoni* and treated at the dose levels of 40 and 20 mg/kg/day ×7, *per os*, a marked hepatic shift was observed and oogram changes were detected in 100, and 50% of the animals, respectively (Pellegrino and Katz, 1968b).

SN 10,275 is known to have a photosensitizing action in man, and this might well proscribe its medical use.

Amphotericin B (Squibb Laboratories). It was reported by Gordon and St. John (1963) that amphotericin B significantly prolongs the life of mice experimentally infected with *S. mansoni* and effectively lowers the mean infection-level of treated animals. These data have not been confirmed by Pellegrino (1965) and by Prata *et al.* (1965) in mice and hamsters treated up to toxic levels. In clinical trials, amphotericin B administered intravenously at the dose level of 1·5 mg/kg/day, for 20 days, was found completely ineffective (Prata *et al.*, 1965).

S-201 (bis-(β-carbhydrazido-ethyl) sulphone, Hoechst Laboratories). Lämmler (1963) reported that S-201 is highly effective against *S. mansoni* infections in mice, when administered parenterally. The dicarbonic acid hydrazide used in a therapeutic regimen involving large parenteral doses (250 mg/kg/day ×6) was only partially effective in rhesus monkeys. Although egg passage in the feces of treated animals was significantly reduced, moderate numbers of live, but stunted worms, were found at necropsy (Bruce and Sadun, 1966).

In our laboratories, the activity of S-201 against *S. mansoni* infections has been confirmed in mice, hamsters and *Cebus* monkeys (Pellegrino and Faria, 1965; Pellegrino and Katz, 1968b).

Hoechst compound S-201 was reported to be effective in patients with *Schistosomiasis mansoni* but its clinical use had to be discontinued due to marked toxic side-effects (Carvalho, 1965).

A-16612 (N-(3-chloro-4-methylphenyl)-N′-(ω-4′-t-amylphenoxyhexyl)-piperazine hydrochloride, Abbott Laboratories). A marked hepatic shift of

S. mansoni worms was observed in mice, starting from the dose of 25 mg/kg/day ×5 *per os* (43·9%) and reaching 99·2% at the schedule of 200 mg/kg/day ×5, when 86·8% of the worms were found dead in the liver. All animals dosed with 200 mg/kg presented oogram changes. At lower dosage schedules (100, 50 and 25 mg/kg) the percentages of animals with altered oograms were 85·7, 57·1 and 33·3, respectively. No antischistosomal activity was demonstrated in infected hamsters even at the dose level of 1000 mg/kg/day ×7. A slight antischistosomal activity was observed in *Cebus* monkeys at doses (500 and 1000 mg/kg ×5) approaching toxic levels (Katz *et al.*, 1967b).

In trials carried out with A-16612 on patients with active schistosomiasis mansoni, no therapeutic activity could be detected with schedules up to a total course of 750 mg/kg, which corresponded to the maximum tolerated doses (Katz *et al.*, 1967b).

It was recently reported (Rosi *et al.*, 1967) that the antischistosomal activity of A-16612 in mice and hamsters is greatly enhanced when the 4-methyl group is replaced by the 4-hydroxymethyl group.

RD 12,869 (6-chloro-5-β-diethylaminoethylamino-8-methylquinoline, Boots Pure Drug Co. Ltd.). It has been recently reported (Pellegrino *et al.*, 1967b) that in mice experimentally infected with *S. mansoni* and treated with RD 12,869 at the dose levels of 120, 60 and 30 mg/kg/day ×5, *per os*, oogram changes occurred in all animals and 100% of the schistosomes were shifted to the liver. At the dose level of 15 mg/kg, 66·6% of the mice presented altered oograms and 82·1% of the worms were found in the liver. No antischistosomal activity could be detected on hamsters dosed with 120 mg/kg/day ×5, administered *per os* or intraperitoneally. Higher dosage schedules were found to be very toxic for infected hamsters. Parasitological cure, demonstrated at necropsy, was achieved in *Cebus* monkeys treated with RD 12,869 at the dose levels of 120, 60 and 30 mg/kg/day ×5, *per os* (Pellegrino *et al.*, 1967b).

The encouraging results obtained in mice and *Cebus* monkeys strongly suggest the desirability of conducting pharmacological clinical trials with this new antischistosomal agent.

Nicarbazin (equimolar complex of 4,4′-dinitrocarbanilide and 2-hydroxy-4,6-dimethylpyrimidine, Merck Laboratories). The data recently reported by Campbell and Cuckler (1967) show that the reproduction of *S. mansoni* in experimentally infected mice is suppressed by treatment with nicarbazin. When fed to mice at 0·2% in the diet, nicarbazin completely suppressed the deposition of schistosome eggs, but did not kill the worms. Schistosomes were not killed even when the drug was fed at 0·3% for 18 weeks, or at 1·0% for 2 weeks (the highest dosage tested). Inhibition of egg production could be detected as early as 2 days after the beginning of nicarbazin treatment. The inhibitory effect was reversible and resumption of egg-laying occurred when the drug was withdrawn. No antischistosomal effect was observed when nicarbazin was administered by gavage as a series of 10 doses at 250 mg/kg (Campbell and Cuckler, 1967).

In our laboratories, the suppressive activity of nicarbazin on mice and

Cebus monkeys experimentally infected with *S. mansoni* has been amply confirmed. In mice the drug was effective only when incorporated in the diet. Oogram changes as well as a slight hepatic shift of worms were observed in mice fed with a diet containing 1·5% of nicarbazin for 5 consecutive days. Relapse occurred shortly after treatment was discontinued and nicarbazin was found completely ineffective when administered by gavage at the dose level of 1000 mg/kg/day, for 5 consecutive days. However, in 2 *Cebus* monkeys dosed *per os* at 400 mg/kg/day ×2 and followed by 200 mg/kg/day ×10, a transient interruption of egg-laying, determined by serial mucosal curettages, was observed (Pellegrino and Katz, 1968b).

Niridazole (1-(5-nitro-thiazolyl)-2-imidazolidinone, Ciba Laboratories; Fig. 20.) The schistosomicidal activity of this new nitrothiazole derivative, synthesized at Ciba Laboratories by Wilhelm and Schmidt, was first evaluated

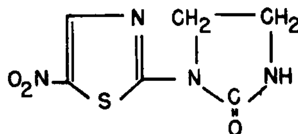

FIG. 20. Chemical structure of niridazole.

by Lambert and co-workers (Lambert and Stauffer, 1964; Lambert *et al.*, 1964; Lambert, 1966; Lambert *et al.*, 1966). The results obtained can be summarized as follows:

(a) In vitro *activity.* Adult *S. mansoni* were not killed by 24 h exposure to concentrations of the drug up to 150 and 200γ/ml. It was found, however, that prolonging the exposure time resulted in a very marked drug action. Adult pairs of *S. mansoni* exposed to 1γ/ml of niridazole for 100 h ceased to lay eggs; 10γ/ml, for the same period of time, produced complete immobilization and death of the worms.

(b) In vivo–in vitro *tests.* The aim was to determine what level of activity is achieved in serum of rabbits following niridazole treatment consisting of a single (500 mg/kg) oral dose. The maximal schistosomicidal action was observed in serum collected 6 h after dosing. This activity corresponded approximately to that observed at a concentration of 1γ/ml of the drug in synthetic medium. Female worms were regularly more sensitive to drug action than males, being completely immobile after about 72 h of contact whereas 96 h was required to immobilize the males.

(c) *Activity on laboratory animals.* A regular schistosomicidal action was observed when mice were treated for 5–10 days with a daily oral dose of 100 mg/kg. In desert-rats a similar activity was observed after treatment at 50 mg/kg, for 15 consecutive days.

The antischistosomal activity of niridazole was further evaluated by Bruce and Sadun (1966) and Sadun *et al.* (1966a) on rhesus monkeys experimentally

infected with *S. mansoni* and on mice infected with *S. japonicum*. It was found that niridazole has a strong prophylactic and chemotherapeutic effect. The dosage levels necessary to bring about satisfactory results in these animals were well below toxic levels. Egg suppression and a significant reduction in worm burden was observed in the animals in which treatment began at the time of exposure or a few days thereafter. No noticeable effect was demonstrated in the animals in which drug administration was initiated 10 or 15 days after exposure. When the drug was given after the worms had reached sexual maturity, a marked therapeutic activity was manifested by the reduction in number, or complete elimination of worms, by the complete suppression of egg deposition, and by a stunting effect in those few worms which survived treatment.

The data obtained in our laboratories (Pellegrino *et al.*, 1966) on the anti-schistosomal activity of niridazole can be summarized as follows:

MOUSE. All schistosomes were shifted toward the liver in mice dosed with 200 and 100 mg/kg/day × 7, *per os*. The hepatic shift was also remarkable at the dose level of 50 mg/kg and, though less pronounced, still significant at the lower schedules of treatment: 32·9 and 20·3 % of schistosomes in the liver of the mice treated with 25 and 12·5 mg/kg/day, respectively. All schistosomes were found dead in the liver of mice treated at the dose levels of 200 and 100 mg/kg/day. There were oogram changes in all mice treated with daily doses of 25 mg/kg or higher.

HAMSTER. The activity of niridazole was less pronounced in hamsters than in mice. The hepatic shift of worms was clearly observed at the dose level of 100 mg/kg/day × 7. There were found oogram changes in all animals dosed with 100, and 80 mg/kg/day.

Cebus MONKEY. Five infected monkeys, treated with niridazole at daily dose levels ranging from 10 to 50 mg/kg, died within 1–3 days after the beginning of treatment. The total dose administered to these animals ranged from 20 to 150 mg/kg. In one *Cebus* dosed with 5 mg/kg/day × 5 there was observed a marked loss of body-weight. This animal died 1 month after treatment. Serial mucosal curettages clearly indicated interruption of egg-laying. Treatment was apparently well tolerated by monkeys receiving 2·5 and 1·25 mg/kg/day × 5. At these dose levels, the higher one produced a temporary interruption of egg-laying. No therapeutical activity, as judged by repeated oograms, could be detected in monkeys dosed with 1·25 mg/kg. The pronounced toxicity of niridazole for *Cebus* monkeys is in sharp contrast with the good tolerance displayed by rhesus (Sadun *et al.*, 1966a) and *Callithrix* monkeys (Pellegrino and Katz, 1968a) when treated at dose levels 20 times higher. The high toxicity of niridazole for infected *Cebus* monkeys was also observed in non-infected animals (Pellegrino and Katz, 1968a).

The mode of action of niridazole on *S. mansoni* worms was studied by Striebel and Kradolfer (1966). Following doses of 10 mg/kg/day × 10, the first alterations in the female genital organs could be seen. Signs of destruction appeared in the distal portion of the vitellogenic gland where the vitelline cells were destroyed. Histological sections revealed vitelline cells completely

depleted of egg-shell substance. A dosage of 20 mg/kg/day × 10 caused a complete arrest of shell formation in the ootype. After a dosage of 50 mg/kg/day × 10, no eggs at all could be found. The destruction of the vitellogenic gland in the female coincided with a reduction in body-length of worms of both sexes and in the size of the ovary. Male worms seemed to be less sensitive. Spermatogenesis was stopped and the spermatocytes were vacuolated at 50 mg/kg.

Faigle and Keberle (1966) have shown that following oral administration, niridazole is absorbed from the gastro-intestinal tract over a period of several hours. A large proportion of the absorbed substance is broken down rapidly. The metabolites are biologically inactive and are excreted in the urine and feces. None or virtually none of the non-metabolized substance is excreted. Niridazole is rapidly absorbed and broken down by schistosomes, and the metabolites accumulate in the parasites. There is probably a connection between this process and the appearance of lesions in the schistosomes. It was found by Hess *et al.* (1966) that niridazole is highly concentrated in the schistosomes, in females more than males, and in eggs more than in any of the mouse tissues, except gastric mucosa.

According to Bueding and Fisher (1967), one of the early changes produced by niridazole is a reduction in the glycogen levels of the male *S. mansoni*. This is not associated with an inhibition of the activity of UDPG-glycogen transferase (which catalyzes glycogen synthesis) nor with a stimulation of glycogen phosphorylase (catalyzing glycogen degradation). On the other hand, as early as 24 h after a single dose of niridazole to mice infected with *S. mansoni*, inactivation of phosphorylase (catalyzed by a phosphatase) by homogenates or extracts of the worms is reduced significantly.

In clinical trials niridazole was found highly effective against the three chief species of schistosomes infecting man. (Cf. reports presented at the Lisbon Meeting, 1965, Round Table on Ciba 32,644-Ba (Ambilhar), Rio, 1966, and Conference on Niridazole, New York, 1967.) Although niridazole is probably the best antischistosomal agent now in current use, the very nature of some of the side effects (hallucinatory phenomena, convulsions, etc.), precludes, in our experience, its use for mass treatment (Katz *et al.*, 1966a, 1967a).

Hycanthone (1-N-β-diethylaminoethylamino-4-hydroxymethylthioxanthone, Sterling–Winthrop Laboratories; Fig. 21). Hycanthone, a hydroxymethyl derivative of Miracil D, was obtained through the biological activity of *Aspergillus sclerotiorum* upon the parent compound (Rosi *et al.*, 1965).

On an equal weight basis, the schistosomicidal activity of intragastrically

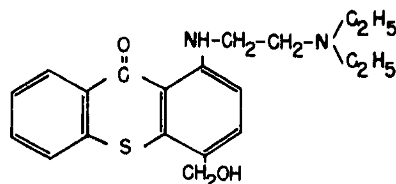

FIG. 21. Chemical structure of hycanthone.

administered hycanthone was 9 times greater in hamsters and 3 times greater in mice than that of lucanthone. The activity of a single dose of hycanthone (sulfamate salt) administered parenterally was equal to that obtained by a 5-day regimen administered *per os*, both for mice and hamsters (Berberian *et al.*, 1967a, b).

The results obtained in our laboratories (Pellegrino *et al.*, 1967a) with oral treatment of hycanthone can be summarized as follows:

MOUSE. A marked hepatic shift of *S. mansoni* worms was observed with all schedules of treatment, reaching over 90% at daily dose levels of 80 and 40 mg/kg/day ×7. The oogram from all mice dosed with 80 mg/kg was found altered. A progressive decrease in the percentage of mice with oogram changes was observed at the dose levels of 40 mg/kg (62·5%), 20 mg/kg (16·7%) and 10 mg/kg (12·5%).

HAMSTER. The antischistosomal activity of hycanthone was far more pronounced in hamsters than in mice. The oogram from all animals dosed with 8 mg/kg/day ×7 was found altered. The percentages of animals presenting oogram changes were 75 and 20%, respectively, for the groups of hamsters dosed with 4 and 2 mg/kg. A significant hepatic shift of schistosomes was observed in hamsters treated at the daily dose levels of 8, 4 and 2 mg/kg. At the highest dose, 60·5% of the worms were found dead in the liver.

Cebus MONKEY. In two monkeys treated with hycanthone, *per os*, for 5 consecutive days, at the dose levels of 10 and 5 mg/kg/day, parasitological cure was achieved. Both animals were necropsied 25 and 16 weeks after the end of treatment. Neither living nor dead worms could be detected and only a few shells and dead eggs were found in press preparations of intestinal and liver fragments (Pellegrino *et al.*, 1967a). Two monkeys were dosed with i.m. injections of hycanthone (sulfamate salt), 3 mg/kg/day ×5, and a single injection of 10 mg/kg. In the first one a persistent interruption of egg-laying was found during the whole period of observation (5 months). In the other animal, relapse occurred 2 months after therapy (Pellegrino and Katz, 1968b).

The results of clinical trials that are being carried out with different formulations of hycanthone, namely, capsules, enterocoated tablets, and a parenteral one (sulfamate salt), are quite encouraging. In 52 patients with active schistosomiasis mansoni, treated with hycanthone (capsules) at the dose levels of 2 and 3 mg/kg/day ×5, the percentage of cure was about 80%, and the side effects observed such as nausea, vomiting, vertigo and headache were of minor clinical significance (Katz *et al.*, 1968). In preliminary clinical trials, a single injection of hycanthone (sulfamate salt, 3–4 mg/kg) seems to be highly effective.

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SHORT REVIEWS

Supplementing the Contributions of Volume 1

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Recent Experimental Research on Avian Malaria*

CLAY G. HUFF

Naval Medical Research Institute, Bethesda, Maryland, U.S.A.

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I. INTRODUCTION

This review supplements the more extensive earlier one (Huff, 1963) and should be read in conjunction with it. The same criteria that were used in the earlier review guided the inclusion or exclusion of papers published since 1962. By necessity, discussion of the results in papers reported herein has been held at a minimum.

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Two symposia have been published on malaria since the earlier review. They represent the material presented at two International Panel Workshops held under the sponsorship of the Walter Reed Army Institute of Research in Washington, D.C. (1964, 1966). Although these reports included work on many phases of malaria, a considerable portion of them dealt with avian malaria. In this period of time the most comprehensive book yet published on the non-medical aspects of malaria appeared (Garnham, 1966).

II. PARASITOLOGY

A. EFFECT OF TEMPERATURE ON EXOGENOUS STAGES

In papers by Chao and Ball (1964) and Ball and Chao (1964) the results of exposing *Culex tarsalis* which had fed upon canaries infected with *Plasmodium relictum* (strain 1*P*1-1) to different temperatures at different stages in the development of the exogenous stages were reported. Briefly summarized, their results demonstrated that (1) temperatures as low as 4°C were not immediately lethal to the parasites, but damage to sporozoite infectivity appeared later, (2) 4°C temperatures for as long as 23 days did not affect 3-day old oocysts but lower temperatures were lethal to young oocysts, (3) temperatures below the optimum (27°C) retarded the development of sporozoites but such sporozoites remained infective for at least 2 months, (4) temperatures above 27°C increased the speed of development of infective sporozoites, and increasing temperatures were most harmful to the earliest stages of development, and (5) sporozoites retained their infectivity for 13 days when the mosquitoes were exposed to 35°C. A more complete review of these experiments is given by Ball (1964).

B. EFFECTS OF ULTRASOUND

Experimental studies of the effects of ultrasound at a frequency of 20000 cycles per second on chicken red cells infected with *P. gallinaceum* were reported by Rutledge and Ward (1967). Gentle sonication released the parasites from the host cells but left them adhering to the free, host nuclei. Greater degrees of sonication yielded complex mixtures of whole cells, fragments of various sizes of host cells and parasites, malarial pigment and artifacts. The amount of treatment required to render the blood non-infective was dependent upon the number of parasites in the blood.

C. SUSCEPTIBILITY; STRAIN DIFFERENCES; GENETICS

A review by Huff (1965) attempted to collect all published experimental results on the susceptibility of mosquitoes to avian malaria up to 1964. Tentative conclusions indicated: (1) that the genera *Aedes* and *Armigeres* reflect their systematic propinquity by their susceptibility to *P. gallinaceum*, (2) that a close relationship of the species *P. fallax*, *P. gallinaceum* and *P. lophurae* is indicated by their capability of infecting species of *Aedes*, and (3) that a similar close relationship between *P. relictum* and *P. cathemerium* is reflected in their infectivity to *Culex*. Since that review appeared the following new mosquito hosts have been reported:

Mosquito	*Plasmodium*	*Reference*
Anopheles stephensi	*giovannolai*	Corradetti *et al.* (1964)
Mansonia crassipes	*polare*	Corradetti and Scanga (1965)
M. crassipes	*gallinaceum*	Niles *et al.* (1965)
*Culex sitiens**	*juxtanucleare*	Bennett *et al.* (1966)
C. gelidus	*juxtanucleare*	Bennett *et al.* (1966)
C. tritaeniorhynchus	*juxtanucleare*	Bennett *et al.* (1966)
*C. annulus**	*juxtanucleare*	Bennett *et al.* (1966)
C. pseudovishnui	*juxtanucleare*	Bennett *et al.* (1966)
C. tarsalis	*hexamerium*	Janovy (1966)

* Proven natural vectors.

The oocysts of *P. juxtanucleare* in *Culex sitiens* were shown by Bennett *et al.* (1966) to be attached to the gut wall of the mosquito by a peduncle. Three strains of this species of parasite (New World, Ceylon and Malaysian) had many features in common, but the Malaysian strain prefers mature erythrocytes, while the other two invade both young and mature erythrocytes; the New World strain is more virulent than the Asian strains; and the gametocytes of the Malaysian strain are normally elongate whereas those of the New World strain are round (Bennett *et al.*, 1966).

Corradetti *et al.* (1966) obtained complete development of *P. gallinaceum* in *Anopheles stephensi* and its transmission by bite to chicks. From a strain thus transmitted they were able, by repeated mosquito transmissions, to raise the percentage of successful transmissions from 10·7 to 47·3–70. This represented experimental demonstration of the part played by the genetic constitution of the parasite in the cycle involving the parasite and its mosquito host (see review of work by Greenberg and co-workers by Huff, 1963). Previously, various authors had demonstrated the effect of the genetics of the parasite in its ability to infect the vertebrate host and of the genetics of the vertebrate and invertebrate hosts to serve as hosts to the parasite.

D. GENETICS OF MOSQUITO SUSCEPTIBILITY

A comprehensive study of the genetic aspects of susceptibility of *Aedes aegypti* to *Plasmodium gallinaceum* was published in 1963 by Ward. Beginning with a strain of this mosquito which was susceptible to infection with *P. gallinaceum* he was able to derive a highly resistant strain by genetic selection. During a period of 26 generations susceptibility was reduced by 98% and, in the absence of selection, did not return to normal levels during 13 generations. He believed that genetic variation could be attributed to the effect of a single, recessive, genetic factor. Further experiments using the same mosquito–parasite combination were reported by Ward (1966). He found no relation between oocyst size and number of oocysts in individual mosquitoes, which confirmed similar studies by Huff (1941) on *P. cathemerium* in *Culex pipiens*. His results also supported Huff's earlier finding of a high correlation between oocyst numbers resulting from successive feedings of the mosquitoes.

E. SPOROZOITES

1. *Penetration*

Sherman (1966a) investigated the effects of various substances and treatments on the penetration of duck erythrocytes by merozoites of *P. lophurae*. Concentrations of folic acid, reduced glutathione, nicotinic acid, and nicotinamide had no effect on penetration or growth; omission of amino acids, purines and pyrimidines had little effect; while the addition of bovine plasma fraction V (albumin), inosine, high concentrations of reduced glutathione and old vitamin preparations lowered the penetration rate, effected a lower growth rate, or both. Penetration of the erythrocytes by merozoites was not changed by pretreatment with trypsin, chymotrypsin or neuroaminidase. Parasite growth was favored by lowering the incubation temperature from 40·5°C to 35·5°C. He concluded that the merozoites of *P. lophurae* show sensitivity to the external milieu and that they lack receptor sites similar to those which function in viral attachment.

2. *Survival*

Further studies have been made on the survival of sporozoites in susceptible and insusceptible hosts by Beckman (1963), who introduced sporozoites of *P. gallinaceum* into chicks as susceptible, and into mice as insusceptible hosts. When blood was drawn from these animals and injected into chicks no infections resulted in chicks receiving blood from the mice and only a few became infected from the bitten chicks. When sporozoites were incubated in whole blood or plasma from mice and chicks, then inoculated into chicks, good survival resulted from those incubated with mouse blood but not as good survival resulted from those incubated with chick blood. Sporozoites were inactivated soon after being injected into mouse skin but not after residence in chick skin. Beckman (1965) then showed that much the same result was obtained when the sporozoites were deposited in excised skin of the two kinds of hosts.

3. *Conservation*

A method for conserving sporozoites of *P. gallinaceum* at temperatures of liquid nitrogen was reported by Weathersby and McCall (1967). When *Aedes aegypti* mosquitoes were shown by sampling to be heavily infected with sporozoites of this parasite they were immobilized in a refrigerator at 4°C, placed in vials tightly closed, immersed in liquid nitrogen and stored. Samplings were made at intervals up to 767 days by removing individual vials, thawing the mosquitoes at room temperature, grinding with mortar and pestle in Hanks' balanced salt solution and injecting intraperitoneally into chicks. Prepatent periods and resulting parasitemias in the inoculated chicks were comparable to the controls which were not refrigerated. The use of this technique will have tremendous advantages over the usual practice of maintaining strains of malaria by continuous passage in animals.

F. EFFECT OF ASSOCIATION WITH VIRUS

An attempt was made by Bertram *et al.* (1964) to test experimentally for a possible effect on the transmission of malaria (*P. gallinaceum*) by *Aedes aegypti* when the mosquitoes were also infected with Semliki Forest virus. In one experiment the mortality in the doubly infected mosquitoes was higher than in those infected with the malarial parasite only. In this same experiment the malarial infection rates were lower in the doubly infected mosquitoes. In further experiments the higher mortality was not observed but the infection rates in the mosquitoes were somewhat less in doubly infected mosquitoes.

G. CAUSE OF DEATH

P. pinottii, which was isolated from a Brazilian toucan, has an unusual pathogenicity for domestic pigeons. Manwell and Stone (1966) attempted to discover the cause of death in these infections. The birds die with hematocrits of 20–48 and with massive hemolysis. Parasitemia at death may vary from 20% to nearly 100% of the red cells. Attempts to demonstrate a hemolytic agent in the plasma were unsuccessful. Artificially induced anemias, although exceeding those produced by the infection, caused only minor, temporary disability. No cerebral capillary occlusions could be found in animals dying of the infection.

H. EPIDEMIOLOGY

Janovy (1966) reported on a two-year epidemiological study of *P. hexamerium* in Barton County, Kansas. Field studies indicated that *Culex tarsalis, Aedes nigromaculis* and *A. sollicitans* were abundant and that they fed regularly on small birds. Janovy believed that annual transmission probably begins in June, before which the mosquito populations were not high. There was an increase in malaria incidence in meadowlarks during March and April which might have been due to relapse in or migration of the hosts. Epidemiological patterns of the parasite were different in meadowlarks and starlings and may have resulted from differences in migratory habits of the birds and differences in ecological association with the mosquito vectors.

III. CULTIVATION

1. *Exogenous stages*

Further progress in the *in vitro* cultivation of the mosquito stages of *P. relictum* was reported by Ball and Chao (1963). They confirmed the work of Weathersby (1954) (reported in my previous review—1963) in showing that, in this combination of mosquito and parasite, the stomach of the mosquito is not necessary for the development of the oocyst. They also showed that (1) sporozoites may be infective without having resided in the salivary glands; that (2) hyperbaric concentrations of oxygen are harmful to the malarial parasites and to the mosquito tissues in which they develop; and that (3) the early exogenous stages, from gametocyte through zygote, are more sensitive to low temperatures, *in vitro* as well as *in vivo,* than to higher temperatures. Summary reviews (Chao and Ball, 1964a, b; Ball, 1964) show that all of the

exogenous stages up to sporozoites can be grown *in vitro* although, as yet, this has not been accomplished in continuous cultures.

2. *Exoerythrocytic stages*

Details of the methods for, and results from, the use of multipurpose chambers fitted with dialysis membranes were reported by Jensen *et al.* (1964). This method was well suited to growing exoerythrocytic stages of *P. fallax* from a variety of chick embryo tissues. These stages could be grown in small numbers for many months and could be studied at high magnifications at all times. An additional advantage of this type of culture was that the host cells underwent relatively less dedifferentiation when compared with cells grown in culture tubes in which there was a gas–liquid face. A review of the methods used in and results obtained from the growth of exoerythrocytic stages in culture was published by Huff (1964).

Davis *et al.* (1966) described procedures which have been especially successful in growing maximum numbers of exoerythrocytic stages of *P. fallax* in turkey embryonic brain cells in conventional tissue culture chambers (T-flasks) containing liquid medium with gas above its free surface. Such cultures facilitated the pursuit of various studies such as the fine structure of, and the action of chemotherapeutic agents upon exoerythrocytic stages. Some of these studies are reported under the section "Exoerythrocytic Stages" of this review.

IV. Biochemistry and Physiology

A. Enzyme Action

Since it has been previously shown by Sherman that the lactic dehydrogenase of *P. lophurae* appears to be physiologically superior to that of the host cell enzyme in the parasitic stage, he (1965) chose to extend his investigations to include glucose-6-phosphate dehydrogenase (these parasites were already known to require reduced glutathione in *in vitro* cultivation). He demonstrated that in erythrocytes infected with *P. lophurae* (29–114% parasitemias) no enzyme deficiency occurred except above the 80% level of parasitemia. There was no evidence of glutathione instability in duck erythrocytes infected with *P. lophurae* in the range of 27–67%. In this parasite–host-cell combination it is believed that an advantage accrues to the parasite when the host cell viability can be maintained during growth, reproduction, and liberation of the parasites.

It had been previously shown by Sherman that a lactic dehydrogenase exists in *P. lophurae* which differs from that of its host cell. Recently he (1966b) chose malic dehydrogenase for further study of enzyme heterogenicity in malarial parasites. He demonstrated by immunological and kinetic studies that the host and parasite enzymes can be distinguished from each other. The amount of the enzyme in *P. lophurae* was about twice that present in the uninfected cell. The parasite seems to synthesize its own malic dehydrogenase, perhaps at the expense of the host cell.

Seeking for the basis of the favorable effect of ATP with pyruvate upon the asexual stages of *P. lophurae* removed from their host cells and developing *in vitro*, Trager (1967b) investigated the possibility that pyruvic kinase and

phosphoglyceric kinase might not be present in the parasites in sufficient amounts to be effective in the glycolytic cycle concerned with formation of ATP. He found, however, that the ATP content of erythrocytes infected with *P. lophurae* was lower than the content of uninfected cells. Pyruvic and phosphoglyceric kinases were found in free parasites and the amount of these enzymes was greater in infected than in uninfected host cells. Thus, the favorable effect of ATP with pyruvate upon parasites developing *in vitro* remains unexplained.

Trager (1967a) compared the action of pyrimethamine, chloroquine, sulfadiazine, and quinacrine on *P. lophurae* developing within erythrocytes with the action on these stages in the absence of their host cells. As expected, these drugs exhibited their antimalarial effects upon the parasite–erythrocyte complex but failed to show harmful effects on the parasites in the extracellular state. Two possible explanations were advanced to account for this difference. First, it might be assumed that the drug is concentrated in the host cell and hence the parasite is exposed to higher concentrations within the cell than it would be in the medium. Another explanation might be that the drugs interfere with a host-cell enzyme system, such as that involved in folinic-acid synthesis to deprive the parasite of a substance essential to it.

B. METABOLISM

Relatively little work has been done on the lipids of malarial parasites. In view of the large amounts of lipid material present in these parasites it is to be regretted that more attention has not been paid to them. Wallace *et al.* (1965) extracted the total lipids of *P. lophurae* (and *P. berghei*) and analyzed them for lipid classes and total fatty acid composition. They separated the phospholipids, sterols, free fatty acids, triglycerides, and sterol esters by thin layer chromatography and demonstrated that the major portion of total lipid appeared as phospholipid. The following chain lengths of fatty acids were recorded: C12:0, C14:0, C15:0, C16:0, C16:1, C17:0, C18:0, C18:1, C18:2, C18:3 and C20:4. The major component fatty acid was C18:1. Wallace (1966) investigated the fatty acid composition of sterol esters, triglycerides, free fatty acids, and phospholipids of *P. lophurae* (and *P. berghei*). A total of 27 fatty acids was observed in the various fractions assayed. A large percentage of the fatty acids was accounted for by the groups with carbon chain lengths of 16 and 18 in all fractions except the sterol esters in which fatty acids with longer chain lengths predominated.

Investigations on phospholipid metabolism of uninfected turkey erythrocytes and of similar cells infected with *P. fallax* in the presence and in the absence of chloroquine were reported by Gutierrez (1966). The cells were incubated with C^{14}-phospholipids, -acetate and -fatty acids. In the presence of the drug the uptake of acetate into fatty acids and phospholipids was depressed in both infected and uninfected erythrocytes. Chloroquine caused an increase of C^{14}-oleic acid. The largest and most consistent effect of the drug was upon the sphingomyelin and lysolecithin fractions.

Since it had previously been shown that duck erythrocytes infected with *P.*

lophurae contained much more folic and folinic acid than erythrocytes of uninfected ducks, Siddiqui and Trager (1964) compared the R_f values of these two growth factors in infected and uninfected erythrocytes. In view of their finding that the bio-autographs of the infected and uninfected erythrocytes gave identical results, they concluded that this finding strengthens the hypothesis that the increase in these growth factors in infected red cells results from an altered metabolism of the host cell rather than from synthesis by the parasites. The bio-autographs of infected and uninfected livers were also identical. These authors (1966) then studied the changes in levels of folic and folinic acids accompanying the development of *P. lophurae* in erythrocytes. They made use of the synchronous cycle of their strain of *P. lophurae* and found that from the uninucleate stages of the parasites to the multinucleate stage (24 h later) there was a change in ratio of folic:folinic acid of 3:1 to 1:2 with no real change in the folic acid. However, when the infected cells were incubated *in vitro* only a small increase in folinic acid was observed.

Since the largest complicating factor now remaining in development of a medium for growth of the extracellular parasites from erythrocytes is the complex, red-cell extract, Siddiqui and Trager (1967) attempted to compare the free amino-acid pool of the blood plasma and erythrocytes from uninfected ducks and ducks infected with *P. lophurae*. They demonstrated an increase in the plasma from infected ducks of 2–3 times the concentration of aspartic acid, proline, valine, leucine, tyrosine, and phenylalanine, and of four times the normal concentration of threonine; the arginine was less than half that of uninfected animals. In the infected erythrocytes there was a reduction of cystine but increases in all other amino acids. There were increases of 100–190 times the amounts of phenylalanine, tyrosine, methionine, and proline. A threefold increase was noted in serine, arginine, and glycine, and an 8–16-fold increase in alanine, threonine, histidine, lysine and valine. With the exception of cystine and tyrosine, the concentrations of which were greater in plasma, the concentrations of the other amino-acids were much greater in infected erythrocytes than in the plasma of the same ducks.

Additional evidence to support the belief that the avian malarial parasite is dependent on its host cell for its required coenzyme A was presented by Bennett and Trager (1967). They reported studies on the conversion of pantothenic acid to phosphopantothenic acid catalyzed by pantothenic acid kinase which is the initial enzymatic reaction in the pathway to coenzyme A from pantothenic acid. They did not find pantothenic acid kinase in *P. lophurae* parasites.

It is well known that malarial parasites grow better *in vitro* when supplied with CO_2, but the reason for this was not well understood until Sherman and Ting (1966) presented evidence indicating that the CO_2 plays a role in metabolism. The parasites (*P. lophurae*) were freed from the host cells and suspended in saline containing ^{14}C, after which they were extracted and the labeled products identified and evaluated. There resulted a 400% increase in CO_2 fixation in infected red cells compared with normal cells. The fixation occurred primarily in the lactic acid, alanine, glutamic and aspartic acids of the infected cells, and in the freed parasites. The authors believe that this newly-found pathway of

carbon dioxide fixation by malarial parasites must be included in consideration of carbohydrate and amino-acid metabolism, along with glycolysis, the Krebs cycle, proteolysis of hemoglobin and free amino-acid pools of the erythrocyte and plasma.

The possibility that changes in levels of pyridine nucleotides may function in the regulation of metabolic pathways in *P. lophurae* was shown by Sherman (1966c). He studied the changes in levels of reduced and oxidized diphosphopyridine nucleotide (DPNH, DPN) and triphosphopyridine nucleotide (TPNH, TPN). During infection the levels of DPN, TPN and DPNH increased 1·5 to 2-fold while the TPNH remained at about the levels found in uninfected erythrocytes. In parasites freed of their host cells the TPNH, DPNH and TPN were found to be about at the level found in uninfected duck erythrocytes but the level of DPN in the parasites was 1·5 times greater. DPN is known to favor the growth and reproduction of parasites freed from their host cells and held *in vitro*.

To test whether malarial parasites lack a pentose phosphate pathway which they use in the red blood cells as a source of reduced triphosphopyridine nucleotide, Herman *et al.* (1966) compared the metabolism of 1-^{14}C- and 6-^{14}C-glucose of chicken blood infected with *P. gallinaceum* with that of the uninfected erythrocytes. Their data indicated that intact chicken erythrocytes infected with *P. gallinaceum* produce more $^{14}CO_2$ from 1-^{14}C-glucose than is produced from uninfected erythrocytes. CO_2 production was accelerated by the addition of TPN or ATP to total red cell and parasite total hemolysate. They believe that a pentose phosphate pathway is absent in *P. gallinaceum* and that such a pathway in the host erythrocyte is utilized by the parasite.

C. HAEMATIN

Because of a possible relationship between hematin and chloroquine-resistant strains of malaria, Sherman *et al.* (1965) undertook amino-acid analyses of hemozoin of *P. lophurae* (and *P. berghei*) and of duck (and mouse) hemoglobins. They found that the amino-acids in hemozoin vary from those found in hemoglobin. They believed their data to be consistent with the idea that in the formation of hemozoin from hemoglobin there is: (1) denaturation and insolubilization of hemoglobin; (2) oxidation of the iron; and (3) partial proteolysis of the globin. They believe that the mechanism involved in chloroquine resistance may be related to ferrihemic acid but that this has as yet not been demonstrated, and that reduced quantities of hemozoin in chloroquine-resistant strains of malaria cannot be due to ferrihemic acid alone since the pigments are not identical.

V. Exoerythrocytic Stages

Discussion of work reported upon fine structure and cultivation of exoerythrocytic stages will be found under the respective sections of this review. An excellent review of the literature on exoerythrocytic stages of malarial parasites in general was published by Bray (1963).

Since 1962, the exoerythrocytic stages of several additional species of avian

malarial parasites have been reported. Corradetti *et al.* (1963) demonstrated these stages in canaries experimentally infected with either infected blood or sporozoites of *P. giovannolai.* They were limited to monocytes and macrophages of the spleen, were found exceptionally in wandering monocytes of the liver and only once in the bone marrow. Garnham and Killick-Kendrick (1964) demonstrated the exoerythrocytic stages of *P. pinottii* in pigeons in which the infections had been prolonged by the administration of quinine. They reported the presence of distinct categories of macro- and micromerozoites without giving statistical proof thereof. Parasites of the pre-erythrocytic cycle of *P.* (*Giovannolaia*) *polare* were also found in the spleen (only) of canaries inoculated 12–16 days prior to sacrifice (Corradetti and Scanga, 1965). Guindy *et al.* (1965) described exoerythrocytic stages in a new species of parasite, *P. garnhami*, from the Egyptian hoopoe (*Upupa epops major*). They were observed in the "reticulo-endothelial cells" of the spleen and liver. Although the authors place this species systematically near *P. elongatum* it does not resemble *P. elongatum* in its preference for the erythrocytic precursors. Nelson (1966) observed exoerythrocytic stages of *P. vaughani* in the lymphoid-macrophage cells of lungs, spleen, bone marrow, liver, and kidney of pigeons. The strain was isolated from the Ceylonese mynah bird (*Acridotheres tristis melanosternus*).

The observations made by Huff *et al.* (1960) by means of time-lapse cinemicrography on exoerythrocytic stages were briefly described in the earlier review (Huff, 1963). More detailed descriptions of the behavior of merozoites, entry of merozoites into host cells, nuclear division in the trophozoites, schizogony, and the inter-relations between host cells and parasites were given by Huff and Weathersby (1963).

Although previous evidence (see Bishop and Birkett, 1948) had been presented which suggested that secondary exoerythrocytic stages of *P. gallinaceum* resulting from a paludrine-resistant strain could transfer this property to erythrocytic stages derived from them, Beaudoin *et al.* (1967) gave a more direct proof of this possibility. They adapted a pyrimethamine-resistant strain of this parasite to tissue culture in which it was grown until all erythrocytic stages were lost. Upon introducing only the exoerythrocytic stages into chickens and subjecting the resulting strain to drug pressure no loss of resistance was found. Hence no erythrocytic stages were involved in the transfer of resistance.

VI. Fine Structure

A. Erythrocytic Stages

Phagotrophy of erythrocytic cytoplasm of the host cell by *P. gallinaceum* similar to that previously observed by Rudzinska and Trager (1957, 1961) in *P. lophurae* was reported by Ristic and Kreier (1964). Meyer and de Oliveira Musacchio (1965) were unable to observe any evidence of phagotrophic action in *P. gallinaceum* and concluded that uptake of nutrient material from the host cell was through the parasite membranes. They observed the organelles in merozoites which have since been called "paired organelles" but described

them as being near the "distal pole" of the merozoite. The reviewer believes that it would be more meaningful to refer to this location as the "anterior pole", since this is the forward end of the organism when it is free-swimming or actively penetrating a host cell.

New light was thrown on the mechanisms by which intracellular, malarial parasites obtain their nutriment, by a series of papers resulting from collaborative work between the Department of Parasitology, Naval Medical Research Institute (Bethesda, Maryland) and the Department of Experimental Pathology, Walter Reed Army Institute of Research (Washington, D.C.). Aikawa *et al.* (1966a) demonstrated the presence of an organelle corresponding in position and structure with the "micropyle" described in sporozoites by Garnham *et al.* (1960). This organelle was found in merozoites, schizonts and gametocytes of *P. fallax*, *P. lophurae*, and *P. cathemerium* and, because of its obvious function, was named a *cytostome*. Early in the intracellular lives of these stages this organelle began to perform an orificial function hitherto undescribed. The membrane of the cytostomal cavity was expanded and large quantities of erythrocytic cytoplasm were engulfed. This engulfed material underwent progressive digestion until very little of it remained except residual hemozoin granules. Aikawa *et al.* (1966b) extended these observations to *P. knowlesi* and *P. cynomolgi* and although they found differences in size of the cytostomes in these simian parasites, they concluded that the process of feeding is essentially the same in the simian as in the avian parasites. In addition to the presence of the cytostome, Aikawa (1966) described the very complex structure of the merozoites of the above three species of avian malaria. The organelles such as the conoid, paired organelles, dense bodies, spherical body, pellicular complex, nucleus, mitochondrion, ribosomes, and endoplasmic reticulum were described and illustrated. The complex changes incident to (1) the transformation of merozoite into trophozoite, (2) schizogony, and (3) reformation of merozoites, were described. Excellent micrographs of mitotic figures, including presumed chromosomes, were presented. In this paper as well as in an earlier one (Aikawa *et al.*, 1966a) the importance of membranes and of membrane formation was emphasized.

B. EXOERYTHROCYTIC STAGES

Concomitant with the studies on fine structure of erythrocytic stages, Hepler *et al.* (1966) carried out studies of exoerythrocytic stages of *P. fallax* grown in tissue culture. They found a close parallelism in structure between these stages and those reported by Aikawa (1966) for the erythrocytic stages, particularly in regard to the sequences of changes during schizogony, the presence of the cytostome, and the changes undergone during transformation of merozoites to trophozoites. The cytostomes of the exoerythrocytic stages were 80–100 mμ in diameter, whereas those of the erythrocytic stages had been shown to be 280–340 mμ (Aikawa *et al.*, 1966a; Hepler *et al.*, 1966). No evidence of ingestion of host cell cytoplasm by the former was observed by Hepler *et al.* (1966). Aikawa *et al.* (1967) observed feeding by cytostomes of *P. elongatum* in a completely graded series of host cells from stem cells, through the erythroblastic series to mature erythrocytes. In host cells lacking hemoglobin, the ingested

material took the form of boluses which did not contain malarial pigment. Since all previous observations of the ultra-structure of exoerythrocytic stages were made on parasites grown in culture, Aikawa *et al.* (1968) made a comparative study of exoerythrocytic stages of *P. gallinaceum in vivo*. Although their observations were confined to the liver of the chick embryo, they found essentially no differences between the parasites in that location and those which had previously been studied *in vitro*. The endothelial cells of the sinusoids, which served as host cells, appeared to suffer no ill effects from the parasitism since the only observable change attributable to the infection was the increased number of lipid droplets in their cytoplasm. Many interesting details of these papers by Aikawa, Hepler, and co-workers have had to be omitted from this review because of limitations of space.

C. MEROZOITES

Some observations on free merozoites were cited above in the work of Meyer and de Oliveira Musacchio (1965). In addition, the papers of Aikawa (1966), Aikawa *et al.* (1966a, 1967) and Hepler *et al.* (1966) present many details of both erythrocytic and exoerythrocytic merozoites of several species of avian malarial parasites. A more detailed study of the pellicular complex of the exoerythrocytic merozoites of *P. fallax* was made by Aikawa (1967) by negative staining and thin-sectioning techniques. These methods clearly revealed this complex to consist of three layers: (a) a thin outer membrane, (b) a thick interrupted inner membrane, and (c) a partial layer of microtubules. The thick interrupted inner membrane proved to consist of a labyrinthine structure covering the entire surface of the merozoite except in the vicinity of the conoid and cytostome. All of these findings, in addition to numerous observations by histological techniques and phase microscopy by other investigators, clearly show the great differences which exist between such motile stages as merozoites and the parasitic, intracellular forms. The complex changes observed in fine structure found during the transformations from merozoite to trophozoite and from schizont to daughter-merozoites add still another dimension to the complexity of malarial parasites.

D. SPOROZOITES AND OOKINETES

Earlier accounts of the fine structure of sporozoites and ookinetes were described in my earlier review (Huff, 1963). Also, see Garnham *et al.* (1960, 1962). The paper by Garnham *et al.* (1963) on the sporozoites of mammalian malarial sporozoites, although not falling within the purview of this report, may be consulted for its bearing on the structure of sporozoites in general.

E. EXOGENOUS STAGES

The papers by Terzakis *et al.* (1966, 1967) on the ultrastructure of the exogenous stages of *P. gallinaceum* throw considerable light on the morphological changes undergone in sporogony. Electron microscopical studies on oocysts in *Aedes aegypti* revealed an extremely complex series of events in the formation and development of sporoblasts and the production of sporozoites.

Formation and fusion of vacuoles in the oocyst cytoplasm precede the formation of sporoblasts. The sequence of events in the formation of sporozoites in the sporoblasts resembles essentially that which has been described for merozoite formation in schizogony (cf. Hepler *et al.*, 1966; Aikawa, 1966). Following extensive nuclear division in the oocyst, the nuclei migrate to a peripheral position in the sporoblast. Early thickenings of the sporoblastic plasma membrane mark the sites of beginning sporozoite formation. *In situ* formation of paired organelles, conoid, dense bodies, elongation of the forming, sporozoite body and migration of nucleus and mitochondria into the peninsular body are all strikingly similar to the steps reported by others for merozoite formation. (Readers are referred to the paper by Vanderberg *et al.*, 1967, on sporozoite formation in *P. berghei*, for comparison with the work reported above on *P. gallinaceum*.)

VII. Immunity

Many of the early advantages offered by avian malaria in the study of immunity such as small size, ready availability, and low cost of the experimental hosts and the ease of transmission by mosquitoes have been overshadowed by the availability of rodent malarias. Moreover, rodent and simian malarias offer the advantage of being parasites of mammals, and therefore, are more comparable to human malarial parasites than the avian malarias. Nonetheless, an appreciable number of publications on immunity to avian malaria have recently appeared.

Initially attention should be called to the pertinent reviews which have appeared. The review by Stauber (1963) emphasized the problems existing in the *in vivo* phases of immunity to intracellular parasites. A considerable portion of this excellent treatise is devoted to immunity to malaria. Zuckerman (1964) reviewed the general theories which apply to autoimmunization and specifically how they apply to malaria. Other pertinent reviews are to be found in Part I of a volume edited by Garnham, Pierce and Roitt (1963) and the chapter by Taliaferro and Stauber (1968).

Publications yielding light on antigens are first considered. Using the fluorescent-antibody technique Voller (1962) showed that sera of *P. gallinaceum* and *P. juxtanucleare* were species- but not strain-specific and that therefore these two species have no common antigens. Sherman (1964), employing the Ouchterlony and immunoelectrophoretic techniques, demonstrated that one of the major antigenic components of the erythrocytic stages of *P. lophurae* is hemoglobin (and that hemozoin is non-antigenic). He demonstrated four to six precipitating antigens in antisera produced in rabbits by inoculation of formalinized erythrocyte-free parasites. His results indicate the value of the immunoelectrophoretic method in distinguishing between parasite and host antigens.

Spira and Zuckerman (1966) utilized the disc electrophoretic technique in differentiating between host cell free extracts of seven different malarial parasites—3 rodent, 3 simian malarias and *P. gallinaceum*. Todorovic *et al.* (1967) showed that antigens isolated and purified from the sera and plasma of

chickens with acute infections with *P. gallinaceum* were proteinaceous. Two antigen–antibody systems were detected. The soluble antigens, when injected into chickens, protected them against *P. gallinaceum*. The antisera agglutinated and lysed infected erythrocytes, *in vitro*. Richards (1966) confirmed the earlier experiments on immunization by sporozoites and also showed that antigen from erythrocytic parasites was capable of producing some immunity against homologous erythrocytic forms. He also found that sporozoite antigen did not give good protection against homologous erythrocytic stages. There is need for more complete studies on the antigenicities of sporozoites and erythrocytic stages.

Two reports were made on the use of fluorescent antibody staining. Voller and Taffs (1963) demonstrated immunofluorescent staining of exoerythrocytic stages of *P. gallinaceum* in sections from infected chickens. There were no cross reactions between the schizonts of *P. gallinaceum* with *P. juxtanucleare* immune serum. They were unable to determine whether blood and tissue stages possess common antigens. Corradetti *et al.* (1964) demonstrated the specific action of the fluorescent antibody technique in distinguishing sporozoites of two species of avian malaria. Fluorescent antibodies were prepared against sporozoites of *P. gallinaceum* and tested against the sporozoites of this species and *P. giovannolai*. There was intense staining against the homologous sporozoites but none against the heterologous sporozoites.

Although it is questionable just how effective agglutinins are in aiding the immune process, the experiments of Kreier *et al.* (1965), utilizing the capillary agglutinin test on erythrocytic stages of *P. gallinaceum* freed from their host cells, extended the findings previously reported by others on *P. lophurae*. They carried out their tests using formalinized parasites and plasma from chickens in various stages of infections and recovery.

Previous workers have shown that, regardless of the species of parasite or its host, splenectomy resulted in a decrease in immunity, which resulted in relapse and recrudescence of the infection. Longnecker *et al.* (1966) found more recently that chicks bursectomized (hormonally or surgically) on the first day after hatching developed higher parasitemias to *P. lophurae* than sham-operated controls, but birds bursectomized at 18 days of age developed no higher parasitemias than the control animals. More severe parasitemia resulted in splenectomized chicks, while thymectomy had no significant effect on the parasitemia.

A study was made on the interrelations between parasitemia (*P. gallinaceum*) from blood-induced infections in chicks and the phagocytic index by Cox *et al.* (1963). The phagocytic index was obtained by the colloidal carbon technique in which this material is injected into a vein; serial samples of blood were taken daily, the sample was treated with ammonium carbonate and the density of the carbon in the sample was measured optically. There was an initial stimulation of phagocytosis within 24 h followed by a steady increase to the 4th day at which parasites appeared in the blood. The phagocytic index then dropped rapidly, concomitant with the increase in parasitemia. The authors postulate that this fall in reticulo-endothelial activity is possibly due to release of toxic substances resulting from rupturing schizonts.

One area in which studies on avian malaria in immunological studies hold an advantage over the mammalian malarial parasites is the ease with which their exoerythrocytic stages can be cultivated. Exoerythrocytic stages of at least three species of avian malarial parasites may be grown abundantly in chick embryos, and more recently Davis *et al.* (1966) have succeeded in growing large numbers of exoerythrocytic stages of *P. fallax* in cell cultures free from erythrocytic stages. Exploitation of this technique may be expected to make possible the analysis of the antigenic potencies of the two forms of asexual stages of the parasite.

VIII. Haemoproteus and Leucocytozoon

Haemoproteus. Baker (1963) reported the transmission of a species of *Haemoproteus* from domestic pigeons by the bite of *Ornithomyia avicalaria.* He showed that microgametocytes required only 5 days for maturation, whereas macrogametocytes required 6 days. Bradbury and Trager (1967) described axoneme formation in *H. columbae*; and the fine structure of the formed microgametes was described by Trager and Bradbury (1967).

Leucocytozoon. While studying an epidemic of *Leucocytozoon caulleryi* in chickens in Taiwan, Pan (1962) proposed a novel theory of gametogony. His theory essentially involves the possibility of two generations of multiplication in the line of development from merozoite to mature gametocytes. In the absence of more detailed descriptions of the various parasites and of the types of cells serving as hosts it is difficult to evaluate his hypothesis. Another interesting study of schizogony and gametogony was made by Desser (1967) in experimentally, sporozoite-induced infections of *L. simondi* in White Pekin ducklings. He described the hepatic schizonts and presented evidence to show three possible courses of their development: (1) they may reinvade hepatic parenchyma cells to initiate another generation of hepatic schizonts, (2) they may penetrate circulating blood cells and grow into round gametocytes, or (3) they may be phagocytized by macrophages and be carried to various sites in the host where they develop into megaloschizonts. He believed that the elongate gametocytes originate from the merozoites of the megaloschizonts. This theory offers a very promising explanation of the interrelations of the two types of schizonts in this infection.

Clark (1964) reported on the frequency of infection and seasonal variation of *L. berestneffi* in the yellow-billed magpie (*Pica nuttalli*). He found throughout the year a significantly higher gametocyte frequency and higher density level in tissue smears than in blood smears. The timed-search method showed that the kidney far exceeded the other organs in number of gametocytes present. Incidence and intensity of infection increased during the spring months with a peak in March; the lowest findings were in August and September. However, appreciable incidence was found in all months of the year. Schizogony and gametocyte development of *L. berestneffi* was described by Clark (1965). Schizonts of the hepatic type only were observed and they occurred in liver, spleen, and kidney, which represents the first report of this type of

schizont in any organ except the liver. Failure to find hemoglobin in the cells infected with gametocytes, together with the morphological characteristics of these cells, led the author to conclude that gametocyte development occurs exclusively in lymphocytes.

Two peaks of gametocyte density in *L. simondi* were demonstrated by Kocan and Clark (1966) in ducks which had either been exposed to black flies for 24 h or intraperitoneally inoculated by ground-up black flies. The first peak occurred on the 10th post-exposure day and was composed entirely of round forms, while the second, which was composed of about 90% elongate forms, occurred on the 14th–16th post-exposure day. Although these results as well as those reported by Desser indicate a sequential relationship between the two types of schizonts, in neither case can one rule out the possibility that two species of parasites with different prepatent periods are involved because of the fact that, in each case, wild-caught vectors were utilized in effecting the parasitism. Fallis and Bennett's (1966) study on epizootiology of infections by *L. simondi* in Algonquin Park, Canada supplied interesting data on transmission, feeding, habitat, longevity, and flight range of the vectors, and length of life cycle of the parasite. Severe and fatal infections were contracted in ducks exposed to flies along a lake shore in May, June and July. Transmission occurred also over the water 100 yards from the shore, in the woods, and occasionally 15 ft above water or ground level.

Two interesting studies have been reported by Kocan and Clark (1966) and Kocan (1967) on anemia in ducks infected with *L. simondi*. In the former, anemia (studied in 34 ducklings) was shown to last for the duration of the initial parasitemia and reached its low point early in the infection. Anemia decreased as the parasitemia decreased. Greatest parasitemia occurred 5–8 days after its detection. Recovery from anemia began in some instances prior to the highest level of parasitemia. Total anemia could not be accounted for by the numbers of parasites observed. It was believed that the short duration of increased hemopoiesis might be accounted for by over-compensation for erythrocyte loss at the end of primary parasitemia. Kocan (1967) tested for agglutinins and hemolysins against erythrocytes from uninfected ducks during the course of infection. During the acute attack agglutinin titers as high as 1:100 and hemolysins as high as 1:64 were observed. Agglutination of their own cells occurred in all birds during the period of hemolysis but only at temperatures above 25°C. No erythrophagocytosis was observed in spleen presses and bone marrow smears. Kocan suspected that intravascular hemolysis might be the result of contact with the anti-erythrocyte factor and hence the direct cause of anemia.

A promising step has been made by Morii *et al.* (1965) in the direction of reducing the number of unknown factors in the life cycles of *Leucocytozoon*. They used chickens which had never been exposed to the bites of midges and laboratory reared *Culicoides arakawae* and *C. circumscriptus*. The midges were fed upon chickens infected with *L. caulleryi* and held in cages in small lots at temperatures of 10°C–30°C for periods of 3–25 days. Sporozoites were dissected from the midges and inoculated in known numbers into clean chicks. A second transmission was carried out in the laboratory.

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Coccidia and Coccidiosis in the Domestic Fowl

P. L. LONG AND C. HORTON-SMITH

Houghton Poultry Research Station, Houghton, Huntingdon, England and Near East Animal Health Institute, Beirut, Lebanon

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I. Introduction

Since the original review was written (Horton-Smith and Long, 1963) a great deal of work has been published. A new species of *Eimeria* has been described and the life cycles and pathogenicity of others have been re-examined. Species of *Eimeria* have been cultivated in the allantoic membranes of the developing chick embryo and some progress has been made in the cultivation of *Eimeria* species in tissue cultures. More work has been published on factors affecting reproduction and pathogenicity of *Eimeria* and on acquired immunity to *Eimeria* infections in the fowl. These subjects are considered in this brief review (which should be read in conjunction with the original review). Because little has been published since 1963 on turkey coccidiosis and cytochemistry of avian coccidia, these subjects have been omitted.

II. The Life Cycles of *Eimeria* Species in Chickens

A. *Eimeria mivati*

This new species was named by Edgar and Siebold (1964), who described four schizogonous generations and showed that the first oocysts were discharged 93 h after infection. This species showed marked similarities to *E. acervulina*, especially in the oocyst characters and prepatent time. When this description was published the life cycle of *E. acervulina* was not fully known,

but it has since been studied by several workers and will be discussed in the next section.

Edgar and Siebold (1964) showed that schizonts of the first generation developed at 36–40 h in the crypts of Lieberkuhn, those of the second generation in the crypts at 55–60 h after infection, and those of the third and fourth generations in the epithelial cells of the villi at about 80 h and 93–96 h after infection respectively. The first gametocytes developed from third-generation merozoites but the major production of gametocytes followed the fourth generation of schizogony.

Edgar and Siebold also showed that chickens which were immune to *E. mivati* were susceptible to eight other species of *Eimeria*, and that chickens immune to eight other species of *Eimeria* were susceptible to *E. mivati*. Reid *et al.* (1965) described *E. mivati* in European chickens, and the species was then isolated in the Lebanon (Long and Tanielian, 1965) and in Britain (Long, 1967a). In the last two papers the life cycle of *E. mivati* was compared with that of *E. acervulina*.

B. *Eimeria acervulina*

Tyzzer (1929) originally described one schizont generation with about 32 merozoites in the epithelial cells of the villi on the third day of infection. Recent work has shown that the sporozoites of *E. acervulina* invade the fundi of the deep glands and that the first generation develops here about 36 h after infection (see Wagner, 1965; Long and Tanielian, 1965; Ball, 1966; Doran, 1966).

Vetterling and Doran (1966) concluded that four generations of schizonts occur; the first 36–48 h, the second 41–56 h, the third 56–76 h and the fourth 80–96 h after infection. They reported that the first generation is situated in the bases of the glands, the second in the necks of the glands, the third at the bases of the villi and the fourth on the sides and tips of the villi. Warren and Ball (1967) considered that at least three generations of schizonts appear before gametogony begins. An additional generation occurs after the initial gametogony which might account for the observed second peak in oocyst production 7–9 days after infection. Long (1967a) described three generations of schizonts similar in morphology and timing to those described by Vetterling and Doran (1966) but considered that four generations of schizonts are necessary to account for the reproduction potential of this species.

The endogenous stages of *E. acervulina* and *E. mivati* are similar; the average sizes and timing of the schizont generations of both species, given in Table I, are based on data collected by Vetterling and Doran (1966) and Edgar and Siebold (1964) respectively. Reference to this Table shows some similarities between *E. acervulina* and *E. mivati*, but the main differences concern the morphology of the first and fourth generation schizonts and the timing of the third and fourth generation schizonts. The first generation schizonts of *E. acervulina* observed by Long (1967a) were 5–8 μ in diameter and contained 8–16 merozoites; those described by Warren and Ball (1967) were 4–6 μ in diameter and contained 8–12 merozoites. The size of the first schizont, as given

TABLE I

Dimensions of the schizonts and merozoites of Eimeria acervulina *and* E. mivati

	1st Generation		2nd Generation		3rd Generation		4th Generation	
	E. acervulina	*E. mivati*	*E. acervulina*	*E. mivati*	*E. acervulina*	*E. mivati*	*E. acervulina*	*E. mivati*
Size schizont (microns)	10·4 × 7·0	10·4 × 10·1	5·2 × 4·3	9·2 × 7·2	4·1 × 4·4	6·6 × 5·0	8·4 × 6·8	12·9 × 11·2
Number of merozoites	12–20	10–30	12–16	16–20	8–10	10–14	28–36	16–20
Length of merozoites (microns)	4·0–4·5	8·6–10·6	3·9–4·5	5·6–8·8	5·2–5·8	5·6–6·7	9·1–10·3	7·5–9·6
Time (h) of appearance of schizonts	36–48	36–45	41–56	55–65	56–72	80	80–96	93–96

by Long and by Warren and Ball, differs considerably from that given by Vetterling and Doran (1966), who described the schizont as being $10{\cdot}4 \times 7{\cdot}0\,\mu$.

C. *Eimeria praecox*

Eimeria praecox was first described by Johnson (1930) and later in greater detail by Tyzzer *et al.* (1932), who concluded that at least two generations of schizonts occur. This parasite, which has the shortest prepatent period of any species of *Eimeria* found in chickens, appears to be restricted to the epithelial cells of the villi in the upper small intestine. Long (1967b) described at least three generations of schizonts occurring before gametogony. The first generation schizonts, which measure 9–10 μ, are found 24–40 h after infection and contain 16 merozoites of about 10 μ in length. The schizonts of the second generation develop about 65 h after infection; these are about 14 μ in diameter and have 16–20 merozoites of about 10 μ in length. The third generation schizonts develop about 83 h after infection, measure about 20 μ and contain about 50 merozoites which are 7–9 μ long. Immature and mature gametocytes were seen in stained sections 83 h after infection. The first oocysts to be produced were detected in the faeces 83½ h after infection, although the peak in oocyst production occurred at about the 6th day after infection. Some small schizonts (8–10 μ diameter) were seen 116 h after infection and may account for further gametogony and oocyst production 24–72 h later. Oocyst production ceased about the 11th day of the infection. The reproductive potential of this species is in inverse proportion to the magnitude of the initial dose of oocysts. For example, the reproduction index (R.I. = number of oocysts produced per oocyst administered) in groups of 3-week-old Brown Leghorn chickens given 10^4, 10^5 or 10^6 oocysts was approximately 313, 174 and 15 respectively. The reproduction was also influenced by the age of the host, e.g. 10-day-old Brown Leghorn chickens given 10^5 or 10^6 oocysts had reproduction potentials of 229 and 23 respectively, whereas in groups of 6-week-old chickens of the same breed given similar doses, the reproduction indices were 2794 and 199. In groups of 6-week-old chickens where the oocyst production was between 200–330 million oocysts per bird there was no mortality or evidence of morbidity.

The maximum reproduction (R.I. = 190000) was obtained with a dose of 250 oocysts. From the three schizont generations described by Long (1967b) a reproduction of approximately 64000 oocysts may be deduced. Clearly a fourth generation schizont is necessary to account for the maximum reproduction obtained. A high degree of resistance to re-infection results from a single dose of oocysts and in this respect *E. praecox* is similar to *E. maxima* (Tyzzer, 1929; Long, 1959).

III. Biological Characteristics of *Eimeria* in the Fowl

A. Site Selection

Species of *Eimeria* usually have a marked predilection for a given site and, in the fowl, have not been found in sites other than the intestines. However, Horton-Smith (1966) showed that *E. necatrix*, which in natural infections

develops its asexual cycle in the small intestine and its sexual cycle in the caeca, can complete the cycle in the caeca when sporozoites are inoculated directly into the caeca. The appearance of the caeca during second generation schizogony of *E. necatrix* was rather similar to that of a haemorrhagic *E. tenella* infection. This work was extended to include *E. brunetti*, *E. acervulina*, *E. maxima*, *E. mivati* and *E. praecox* (see Horton-Smith and Long, 1965, 1966; Long, 1967b). Similarly it was found that *E. brunetti* and *E. mivati* completed their life cycles in the caeca but *E. acervulina*, *E. maxima* and *E. praecox* did not, which indicated that these last three species are more restricted in their sites of development. In natural infections none of these species invades the caeca at any stage of their life cycle, whereas *E. brunetti*, *E. mivati* and *E. necatrix* do. An example of extreme rigidity in site selection was described by Long (1967b); *E. praecox* sporozoites inoculated into the caeca did not develop here but, in several tests, gametocytes and oocysts were found in the upper intestine (the usual site) at the expected time.

Sporozoites of *E. tenella* hatched *in vitro* and, inoculated into the allantoic cavity of chick embryos 9–11 days old, invaded the allantoic membranes and completed their development with production of oocysts (Long, 1965). *Eimeria brunetti*, *E. mivati* and *E. necatrix* also developed in this site but *E. acervulina*, *E. maxima* and *E. praecox* from the chicken and *E. stiedae* from the rabbit did not (Long, 1966); *E. tenella* failed to grow in turkey embryos. In the chick embryo, *E. brunetti* and *E. mivati* completed their life cycles but *E. necatrix* did not develop beyond second generation schizogony. It is interesting to note that those species which developed partly or wholly in the embryo allantois could also complete their life cycles in the caeca.

An immediate practical application of these techniques involves the recognition and separation of *Eimeria* species in the fowl. Long and Tanielian (1965) were able to identify and separate *E. mivati* from *E. acervulina* by using these techniques. It might be possible to separate *E. tenella* from *E. necatrix* or *E. brunetti* from *E. maxima* by using either of the techniques. A summary of these findings together with other major characters of the eight species of *Eimeria* of the fowl is shown in Table II. Horton-Smith and Long (1963) discussed the then recent observation that sporulated oocysts injected parenterally would initiate infections at the usual (intestinal) site (Landers, 1960; Sharma and Reid, 1962; Davies and Joyner, 1962); in all these studies much lighter infections occurred after parenteral compared with oral inoculation. Sharma (1964) found no delay in the life cycles and claimed that oocysts were produced at the usual times. This was not in agreement with the findings of Davies and Joyner (1962) who noted some delay in the appearance of oocysts and suggested that sporozoites released at the parenteral site may migrate to their normal site for development. Patnaik (1966) reported for *E. tenella* a delay of 2–3 days in the appearance of oocysts in the faeces and suggested that it was due to the delay in excystation of the sporulated oocysts after inoculation at the parenteral site. He placed sporulated oocysts in millipore chambers grafted within muscles and found that the sporozoites excysted within the chambers and produced infection at the caecal site. The millipore chambers became infiltrated with leucocytes and he suggested that these cells produced enzymes capable of

TABLE II

A summary of some major characters of seven species of Eimeria *of the fowl*

Species	Development in embryo allantois	Development in caeca after sporozoite inoculation	Development in caeca during normal infection	Mean oocyst dimensions (In μ)	Prepatent time (h)	Site of early schizogony	Degree of pathogenicity	Immunity after single exposure to infection
E. tenella	+	+	+	22 × 19	138	Fundi of deep glands	+ + + +	No
E. necatrix	+*	+	(+)	20·4 × 17·2	138	Fundi of deep glands	+ + + +	No
E. mivati	+	+	(+)	15·9 × 13·7	93	Fundi of deep glands	+ +	No
E. acervulina	—	—	—	17·4 × 13·2	97	Fundi of deep glands	+ +	No
E. brunetti	+	+	(+)	24·6 × 18·8	120	Villus epithelium	+ + +	No
E. maxima	—	—	—	30·8 × 23·3	121	Villus epithelium	+ + +	Yes
E. praecox	—	—	—	20·4 × 17·4	83½	Villus epithelium	Weak	Yes

+* = development including 2nd generation schizogony but no gametogony.
(+) = late life cycle stages produced.

causing excystation. He also found macrophages containing engulfed sporozoites within the millipore chambers and postulated that they might carry the sporozoites to various parts of the body and that only those which reached the caeca developed further. This theory was supported by the consistently low infections produced by injection of sporozoites by parenteral routes.

B. *In vitro* CULTIVATION

Patton (1965) reported that sporozoites of *E. tenella* invaded mammalian fibroblasts, epithelial cells and avian fibroblasts grown in tissue culture and developed into what appeared to be first generation schizonts. He observed multiple invasion of tissue cultured cells by sporozoites and noted that only a small number of trophozoites developed to schizogony. The schizonts observed had merozoites 7–8 μ in length, i.e. longer than the first generation merozoites but smaller than the second generation merozoites seen in normal infections. He also found that an incubation temperature of 41°C was necessary for development; none occurred at 37°C.

Strout *et al.* (1965), working with *E. acervulina*, used chick embryo kidney cells, chick embryo fibroblasts, mouse fibroblasts, human amnion cells and HeLa cells and found that sporozoites invaded all these cells. Trophozoites were seen after 18–24 h and in a few instances some segmentation occurred but there was no development into mature schizonts. Multiple invasion of cells by up to 16–20 sporozoites was observed. Doran and Vetterling (1967), working with the turkey coccidium *E. meleagrimitis*, reported development of some parasites to mature schizonts in bovine kidney cells. They also found some development of the second generation schizonts. Although the schizonts were almost twice the normal size the timing of the stages was identical. Invasion of porcine kidney cells also occurred but no development ensued. Multiple invasion of cells by sporozoites was common and when infection with up to four sporozoites occurred they were usually found side by side and were orientated in the same direction. The work so far carried out on tissue culture of *Eimeria* is encouraging and is being intensified, but it is still a long way from successful culture of the entire endogenous cycle. Patton's observation that a temperature of 41°C is necessary, and the fact that he and Doran and Vetterling obtained the best results with mammalian cell lines (bovine kidney) and rather poor results with chicken and porcine cell lines, are of interest. All workers have found that sporozoites need to be carefully washed free from coccidial debris (oocysts and sporocysts) to avoid toxic effects on the tissue cultures, a finding parallelled by the toxic effects of coccidial material inoculated into rabbits (Sharma and Foster, 1964; Rikimaru *et al.*, 1961).

IV. FACTORS AFFECTING THE REPRODUCTION AND PATHOGENICITY OF *Eimeria* IN THE FOWL

A. EFFECT OF AGE OF HOST

Some authors suggest that young chickens are more susceptible than older ones (Tyzzer, 1929; Karmann and Presch, 1933); conversely, others suggest that the reverse is true (Tyzzer *et al.*, 1932; Mayhew, 1934; Jones, 1932;

Horton-Smith, 1947). It is important to distinguish between susceptibility of fowls to the clinical disease and the susceptibility of fowls to coccidial infection as measured by oocyst production. It may be that although the reproduction of the parasites is less in the young birds the clinical effects are greater. Krassner (1963) pointed out that there are more cells available in older birds and that a dose of 10^6 oocysts may be too high for young chickens. Although this dose may have a considerable effect on the condition of the young bird, only a percentage of the parasites are able to develop and produce oocysts. Judged by oocyst production, older birds are clearly more susceptible to the parasites than younger ones. This has recently been shown to be true with a number of species of *Eimeria*, e.g. *E. maxima* (see Long, 1959), *E. acervulina* (see Krassner, 1963), *E. praecox* (see Long, 1967b) and *E. tenella* (see Rose, 1967a). Increased oocyst production in older chickens is due, at least in part, to better excystation of the oocysts in the alimentary tract (Doran and Farr, 1965; Rose, 1967a). This is probably one factor and acts in conjunction with the availability of more intestinal cells, and possibly better physiological conditions in the intestines of older birds. One of the difficulties encountered in this type of work is that of keeping chickens completely free of infection for long periods of time and, clearly, special isolation facilities are essential.

B. BREED OF HOST AND SUSCEPTIBILITY TO COCCIDIAN INFECTIONS

As previously noted, different strains of chickens vary in their susceptibility to *E. tenella* and resistance can partially be controlled by hereditary factors (Rosenberg, 1941; Champion, 1954). Patterson *et al.* (1961) found the differences in the mortality caused by four species of *Eimeria* in several strains of White Leghorn chickens. They concluded also that selection for resistance to one disease (e.g. chronic respiratory disease and visceral lymphomatosis) did not necessarily result in resistance to others.

Over the past few years we have observed differences in the susceptibilities of different breeds to some *Eimeria* infections. This has been examined in greater detail using mortality, depression of body weight gain and oocyst production as criteria for measuring possible effects on the host. Decline in body weight gain was accompanied by increased reproduction of the parasites and, in general, mortality rates were also associated with increased reproduction of the parasites. A strain of Light Sussex chickens was more susceptible than a strain of Rhode Island Reds to *E. acervulina, E. brunetti, E. maxima* and *E. mivati* but was not more susceptible to *E. tenella*. The Brown or White Leghorn strains used were much more susceptible to several *Eimeria* species than the Rhode Island Reds (Long, 1968).

V. The Effect of Parasitism on the Host

Stephens (1965), working with *E. necatrix*, observed an increase in the erythrocyte sedimentation rate (E.S.R.) in infected chickens on the 6th day of

infection, which returned to normal on the 16th–18th days of the infection. The increases in E.S.R. were inversely related to the packed cell volume (P.C.V.) recordings. The light transmittance of the plasma of infected birds was also increased and this may be due to the inability of infected chicks to absorb carotenoid pigments. The light transmittance of the plasma of infected birds was also related to the rate of weight gain of infected chickens, indicating that the birds failed to absorb essential nutrients via the intestines. There was also a decrease in hydrogen ion concentration of gut contents on the 6th day after inoculation. A study of infection with *E. maxima* by Stephens *et al.* (1967) showed that there was less effect on packed erythrocyte volume than with *E. tenella* or *E. necatrix*, although there was a lower value 6–8 days after infection. Haemoconcentration measured by P.C.V. was found on the 14th day after inoculation. Apart from depression of body weight gain the most dramatic effect of the disease was the elimination of the yellow pigment from the plasma. These values were similar to those of Douglas (1966), who reported decreased blood xanthophyll levels in chickens infected with *E. maxima*. The maximum effects of *E. maxima* on weight gain, pH of gut contents, E.S.R., P.C.V. and light absorption of plasma occur on the 6th–8th days, which is the period associated with the gametogonous stages of the life cycle.

Bertke (1963) studied renal clearance in chickens infected with *E. tenella*, using uric acid as a marker. He observed that the rate of clearance was greatest 2–4 days after infection. He concluded that renal clearance measurements were indicative of systemic changes caused by the parasite and that infected chickens were in a state of shock. The changes were possibly caused by toxic material produced by or because of the response to the presence of the parasite, which indicates that death from caecal coccidiosis may not be caused solely by caecal damage or caecal haemorrhage.

These studies indicate that in addition to blood loss, which is considerable in acute *E. tenella* and *E. necatrix* infections, the parasites cause many other effects on the host. Preston-Mafham and Sykes (1967) have shown that changes occur in the permeability of the intestinal wall during active infection with *E. acervulina* and that these changes are concurrent with a depression in the absorption of L-histidine, D-glucose and fluids. Working with the same parasite Pout (1967) showed that villus atrophy occurred during the peak of infection; the villus height of infected intestinal tissue was appreciably shorter than in the controls. The smaller surface area resulting could be one reason for reduced absorption of nutrients and liquids.

Toxin production by coccidia has not been demonstrated conclusively since toxic effects have only been shown by inoculating rabbits with extracts prepared from the parasites. Daugherty and Herrick (1952), however, demonstrated that a substance from the caecum during the acute phase of caecal coccidiosis was capable of reducing the capacity of chick brain to utilize glucose. We have inoculated large amounts of oocyst extracts into chickens without any apparent effect. However, it may be that toxic products are produced only by the interaction of the parasite and the host cell and that these toxins are extremely labile.

VI. Immunity to *Eimeria* Infection

In previous reviews on immunity to coccidiosis (Horton-Smith and Long' 1963; Horton-Smith *et al.*, 1963a), it was reported that infection of chickens by coccidia stimulates a high degree of resistance to reinfection. Acquired resistance is accompanied by the presence of serum antibodies but similar antibodies can be produced in chickens inoculated with extracts of the coccidia. It was not possible to protect chickens by the passive transfer of immune sera nor was it possible to transfer protection passively from immune hens to their progeny. More recently Long and Rose (1965) attempted active and passive immunization of chickens infected with sporozoites by the intravenous route. Sporozoites of *E. tenella* inoculated intravenously produce infection at the usual site although there is considerable loss of parasites. When immune serum (or globulin) was introduced 4½ h before the introduction of sporozoites, a high degree of protection developed. If the immune serum (or globulin) was introduced 19 h after infection with sporozoites there was little or no protection. The results support the view that serum antibodies are effective *in vitro* and *in vivo* provided that the invasive stages are exposed directly to antibody, but when the parasites have invaded the epithelium of the intestine, circulating antibody is not effective.

Lysins found in immune sera are active *in vitro* against sporozoites and merozoites (Long *et al.*, 1963). Burns and Challey (1965) confirmed that lysis of merozoites of *E. tenella* occurred in immune but not normal sera. However, they considered that normal sera lysed sporozoites of *E. tenella*. This finding is in accord with the view of Augustin and Ridges (1963) that normal turkey sera lyses the sporozoites of *E. meleagrimitis*. Herlich (1965) obtained results similar to those reported by Long *et al.* (1963), and showed that immune serum lysed both sporozoites and merozoites of *E. tenella in vitro* but normal serum and *E. acervulina* immune serum did not. Herlich also found that intestinal and caecal tissue extracts were ineffective against these stages.

Horton-Smith *et al.* (1963b) showed that sporozoites of *E. tenella* harvested from the caecal lumina of immune fowls produced in susceptible fowls infections of similar magnitude to infections produced by sporozoites harvested from non-immune chickens. They observed that both sporozoites and second generation merozoites invaded the caecal epithelium of immune chickens and reached their normal site for development. No growth occurred and the parasites were not seen at the 72nd h of the infection. Leatham (1966) and Leatham and Burns (1967) confirmed these results and further observed that sporozoites recovered from immune caecal tissue 48 h after inoculation were not infective to susceptible chickens. Leatham also found that immunity to clinical symptoms of *E. tenella* diminished between 42–63 days after immunization, and immunity against fatal *E. tenella* coccidiosis lasted only up to 105 days after immunization.

The work so far carried out suggests that cell factors are involved in mediation of immunity to coccidiosis. Recently, some work has been concerned with the role of the thymus and bursa of Fabricius in immune mechanisms in fowls. There appear to be at least two populations of immunologically competent

cells in the fowl, one derived from the thymus and the other from the bursa of Fabricius (Szenberg and Warner, 1962). Cells under the influence of the thymus have been implicated in the mediation of cell-based immune responses (e.g. homograft reaction) and cells influenced by the bursa, in antibody production. Long and Pierce (1963) and Pierce and Long (1965) found that "hormonally bursectomized" chickens had no detectable serum antibodies yet became immune to *E. tenella* infection in the same way as normal chickens. However, surgical thymectomy at one day old retarded the rate at which chickens became immune to *E. tenella* without influencing antibody production. Burns and Challey (1965) also found that lytic antibody activity was significantly lower in surgically bursectomized resistant chickens, although it is well known that surgical bursectomy is less effective than hormonal bursectomy in suppressing antibody production. Rose (1968) examined the role of the spleen in the development of acquired resistance to *E. tenella* and found that splenectomy alone, or in conjunction with hormonal bursectomy, did not affect the acquisition of immunity. Similar work with *E. meleagrimitis* (Warren, E. W. and Ball, S. J.—personal communication) also indicated that the spleen plays no part in the acquisition of resistance to this infection in turkeys.

These results strongly suggest a cellular basis for the mediation of immunity to coccidiosis in chickens. Experiments associated with immunity to coccidiosis in cattle indicate that similar mechanisms may be involved (Fitzgerald, 1964).

Work in progress includes further studies on the roles of the thymus and the leucocytes, particularly lymphocytes, in the development of immunity to coccidiosis.

It has always been believed that immunity to *Eimeria* is species-specific; immunity to one species did not protect against infection with another. Rose (1967b) studied cross-immunity between *E. tenella* and *E. necatrix*. By producing sporozoite-induced infections of *E. necatrix* in the caeca she was able to show considerable cross-immunity between these two species. In a study on cross-immunity between *E. maxima* and *E. brunetti* Rose (1967c) found that chickens immunized against *E. maxima* had a considerable degree of resistance to infection with *E. brunetti*. Chickens immunized with *E. brunetti* also showed protection against challenge with *E. maxima*, although the degree of protection was not as great as those chickens immunized with *E. maxima* and challenged with *E. brunetti*.

These studies indicate that the specificity of acquired resistance may not be so rigid as suggested by earlier reports. More work using other species of *Eimeria* is in progress.

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The Infectious Process, and its Relation to the Development of Early Parasitic Stages of Nematodes

W. P. ROGERS AND R. I. SOMMERVILLE

Waite Agricultural Research Institute and Department of Zoology, The University of Adelaide, Adelaide, South Australia

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I. Introduction

Our original review (Rogers and Sommerville, 1963) dealt with the biology of infective stages of nematodes. The present review, which should be read in conjunction with the original review, briefly covers recent work on the physiology of development of early parasitic stages from the infective stage. There is now much evidence to support the view (Rogers and Sommerville, 1963) that the infective stage (in nematodes which infect the host *per os*) must receive from the host a stimulus which induces early developmental changes (Section II). It is presumed that the stimulus acts on a "receptor" of the infective stage and leads to possible neuro-endocrine activity which directly or indirectly affects target areas concerned in development (Section III). The host may also affect the development of early parasitic stages directly, e.g. by providing nutrients and by the actions of its enzymes on the coverings of infective stages (Section IV).

This area of research is of special interest to us because it may lead to a more general understanding of the relationship of the parasite to its host and help to explain the phenomenon of parasitism. Moreover, the early development from the infective stage may provide a useful model for the study of the control of differentiation.

II. Stimuli which Induce Development

The development of early parasitic stages immediately after infection must depend on (a) the presence of functional components of the developmental machinery in the infective stage, and (b) the provision of the substrates on which the machinery acts. For many species the host must play a part in (a) by providing a stimulus which in some way starts the mechanisms governing development. The nutrients (b) may be provided initially from endogenous reserves, but in most parasites the host must provide these also. It may be difficult always to make a clear-cut division between these functions of the host, but as a working hypothesis we accept the view that the stimulus from the host may act as a physiological trigger; i.e. the stimulus, after acting for the appropriate time, sets in motion events which take place even when the stimulus is withdrawn.

Work on this topic has been concerned largely with infective agents which enter the animal host *per os* and on root diffusates which facilitate infection of plants. As yet, the role of the animal host in stimulating the early development of parasitic stages of nematodes which enter the host parenterally has not been critically examined, although studies of the culture of such forms as *Nippostrongylus brasiliensis in vitro* have elucidated the general nutritional needs.

A. The Infection of Animals

The hatching of infective eggs and the exsheathment of infective juveniles have been taken as the first obvious signs of the development of early parasitic stages. There is now evidence from a variety of species that infect the host *per os* that this development starts as the result of a signal from the host, and that this stimulus acts as a physiological trigger (Rogers, 1966c).

Further work on the factors which induce hatching or exsheathment has been carried out in recent years, but it has been concerned with added detail within a generally accepted framework rather than with the establishment of new principles.

1. *Carbon dioxide and related substances*

In many experiments infective agents have been exposed to the range of substances which occur in carbon dioxide–sodium bicarbonate buffers. It is uncertain which of the components of this system are important, though there is evidence that ions generally and the bicarbonate ion in particular are not the directly active entities. The view that the efficiency of the signal for exsheathment of infective juveniles of *Haemonchus contortus* is determined by the rate of conversion of carbon dioxide to carbonic acid (Taylor and Whitlock, 1960a, b) has been supported by recent work (Whitlock, 1966). Notably, Silverman and Podger (1964) found that 100% carbon dioxide was most effective in inducing exsheathment of *H. contortus* in 1% ammonium hydroxide.

(a) *The hatching of eggs.* The method of hatching infective eggs used by Hass and Todd (1962) was essentially that described by Fairbairn (1961), i.e. a gas phase of 5% carbon dioxide in nitrogen, sodium bicarbonate $3{\cdot}3 \times 10^{-2}$M, sodium chloride $3{\cdot}3 \times 10^{-2}$M, sulphur dioxide 2×10^{-3}M at 40°C. The chief

significance of this work is that the eggs of seven species of ascarids, *Heterakis gallinae*, *Trichuris suis* and *T. ovis* all hatched freely under the same conditions.

The results obtained with eggs of *Ascaris suum* by Jaskoski and Colucci (1964) need some comment. In this work a high proportion of the eggs hatched under a gas phase of 100% carbon dioxide, whereas Rogers (1958, 1960) found that concentrations of dissolved carbon dioxide plus undissociated carbonic acid above $0{\cdot}6 \times 10^{-3}$M at pH 7·3 inhibited the hatching of eggs. This discrepancy may be due to the fact that the eggs used by Jaskoski and Colucci had been "deshelled" in sodium hypochlorite. Normal eggs did not hatch under the conditions used in these experiments.

For the first time the effects of carbon dioxide on the hatching of infective eggs of species other than ascarids have been examined. Threfall (1966) who used infective eggs of the strongylid *Cyathostoma lari* got good results in buffers (pH 7·2–8) containing 0·2M sodium dithionite under a gas phase of 5% carbon dioxide in nitrogen. Under somewhat similar conditions with sulphur dioxide instead of sodium dithionite, eggs of *Trichuris ovis* and *T. suis* also hatched freely (Hass and Todd, 1962).

Contrasting results have been obtained with two species of oxyurids. Eggs of *Heterakis gallinae* hatched well under 5% carbon dioxide in nitrogen in the presence of sulphur dioxide (Hass and Todd, 1962). However, the eggs of *Aspiculuris tetraptera* hatched best at 37°C, pH 7–8, under air (Anya, 1966). Carbon dioxide had little effect under aerobic conditions, but the addition of 5% carbon dioxide to a gas phase of nitrogen increased the proportion of eggs which hatched from about 20% to 40%. Reducing agents had little effect (Section II).

(b) *The exsheathment of juveniles.* In the past, studies on the mechanism of exsheathment have been largely confined to species of the Strongyloidea. For members of this group which infect the host *per os* the role of carbon dioxide as a component of the stimulus for exsheathment has been broadly defined. At one extreme, juveniles of *Haemonchus contortus* have a definite requirement for carbon dioxide. At the other extreme the infective juveniles of *Dictyocaulus viviparus* and *Nematodirus battus* do not require carbon dioxide as a component of the stimulus (Silverman and Podger, 1964; Christie and Charleston, 1965). In between, the exsheathment of *Trichostrongylus colubriformis* is enhanced by carbon dioxide (Rogers, 1960), though it is not essential (Silverman and Podger, 1964).

Recent work has shown that carbon dioxide is an important component of the medium for exsheathment and moulting in groups other than in Strongyloidea. Thus Meerovitch (1965a, b) found that high concentrations of carbon dioxide favoured the complete moulting of intestinal forms of *Trichinella spiralis in vitro*. Ecdysone, in the form of extracts from the wax moth *Galleria mellonella*, had the same effect when added to cultures at low tensions of carbon dioxide. Third-stage juveniles of *Dirofilaria immitis* were found to exsheath freely in complex media when 5% carbon dioxide was present in the gas phase (Sawyer, 1965).

Further work on this topic is needed. Little is known of factors which govern the exsheathment of juveniles which enter the host parenterally. And in all

TABLE I

Effect of carbon dioxide on dormant stages of a variety of organisms

Organism		Effect of CO_2	Other necessary components	Reference
Eimeria arlongi	Protozoa	Induces excystment	Temperature, reducing conditions, trypsin and bile	Jackson, 1962
Naegleria gruberi	Protozoa	Induces excystment	None?	Averner and Fulton, 1966
Fasciola hepatica	Platyhelminthes	Induces excystment	Temperature, reducing conditions, bile	Dixon, 1966
Moniliformis dubius	Acanthocephala	Activation of cystacanth	Temperature, bile	Graff and Kitzman, 1965
Coccidioides immitis	Eumycete	Conversion of arthrospores to spherules	A defined medium and anionic surface active agent	Lones and Peacock, 1960
Trifolium subterraneum	Leguminosae	Breaks dormancy in the seed	None?	Ballard, 1958

studies more consideration of the action of carbon dioxide under physiological conditions is required. It is regrettable, for example, that some workers have restricted their experiments to the use of 100% carbon dioxide, a concentration rarely if ever met by infective juveniles *in vivo*.

(c) *Carbon dioxide and development in other organisms.* Examples of some other organisms in which a resting or dormant stage is known to be activated by carbon dioxide are given in Table I. Carbon dioxide acts not only on dormant stages; it can induce differentiation and morphogenesis in the "vegetative" stages of microorganisms, invertebrates and isolated vertebrate cells (see Bartnicki-Garcia *et al.*, 1964; Loomis, 1964). The organisms listed in Table I are very diverse, and no information appears to be available for any of them on the way in which carbon dioxide acts. Clearly it is capable of directing growth processes, and in some examples, such as *Eimeria arlongi* and *Fasciola hepatic*, it may do so in ways which are analogous to its role in infective eggs, or ensheathed infective juveniles. The relevance of the report that carbon dioxide increases the size of puffs on salivary gland chromosomes in third-instar *Drosophila melanogaster* (Burdette and Anderson, 1965) is not known, although it gives rise to some intriguing hypotheses.

2. *The effect of pH*

Hydrogen ion concentration may affect the efficiency of the stimulus for exsheathment or hatching independently of its effect on the concentration of dissolved carbon dioxide or on the redox potential. However, the optimum pH for a given species may be affected by the concentration of dissolved carbon dioxide and by the presence or absence of reducing agents (Rogers, 1960); hence, it is difficult to designate the optimum pH for the stimulus for some species. Nevertheless, the range of hydrogen ion concentrations which can be used in providing an efficient stimulus *in vitro* may be related to the site *in vivo* where the stimulus is most likely to act.

For infective juveniles of *H. contortus*, which receive the stimulus for development in the rumen of the host, neutral pH values were found to be the most satisfactory (Rogers, 1960; Silverman and Podger, 1964). *Trichostrongylus axei* may be of this category also, though effective exsheathment in this species could be obtained at lower pH than with *H. contortus* (Rogers, 1960). Juveniles of *Dictyocaulus viviparus*, *Trichostrongylus colubriformis*, *Nematodirus battus* and *Trichostrongylus retortaeformis* evidently receive the stimulus in the stomach of the host because acid media are effective (Silverman and Podger, 1964; Rogers, 1960; Christie and Charleston, 1965; Bailey, 1967).

For the hatching of eggs neutral pH values have been found satisfactory. The most recent detailed studies have been carried out by Anya (1966), who used eggs of *Aspiculuris tetraptera*. Optimum hatching was obtained at about pH 7·35, and moderately alkaline pH values were more effective than high hydrogen ion concentrations.

3. *Reducing agents*

There seems to be good evidence that reducing conditions enhance the hatching of infective eggs of ascarids, *Trichuris* spp., *Heterakis gallinae* and *Cyathos-*

toma lari (Hass and Todd, 1962; Threfall, 1966). The eggs of *Ascaris lumbricoides* have been most critically examined and the reducing agent was just as necessary as carbon dioxide to give high activity (Fairbairn, 1961; Rogers, 1960). The experiments of Jaskoski and Colucci (1964) in which hatching was obtained in the absence of a reducing agent do not affect this issue; in these experiments it was necessary to treat the eggs with sodium hypochlorite if hatching was to be obtained.

Anya's work (1966) with eggs of *Aspiculuris tetraptera* showed that under aerobic conditions sodium dithionite or cysteine did not affect hatching. However, as it seems likely that the conditions under which these experiments were conducted did not approach those which would prevail *in vivo*, the action of reducing agents on the hatching of eggs of this species should be examined further.

The role of reducing conditions in the exsheathment of juveniles is not clear. Early experiments (Rogers, 1960) suggested that reducing agents enhance exsheathment of *Haemonchus contortus* but do not affect exsheathment of *Trichostrongylus colubriformis*. In general, the effect of reducing agents was decreased when high concentrations of carbon dioxide were used and when the pH was decreased. Other workers (Taylor and Whitlock, 1960a, b; Silverman and Podger, 1964) showed that reducing agents were not necessary for the exsheathment of *H. contortus*, but in some of these experiments the pH may have been low and in others 100% carbon dioxide was used.

Recent experiments on the inhibition of the action of carbon dioxide as a stimulus for the exsheathment of *H. contortus* and *T. colubriformis* (Rogers, 1966a, b) throw some light on the action of reducing agents in exsheathment. An oxidizing agent (10^{-4}N iodine) inhibited the action of carbon dioxide as a stimulus and this inhibition could be reversed by subsequent treatment with a reducing agent (hydrogen sulphide-water or sodium dithionite). It would seem possible that under natural conditions response to carbon dioxide might be decreased as the juveniles aged, in which case the inclusion of a reducing agent in the stimulating medium might be expected to enhance exsheathment.

The importance of reducing conditions in rumen fluids as a medium for the exsheathment of *H. contortus* (Rogers and Sommerville, 1960) was confirmed by Silverman and Podger (1964), and reducing agents (sodium dithionite was more effective than cysteine) even enhanced the activity of "fresh" rumen fluid. As might be expected, the effect of reducing agents was less marked when the rumen fluid was saturated with carbon dioxide.

4. *Temperature*

(a) *Temperature and infection of homoiothermic animals.* In their free-living existence, infective stages may encounter a wide range of temperatures. Infective eggs of *Ascaris suum* respond to increased temperature by a reduction in metabolic rate, a "capacity adaptation" which is probably widespread in dormant or semidormant structures such as infective eggs or infective juveniles (Wilson, 1967). But dormancy breaks down when these infective stages encounter the relatively high and constant temperatures of homoiothermic animals,

and temperature is often an important part of the stimulus for development. Some information is available about the effect of temperature on exsheathment of infective juveniles (Rogers and Sommerville, 1960) and the hatching of infective eggs (Fairbairn, 1961; Anya, 1966).

There is little or no exsheathment of *Trichostrongylus axei* below 30°C, and the optimum temperature for exsheathment is about 40°C and thus within the range of body temperature of the sheep host (Waites and Moule, 1961). When infective juveniles of *T. axei* were "triggered" to exsheath by exposure to a suitable stimulus for 10 min at 38°C, more than 70% exsheathed during subsequent incubation in water at 38°C (Rogers and Sommerville, 1960; Table II). But there was no exsheathment of worms exposed to the stimulus

TABLE II

The effect of temperature on exsheathment of Trichostrongylus axei (Rogers and Sommerville, 1960)

Exposed to Stimulus 10 min. ("Trigger")	Suspended in water 120 min
Temperature: 38 C 0% exsheathed	→ Temperature: 38 C 71% exsheathed
	→ Temperature: 14 C 32% exsheathed
Temperature: 14 C 0% exsheathed	→ Temperature: 38 C 0% exsheathed
	→ Temperature: 14 C 0% exsheathed

for 10 min at 14°C, when subsequent incubation was at this temperature or at 38°C. These experiments showed that the stimulus could be effective only if the worms exposed to it were held at relatively high temperatures. And because there was no visible change in these worms after 10 min, it was concluded that temperature was critical during some early stage or stages, perhaps reception of the stimulus or closely associated processes. Later stages, such as the breakdown of the sheath, continued at 14°C, although slowly. Most probably the enzyme responsible is a leucine aminopeptidase. A crude assay of exsheathing fluid from *H. contortus*, which would have contained this enzyme, showed activity to be present at temperatures below 20°C (Rogers and Sommerville, 1960). Bailey (1967) has shown that the infective juvenile of *Trichostrongylus retortaeformis* does not respond to the stimulus to exsheath at temperatures of 30°C or less, but in properly stimulated worms, the final fracture of the sheath can take place at 15°C.

There is little or no hatching of infective eggs of *Ascaris lumbricoides* and *Aspiculuris tetraptera* at temperatures below about 30°C (Fairbairn, 1961; Anya, 1966), but the curves which relate hatching and temperature climb steeply to about 40°C, like the curves which relate temperature and exsheathment. It is difficult to compare these results on the hatching of infective eggs

with those for eggs which hatch "spontaneously". Eggs of many free-living nematodes probably hatch in this way, but not much is known about this. Eggs of the free-living species *Aphelenchus avenae* develop and hatch at any temperature between 10°C and 38°C (Taylor, 1962). By comparison, eggs of *Ascaris lumbricoides* and *Aspiculuris tetraptera* have a much narrower thermal range for hatching, because they do not respond to temperatures below about 30°C.

These examples show that the hatching of infective eggs and exsheathing of infective juveniles take place when the temperature is raised and other components, such as suitable concentrations of carbon dioxide and hydrogen ions, are present. But dormant fourth-stage juveniles of *Phocanema dicepiens*, which live in fishes, will commence to develop, form a new cuticle and occasionally moult in physiological saline alone, if the temperature is raised to 35°C (Townsley *et al.*, 1963). The worms die during or shortly after moulting when they have been exposed to this treatment for 3–6 days. They will complete the moult and survive if instead of saline a medium containing nutrients is used, but it is clear that nutrients are not needed to initiate the processes of development. The particular components of the nutrient medium which enable the moult to be completed are unknown. Physiological saline (presumably sodium chloride, about 0·85% w/v) is toxic to many organisms and the addition of other ions to "balance" the salt solution may be sufficient to enable the moult to go to completion.

The free-living stages of *Nippostrongylus brasiliensis* have been grown from the egg to the infective stage in chick embryo extract incubated at 20–25°C (Weinstein and Jones, 1957). If the cultures were left within this range of temperature all development ceased. But if the temperature of the medium was raised to 37°C, development was resumed, and the infective juveniles differentiated to a stage which corresponded to that of moderately developed juveniles in the rat's lung. Development went no further unless additional nutrients were supplied. Although it is possible that the rise in temperature was the only stimulus needed to restart development, the chick embryo extract may well have supplied a component or components which, together with high temperatures, provided the necessary stimulus.

(b) *Temperature and infection of poikilothermic animals.* The examples we have discussed were concerned with infective stages which parasite homoiothermic animals. Many nematodes live in poikilothermic animals, but very little information is available about the ways in which development of these parasites is triggered.

Microfilariae of the filariid worms *Dirofilaria immitis* and *Macacanema formosana* are released into the blood of their hosts (dogs and monkeys respectively), but do not develop until they are ingested along with a blood meal by a mosquito. It has been possible to induce microfilariae of both species to undergo *in vitro* some of the morphological changes which follow infection of the mosquito (Sawyer and Weinstein, 1963; Wood and Suitor, 1966). The media were complex, but development took place only if the temperature was less than the temperature of the definitive host. *M. formosana* did not develop above 22°C and *D. immitis* failed to develop at temperatures higher than 27°C.

Evidently, in these species relatively low temperatures form a part of the stimulus which makes the microfilariae commence development. Alternatively, the high temperatures of the definitive host could be regarded as part of a mechanism which suppresses development until favourable environments are attained.

(c) *Diapause and the hatching of eggs.* Diapause has been defined as "a stage in the development of certain animals during which morphological growth and development is suspended or greatly retarded" (Andrewartha, 1952). Physiological development may also be suspended, but many species, if adequately stimulated by, for example, low temperatures, will resume development. This is called "diapause development": it may last for many weeks and is a preparation for the "active resumption of morphogenesis".

Probably the infective stage of *Nematodirus battus* is in diapause (Thomas and Stevens, 1960; Christie, 1962). This nematode is parasitic in sheep. Eggs are deposited on pasture in spring and early summer, and the juvenile develops until it becomes an ensheathed infective stage, still enclosed within the egg. Hatching of the egg is delayed, sometimes until the following spring.

Eggs seem to be able to hatch at any temperature from just above zero to about 26°C, but hatching is very slow except at temperatures between 8 and 15°C. At constant temperatures within this range, a major proportion of the eggs hatched within 8 weeks. Christie (1962) has suggested that his observations can best be interpreted in terms of diapause. Hatching was assumed to involve two processes, a "cold process", which has been called "diapause development", and a "warm process", which is of much shorter duration and succeeds diapause development. The warm process might be the breakdown of egg membranes, or some other process concerned in escape from the egg.

Diapause development was fastest at 8–15°C, but could take place at temperatures from about zero, or just below, to 26°C. Thomas and Stevens (1960) did not examine a wide range of temperatures in their hatching experiments and they concluded that eggs hatched only after exposure to temperatures about 0°C for several months, followed by exposure to temperatures about 20°C for a shorter time. But Christie (1962) showed that hatching was quicker at constant temperatures of 8–15°C. The much slower hatching induced by temperatures close to zero might be expected if diapause development was very slow about 0°C and faster at slightly higher temperatures. Christie's data suggest that the temperature range at which diapause development takes place extends to all temperatures at which the "warm process" can operate. The time required to complete diapause development cannot be determined under these circumstances because results are expressed as the proportion of individuals which hatch, and an alternative index must be found (Lees, 1955).

The use of the term diapause implies that some stimulus, in this instance temperature, induces development by speeding up processes which operate fastest at relatively low temperatures. No information is available on the nature of these processes in *N. battus*. Furthermore, we do not know whether the basic mechanisms which regulate differentiation and morphogenesis of the parasitic stages are involved, or whether the only process affected is hatching. It is perhaps significant that successful infection of the sheep host can be made with

infective juveniles which have been artificially hatched (Gibson, 1958), but direct comparison between infectivity of worms which have hatched normally and those which have hatched artificially has not been made.

B. THE INFECTION OF PLANTS

The infective stages of some of the most highly specialized parasites of plants require very specific stimuli before they will hatch or moult, but the nature and mode of action of these stimuli are largely unknown.

1. *Root diffusates and moulting*

Pre-adults (i.e. fourth-stage juveniles) of some species of *Paratylenchus* require an external stimulus to induce moulting, and this is supplied by an unknown component in root diffusate from host plants (Rhoades and Linford, 1959; Fisher, 1966). In Fisher's experiments, worms which had been exposed to the diffusate moulted 8–13 days later. It was not necessary for the root diffusate to be present all the time. Exposure for as little as 24 h "triggered" moulting in many individuals, implying that the root secretions stimulated a receptor, which in turn restarted internal mechanisms governing development. Alternatively, the active principle in root diffusate might replace a missing component in a sequence of internal secretions, a role suggested for eclepic acid in the hatching of *Heterodera rostochiensis* (Rogers, 1960). Some evidence for the location of internal mechanisms was provided by ligating worms before exposing them to root diffusate. Moulting in ligated worms was restricted to the anterior part of the worm, which suggested that a complete regulatory mechanism was located in that region.

2. *Hatching in the genus* Heterodera

(a) *The effect of low temperatures.* Low temperatures stimulated hatching of *Heterodera avenae* (Cotten, 1962; Fushtey and Johnson, 1966). The best hatch was obtained from cysts which had been exposed to 7°C for twelve weeks, and subsequently incubated at 21°C for 6 weeks. The parallel with the effect of low temperatures on hatching of *Nematodirus battus* is obvious, and suggests that the eggs may be in diapause.

(b) *Root diffusates and hatching.* The hatching process in *Heterodera rostochiensis* and *H. schachtii* is stimulated by root diffusates (Shepherd, 1962). The active principle is believed to contain an unsaturated lactone ring, although tests on a purified hatching factor for α- and β-unsaturated γ-lactones were negative (Clarke and Widdowson, 1966). Some compounds with this structure, e.g. the cardiac glycosides, have high biological activity, and Ellenby and Gilbert (1957, 1960) have proposed that the hatching factor, like the cardiac glycosides, may influence ion transport. The way in which this might stimulate the worm and so lead to hatching is unknown. Rogers (1960, 1962) has suggested that the hatching factor may be able to enter directly into a sequence of internal secretions, perhaps by replacing some missing component. The mechanism ultimately activated may be the same as that which carbon dioxide

effects in other species. Clarke and Shepherd (1964) have suggested that many synthetic hatching agents may function by acting as electron acceptors. They believe the central problem in the mechanism of hatching concerns the way in which the metabolism of the juvenile is adjusted from a dormant to an active state: this might be done by regulation of the availability of oxygen or by interaction between the hatching agents and the electron transport system. But Ellenby (1957) found no evidence that juveniles liberated from the egg of *H. rostochiensis* by mechanical means were dormant. The hatching stimulus did not "wake" the juveniles; most were pressed against the egg membranes like "a spring in a box". Ellenby and Smith (1967) have defined dormancy in *H. rostochiensis* as a state in which the cyst does not respond to the presence of a hatching stimulus, but the significance of this in terms of the physiological state of the juvenile is unknown.

3. *Infection and development in* Meloidogyne javanica

In most of the examples discussed, the first signs that development of an infective stage has been restarted are hatching or moulting. As yet very little has been done to establish other ways in which the earliest changes associated with the initiation of the parasitic life might be measured.

Infection of the host plant by *Meloidogyne javanica* is not followed immediately by a moult. Other changes take place, for example, in the oesophageal glands and mobility. The stimulus which triggers developmental processes in the infective stage, and so leads to these changes, is unknown, but Bird (1967) has argued that the controlling mechanisms might be more readily studied in a species like *M. javanica*, because the complex series of physiological processes associated with moulting do not intrude. Species such as *Nippostrongylus brasiliensis* and *Strongyloides* spp. are similar, and might be studied in this way.

III. Internal Mechanisms which Govern Development

It has been postulated (Rogers, 1962; Rogers and Sommerville, 1963) that the stimulus from the host reacts with a "receptor" in infective stages. The "activated receptor" then sets in train the events which lead to exsheathment, hatching, and other developmental changes of the early parasitic stages. In a hypothetical scheme which might explain the action of the stimulus (Fig. 1) it is envisaged that the "activated receptor", probably through some intermediary system, affects target cells and so leads to the secretion of exsheathing and hatching enzymes and to the morphogenesis and differentiation of early parasitic stages. It is also envisaged that there is a long-acting phase of the stimulus in which carbon dioxide is concerned in more conventional biochemical mechanisms for the provision of energy (Sommerville, 1964; Rogers, 1966a).

The various phases of this hypothetical scheme are discussed below under the headings: A. "The nature of the receptor", B. "Intermediary mechanisms" and C. "Terminal mechanisms".

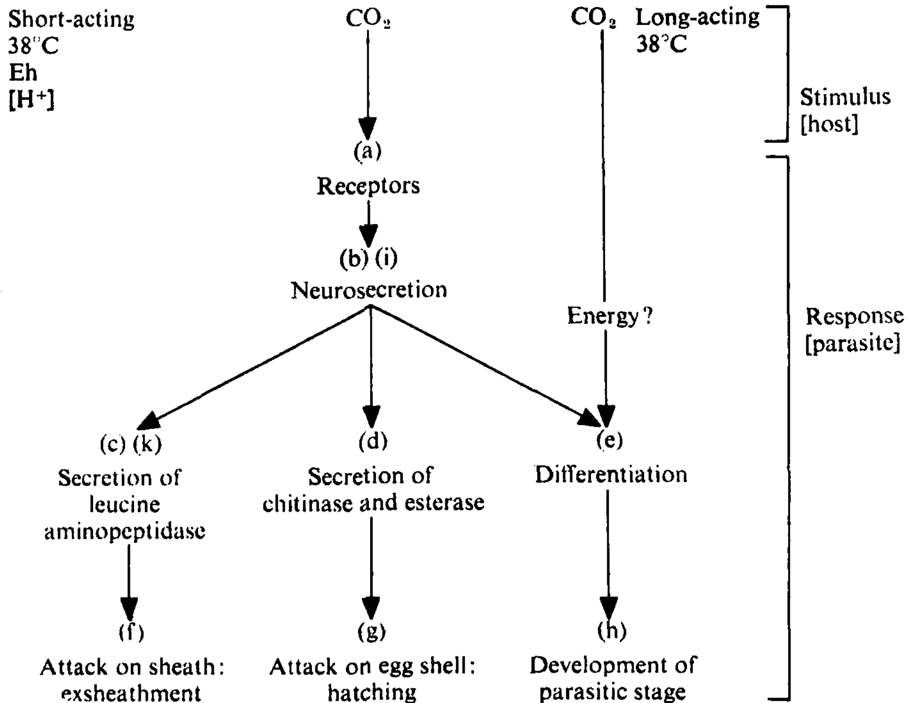

FIG. 1. Model which relates the stimulus from the host and the early development of parasitic nematodes which infect the host *per os*. Adapted from Rogers, 1966a. Data (a), (d), (g) from Rogers, 1958; (a), (c), (f) from Rogers, 1965; (b) from Rogers, 1968; (a), (e), (h) from Sommerville, 1964, 1966. Although a reference to Davey and Kan, 1967—(i), (k)—is also included, it must be emphasized that in this instance there is no evidence that the stimulus from the host is carbon dioxide.

A. THE NATURE OF THE RECEPTOR

The concept of a "receptor" in this context is a broad one. It could be a discrete structure (e.g. a chemical sensory organ) or, more likely, it could refer to a compound or group of compounds distributed more widely in certain cells (e.g. some neurones or the nuclei of some cells) in the organism. There is as yet no evidence implicating any special region of the infective juvenile as a "receptor" beyond the results (Sommerville, 1957; Rogers and Sommerville, 1960) which indicated that some region, more than 15 μ wide, between the base of the oesophagus and the nerve ring, was involved in the reception of the stimulus and in storing and releasing exsheathing fluid in juveniles of *Trichostrongylus axei*.

Rogers (1966a, b) tested one of the many possible models for the activation of a "receptor" with carbon dioxide. Brief exposure of juveniles of *H. contortus* and *T. colubriformis* to an oxidizing agent (10^{-4} N iodine) inhibited exsheathment, and this inhibition could be reversed by treatment with a reducing agent (hydrogen sulphide–water). Further, infection with *Nematospiroides dubius*

could be reversibly inhibited by treating the infective juveniles in the same way. This was not surprising; if exsheathment of this organism could be controlled in this manner, infection would certainly be influenced. What was more to the point, however, was the finding that infection with juveniles of *N. dubius* from which the sheaths had been removed with sodium hypochlorite was inhibited by treatment with 10^{-4}N iodine, and that this inhibition was also reversed by treatment with the reducing agent.

These results obtained from experiments *in vivo* suggested that the stimulus provided by carbon dioxide influenced features of development in early parasitic stages as well as exsheathment.

B. INTERMEDIARY MECHANISMS

1. *Neurosecretion in nematodes*

It is generally accepted that hormones regulate growth, development, reproduction and many other body functions in invertebrates, although evidence is available for only a small number of species. A direct or indirect source of some of these hormones is believed to lie in secretory neurones, in which the association of neuronal and glandular activity enables the cell to carry on its special function, "the release of hormonal agents in response to integrated nervous influences" (Bern, 1963, p. 351). It would not be surprising, therefore, if neurosecretion was important in the processes which govern development and other activities in nematodes by forming part of the link between external stimuli and terminal mechanisms.

Little is known about neurosecretion in nematodes. Neurosecretory cells, identified in *Ascaris lumbricoides* by the use of histochemical techniques, are located in ganglia about the circumenteric nerve ring, or in peripheral sensory structures at the anterior end (Gersch and Scheffel, 1958; Ishikawa, 1961; Davey, 1964). Electron microscopy has shown the presence of bodies similar in appearance to neurosecretory granules, in axons of the ventral nerve just posterior to the secretory pore in juveniles of *Haemonchus contortus* (Fig. 2; Rogers, 1968). The fate and function of these neurosecretory granules is unknown.

In *Phocanema dicepiens*, the paraldehyde-fuchsin technique has been used to follow the secretion of granules in ganglionic cells associated with the circumenteric nerve ring (Davey, 1966; Davey and Kan, 1967). These granules, which are believed to be neurosecretory, did not appear until the juvenile was removed from fish muscle and exposed to temperatures about 36°C and, preferably, a source of nutrients (Section II. A.4). After this treatment commenced, the intensity of staining increased, and then declined. This cycle has been linked with final shedding of the old cuticle on the following grounds: (1) the ventral ganglion and part of the excretory system are intimately related, and neurosecretion has been observed in the ventral ganglion; (2) there is a cycle in the appearance of neurosecretory material which parallels a cycle of synthesis and release of leucine aminopeptidase in the excretory system, and this enzyme is probably concerned in the final shedding of the old cuticle (Section III. C); (3) *P. dicepiens* can be prevented from shedding the cuticle

and if this is done, stainable material does not appear in the ganglionic cells and leucine aminopeptidase does not appear in the excretory system.

There seems to be no evidence that the cycle of neurosecretion is associated with deposition of the new cuticle. Posterior halves of ligated worms formed cuticle as readily as the anterior halves. This could mean that the formation of new cuticle is under localized control.

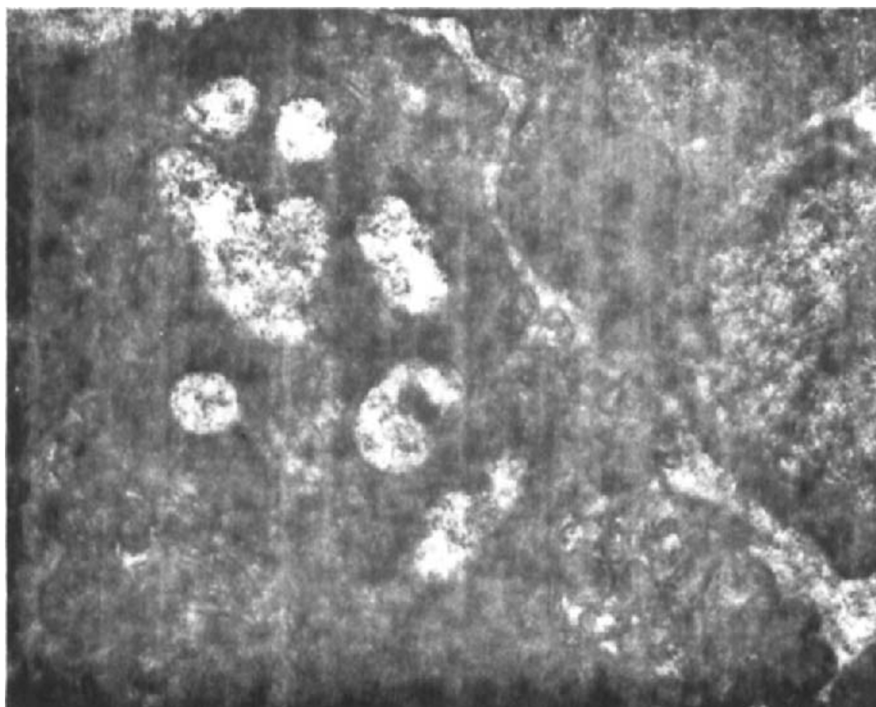

FIG. 2. Transverse section (× 33000) taken just posterior to the excretory pore of an infective juvenile of *Haemonchus contortus* (Rogers, 1968). An axon containing neurosecretory granules is shown bordered by groups of axons which fuse posteriorly to form the ventral cord.

Conclusions about the neurosecretory status of particular cells cannot be based on morphological grounds alone, whether by the light microscope or at the ultrastructural level: definition must be by function rather than by structure (Bern, 1966). But support for observational data in animals such as nematodes is extremely hard to obtain. In arthropods the usual approach is by micrurgical techniques, but nematodes are usually small and internal pressures are high, so that these techniques are very difficult to apply (Davey, 1966). A rewarding alternative approach is to use extracts of organs and structures believed to contain neurosecretory material (Davey and Kan, 1967). For example, extracts of the "heads" of *P. dicepiens* have induced synthesis of leucine aminopeptidase in excretory cells. Experiments like these are limited to nematodes which are

relatively large when they moult, or preferably, which have big infective stages, and species such as *P. dicepiens* or *Amplicaecum robertsi* (Sprent, 1963) are very suitable.

2. *Differentiation of the early parasitic stage of* Haemonchus contortus

The most obvious events during the early stages of the infection of the sheep by *Haemonchus contortus* are exsheathment and then the differentiation of the fourth stage. Exsheathment (Section II. A.1) takes place quickly in the host's rumen, but the changes which the post-infective third-stage juvenile undergoes are slower. They probably commence in the rumen, but they are completed in the glands of the abomasal mucosa about 48 h after infection. The most obvious changes during development of the third stage are the formation of a new mouth and cuticle. At the third moult, the old cuticle is discarded and the fourth-stage juvenile commences to ingest food (see Sommerville, 1964).

The changes which take place in the sheep, and which terminate in the third moult, can be produced *in vivo* in a non-nutrient medium, provided carbon dioxide is present (Silverman, 1962, 1965a, b; Silverman *et al.*, 1962; Silverman *et al.*, 1966; Sommerville, 1964, 1966).

(a) *The role of carbon dioxide*

When the infective stage of *H. contortus* was incubated in a balanced salt solution at pH 6 and 40°C for 72 h, the proportion of juveniles which reached the early fourth stage increased as the pCO_2 in the gas phase was increased to about 430 mm Hg (Sommerville, 1966). At higher concentrations development was slower, and it usually stopped when the gas phase was carbon dioxide alone. In these experiments, the concentration of sodium bicarbonate (and hence of total salts) had to be increased as the partial pressure of carbon dioxide rose, but there was no evidence that this had an adverse effect on development of the third stage or that bicarbonate ion was a necessary component of the medium. Development can take place at high hydrogen ion concentrations in the presence of carbon dioxide (Silverman *et al.*, 1966), and carbonic acid would almost certainly be undissociated under these conditions.

It seems, therefore, that either undissociated carbonic acid or dissolved gaseous carbon dioxide provides the stimulus for development. But carbon dioxide was required for at least 30 h in a culture which was terminated at 72 h. Only a very small proportion of juveniles attained the fourth stage if exposed to carbon dioxide for shorter periods and many probably needed it for most of the time. This is quite different from exsheathment, where carbon dioxide probably functions as a "trigger", and it suggests that carbon dioxide may have a direct effect upon metabolism (Rogers, 1966a).

This hypothesis implies that carbon dioxide could be important in two ways (Fig. 1). First, it could act on a receptor, which leads to activation of other systems, for example neurosecretory systems, which in their turn affect target organs. When the infective stage has been stimulated in this way it is potentially capable of resuming development. But other requirements have to be met. These would include energy and a favourable external environment (see below).

Carbon dioxide may have a function in the production of energy or it may affect metabolism in some way so that endogenous reserves can be used.

In Sommerville's (1966) experiments the infective juveniles were exsheathed artificially without any adverse effect on development to the fourth stage. But it is obvious that both the short and long term requirements of carbon dioxide would have been met by the culture system: in this situation the way in which the sheath is removed was irrelevant.

(b) *Ancillary requirements for development*

(i) *Temperature, pH, osmotic pressure and ionic components.* Development of *H. contortus* to the fourth stage requires temperatures in the vicinity of the sheep's body temperature, which commonly ranges between 39° and 40·5°C (Waites and Moule, 1961). Lower temperatures, for example 37°C, are much less favourable (Sommerville, 1966).

Silverman *et al.* (1966) found that development proceeded over a wide range of hydrogen ion concentrations, but it was much slower about pH 7. Unfortunately the partial pressure of carbon dioxide used in the gas phase was not stated. Sommerville (1964) claimed that development was adversely affected at pH 2 compared with pH 5, under gas mixtures containing 5, 25 or 50% carbon dioxide in air. The worms appeared to react unfavourably to low pH values. This might seem surprising, in view of the low pH values to which the juveniles might be exposed in the abomasal mucosa. But it should be emphasised that the "system" used *in vitro* would be very different from the normal environment. Probably the mucous and other secretions of the cells in the gastric mucosa protected the juveniles in some way.

Apart from any direct effect on the juveniles, pH would have an effect on the concentration of undissociated carbonic acid in the medium. At a given concentration of carbon dioxide, most development took place at the lower end of the effective range of the bicarbonate–carbon dioxide buffer system, at pH values where the concentration of undissociated carbonic acid would be greatest. Exsheathment of *H. contortus* responds in a similar way to changes in pH of the medium (Rogers, 1960).

The osmotic pressure of the medium was usually about 300 milliosmols. Relatively large changes in osmotic pressure (200–400 milliosmols) had little or no effect on development. Outside this range, development was adversely affected. At osmotic pressures in excess of 400 milliosmols, non-ionic additives produced the same effect as increased salt concentrations (Sommerville, unpublished data).

All the media used in these experiments with *H. contortus* are balanced salt solutions (Silverman *et al.*, 1966). Sommerville (1966) based his medium on a Krebs–Ringer salt solution, modified by the exclusion of phosphate ion, and buffered with bicarbonate-carbon dioxide. Addition of phosphate ion to this medium did not appear to be advantageous. Deletion of calcium and magnesium salts singly or together gave a significant but small reduction in the number of worms which reached the fourth stage, but deletion of potassium was disastrous: few or no worms developed and all were adversely affected (Sommerville, unpublished data). Potassium may play a very special role in the

development of *H. contortus*, quite apart from its general biological functions. Or the cuticle may be relatively more permeable to this ion than to calcium and magnesium ions. The leakage of components from cells or organisms in culture systems is well known, and has been described for *Caenorhabditis briggsae* (see Rothstein, 1965). Clearly this must be kept in mind when assessing the requirements of nematodes, even in very simple systems.

(c) *Tissue extracts and development*

A heat-stable polypeptide extracted from liver stimulates the conversion of the third-stage infective juveniles of *Dictyocaulus viviparus* to the fourth stage (Silverman, 1965b; Silverman *et al.*, 1966). This extract has been named "ecdysis stimulating factor", although it seems that it speeds up development of the third stage generally, and is not solely concerned with the ecdysis. "Ecdysis stimulating factor", when added to suspensions of *H. contortus* in a balanced salt solution, "accelerates conversion rates by twenty-four hours" (Silverman *et al.*, 1966). Apparently the effect of the extract is not as great as it is with *D. viviparus*, but further details of these experiments with *H. contortus* are not available.

Rogers (1960, 1962) and Rogers and Sommerville (1963) have suggested that one way in which a host might help the development of a parasite is by the provision of substances which replace missing internal secretions. The organic extracts examined by Silverman and his co-workers might function in this way, and the small amounts which are required lend some support to this idea. Another more speculative possibility arises from Rothstein's (1965) view of the culture medium as a "reservoir for metabolic intermediates". The complex components extracted from liver may contain substances identical with intermediates which normally leak out of the developing parasite. In the host an equilibrium may be set up so that the amounts which are lost are regained from the environment. Obviously this would not be possible *in vitro*, unless the organic component was already present in the medium, or if the organisms were very crowded.

If leakage of metabolic intermediates is significant, worms in more concentrated cultures might develop faster than those in less concentrated cultures. Crowding has been stated to enhance the conversion of the third stage *D. viviparus* to the fourth stage (Silverman *et al.*, 1966), but these cultures already contained a supplement. Under certain conditions, 10000 or more third-stage juveniles per ml of medium led to a retardation of development of *H. contortus* (Sommerville, 1966), but the range of concentrations in these experiments was probably not adequate to assess this hypothesis satisfactorily.

C. TERMINAL MECHANISMS

So far only the more obvious developmental changes, *viz.* hatching of infective eggs and the exsheathment of infective juveniles, which take place as a result of the stimulus from the host, have been examined. Moreover the biochemistry of these processes has been examined in a few species only, so it is not possible to generalize on the results which are at present available.

1. *Hatching of infective eggs*

Little has been added to the early work on this topic in which it was shown that the stimulus for the hatching of eggs of *Ascaris lumbricoides* led to the release of "hatching fluid" which contained a chitinase and esterase (Rogers, 1958). Recent work has been concerned with the esterase. With phenylpropionate as substrate Rogers (1963) showed that optimal activity was obtained at pH 7–7·5 in 0·01 M phosphate buffer. The enzyme was little affected by 0·01 M diaminoethanetretra-acetic acid, di*iso*propylfluorophosphate or ethylmaleimide. Jaskoski and Colucci (1964) found that this enzyme was active against olive oil–gum arabic at pH 6·5.

2. *Exsheathment of juveniles*

It appears that leucine aminopeptidase is the enzyme responsible for the exsheathment of *H. contortus* and *T. colubriformis* (Rogers, 1965). The enzyme was found in "exsheathing fluid" of juveniles which had been stimulated. Purified fractions of exsheathing fluid which contained leucine aminopeptidase were active against isolated sheaths. Fractions which did not contain the enzyme were inactive.

The leucine aminopeptidase in exsheathing fluid was similar in its biochemical characteristics to the mammalian enzyme, but mammalian leucine aminopeptidase did not attack sheaths. Although the enzymes produced by the two species of juveniles, like the mammalian enzyme, attacked the low molecular weight compounds L-leucinamide and L-leucyl-B-naphthylamide, they were specific to their own natural substrates, i.e. the leucine aminopeptidase from *H. contortus* attacked isolated sheaths from the same species, but it did not attack sheaths from *T. colubriformis* and *vice versa*.

3. *Leucine aminopeptidase and moulting generally*

Histochemical tests on two species have revealed cyclic fluctuations in the activity of leucine aminopeptidase at the time of moulting. In *Phocanema dicepiens* (Davey and Kan, 1967) the excretory gland became positive after 1 or 2 days in culture, but was almost negative on the 3rd day, when leucine aminopeptidase activity could be detected in the space between the two cuticles. Presumably leucine aminopeptidase was released from the excretory pore and attacked the cuticle at the anterior end, so that a "cap" was shed. The nematode would then be free to discard the cuticle.

In *Xiphenema index* some of the vacuoles in the epidermal chords gave positive histochemical tests for leucine aminopeptidase (Roggen *et al.*, 1967). The positive vacuoles were most numerous immediately before moulting, and towards the end of the moult there was a slight positive reaction in the space between the new cuticle and the old one. Although "leucine aminopeptidase" is ubiquitous in some nematodes, such as *X. index* and *Ascaris lumbricoides* (see Lee, 1962), these observations are significant because they are concerned with cycles which are associated with moulting.

In trichostrongylids such as *H. contortus* and *T. colubriformis*, leucine aminopeptidase has a clearly defined function. It is released as the terminal

process in a moult which has been interrupted. In most nematodes, the cuticle is discarded soon after it becomes free of the epidermis. Is leucine aminopeptidase involved in moults like these, or is it important only in exsheathment?

P. dicepiens from the muscles of fish is also an infective stage, but the growth processes have been interrupted earlier than they have in *H. contortus*, and there is only one cuticle. During the formation of the new cuticle the old one becomes separated from the underlying epidermis where the new cuticle is being formed (Davey, 1965). Subsequently leucine aminopeptidase is released from the excretory cell or gland and probably attacks the old cuticle. This process is evidently similar to that which occurs in *H. contortus.*

In *X. index* the enzyme disappears from the epidermis at the same time as the old cuticle is loosened. Roggen *et al.* (1967) suggest that leucine aminopeptidase is concerned in the detachment of the old cuticle, as well as being involved in the synthesis of the new one. In *Meloidogyne javanica* most of the old cuticle is broken down and reabsorbed during the moult, but it is not known whether leucine aminopeptidase is concerned (Bird and Rogers, 1965).

IV. Factors which Affect Development Directly

Substances from the host may initiate development by direct action on the infective stage. Thus the host's digestive enzymes might remove protective membranes of low permeability, or the host might provide substances which serve as sources of energy or as special metabolites for the developing parasite.

The infective stage of *Trichostrongylus colubriformis* does not have an absolute requirement for carbon dioxide to make it exsheath, but will do so in the presence of pepsin (Silverman and Podger, 1964). The pepsin attacks the sheath directly, and this can be shown by placing sheaths which have been dissected from infective juveniles into a solution of pepsin (Sommerville, unpublished data). Exsheathment of *Dictyocaulus viviparus* also requires pepsin, and carbon dioxide has no effect (Silverman and Podger, 1964). Probably internal processes in these infective stages are also activated when they exsheath under natural conditions. During infection of the sheep, *T. colubriformis* would be exposed to high levels of carbon dioxide at high hydrogen ion concentrations: under these conditions it would release a leucine aminopeptidase (Rogers, 1965), but the relative importance of pepsin and leucine aminopeptidase in releasing the juvenile from the sheath in the host is unknown. Silverman and Podger (1964) have obtained indirect evidence that "exsheathing fluid" is released when *D. viviparus* exsheaths in solutions of pepsin at high hydrogen ion concentrations. This species may require low pH values to stimulate developmental processes, but no information is available about this.

Berntzen (1966) has tested a variety of "enzyme and hormones found in the intestinal tract" in an effort to induce *Trichinella spiralis* to complete the final moult. It was found that exsheathment was induced by the "mixed enzymes co-carboxypeptidase A and co-carboxypeptidase B". The implication is that these broke the cuticle by attacking it directly. The relevance of this procedure

to the situation in the host is unknown: there are considerable difficulties in correlating results *in vitro* with results obtained *in vivo* (Shanta and Meerovitch, 1967).

It has been suggested that some synthetic hatching agents may intervene directly in metabolic processes in infective juveniles of some *Heterodera* sp. If hatching agents act as electron acceptors, they could provide a means of by-passing a rate-limiting step in the electron transport chain (Clarke and Shepherd, 1964).

Probably many of these stimuli which seem to act directly also restart internal secretions, as Dixon (1966) has suggested for excystment of the trematode, *Fasciola hepatica*. Or by removing external membranes, they may expose the infective stage to other environmental components which in turn activate internal mechanisms, and lead to development.

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Parasitic Bronchitis

D. POYNTER

Research Division, Allen and Hanbury's Ltd., Ware, Hertfordshire, England

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I. Introduction

Since the publication on parasitic bronchitis in Volume 1 of this series, two papers have appeared which summarize work carried out in this area. Poynter and Selway (1966) reviewed diseases caused by lungworms and included the genera *Dictyocaulus*, *Protostrongylus*, *Muellerius*, *Cystocaulus*, *Metastrongylus* *Filaroides*, *Aelurostrongylus*, *Crenosoma*, *Bronchostrongylus* and *Angiostrongylus*; whilst Forrester *et al.* (1966) have published a contribution towards the bibliography on the lung nematodes of mammals and assembled an extensive collection of references covering all aspects.

This review considers the work carried out on *Dictyocaulus viviparus* during the last 4 years. An attempt has thereby been made to bring up to date the review published in Volume 1 (1963), which should be read in conjunction with the present review.

II. The Parasite in the Pasture

Parasitic bronchitis is known to be a seasonal disease and this has been confirmed by Frick (1964), who reported a seasonal fluctuation in the faecal count of *D. viviparus* in cattle, with a peak in August. The epidemiology of the disease in the Omsk district of the U.S.S.R. was studied by Dmitriev (1964) with results on the annual incidence from 1958 to 1962. In 1960 there were 5 140 reported cases and 213 deaths out of a total population of about one million. The disease was correlated with years of high rain and snow fall, the peak month being September.

Grabner *et al.* (1965) in East Germany carried out pasture examinations and recovered larvae from a well drained pasture with soil pH 6·8–7·0 from December to April. With soil pH 6, larvae disappeared at the end of February. It was estimated that 2% of larvae were able to survive the winter. Of eleven calves

placed on a spring pasture for 6 weeks, two were found to harbour lungworms, showing that some larvae had overwintered and retained infectivity. In herds found to be infested in April and May, larvae could be demonstrated in the faeces until January. It was confirmed that the parasite may overwinter on the pasture and also persist in the lungs of infected animals.

Gurchiani (1964) has given an account of the seasonal and age dynamics of lungworms in buffaloes in Eastern Georgia, S.S.R. Wertejuk (1965) correlated reports of veterinary surgeons on *D. viviparus* infection of cattle in Poland during 1963. The disease was commonest in the north and accounted for 1 171 deaths in that year.

Mikes (1966) attempted to prevent outbreaks of disease in young cattle by the implementation of various grazing techniques. He stated that a 4-day cycle on fenced pastures with regular rotational management of these pastures was the most effective.

Buchwalder (1964) found that various disinfectant solutions killed the free-living stages of *D. viviparus*, and Enigk and Duwel (1961) recorded that chemical fertilizers had no adverse effect on larvae.

Hiepe (1964) in East Germany pointed out that the expansion of intensive farming has rendered the problem of lungworm disease in cattle an acute one. He suggested that infected animals be isolated, that any simultaneous bacterial infections be treated and that anthelmintics be given. He also advocated pasture rotation with pastures being used for 4–6 days and then rested for 40. It is apparent that many of the suggestions made for management are not really applicable to modern farming practice.

III. Treatment

The majority of papers recently published on parasitic bronchitis deal with its treatment, and many are from Russia and East Germany. Since intra-tracheal treatment with iodine and free access to phenothiazine and salt can hardly be said to represent "advances in parasitology", they are not considered in this brief review.

Wick (1963) has recommended treatment with a mixture of trichlorophen, atropine sulphate and pyridine aldoxime methiodide, which he found useful in four herds in East Germany. Geipel (1963) also used this mixture. He found that intramuscular injections of 5 ml/100 kg body weight (i.e. 25 mg trichlorophen) successfully alleviated the coughing except in calves with intense symptoms.

Teterin (1966) used trichlorophen and diethylcarbamazine for the treatment of lungworm disease and reported high efficacy.

Enigk and Duwel (1963b) found that an oral dose of thiabendazole at 200 mg/kg expelled an average of 65·4 % adult *D. viviparus*, but had no effect on larval stages. Enigk *et al.* (1964) confirmed this observation and reported that although at 200 mg/kg the efficacy varied from 23 to 95 % against mature worms, no action was seen against immature worms. In the field at the same level a satisfactory result was seen against light infections but not against heavy infections.

Ross (1966) also worked with thiabendazole. At 110 mg/kg orally little

effect was seen on either larval or adult forms. A single intratracheal injection of 2–4 g of 2 μ particle size aqueous suspension was 39–46 % effective against 15-day-old larvae and 61–74 % effective against adults. On the other hand, diethylcarbamazine intramuscularly for 3 days and a single dose of methyridine either orally or intraperitoneally were 99·8 % and 95 % effective against larvae and adults respectively. A single intramuscular injection of 2 μ thiabendazole had a variable effect against 16-day-old larvae, 0–99·7 %, but no effect on adults. By the intravenous route 110 mg/kg of a 2 μ suspension was lethal. At 37 mg/kg it was tolerated but there was no effect against larvae. Three daily intramuscular injections of 5 μ thiabendazole at 110 mg/kg, 66 mg/kg, 44 mg/kg or 33 mg/kg were 98·4 % effective. Three daily intraperitoneal injections at 44 mg/kg had no effect on larvae but three daily intramuscular injections at 44 mg/kg were 95·5 % effective against adults.

Rubin and Ames (1966) also experimented with intratracheal injections of thiabendazole and found that they showed little promise.

Enigk *et al.* (1966) investigated the efficacy of tetramisole against *D. viviparus*. They used solutions of 5 %, 7·5 % and 10 % administered subcutaneously at 10 mg/kg of active substance. By total tracheotomy and the recovery of worms from the bronchial mucus it was demonstrated that tetramisole eliminated 88·4 % of a 24-day-old infection and 84·7 % of a 16-day-old infection. The drug acted as a vermifuge. There were some signs of toxicity and the injection reactions were detectable for 1 week in half of the calves.

Forsyth (1966) in Australia reported favourably on tetramisole. He used two dose levels (13·2 mg/kg orally or 10 mg/kg subcutaneously), both of which were highly effective. Of 11000 calves treated in New Zealand and Australia, no deaths have been attributable to tetramisole.

Reinders (1966) used tetramisole in 89 calves aged about 6–9 months. He used a single large injection and reported high efficacy against gastro-intestinal nematodes and lungworms.

Kaemerer and Budden (1966) have commented on the side effects of tetramisole. Cattle dosed at 10 mg/kg intramuscularly or subcutaneously showed unrest, salivation, increased respiratory rate, yawning, and frequent defaecation and urination. These symptoms lasted for 1–2 h.

Reports have also appeared on older well-established anthelmintics. Olteanu (1963) confirmed the use of diethylcarbamazine, finding that three i/m doses of 25 mg of active substance per kg body weight given at 24-h intervals were 90–99 % efficient. Enigk and Duwel (1963c) carried out tests in 12-week-old calves each infected with 4000 larvae. Tracheotomies were performed so that any worms eliminated could be collected. The animals were slaughtered and remaining lungworm burdens estimated. The authors used cyanacethydrazide, diethylcarbamazine, methyridine and thiabendazole, but considered these to be less satisfactory than an aerosol of Ascaridol which, when administered for 15 min, was stated to kill all worms.

Scheffler (1965) used diethylcarbamazine at 20 or 40 mg/kg i/m at an interval of 24 h. He injected 80 diseased cattle between the ages of 5 and 15 months and at post mortem examination 8–10 days later found no effect. He did, however, recommend the drug as prophylactic treatment in young cattle.

Vodrazka (1960) compared diethylcarbamazine and cyanacethydrazide in calves and found 67·8% and 65·5% efficacies respectively.

Walley (1963) reported on the anthelmintic activity of methyridine against *Dictyocaulus* spp. in cattle and sheep, finding it of greatest use against adult worms. Enigk and Duwel (1963b) also found methyridine useful, and Grunder (1963) reported it effective in mild infections but less so in heavy infections.

Hiepe (1964) recommended cyanacethydrazide, methyridine and diethylcarbamazine for the treatment of lungworm disease, whilst Zotov (1966), using cyanacethydrazide as a sterile aqueous solution injected s/c (20 mg/kg) three times at daily intervals, claimed that 82% of the animals were cured.

Work has also been published on unusual anthelmintics. Loepelmann and Wolter (1964) found Benecid to be of use against *D. viviparus* when given in repeated doses of 15 mg/kg over several days. Subcutaneous and oral administration were effective but the intratracheal injection of the drug was without activity.

Klosov (1964) tried an aluminium iodide aerosol on a large number of farms in the Ukraine and claimed a cure rate of 80–100%. [In a letter to the Editor, the writer considers diethylcarbamazine still to be the drug of choice.]

IV. Immunity

Perov (1966) carried out an experiment in calves which confirmed that previous exposure to *D. viviparus* evoked protection.

In calves Cornwell (1962a) showed that immunity to *D. viviparus* could be produced by the parenteral administration of fourth-stage larvae, but that the immunity resulting was not as high as that following natural infection or vaccination with irradiated larvae.

Robinson (personal communication) has carried out some interesting experiments on the immunization of calves with dead larvae. He has observed that antigens prepared from fourth-stage larvae, cultured by existing *in vitro* methods, are inferior to third-stage larval preparations. Killed third-stage larvae antigens either freshly prepared or stored gave almost complete protection when injected subcutaneously into calves, but Robinson states that a large amount of antigen had to be used and that such a vaccine will have little practical use in the field.

Wilson (1966) used the rate of elimination of *D. viviparus* larvae from the guinea-pig lung to carry out experiments on acquired and passive immunity in this rodent. It was found that the passive transfer of guinea-pig immunoglobulins conferred a strong immunity. The globulins responsible for immunity were present in a serum fraction containing electrophoretically fast moving globulins and anaphylactic activity. Guinea pigs were not protected by artificial immunization with adult worm homogenate even though precipitating and anaphylactic antibodies were produced. Artificial immunization may have failed because α_1 and α_2 antibodies were produced against irrelevant antigens or because blocking α_2-antibody was produced in excess.

Pagirys *et al.* (1966) claimed to have developed a specific intradermal test for the diagnosis of *D. viviparus* infections which is positive as early as 10 days

after exposure. However, no details of the manner in which the allergen was prepared are given.

Cornwell (1963a) found that all stages of the parasite *D. viviparus* contained similar somatic antigens which were capable of detecting CF antibodies in sera. He also found that the adult worm antigen and metabolic products of adult worms were very similar in their capacity to fix complement in the presence of positive sera. These reactions were attributed to the lipid and protein fraction but not the polysaccharide fraction.

As early as 1939 Kotlan reported that when sheep and goats shared pastures with cattle, no lungworm infection was found in the cattle. He infected a calf with 10000 *D. filaria* and 17 days later recovered only 3 immature worms.

Parfitt (1963) experimented with *D. filaria* in calves and obtained clinical husk. He used infections of 30000 larvae each given as single doses of 10000 separated by intervals of 7 days. Out of six calves, three recovered without patent infection. The other three died, the infection in one reaching patency.

Parfitt and Sinclair (1967) found that calves which had previously been infected with *D. filaria* were more resistant to a challenge infection of *D. viviparus* than previously uninfected animals. The reduction in the *D. viviparus* worm burden was 67% at 10 and 24 days and 76% at day 30 after challenge. The difference was attributed to three possible causes, fewer worms becoming established in the lungs of vaccinated calves, a slower growth rate and possibly a greater loss from the lungs. One calf died of its *D. filaria* infection and some mature worms were recovered from its lungs. Using the gel-diffusion test it was found that precipitins to *D. filaria* antigen were present in the vaccinated animals and that one of these cross-reacted with *D. viviparus* antigen.

Hildebrandt (1962) reported that in two calves an immunity to a primary *D. filaria* infection was to some extent effective against *D. viviparus*.

Enigk and Hildebrandt (1964) also infected calves with *D. filaria*. They used infections of 3000 to 12500 larvae and reported that although a few larvae developed to the fifth stage, none attained sexual maturity. The prepatent infections did, however, produce disease. Two 8-month-old calves were each given 12500 *D. filaria* larvae and the infections did not become patent. Seventy-two days later each calf was given 7400 *D. viviparus* larvae. Although these infections became patent the numbers of larvae per g of faeces was much less than that seen in control calves. It was also noted that *D. viviparus* infections in red and fallow deer appeared to evoke protection against *D. filaria*. Lucker *et al.* (1964) also found that *D. filaria* did not produce patent infections in calves although respiratory distress was seen. Their experiment also suggested that some degree of immunity to *D. viviparus* was established by the *D. filaria* infection.

It is apparent, then, that parasitic bronchitis may be produced in calves by *D. filaria*, and furthermore that exposure to this parasite evokes some resistance to *D. viviparus*.

Sinclair (1967), using guinea pigs, has made a similar observation and noted that previous exposure of these animals to *D. filaria* evoked protection to *D. viviparus*.

An interesting experiment was carried out by Demski (1966), who gave each

of six 7-month-old calves two i/v doses of *Rhabditis axei* at 4-week intervals. Upon subsequent challenge with 9000 third-stage *D. viviparus* larvae the infections became patent and the calves showed faster respiratory rates, more severe lesions and a higher eosinophilia than non-sensitized controls. The lungworm burdens in the sensitized animals were, however, less than those seen in the controls. The introduction of non-specific nematode material apparently evoked a response.

Critical work with anthelmintics has also thrown light upon the resistance of calves to *D. viviparus*.

Duwell (1965) made an interesting observation when he administered phenothiazine to calves at the rate of 1·5–2 g per animal per day throughout their first season on pasture. The numbers of lungworm larvae and eggs of gastro-intestinal helminths remained low and, as opposed to the controls, no cases of lungworm disease were seen. Treatment was not continued in the second year and no disease occurred. Furthermore when heifers the animals were challenged with 10000 lungworm larvae and were shown to be immune.

Kendall (1965) made some relevant observations on the stages of *D. viviparus* required for resistance when he used diethycarbamazine to terminate the infection at as early a stage as possible, and then subjected the calves to challenge. He found that the effect of treatment was to increase the number of worms which remained after challenge by about four times those found in the untreated previously infected animals. Although a considerable degree of resistance remained in the treated animals it was found that the chemotherapeutic interference with the development of the immunizing infection caused a marked reduction.

A different result was recorded by Cornwell (1963b), who found no significant difference between the resistance of calves which had been treated with diethylcarbamazine at 6–9, 14–17 or 25–28 days after infection and those in which the immunizing infection had been allowed to proceed to maturity. Cornwell (1963b) used smaller doses of diethylcarbamazine than Kendall (1965), who explained the apparent discrepancy by suggesting that in Cornwell's experiments too much antigenic material remained for a marked effect to be demonstrable.

Michel (1962) studied the development of resistance to the establishment of newly acquired worms in calves following the administration of a fairly large dose of infective larvae of *D. viviparus*. Resistance rose to a high level about 10 days after administration of the immunizing infection, continued to rise at a slower rate for a further 100 days and then began to decline.

In 1965 Michel and Mackenzie published a comprehensive account of the duration of acquired resistance to infection with *D. viviparus*. This they studied by immunizing calves by either two doses of normal infective larvae or two doses of x-irradiated larvae. The calves were challenged at 3, 6, 12, 18 and 27 months.

Using normal larvae for immunization it was shown that a strong resistance to the establishment of challenge was apparent 3 months later. This resistance diminished during the next 3 months and was no longer in evidence after 12 months. It was also observed that the growth of the worms during the first 10

days was greatly retarded. This effect decreased with the passage of time but was offset by an interference with early growth due to the increasing age of the host. There was a great reduction in the number of worms present between the 11th and 29th day after challenge, and a large proportion of those worms which were not lost by the 29th day failed to develop beyond the early fifth stage. Further, the worms which persisted and were not inhibited in their development did not grow as fast as those in control animals. As a consequence of these factors none or very few larvae appeared after challenge in the faeces of infected animals.

When x-irradiated larvae were used for vaccination there were no symptoms of disease and no larvae appeared in the faeces. The resistance to the establishment of the challenge infection given after 3 months was not as marked as that following infection with normal larvae, but the growth of worms during the first 10 days was affected in the same way as after normal infection. Little or no reduction was seen in the number of worms present between the 11th and 29th days after challenge. Worms were inhibited in their development but the proportion affected was smaller than in calves immunized by the administration of normal larvae. Little effect was noted on worms not inhibited in their development, and in vaccinated animals challenge worms grew and reproduced.

It is interesting that Kendall's work (1965) and that of Michel and Mackenzie (1965) suggests that the later stages of *D. viviparus* are necessary for complete resistance, and surprising that in his experiments Robinson (personal communication) found that antigens prepared from third-stage larvae were superior to those prepared from the later stages. Apparently the existing *in vitro* methods are unsatisfactory, or perhaps the presentation of the material is critical and direct introduction into lung tissue is needed.

V. VACCINATION

The large experiment of Michel and Mackenzie (1965) has already been mentioned. Nelson (1964) has reviewed the place of vaccination against bovine parasitic bronchitis and made observations on reports from the field.

Tomanek and Prochazka (1965) have confirmed the earlier observations of Poynter *et al.* (1960) on the influence of ionizing radiations on *D. viviparus* larvae in guinea pigs.

Blindow (1966) carried out a field trial of the x-irradiated vaccine in the German Federal Republic. A total of 35 farms were used and one or two doses of vaccine were given to 650 calves and 551 young bulls which had grazed for a short time during the previous year. After introduction to pasture 9 calves and one bull developed clinical disease and 11 calves and one bull became carriers. The author concluded that the minimum age for vaccination should be 12 weeks, that a single dose was not effective and that attention must also be paid to gastro-intestinal strongyles. In the U.S.A. Lucker and Vegors (1964) concluded that the rate of irradiation was not of prime importance in the efficacy of immunization, and they attributed the variable results they obtained to individual calf variation.

Menear and Swarbrick (1968) used the commercial vaccine on 100 calves

under field conditions. They found that small numbers of larvae could be recovered from faecal samples taken from clinically normal vaccinated animals that had met a challenge. Further contamination of an originally infested pasture by these animals was probably the cause of a breakdown in younger vaccinated animals suddenly introduced to that pasture.

Downey (1965) carried out two trials on the x-irradiated vaccine which were performed under grazing conditions. He found that a severe pasture challenge which caused an 80% mortality in control calves caused the death of only 18% of vaccinates. He pointed out that had all the calves been vaccinated then pasture reinfestation would have been less severe and in these circumstances there might well have been no deaths from parasitic bronchitis.

Edds (1963) in the U.S.A. has also reported favourably on the x-irradiated vaccine, whilst Vercruysse *et al.* (1963) have presented the results they obtained in Belgium. Cornwell (1962b) reported his results in vaccinating 134 calves under farm conditions and found the vaccine to be safe. He found the most striking result of vaccination to be the great reduction in the degree of patency resulting from challenge. Natural exposure to pasture challenge produced only mild symptoms. Sudden heavy challenge was able to overcome resistance but experimental challenge with 5000–10000 larvae resulted only in mild symptoms without any apparent loss in condition. In Germany the vaccine has been found effective by Enigk and Duwel (1963a, b) and Duwel (1963).

VI. Pathology

An account of recent pathological studies on the lungs of calves infected with *D. viviparus* was given by Poynter (1966) in Volume 4 of this series. Of particular note is the paper by Jarrett and Sharp (1963), who compared the course of infections with normal and irradiated larvae describing the development of the lymphoreticular broncho-occlusive lesion, and that by Michel and Mackenzie (1965).

Perov (1965) confirmed the observation of Poynter *et al.* (1960) that the larvae of *D. viviparus* were capable of reaching the calf lung within 24 h. Perov (1966) also infected calves with doses of larvae increasing from 50 to 200 per day (a total of 2000–5000). Clinical symptoms developed within 7 days, focal pneumonia by the 16th and pulmonary oedema after 42–20 days.

Reinders (1966) made a pertinent observation when he recorded that coughing and decline of condition in calves at grass may be caused not only by *D. viviparus* but by parainfluenza A3 virus.

Wallis (1963) recorded two outbreaks of husk in cattle in Britain, which are of interest in that they occurred in partially immune animals. Although appreciable lung damage occurred the infections did not become patent and anthelmintic therapy was of no use.

Mackenzie and Michel (1964) made further experiments on the eosinophil leucocyte response to the x-irradiated vaccine. A significant rise in circulating eosinophils was observed and on challenge a massive rise took place. Challenge with gastro-intestinal species did not produce the secondary rise but it was seen after challenge with *D. filaria*. The secondary response to *Dictyocaulus*

spp. was also elicited by larvae given by the s/c or i/v routes. Later Michel and Mackenzie (1965) confirmed their observations with regard to *D. viviparus* and further noted that the blood eosinophilia was paralleled by dense infiltration of tissues by eosinophils. This occurred especially in the intestinal wall, mesenteric lymph nodes, liver, heart and lungs, and was not dependent on the presence of lungworms or larvae in the affected sites. Eosinopoiesis was observed in bone marrow. Tissue eosinophilia after challenge was more pronounced in immunized animals than in controls.

Mackenzie (1966) recently reviewed "fog fever" and pointed out that the term has been used loosely to cover a clinical syndrome of respiratory distress and emphysema which may have more than one aetiology and which may rise in a variety of circumstances. He emphasized that the classical picture in Britain is seen in the late summer and autumn months in adult cattle recently transferred to graze on lush wet pasture. In the U.S.A. and Canada the disease is known as acute bovine pulmonary emphysema and is seen when cattle are moved in the fall from dry mountain pasture to lower irrigated fields. The disease is then often associated with a change in forage, and in this connection it is of interest to note that Dickinson *et al.* (1967) have reproduced it experimentally by the oral administration of large doses of the amino acid DL-tryptophane.

Lungworm infection was considered to be one of the main factors in fog fever, and although it cannot be disregarded it is apparent that other initiating causes do operate and are possibly of more importance.

It is known that in parasitic bronchitis the faecal larval count is not directly related to the severity of the disease, and this point has been stressed by Kassai and Hollo (1962).

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Experimental Trichiniasis

JOHN E. LARSH, Jr

Professor of Parasitology, Schools of Public Health and Medicine, University of North Carolina, Chapel Hill, and Professor of Parasitology, School of Medicine, Duke University, Durham, North Carolina, U.S.A.

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I. Introduction

In the summary of the first review of this subject in Volume 1 (Larsh, 1963), it was predicted "that during the next decade or so interest in this field will remain high and the volume and depth of work will provide ample justification for another review". Although the prediction has proved to be correct, the period required to accumulate advances worthy of review was underestimated. After just five years, advances have been made in a number of the problems listed for probable future attention. Due to space limitation, in the present review (which should be read in conjunction with the original review) consideration will be confined to: (1) new methods for separating the phases of the life cycle, (2) new knowledge of hypersensitivity, and (3) new concepts of the mechanism of acquired immunity.

II. Selected Advances

A. New Methods for Separating the Phases of the Life Cycle

Although irradiation has been used for many years to separate phases of the life cycle of *Trichinella spiralis* (Larsh, 1963), the first report of the use of a chemical (*viz.* thiabendazole) for this purpose did not appear until five years ago (Campbell *et al.*, 1963). In a later report (Campbell and Cuckler, 1964), it was shown that in mice a single oral dose of 150 mg/kg of thiabendazole was about 100% effective in eliminating an intestinal infection if given not later than 24 h after infection. At 48 h, a higher dose (250 mg/kg) was required for even partial eradication, but between 4 and 11 days of infection complete

eradication was achieved by providing a continuous high dosage (1·0%) in feed. When this dose in the feed was reduced (0·05%), the chemical produced complete inhibition of reproduction without eradicating the worms. Therefore, by regulating the dosage, and means and time(s) of administration, this chemical provides a convenient tool for eliminating one stage (phase 3; migrating and encysting larvae), two stages (phase 2, adults; and phase 3) or, if used prophylactically, all three stages of the life cycle. In all probability, the elimination of phase 3 by this method is less harmful to the adult worms than the irradiation method. Moreover, there is no question of the superiority of this chemical treatment over irradiation for elimination of phases 2 and 3. The pre-adult worms of the immunizing infection(s) are not subject to injury prior to administration of the chemical, whereas the doses of irradiation required for this purpose produce marked stunting of growth within 24 h after infection (Gould *et al.*, 1957). By use of this chemical procedure, it is possible to study the effects of phase 1 (pre-adults) alone or the combined effects of phases 1 and 2 on the production of pathologic conditions and host responses (hematologic alterations, biochemical changes, serologic responses, immune reactions, etc.). Advantage has been taken of its use in immunity studies as illustrated by the following examples.

Four heavy infections (1100–1700 larvae) were inoculated into mice on alternate days and in each case a single oral dose of 250 mg/kg of thiabendazole was given 24 h after infection to eradicate 95–100% of the pre-adults (Campbell *et al.*, 1963). At 28 days after the first infection, these immunized-treated mice, and various controls, were given a challenging infection with 1500 larvae. The immunized-treated animals harbored 63% fewer adult worms than treated or untreated non-immunized controls at seven days after challenge. In a similar experiment, 72% fewer adult worms were recovered from the immunized-treated group than from the treated non-immunized mice.

Campbell (1965) used a single oral dose of thiabendazole (300 mg/kg) to eradicate the intestinal infection 20 h after one large immunizing infection. After challenge 28 days later, these mice had 54% fewer adult worms after 7 days and 53% fewer muscle larvae after 28 days than non-immunized controls. These reductions were 69% and 91%, respectively, in another experiment, in which the reproduction of the adult worms was suppressed from the fourth day onward by use of medicated feed (0·1% on days 4–20 of the immunizing infection, and 1·0% on days 21–28). Inasmuch as the adult worms were not eradicated during the immunizing period, these results suggest that a stronger immunity was produced by the combined phases than by phase 1 alone in the first experiment.

Most workers agree that larvae used for infection undergo a series of molts during development in the intestine and that the adult stage is not reached until 29–36 h (Ali Khan, 1966). Therefore, the strong immunity provided by the above immunizing infection terminated after 20 h can be attributed to the phase 1 (pre-adult) worms, which confirms previous reports (Larsh, 1963). Therefore, both this stage alone and, as shown in other studies (Larsh, 1963; Denham, 1966b), the adults alone are immunogenic, but there is as yet no conclusive evidence that phase 3 alone can immunize a host. Also, it is clear

that under the conditions of one of the above experimental designs, a single large immunizing infection produced striking immunity, hence repeated infections are not necessary.

Methyridine also has been used in studies of this type. A single subcutaneous dose of 500 mg/kg injected into mice at 24, 72, or 120 h after infection was very effective within 24 h in eliminating the intestinal forms of *T. spiralis*, and in another experiment similar results were obtained when the chemical in a single dose as low as 200 mg/kg was injected 3 days after infection (Denham, 1965). By giving a single subcutaneous injection (500 mg/kg) at 7, 48, 72, or 96 h after infection and two additional ones within 18 h, it was possible to test the effect of single or triple immunizing infections on producing immunity against a challenging infection (Denham, 1966a). Acquired immunity based on a significantly reduced burden of muscle larvae 35 days after challenge was demonstrated in those given a single immunizing infection terminated at 72 or 96 h, and in those given three such heavy infections terminated after 7 h. Accepting the opinion of most workers that the adult stage is not reached until 29–36 h after infection (Ali Khan, 1966), these results, as those above with thiabendazole, confirm the fact that phase 1 pre-adult worms alone are capable of producing immunity as well as phases 1 and 2 combined (Larsh, 1963).

B. NEW KNOWLEDGE OF HYPERSENSITIVITY

1. *Immediate (humoral) hypersensitivity*

Antigens released during infection cause the production of specific antibodies after an induction period of usually 7–10 days. Once the host is sensitized, an immediate sensitivity response will be provoked only by contact of these antibodies with the provocative antigen or a substance closely related to it chemically. This response in a sensitized host usually occurs within minutes, hence is said to be of the immediate type. The histopathology of an immediate type response is characterized by increased blood vessel permeability and edema, sometimes accompanied by extravasation, spasm, and constriction of arterioles. Finally, this type of sensitivity can be transferred passively with plasma or serum but not with cells, and with one exception (atopic hypersensitivity), the antibodies can be detected *in vitro* by conventional serological techniques, such as the complement-fixation and precipitin tests. Of the various types of immediate hypersensitivity (anaphylaxis, the Arthus reaction, etc.), anaphylaxis has been given most attention by those working with *T. spiralis*.

In anaphylactic hypersensitivity, the injury of mast cells causes the release of histamine and other pharmacologically active amines, which initiate a variety of tissue responses, including increased tissue permeability. The subcutaneous inoculation of metabolic or somatic antigens, or both, into mice actively sensitized to *T. spiralis* by infection or injection of somatic antigens, caused marked local reactions (Briggs, 1963). Mast cells were disrupted and released granules into the supporting ground substance. These granules showed a striking metachromasia. Sensitivity of this type developed about 2 weeks after a light initial infection and persisted for at least 15 months. Antibodies transferred from mice or rabbits produced much less striking reactions in

recipients. Mice infected with 50 larvae exhibited generalized, dose-dependent responses within 1 h after metabolic or somatic antigens were injected intravenously (Briggs and DeGiusti, 1966). Alterations in behavior were characteristic, such as change in rate of respiration, prostration, and no response to touch or sound. Visceral congestion, especially of the small intestine, was noted in most of those that died or were killed. As would be expected, the degree of sensitivity was dependent on the number and duration of infections. After initial infection, slight sensitivity to exogenous somatic antigen was evident after 5–7 days, it was more pronounced during the second week, and the peak sensitivity occurred between the second and fourth weeks. When the animals were challenged 8–10 weeks after initial infection, the sensitivity was evident within 3–5 days and was more severe. These sensitivity patterns were associated with loss of adult worms from the small intestine both after initial and challenge infections, and cortisone was shown to inhibit the reactivity to exogenous antigen and the elimination of adult worms after initial infection.

Other interesting studies have been reported on the detection of sensitizing antigens in *T. spiralis* by use of the Schultz–Dale (SD) test. Guinea-pigs given an initial infection and those injected subcutaneously with five doses of ES (metabolic) or Sonicate larva antigens developed sensitivity within 25–32 days, as revealed by positive SD tests with ES or Sonicate antigens (Sharp and Olson, 1962). Cross reactions occurred with *Ascaris* and *Toxocara* larva test antigens when tested with sera from the infected animals and those injected with ES antigen, whereas the Sonicate-injected animals cross reacted only with the *Ascaris* test antigen. Moreover, intracardiac challenge with Sonicate antigen produced anaphylactic responses in animals infected separately with each of the three parasites. Therefore, it would appear that both group and specific antigens are involved in these immediate hypersensitivity reactions. Further evidence for the presence of common sensitizing antigens among these parasites was obtained in SD tests with both adult and larva extracts of *Ascaris* and *Toxocara* and larva extracts of *Trichinella* (Ivey, 1965). In this case, there was an indication of the relative amounts of specific sensitizing antigen in the heterologous and homologous parasites and in the adult and larva extracts. However, precise estimates of common antigen concentration in heterologous preparations were not possible due to variations among animals as to the proportions of homologous–heterologous antigens needed for reactions. Cross-reactions between these three parasites also have been detected by use of the passive cutaneous anaphylaxis (PCA) technique (Ivey and Slanga, 1965). The results were similar to those obtained by the use of the SD test, but the PCA tests were less sensitive. Studies with both rabbit and guinea-pig antisera indicated that antibodies detected by PCA may differ from those detected by the bentonite flocculation or hemagglutination tests. It is of interest that sera from human cases of trichinosis produced PCA reactions in guinea-pigs and monkeys (Calero *et al.*, 1966). Histological study of the lesions revealed that the most striking feature was marked rupture of mast cells and local necrosis. A recent report indicates that a modified PCA procedure is more suitable for such studies (Ivey, 1967). In this case, antibody for sensitization is injected by the intracutaneous route, followed in 18–20 h by intravenous injection of the

indicator dye and, immediately thereafter, by the injection of the challenge antigen intracutaneously into the site of sensitization. Less than 1 μg of antigen provokes a reaction with small amounts of antibody, hence this test offers an advantage over the earlier ones that required large amounts of either antigen or potent antiserum.

2. *Delayed (cellular) hypersensitivity*

The basic immunological factors are the same as those involved in the immediate type reaction, i.e. specific antibodies are produced after an induction period, and a response can be elicited in a sensitized host only by the provocative antigen. However, in this case, after interaction of the antibody and antigen, there is no evidence of a response for several hours at least, hence it is said to be delayed. Moreover, the histopathology is characterized by cellular infiltration, hence skin reactions are typically indurated. Finally, delayed sensitivity can be transferred passively only with lymphoid cells or their derivatives, and there is no *in vitro* method for detecting these antibodies. Despite the theories that these are serum-borne immunoglobulins, most workers consider them to be intracellular. Competent cells for transfer of the sensitivity can be obtained from lymph nodes, spleen, aseptically induced peritoneal exudates, and other sources.

Both a protein extract (Melcher's acid-soluble protein fraction) and a crude saline extract of *T. spiralis* larvae can produce a tuberculin type delayed hypersensitivity in guinea-pigs (Kim, 1966). The protein extract antigen in equal volume with Freund's complete adjuvant was used in doses from 9 μg (0·69 μgN) to 60 μg (4·62 μgN) for intradermal sensitization. Seven days after the sensitizing injection, the animals were skin tested with doses from 10 μg (0·77 μgN) to 60 μg (4·62 μgN). Within 6 h, all animals showed a typical indurated reaction, which reached a peak size at 18 h. The saline extract plus adjuvant was used in doses from 1:1000 (3·3 μgN) to 1:100 (30–33 μgN) for both sensitizing and test doses. The timing of the reactions was similar to that with the protein extract, and at 7, 10, and 14 days after sensitization these were comparable for like doses. Perivascular mononuclear infiltration in test sites was demonstrated in all animals that exhibited intense skin reactions with either antigen. Spleen cells were collected at the height of the skin reaction, from donors sensitized with the extract, and injected intraperitoneally in determined doses (2×10^8–5×10^8) into recipients. At 3–6 days later, skin reactions proved to be similar to those of the donors. Circulating precipitating antibodies were not detected in the sera of the donors by microimmunoelectrophoresis or micro double diffusion (Ouchterlony) tests, and active systemic anaphylaxis was not demonstrated after intravenous injection of a shocking dose of antigen. However, the sera of a few donors skin tested 7 days after sensitization with saline extract and adjuvant produced passive cutaneous anaphylaxis (PCA) in test animals, and the sera of all of them skin tested 14 days after sensitization produced this reaction.

The optimal time for transfer of this sensitivity was determined in other studies with the saline extract antigen and adjuvant (Kim *et al.*, 1967a). One

injection of antigen (76 μg protein) was used to sensitize the donor guinea-pigs, and the same amount was used later for skin testing. At 5 days after sensitization, all animals had strong delayed reactions, and humoral antibody was not detected by PCA or the ring precipitin test. Similar skin reactions and negative precipitin tests were noted in those tested at 7 days, but the sera of 61 % of the animals produced a positive PCA reaction. When the sensitizing antigen dose was increased five times (380 μg protein) and the skin test dose was increased 2·5 times (190 μg protein), the size of the skin reaction was not larger, but smaller. At 14, 21, and 28 days after sensitization, the skin reactions were either of a mixed delayed-Arthus or predominantly an Arthus type, and humoral antibody was detected by both tests in at least 76 % of the animals.

Lymph node cells were removed from donors at the peak of the skin reaction and injected intraperitoneally into recipients in a determined dose (8×10^7 1×10^8). Recipients skin tested 3 days after transfer of the cells, from donors sensitized 7 days before they were skin tested and the cells collected, showed the maximum reaction, and the next reactions in order of size were noted at 2 and 21 days. The cells from donors sensitized 5 days before skin testing and cell collection were also effective 3 days after transfer to the recipients. However, cells from the donors sensitized 14–28 days before the skin test and cell collection were ineffective at all periods (1–21 days) tested after transfer.

The antibody response of recipients was also determined (Kim *et al.*, 1967b). The transfer of lymph node cells from donors that had been sensitized with saline extract and adjuvant 5–7 days before skin testing and cell collection produced an antibody response detected by PCA at 10 days (7 days after skin testing the recipients). The use of cells from 7-day donors that had not been skin tested delayed the appearance of such antibody (14 days after skin testing the recipients). All recipients that were not skin tested failed to produce detectable antibody between 1 and 31 days after cell transfer, regardless of the source of cells. Finally, the ring precipitin test, even with undiluted serum, failed to detect antibody in the sera of any recipients. Various observations proved that the antigen used to skin test the recipients, which was the same, including the dose, as that used to sensitize and skin test the donors, caused the production of the antibody demonstrated by PCA: (1) antibody was present 7 days after skin testing normal animals not given cells, (2) antibody was detected in skin tested recipients after transfer of cells from unsensitized, non-skin-tested donors, and (3) as stated above, transferred cells failed to produce demonstrable antibody in non-skin-tested recipients.

Although it is not known whether the same cells produce both humoral and cellular antibody, these and other results of this study show again the relation between the cellular transfer of sensitivity and the later production of humoral antibody by the transferred cells and/or their progeny. The skin test of the recipients had a booster effect on the production of humoral antibody, and there was a much more rapid response in those that received cells from skin tested donors. Therefore, there is a suggestion that repeated injections of the antigen at intervals accelerated the priming of the cells for humoral antibody production. On the other hand, it is clear that much less stimulation was needed to prime the cells for the transfer of delayed sensitivity. In any case, these results

are consistent with the fact that the nature and dose of antigen, and the timing of injections, are important factors in humoral antibody production. In regard to the role of lymphocytes in humoral antibody production, recent evidence from electron microscope studies adds support to this view (Jamuar *et al.*, 1968). Lymphocytes from lymph nodes of guinea-pigs sensitized with saline extract and adjuvant and skin tested 7 days later had a larger nucleo-cytoplasmic ratio and more ribosomes than cells from normal animals. Moreover, chromatin was scattered in the nucleoplasm as well as being compact in patches around the eccentric nucleus. After incubating sensitized cells with the antigen for 5 days, unusual enlarged cells were observed with a great increase in ribosomal content and the appearance of the ribosomes in an aggregated form as polysomes. The endoplasmic reticulum was increased and the vesicles were lined with ribosomal granules, indicating active protein synthesis. Lymphocytes from non-sensitized hosts did not show such changes after similar incubation.

C. NEW CONCEPTS OF THE MECHANISM OF ACQUIRED IMMUNITY

As brought out in the earlier review (Larsh, 1963), there is considerable information on various aspects of acquired immunity in experimental hosts, especially mice. The results of certain of these studies in mice opened the way for the formulation of a working hypothesis to explain the mechanism for expulsion of worms from the small intestine (Larsh, 1967a). These will be reviewed briefly before presenting the results of recent studies that proved the validity of the hypothesis.

For at least 11 days after initial infection of old mice, the majority of adult worms is located in the anterior half of the small intestine. A tissue response in this region is not evident until about 4 days after infection despite obvious mechanical damage produced by the worms. After the initiation of a mild inflammation at about 4 days, it develops into an acute phase, characterized by infiltrating polymorphonuclear leukocytes, and reaches a peak at about 8 days. At about 10 days, a subacute (or chronic) phase of inflammation develops, with a mixture of infiltrating mononuclear cells, and this lasts for many days. A few days after the peak of the acute response (11–14 days after infection), a significant number of worms is eliminated. In immunized mice, the pattern of the intestinal inflammation, including the cellular components, is similar, except that it is initiated sooner (within at least 12 hours), runs a more rapid course (peak of the acute response noted at about 4 days), and is much more severe. These events are associated with expulsion of worms, which occurs about 1 week earlier than in non-immunized mice, i.e. between 5 and 7 days after challenge. This characteristic tissue response is a panmucosal one and, therefore, is about the same in degree throughout large areas of the mucosa and submucosa as it is in areas adjacent to the worms. The response varies in intensity as to the degree of immunity demonstrated. It is most striking in mice given repeated stimulating infections with untreated larvae before being challenged, it is intermediate in degree in those stimulated with larvae irradiated to prevent sexual maturity of the developing adults, and is least in mice

stimulated with larvae irradiated to prevent development beyond the pre-adult stage. Moreover, the immunity and inflammation are more striking in old mice than in young ones.

The initial demonstration of the association of intestinal inflammation and expulsion of adult worms suggested that the former was directly responsible for the latter. Therefore, it was decided to determine the fate of the worms after inhibiting or preventing the inflammation. Cortisone proved to be ideal for this purpose. Mice were selected for study after they had been strongly immunized by repeated stimulating infections to rule out any effect cortisone might have on the development of this immunity. After daily injections, the intestinal inflammation was prevented almost entirely for at least 9 days after challenge, and the adult worms persisted for long periods. Notwithstanding the fact that cortisone might have exerted an immunosuppressive effect against humoral factors, these results strengthened the view that the inflammation is responsible for the elimination of the worms. The cortisone-treated mice showed a prompt and striking reduction in spleen size and a pronounced reduction in circulating lymphocytes. In the absence of information on the possible suppressive effect of cortisone on the humoral factors, these observations raised a suspicion that delayed hypersensitivity might be responsible for initiating the inflammatory response.

It was decided to test this suspicion by use of another treatment to produce a striking lymphopenia. The selection of whole-body X-irradiation proved to be a good choice. Again, to avoid the effects the treatment might have on the development of immunity, mice were selected for study after they had been strongly immunized by repeated stimulating infections. When the challenging infection was given 4 days or 8 days after irradiation (450 r), the intestinal inflammation at 7 days was less than that in untreated, non-immunized mice, and there was no loss of worms. At this time the splenic measurements of the irradiated mice indicated severe damage to the lymphoid-macrophage system, and these animals had an 80–90% reduction in circulating leukocytes due largely to a severe lymphopenia. Moreover, there was no evidence of interference with humoral antibody titers. Therefore, the fact that X-irradiation if sufficient to destroy lymphoid tissue does not affect an established immediate (humoral) hypersensitivity, but strongly depresses tuberculin (cellular) reactivity, strengthened the early suspicion that delayed (cellular) hypersensitivity might be the cause of the intestinal inflammation and thereby the subsequent loss of worms.

Ample justification for the suspicion that delayed sensitivity might be involved was obtained by repeated failures to cause a significant loss of adult worms from a challenging infection in mice injected by various schedules and routes with high-titered antiserum from rabbits or mice (Larsh, 1967a). At this point, it was decided to test this hypothesis directly by passive transfer of lymphoid cells.

In two experiments, the donor mice, 9 weeks of age, were infected with 400 *T. spiralis* larvae (Larsh *et al.*, 1964). After 28 days, each was injected intraperitoneally with 1 ml of warm mineral oil, and killed 3 days later for collection of peritoneal exudate cells. The recipients, 13–15 weeks old, were each injected

intraperitoneally with a dose of cells ($3{\cdot}6 \times 10^6$ or $2{\cdot}9 \times 10^6$), and 21 days later they were challenged with 100 or 150 larvae. At 11 days after challenge, the recipients harbored significantly fewer adult worms than controls of the same age and sex given the same infection.

These results were confirmed later by use of a similar experimental design (Larsh *et al.*, 1966). Also, it was shown by the ring skin-graft procedure that these mice are an isologous strain, and that there was no demonstrable effect on worm expulsion in controls given peritoneal exudate cells from uninfected donors. Finally, serological results with five different tests (bentonite flocculation with Melcher's or metabolic antigens, indirect hemagglutination, latex agglutination, and the indirect fluorescent-antibody test) ruled out the possibility that the transferred cells produced a humoral immunity, and the studies of the intestinal histopathology revealed an inflammation similar to that noted in various earlier studies.

Therefore, the evidence warrants the conclusion that the transferred lymphoid cells and/or their progeny are responsible for a specific delayed hypersensitivity response after union with a parasite antigen in the upper small intestine. Such reactions cause local cellular injury, which initiates an allergic inflammation (Chase, 1959). Among other alterations, in acute inflammation there is a decreased tension of oxygen, an increased concentration of CO_2, and the accumulation of organic acids, especially lactic acid (Raffel, 1961). Therefore, it seems logical that such an altered biochemical environment would be harmful to resident parasites and cause their withdrawal. There are at least two different observations to support the suggestion that *T. spiralis* adults are driven from the inflamed tissue due to the creation of an unfavorable environment. In the first place, many are not killed, since after leaving the inflamed areas of the small intestine they live for a time in the mucosa of the large intestine (Larsh *et al.*, 1952). Secondly, intestinal inflammation produced by other agents (*Ancylostoma canium*; *Salmonella typhimurium*) can drive out the adults of *T. spiralis* in significant numbers even when the specific immunity is feeble (Cox, 1952; Brewer, 1955; Goulson, 1958).

It is important to emphasize that the specific immunological reaction of the provocative antigen and cellular antibody is the primary event, and that non-specific inflammation is initiated later in response to the local tissue injury. Recent evidence indicates that the interaction of sensitized lymphoid cells with specific antigen might give rise to pharmacologically active substances that mediate the secondary vascular response. In fact, one such substance (lymph-node permeability factor) has been shown to cause diapedesis of leukocytes and increased permeability to protein (Schild and Willoughby, 1967). Moreover, recent *in vitro* studies have shown that the reaction of sensitized lymph-node cells with antigen causes a cytotoxic effect on fibroblasts used as target cells (Ruddle and Waksman, 1967). These findings provide a possible explanation for local cell damage in the body that triggers the delayed non-specific inflammation. For example, in the case of the specific reaction of sensitized cells and *T. spiralis* antigen, it is likely that adjacent parenchymal cells in the mucosa and submucosa are killed or injured as innocent bystander cells. These two recent reports encourage the speculation that both killed, or

injured, sensitized cells and bystander cells are responsible for triggering the delayed allergic inflammation; perhaps, in both cases, by the release of pharmacologically active substances. In any event, the point of emphasis here is that inflammation is not an acquired phenomenon; hence its initiation, rate of development, and intensity are governed by the acquired primary response.

When proof was presented that the mechanism for expulsion of adult *T. spiralis* is triggered by a specific delayed sensitivity reaction, it was predicted that this phenomenon would be shown to be a consequence of many parasitic infections (Larsh, 1967a). Therefore, it is of interest to note that the transfer of lymphoid cells and consequent immunity against a challenging infection has been demonstrated in recent studies of four other helminths: *Trichostrongylus colubriformis*, *Ancylostoma canium*, *Hymenolepis nana*, and *Fasciola hepatica* (Larsh, 1967b). In this connection, it is also interesting to note that granuloma formation around *Schistosoma mansoni* eggs in the lungs of mice is a manifestation of delayed sensitivity that was transferred from infected mice to isologous recipients by spleen and lymph node cells but not by serum (Warren *et al.*, 1967), and that thymectomy soon after birth resulted in a greatly diminished granuloma formation around the eggs (Domingo and Warren, 1967). In the case of protozoans, delayed sensitivity has been demonstrated in guinea-pigs injected with leishmanial antigens and transferred to recipients by lymph-node cells (Boysia, 1967); also there is indirect evidence that delayed sensitivity might be responsible for immunity to *Eimeria tenella* (Long and Pierce, 1963). Therefore, inasmuch as this sensitivity is known to develop in hosts bitten by various bloodsucking arthropods (Andrews, 1962), it is now clear that representatives of all three major groups of animal parasites are capable of causing this characteristic host response. However, except for *T. spiralis*, further research is needed to prove its role in immunity.

III. Summary

This brief review includes selected advances in experimental trichiniasis that have been made during the 5 years after the full length review was published in Volume 1.

The use of thiabendazole and methyridine to separate phases of the life cycle is discussed. By regulating the dosage of thiabendazole, and the means and time(s) of administration, it is possible to eliminate phase 3 (migrating and encysting larvae), or phases 2 (adults) and 3, or all three phases (by prophylaxis). Chemical treatment has advantages over the use of irradiation for such studies, hence the way is open to use this approach to study the effects of a single phase or combined phases on the production of pathological conditions and various host responses.

The recent work on both immediate and delayed hypersensitivity has resulted in a much better understanding of the allergic phenomena in this infection. The injection of exogenous larva antigens into infected mice causes local or systemic anaphylactic responses, depending on the route of injection. The degree of sensitivity is related to the number and duration of infections and is associated with loss of adult worms. Results with the Schultz–Dale test and the

passive cutaneous anaphylaxis test (PCA) indicate the presence of common sensitizing antigens in *Trichinella*, *Ascaris* and *Toxocara*. Only small amounts of larva antigens are needed to produce delayed skin reactions of the tuberculin type. This sensitivity develops within 5–7 days, and after transfer of spleen or lymph-node cells it can be detected within 3 days in recipients. Finally, studies of the antibody response of such recipients show again the relation between the cellular transfer of sensitivity and the later production of humoral antibody.

A brief review is given of results from earlier studies that led to the formulation of a working hypothesis that delayed hypersensitivity is responsible for expulsion of adult worms. After this, recent evidence is presented that proves the hypothesis, and encourages the belief that this phenomenon may play a prominent role in immunity to other tissue-invading parasites.

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Larvae and Larval Development of Monogeneans

J. LLEWELLYN

Department of Zoology and Comparative Physiology, The University, P.O. Box 363, Birmingham 15, England

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I. Introduction

The five years preceding the preparation of my review (Llewellyn, 1963) showed a great increase of available information about monogenean larvae. Since then relatively few new forms have been described and effort has been concentrated on some aspects of the biology of larvae and on larval development of a few species. In particular outstanding contributions to the biology of larval monogeneans have been made by Dr G. C. Kearn in England and Dr C. Combes in France, and this short review (which should be read in conjunction with my original review) owes much to their researches.

II. Structure

Combes (1967) used a silver nitrate technique to investigate the ciliated epidermal cells of larval *Polystoma integerrimum*, *P. pelobatis* and *P. gallieni*, and found their total number (55) and distribution to be exactly as Bychowsky (1957) had described for *P. integerrimum*. In these three forms, by the same technique, Combes discovered a well-marked bilateral series of silver nitrate-staining bodies which he regarded as sensilla of special but unknown function. Possibly, such techniques (using a silver salt that does not precipitate in sea-water) may reveal sense-organ patterns of taxonomic and phylogenetic importance.

The nature of the haptoral sclerites of monogeneans and their larvae was investigated by Lyons (1966), who found that the marginal hooks and hamuli are keratinous and arise in deeply-situated sites, i.e. are not superficial "cuticularizations". The larval hooks of *Gyrocotyle*, *Amphilina* and *Archigetes* are also keratinous, so that to known similarities in the shapes of hooks in monogeneans, gyrocotylideans and cestodes has been added newly discovered biochemical similarity.

Adult microbothriids are devoid of haptoral sclerites, and Bychowsky (1957) questioned their validity as monogeneans. However, Kearn (1965) hatched the eggs of *Leptocotyle minor* and found that the larval haptor bears six slender marginal spicules, each borne on a papilla. The spicules soon disappear in post-larval development, the parasite attaching itself to the dermal denticles of its host, *Scyliorhinus canicula*, by secreting a cement. Hooks could not penetrate the denticles, and Kearn regarded the spicules as vestiges of ancestry rather than functional larval organs. If the spicules are homologues of the (marginal) haptoral hooks of other forms, it seems probable that microbothriids are indeed monogeneans.

Sclerites described as "additional hooks" were observed by phase-contrast microscopy in 18 species of *Dactylogyrus* by Mizelle and Price (1963, 1965). These structures occupy constantly position "4A" in the scheme of numbering used by these writers, and thus in the interval between marginal hooks II and III in the system proposed by Llewellyn (1963). Gussev (1967) regarded this eighth pair of marginal hooks as additional tendons associated with one of the pairs of marginal hooks, but corresponding sclerites found in this position in three species of *Dactylogyrus* were regarded by Kearn (1968) as undeveloped ventral hamuli because they were associated topographically with marginal hooks II. Thus the "extra" hooks described by Mizelle and Price in dactylogyrids are almost certainly not marginal hooks but are vestigial ventral hamuli. (Kearn has shown that the prominent hamuli of dactylogyrids are in fact the *dorsal* hamuli—see p. 378).

"Extra" haptoral hooks (i.e. hooks in excess of the basic 16 marginals plus 4 hamuli lying between marginals II and III) had been described in some species of *Polystomoides* (e.g. by Price, 1939), but were overlooked in the previous review (Llewellyn, 1963). Since then similar "extra" hooks have been described in a new species *P. chabaudi* by Euzet and Combes (1965), and these hooks were said to be similar in shape and size to the postero-laterals (= "II" of Llewellyn, 1963), but were regarded as "inter-posterieurs". If the posterior hooks (="I" of Llewellyn, 1963) are regarded as marginal hooks, then *Polystomoides chabaudi* and some other species of *Polystomoides* have 18 *marginal* hooks, differing from nearly all other monogeneans where the basic number does not exceed 16. However, an "extra" pair of hooks was described in another polystomatid, *Protopolystoma xenopi*, by Thurston (1964), these hooks being situated, according to the scheme of Euzet and Combes, between the "postéro-lateraux" and the "inter-postérieurs" (i.e. making them "posterieurs"), and in Llewellyn's scheme between marginals I and II. A fundamental difference between marginal hooks and hamuli is that the former are primarily a part of the attachment apparatus of the newly-hatched larva, while the latter are essentially post-larval developments (though sometimes appearing precociously), and so the identities of the extra hooks might be expected to emerge from a developmental study. In fact, Dr Thurston (private communication) has observed that during the embryonic development of *P. xenopi* the haptor acquired 16 equally spaced hooklets as in *Polystoma*, but at that stage no hamuli; 3 days later, and still a few days before hatching, the "fine anchors" (hamuli) appeared. There can be little doubt then that the

"extra hooks" of *Polystoma xenopi* belong to the hamulus series of sclerites rather than to the marginal, but are not direct homologues of the second pair of hamuli ("outer" in entobdellids, "dorsal" in amphibdellids, tetraonchids etc.) of other monogeneans.

Thus the scheme of nomenclature for haptoral hooks proposed in the earlier review (Llewellyn, 1963) is able, without modification, to accommodate the subsequent reports of "additional" hooks, and its general adoption could be a useful tool in assessing the inter-relationships of monogeneans.

III. Host-finding by Oncomiracidia

In a series of elegant experiments Kearn (1967a) has investigated how the larvae of *Entobdella soleae* find their flatfish host, *Solea solea*. Skin-covered scales of various fishes were exposed simultaneously to free-swimming larvae, and there was highly selective "homing" onto the scales of the "correct" host *Solea solea*. By presenting successively "normal" epidermis (in the absence of dermis), then epidermis devoid of mucus-secreting cells (conjunctiva), then agar impregnated with epidermal mucus, Kearn showed conclusively that larval *Entobdella soleae* display a narrowly-specific chemotactic response to the skin mucus of *Solea solea*.

Combes (1966) carried out experiments with living tadpoles of *Rana temporaria* and *Pelobates cultripes* and the free-swimming larvae of their respective monogenean parasites *Polystoma integerrimum* and *P. pelobatis*. In simultaneous exposures of the two species of tadpole first to larval *P. pelobatis* and then to larval *P. integerrimum* a preference to the "correct" host of about 13:1 was found in *P. pelobatis* and of about 2:1 in *P. integerrimum*. However, this was not the only expression of specificity to operate: having invaded a "wrong" host, the "foreign" parasites were unable to persist on the gills and were lost progressively before reaching the stage at which they would have migrated to the bladder. Narrower specificity towards older hosts was reported by Konovalov (1967), who found that adult *Esox lucius* were parasitized by only *Tetraonchus monenteron*, whereas 1-year-old pike harboured *Dactylogyrus borealis*, *Gyrodactylus decorus*, *G. cernuae* and *G. phoxini*, these parasites normally being specific to other fishes.

IV. Invasion Route

In some highly specialized monogeneans, the gyrodactylids, invasion of a new host is by the direct transfer of adult forms to the skin of another fish when two hosts are closely situated, but in most monogeneans it is the larva which invades. The larvae of some oviparous monogeneans lack a ciliated epidermis and invasion is "passive", the larvae remaining on the bottom of the sea or pond until a suitable host comes to rest nearby. This is true of species of *Acanthocotyle* infecting *Raia* (see Kearn, 1967b), *Squalonchocotyle torpedinis* infecting *Torpedo marmorata* (see Euzet and Raibaut, 1960), and *Sphyranura oligorchis* infecting salamanders (see Alvey, 1936).

Kearn (1963) investigated the precise locations and sizes of individual *Entobdella soleae* on the skin of soles kept under conditions that promote the build-up of a dense population of parasites through re-infection. It was

established that the free-swimming larvae become attached to the upper surface of the anterior region (i.e. the part exposed when the fish is at rest in mud on the sea-floor) and remain there until about the time they become sexually mature, when they migrate to the underside of the fish and spread over the entire surface.

Prost (1963) found that there were two routes for *Dactylogyrus anchoratus* invading young carp. In young fishes up to about a month old, 10 mm long, invasion was sometimes by way of the mouth, but usually the operculum: after swimming at random for some time the larvae made a sudden movement and became attached to the skin of the fish, later migrating and aggregating near the bases of the pectoral fins before entering the ventral ends of the opercular openings. In older fishes, passive entry into the buccal cavity was the more frequent route of invasion, as Paperna (1963) found for *D. vastator* invading young carp (8 mm long). During the course of her work Prost confirmed some observations previously made by Izjumova about the failure of dactylogyrids to settle on the gills of carp less than about 7 mm long, when the structural differentiation of the gills is insufficient to provide a suitable anchorage for the parasites.

The invasion of two freshwater fishes (*Rutilus rutilus* and *Esox lucius*) by their respective monogenean parasites (*Neodactylogyrus crucifer* and *Tetraonchus monenteron*) and of the marine host *Morone labrax* by *Diplectanum aequans* was investigated by Kearn (1968), who kept the fishes in small aquaria so as to encourage re-infection and provide parasites at various stages of growth. The youngest parasites, differing from the larvae only in the absence of the ciliated epidermis, were found in scrapings of the skin and the lining of the buccal cavity, and it was concluded that the invasion route could be by attachment to and subsequent migration over the general body skin, or by passive ingress with the gill ventilation current and attachment to the buccal skin.

It was long supposed that at the metamorphosis of their hosts juvenile *Polystoma integerrimum* migrate from the gills of the tadpole to the bladder through the alimentary canal. However, Combes (1967) failed to find any migrating forms in the gut of tadpoles when according to this supposition they should have been there, but he discovered that juveniles migrate at night over the ventral skin of the tadpole to the cloaca and thence directly to the bladder the migration to the cloaca lasting only about 1 minute or so. When metamorphosing tadpoles with externally migrating juveniles were exposed to the newly-hatched larvae the latter went to the gills, even though the customary sojourn on the gills had been shown not to be a necessary preliminary to development in the bladder. Combes concluded that the two migrations (one to the gills and the other to the bladder) are controlled endogenously and not by stimuli from the host.

The free-swimming larvae of *Protopolystoma xenopi* do not invade tadpoles of *Xenopus* until about the time of the metamorphosis of the toad, and then they enter the cloaca directly, without first invading the gills (Thurston, 1964), resembling *Polystoma nearcticum* invading *Hyla*, and differing from *P. integerrimum* invading *Rana temporaria*. There is a gill-dwelling stage in *P. nearcticum*

but this consists of neotenics belonging to a different generation (Paul, 1938). A strange feature of the invasion of *Xenopus* by *Protopolystoma xenopi* is that the larval parasite passes from the cloaca to the kidney before returning to the bladder, a diversion reminiscent of the path followed by *Gorgoderina vitelliloba* during its invasion of the bladder of *Rana temporaria* as described by Lees and Mitchell (1964).

Using small specimens of *Rutilus rutilus* and *Abramis brama* as hosts, Bovet (1967) observed with a stereomicroscope that the larval *Diplozoon paradoxum* were drawn into the mouth with the gill ventilating current; they never crept over the fish's skin. The apparently passive nature of the oral invasion was a positive behavioural response, for the ciliary beating of the randomly-swimming larvae stopped in the currents of the catchment cone of the inhalant respiratory stream, allowing larvae to be swept towards the mouth. It sometimes took up to a dozen sucking efforts to draw a larva into the buccal cavity. Only about one in ten of larvae entering the buccal cavity attached to the gills, and even these were sometimes removed by "coughing" of the fish. Bovet claimed that these unsuccessful attempts at invasion did not imply immediate losses to the parasite population, even though the larvae had lost their cilia; in nature such larvae would settle on the bottom of the lake, and as *Abramis brama* is a bottom feeder, the larvae might be inhaled again and get a second chance of becoming attached.

Bychowsky and Nagibina (1967) have produced convincing circumstantial evidence that in some monogeneans the invasion of a new host is carried out not by a free-swimming larva, but through the agency of an intermediate host. Adult and near-adult forms of two narrowly-specific gastrocotylids *Pricea multae* and *Gotocotyla* sp. lived in great abundance on their respective predatory fish hosts *Scomberomorus commersonii* and *Sawara niphonica*, but young larval forms were not found. However, young gastrocotylids were found on a variety of small fishes (29 species belonging to 16 families) throughout the year. All these young parasites had approximately the same structure and similar haptors. The smallest moved actively over the gill lamellae, but lost mobility as they grew bigger and eventually settled at the distal ends of the primary lamellae, where most of them became embedded by their haptors. The host tissue responded to form a parapet surrounding one, two or three of these larvae, so that only about the anterior third of the bodies of the parasites projected. No degenerating larvae were found, nor empty craters that might have housed larvae. Because of this, and because no adult forms were found, it was supposed that these juveniles live for some time embedded in the gills in a state of suspended development, and that the larvae might be the juvenile forms of species of *Pricea* and *Gotocotyla* which live on large predatory fishes where no young juvenile forms had been found. This interpretation gained support when it was found that the distinctive haptoral sclerites (marginal hooks I and the hamuli) of the young parasites from the small fishes and those of growing adult specimens of *Pricea* and *Gotocotyla* parasitic on the larger predatory fishes could be arranged in a continuous series. It appears that in the life cycles of *Pricea* and *Gotocotyla* a non-specific intermediate fish host facilitates invasion of the specific definitive host.

V. Post-oncomiracidial Development

Bychowsky (1957) and Llewellyn (1957, 1963) concluded that comparative studies of the newly-hatched larvae of monogeneans would provide the best clues to a "correct" phylogenetic classification, but more recently it has been suggested that such studies should be supplemented by studies of the patterns of development, especially those of the post-larval stages (Llewellyn, 1965). The ontogenetic development of monogeneans usually includes three consecutive stages: (1) a marginal hook stage; (2) a hamulus stage; (3) a sucker/clamp or pseudohaptor stage. Occasionally stages may be omitted, but there is never any reversal of the sequence. The clamps may appear early, e.g. in *Diplozoon paradoxum* (see Bovet, 1967) and in the microcotylid *Polylabris diplodi* (see Euzet and Cauwet, 1967). In *P. diplodi* the hamuli are reduced to slender rods ("tigelles"), whereas in the larva of *Microcotyle donavini* (see Euzet and Marc, 1963) well-developed hamuli are present, but not clamps. The precocious appearance of the first (= posteriormost) pair of clamps may be related to degeneration of the hamuli. Diclidophorids appear not to have any hamuli (e.g. *Diclidophora denticulata* as described by Frankland, 1955), and here it seems that what is normally a third phase in the kind of attachment organ (the clamps) has suppressed and superseded the hamuli.

According to Euzet and Marc (1963) the post-larval development of *Microcotyle donavini* resembles that described by various authors for *M. spinicirrus*, *M. gotoi* and *M. chrysophryii*, and includes some degeneration of marginal hooks I ("crochets en fléau") and the hamuli at about the 5 pairs of clamps stage—before the complete disappearance of the languette bearing marginal hooks I and II and the hamuli at about the 6 or 7 pairs of clamps stage.

The post-larval development of *Acanthocotyle lobianchi* has been described by Kearn (1967b), who confirmed that the pseudohaptor of the adult develops from the larval body anterior to the larval haptor, where ten transverse folds develop on the ventral surface. The sclerites characteristic of the haptor of the adult are formed each from a single cell lying beneath the folds. The pseudohaptor becomes functional when the larva has lengthened by a factor of 4 from about 150 μ to 600 μ, and until then the larval haptor serves for attachment. There is no sign of hamuli at any stage of development.

The early post-larva of *Tetraonchus monenteron* uses the larval haptor before the development of the hamulus apparatus (Kearn, 1968), which first appears as two pairs of primordial hamuli in front of the larval haptor, but later is more posterior and becomes very complex before being used for attachment (Kearn, 1966).

In the initial stages on *Rutilus rutilus*, the larva of *Neodactylogyrus crucifer* uses its marginal hooks for attachment to the skin, but the haptor later becomes transformed as an adaptation to attachment between adjacent secondary gill lamellae (Kearn, 1968). During this process marginal hooks III and IV become dorsally directed, as does one pair of hamuli, the other pair remaining ventral and undeveloped. At the same time the marginal hooks, contrary to what happens in most monogeneans, continue to grow and, in conjunction with the dorsal hamuli, eventually constitute an apparatus which in the adult worm

facilitates attachment to the secondary gill lamellae of the host in contact with both ventral and dorsal surfaces of the haptor.

Three other dactylogyrids, *Dactylogyrus amphibothrium*, *D. auriculatus* and *D. sphyrna*, all show post-larval development similar to that of *Neodactylogyrus crucifer*, but in *D. sphyrna* marginal hooklet III grows especially long to act in conjunction with the dorsal hamulus as a pincer apparatus (Kearn, 1968; Glaser, 1965).

Some features of post-larval development of *Diplectanum aequans* were described by Paling (1966), and in more detail by Kearn (1968). The metamorphosis of the haptor, including the dorsal migration of marginal hooks III and IV, is generally similar to that in dactylogyrids, but in *Diplectanum* the ventral hamuli develop faster than the dorsal hamuli. Transverse bars are developed in association with the hamuli, and later pseudohaptoral squamodiscs (=plectana) form in front of the haptor, one squamodisc being directed dorsally and the other ventrally. The armature of the squamodiscs is derived from the epidermis, in contrast to the more deeply-seated origin of the sclerites of the pseudohaptor of *Acanthocotyle* (as described by Kearn, 1968). In the adult *Diplectanum* the squamodiscs act in conjunction with the hamuli of the same surface of the body to form a pincer-like attachment apparatus (Paling, 1966).

Kearn (1968) found an unidentified diplectanid on the gills of *Solea variegata*, and this differed from *Diplectanum aequans* in that the ventral hamuli and marginal hooks II remained ill-developed.

VI. Conclusions

Recent research on the larvae and larval development of monogeneans has yielded information about the distribution of larval sensilla that may be of taxonomic and phylogenetic interest, the origin and chemical nature of haptoral sclerites, the presence in microbothriids of haptoral spicules that may be homologous with the marginal hooklets of other monogeneans, the presence of an "extra" pair of haptoral marginal hooks (which, however, in one case have been shown to be vestigial ventral hamuli, and in the other to be an extra pair of unusually-situated hamuli), and the establishment of the homology of the single pair of hamuli in dactylogyrids with the *dorsal* hamuli of those monogeneans in which two pairs of hamuli are present.

Some larvae find their hosts by chemotactic response to the mucus secreted by the epidermal cells of their hosts. An invasion of unusual hosts may take place when the hosts are young, but in these cases specificity becomes more restricted with the increasing age of the host.

Among monogeneans there is a fairly wide range of ways of invading new hosts, including the direct transfer of adults, the attachment of a non-ciliated larva to the limbs or bodies of hosts which come to rest over the larvae, invasion via the skin in both skin and some gill parasites (which gain access to the gills by the opercular opening), invasion with the inhalant respiratory current through the mouth, and invasion by the ingestion of an intermediate host.

In some monogeneans degenerate hamuli may be associated with the

precocious development of the clamps of the adult, and in others (acanthocotylids) the hamuli seem to have disappeared. The dorsal hamuli of forms with two pairs of hamuli have their origin as ventrally-directed structures, which later rotate to face dorsally, an orientation also taken up by marginal hooks III and IV. In some monogeneans the ventral pair of hamuli remains undeveloped with the dorsal hamuli constituting the single pair of "median hooks" of the adult.

The biggest gaps in our knowledge about the inter-relationships of the major groups of monogeneans concern, on the one hand, the entobdellid–amphibdellid–diplectanid–dactylogyrid line (with hooks I of the oncomiracidium centrally-placed, and 2 pairs of hamuli), and, on the other, the monocotylid line (with none of the marginal hooks centrally placed, and a single pair of hamuli). It is likely that more information about the larvae and larval development of tetraonchoidids (adults with 16 marginal hooklets, none central, and one pair of hamuli—see Bychowsky *et al.*, 1965) and *Euzetrema knoepffleri* Combes, 1965 (adult with 16 marginal hooklets, 2 of them central, and two pairs of hamuli) would yield valuable information for solving these problems. Attempts are being made to pursue this line of enquiry (Llewellyn and Kearn, work in progress).

This new information generally substantiates a classification of monogeneans proposed previously (Llewellyn, 1965) and permits some elaborations: dactylogyrids are regarded as offshoots of diplectanids (i.e. with 14 marginal hooks—I centrally placed and VIII lost—and with the hamulus apparatus having reduced or no ventral components, and without squamodiscs); acanthocotylids are regarded as offshoots of the entobdellid line (with centrally-placed marginal hooks I) in which the hamuli have been lost and replaced by a pseudohaptor; and microbothriids are regarded as having soon branched from protomonogeneans, replacing the marginal hooks (six of which remain, however, as vestigial rods) with a cement-secreting apparatus.

All this supports the view that the protomonogenean may have resembled the hooked larva of modern monogeneans. The absence of hooks in udonellids, together with some other differences, led Ivanov to remove this group from the Monogenea, but van der Land (1967) restored it as the sub-class Udonellida alongside the Monogenea *sensu stricto* and the Gyrocotylida, which Llewellyn (1965) regarded as endoparasitic monogeneans that have lost the gut.

Stunkard (1967) rejected Llewellyn's (1965) view of the derivation of cestodes from monogeneans, suggesting that such a view requires anachronistic postulates that are at variance with essential phylogenetic criteria. Stunkard's arguments are based on his belief that the hooks of the cestode oncosphere are *anterior* and serve not for attachment but for progression and, being anterior, could not correspond with the posteriorly-situated hooks of larval monogeneans. Stunkard had explained his interpretations of the orientation of cestodes in another paper (Stunkard, 1962), where he cited several examples of cyclophyllidean oncospheres which move with the hooks in front. These observations are not in dispute; but the choice as representative cestode larvae of non-ciliated cyclophyllidean oncospheres from ingested eggs which hatch in an intermediate host seems odd when in the same paper Stunkard referred to

the free-living coracidium of a pseudophyllidean swimming with the hook-bearing end at the rear—only *later* does it move through the tissues with the hooks in advance. Cyclophyllideans are essentially parasites of terrestrial vertebrates which use mainly terrestrial intermediate hosts, and the absence of cilia in the newly-hatched larva appears to be a loss from an ancestral stage in which the protocestode free-swimming larva (resembling e.g. juvenile turbellarians, monogenean oncomiracidia, and digenean miracidia) invaded an aquatic host, a condition which survives in many modern pseudophyllideans. But in any case the backward movement of an oncosphere to use efficiently an inherited hook apparatus for a new job requires no special explanation; the cercariae of some digeneans are known to swim "tail first", and it is quite unnecessary to invoke analogies with the "reversals of polarity" which are thought possibly to occur during the development of some organisms.

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