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VOLUME 29

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PREFACE

This volume starts with a paper by David Godfrey and his colleagues on a topic that they have made very much their own—the study of isoenzymic variants within the rather enigmatic subgenus *Trypanozoon* of the genus *Trypanosoma*. As a result of an immense amount of work over several years, they have analysed (with the aid of a computer) the electrophoretic profiles of 11 enzymes from (literally) hundreds of populations of trypanosomes. The results of this monumental task, a summing up of years of work, very probably provide a definite answer to the question, debated ever since the original descriptions of the species at the turn of the century, of the relationship between the members of the so-called *T. brucei* group.

David Kemp, Alan Cowman and David Walliker take us into the molecular minefield of a topic that they, too, have lately made very much their own—the genetics of the genus *Plasmodium*. They discuss antigenic diversity and its significance in the topical field of vaccine production, chromosome size polymorphisms, meiosis and genetic recombination, and—another topical and important aspect—drug resistance.

Masamichi Aikawa and Carter Atkinson review a fairly recently developed technique, immunoelectron microscopy, and its applications within the discipline of parasitology. After a full technical, explanatory introduction, they discuss applications of the process to parasites both protistan (*Plasmodium*, *Toxoplasma*, *Sarcocystis*, *Eimeria*, *Theileria* and *Trypanosoma*) and helminthic (*Trichinella* and other nematodes, *Schistosoma* and *Fasciola*). The review ends with detailed appendices describing the techniques involved.

Jane Huffman and Bernard Fried write on a topic more purely helminthological, reviewing an interesting group of parasites which perhaps suffer somewhat from underexposure—the echinostomes. The authors first help to clear up the rather confused systematics of the group, partly caused by the lack of host specificity, and then comprehensively review the biology, immunology, epidemiology, physiology and biochemistry. The use of members of the group as easily maintained laboratory models is stressed.

Finally, Hilary Hurd ends this volume with a subject which should interest both protistologists (as we must learn to call them) and helminthologists—the complex and fascinating interactions between parasites and their invertebrate hosts. This, again, is a topic which tends to be overlooked. Volumes have been written (including many reviews in this series of *Advances*) about the relationships between parasites and their vertebrate hosts, but this review considers the much less fashionable, though biologically no less important, aspects of their relationships with invertebrates—including those which serve as vectors of parasites of man and domestic animals. Dr Hurd has synthe-

sized information from a wide variety of sources and has attempted a conceptual rather than descriptive treatment. The complex manner in which host and parasite interact to limit harmful effects but to perpetuate the relationship are indicative of highly co-evolved associations.

J. R. BAKER
R. MULLER

CONTENTS

CONTRIBUTORS TO VOLUME 29 v
PREFACE vii

The Distribution, Relationships and Identification of Enzymic Variants within the Subgenus *Trypanozoon*

D. G. GODFREY, R. D. BAKER, L. R. RICKMAN AND D. MEHLITZ

I. Introduction 2
II. Materials and Methods 3
III. Observations and Comments 7
IV. Strain Groups 22
V. The Dispersal of the Subgenus *Trypanozoon* 35
VI. Conclusions 38
Acknowledgements 40
References 40
Appendix 44

Genetic Diversity in *Plasmodium falciparum*

D. J. KEMP, A. F. COWMAN AND D. WALLIKER

I. Introduction 76
II. Antigenic Diversity in *Plasmodium* 78
III. Chromosome Size Polymorphisms 101
IV. Meiosis and Genetic Recombination 108
V. The Genetic Basis of Drug Resistance 116
VI. Outlook 126
Acknowledgements 128
References 128

Immunoelectron Microscopy of Parasites

M. AIKAWA AND C. T. ATKINSON

I. Introduction 151
II. Methods 152
III. Applications to Parasitology 160
IV. Conclusions 194
Acknowledgements 195

References 195
Technical Appendix 210

Echinostoma and Echinostomiasis

J. E. HUFFMAN AND B. FRIED

I. Introduction and the Genus *Echinostoma* 215
II. Biology of 37-Collar-spined Echinostomes 224
III. Infectivity Studies in First Intermediate Snail Hosts 228
IV. Infectivity Studies in Second Intermediate Hosts 232
V. Infectivity, Growth and Development in Vertebrate Hosts 238
VI. Immunology of Echinostome Infections in Vertebrate Hosts 246
VII. Clinical and Pathological Effects of Echinostomes 248
VIII. Epidemiology and Disease of Echinostomes 253
IX. Physiology and Biochemistry of *Echinostoma* 254
X. Concluding Remarks and Future Prospects 258
References 260

Physiological and Behavioural Interactions Between Parasites and Invertebrate Hosts

H. HURD

I. Introduction 272
II. Parasitic Castration 273
III. Host Growth 286
IV. Host Sex 290
V. Parasite Modification of Host Behaviour 291
VI. Host–Parasite Synchrony 299
VII. Endocrinological Interactions 300
VIII. Conclusions 304
Acknowledgements 305
References 306
Appendix 317

The Distribution, Relationships and Identification of Enzymic Variants within the Subgenus *Trypanozoon*

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I. Introduction 2
II. Materials and Methods 3
A. Trypanosomes 3
B. Enzyme electrophoresis 4
C. Numerical analyses 6
III. Observations and Comments 7
A. General 7
B. Dendrogram 9
C. Cladogram 11
D. Distribution 14

ADVANCES IN PARASITOLOGY VOL 29
ISBN 0-12-031729-X

E. Mixed populations 16
F. Infrequent enzyme patterns 18
G. Limitations 20
IV. Strain Groups 22
A. *T. b. rhodesiense* 23
B. *T. b. brucei* 27
C. *T. evansi* 30
D. *T. b. gambiense* 32
E. Unidentified zymodemes 33
V. The Dispersal of the Subgenus *Trypanozoon* 35
A. East Africa 36
B. West Africa 37
VI. Conclusions 38
Acknowledgements 40
References 40
Appendix 44

I. Introduction

The morphological similarity of the behaviourally different kinds of trypanosome (*Trypanosoma* spp.) within the subgenus *Trypanozoon* has led to considerable uncertainty regarding the taxonomic status of each (see Hoare, 1972). The uncertainty is only beginning to be resolved by studies on enzyme polymorphism and deoxyribonucleic acid (DNA) (e.g., Godfrey and Kilgour, 1976; Gibson *et al.*, 1980, 1985; Borst *et al.*, 1981, 1987; Tait *et al.*, 1985; Paindavoine *et al.*, 1986; Godfrey *et al.*, 1987). On the other hand, epidemiology has benefited from an appreciation of the geographical and hostal distribution of the different genetic forms (e.g., Gibson *et al.*, 1978, 1980, 1983; Mehlitz *et al.*, 1982; Gibson and Gashumba, 1983; Gibson and Wellde, 1985; Otieno and Darji, 1985; Boid, 1988).

So far, much of the characterizing work has been based on the enzyme polymorphisms seen after electrophoresis and specific staining. Since each enzyme in a sample may appear as several isoenzyme bands, the use of a number of enzymes results in a complex profile for every trypanosome population examined. Although this genetically controlled profile remains consistent in a clone, the wide variety encountered means that the assessment of relationships is best addressed by mathematical methods (Gibson *et al.*, 1980; Tait *et al.*, 1984, 1985). It is possible that a new enzyme profile may be generated after hybridization between two different isolates (Jenni *et al.*, 1986; Sternberg *et al.*, 1988), although the frequency of this phenomenon in nature remains unknown (Cibulskis, 1988).

One purpose of this review is to present and compare the enzyme profiles of a large number of trypanosome populations from many countries. The results were obtained over some years by associated workers using thin layer

starch gel electrophoresis under similar conditions with the same range of enzymes. Many published results are presented again here for comparision and for inclusion in the numerical analyses, which were used to group related trypanosomes and to devise a practical identification system.

The evaluation of any group rests on comparing its epidemiological and other non-enzymic attributes with those of other groups. Greater confidence can be placed on validity if similar groupings are produced by different numerical approaches. Consequently, two methods were used to determine relationships and groups. A similar dual approach has demonstrated three genetically distinct groups in *T. congolense*, which were later confirmed by differences in satellite DNA (Gashumba *et al.*, 1988; Gibson *et al.*, 1988). Unfortunately, the divisions within the subgenus *Trypanozoon* were not so striking and required detailed consideration, as described below.

II. Materials and Methods

A. TRYPANOSOMES

1. *Isolation and preparation*

Summaries of the origins of the primary isolates used are listed in Table A3. The references quoted in the text and tables (see Appendix) describe in detail the means of isolating and preparing the trypanosomes. In brief, the organisms were isolated from the original host by inoculating rodents, before cryopreservation and subsequent further multiplication in rodents to provide sufficient material for examination. Sometimes this was achieved by immunosuppression of the animals with cyclophosphamide or γ-irradiation. The bloodstream forms were obtained from the host's blood by column separation and centrifugation (Lanham and Godfrey, 1970), followed by extraction of the water-soluble enzymes. The extracts were stored in liquid nitrogen.

The observations on a number of stocks have been reported previously (Table A2). Most of the new results were obtained from isolates collected in Zambia and West Africa; details of these in relation to local epidemiology will be published later.

2. *Definitions*

For simplicity, the term "animal", when used in this publication to describe the original host, refers to a mammal other than man (or, in one case, to a bird).

Some primary isolates contained several populations of *Trypanozoon*, as shown by differences in enzyme profiles (Tables A3 and A5), and it is likely that other mixtures were undetected. The term "population" is used to describe the trypanosomes harvested for analysis on a particular occasion.

The generalizations in the text regarding location of isolates, etc., in East or West Africa are related to an imaginary line running from north to south down the middle of the continent. This simple division permits a clearer view of the observations than if further geographical terms had been included, such as central and southern Africa.

B. ENZYME ELECTROPHORESIS

1. *Techniques*

The methods for electrophoresis and specific enzyme staining in thin layer starch gel are described by Bagster and Parr (1973), Kilgour and Godfrey (1973) and Gibson *et al.* (1978).

The results were obtained with the following 11 variable enzymes, listed from the least to the most polymorphic: EC 5.3.1.9, glucose phosphate isomerase (GPI); EC 3.2.2.1, nucleoside hydrolase (NH); EC 1.1.1.103, threonine dehydrogenase (TDH); EC 1.1.1.42, isocitrate dehydrogenase (ICD); EC 1.1.1.37, malate dehydrogenase (MDH); EC 2.7.5.1, phosphoglucomutase (PGM); EC 2.6.1.1, aspartate aminotransferase (ASAT); EC 2.6.1.2, alanine aminotransferase (ALAT); EC 3.4.11, two peptidases, (i) substrate L-leucyl-L-alanine (PEP2) and (ii) substrate L-leucylglycineglycine (PEP1); and EC 1.1.1.40, "malic" enzyme (ME).

2. *Terminology*

After electrophoresis and staining, each enzyme gave a pattern consisting of one or several isoenzyme bands. Unlike earlier work, every pattern was coded with an arabic instead of a roman numeral after the enzyme abbreviation (e.g., ALAT-1, ALAT-2) (Fig. 1). This change occupies less computer space and avoids the errors that may arise when entering complex roman numerals.

Enzyme patterns were numbered in sequence as new ones were found. During the separate investigations, the same pattern was at times coded differently. The coding is now standardized but with the consequence that the sequence is incomplete with certain enzymes (Fig. 1); better rationalization is not possible because many pattern numbers are already published.

In a trypanosome population, the combination of patterns for the 11 enzymes is the "enzyme profile"; populations with the same profile belong to

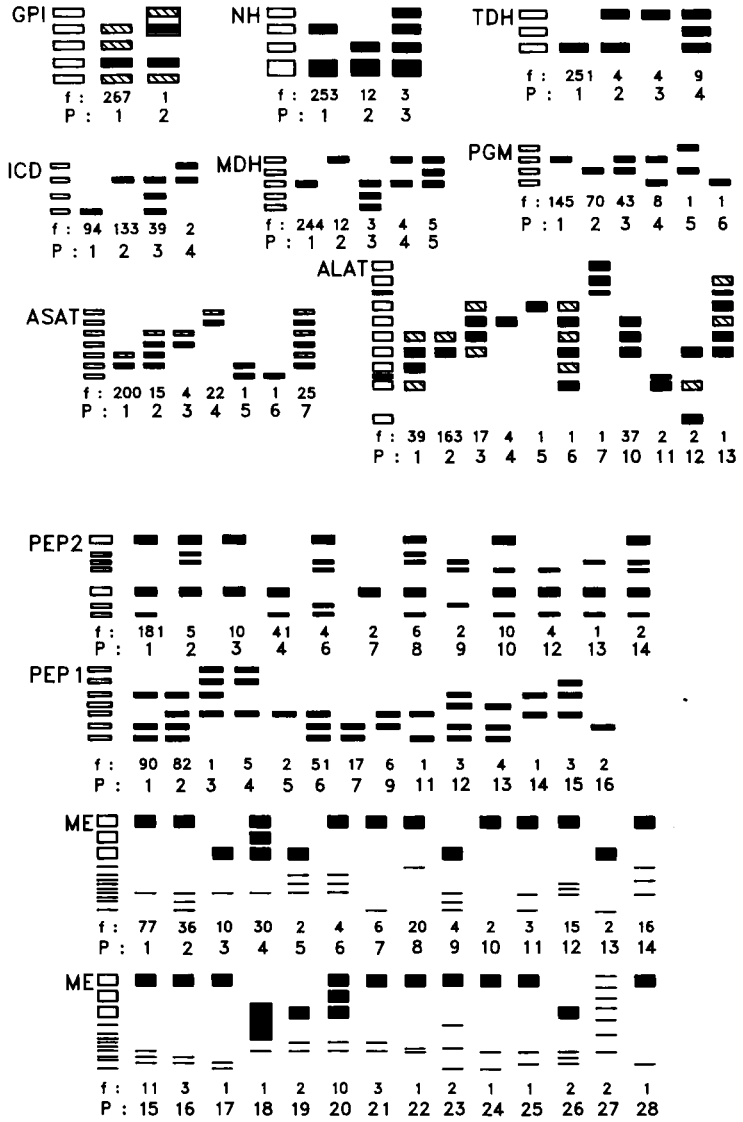

FIG. 1. Diagrams of enzyme patterns found in *Trypanozoon*. P, pattern number; f, number of zymodemes (total = 268) with pattern. All isoenzymes migrated towards the anode, represented by the top of each diagram. Faint or inconsistent bands are hatched. Only the bands that stained consistently were used in the numerical analyses. The full names of the enzymes are given in the text (Section I B 1, p. 4).

the same "zymodeme" (Z). It should be noted that two populations with identical profiles for a range of enzymes may differ if a further enzyme is introduced; they would then be separated into different zymodemes. For the same reasons as already given for missing pattern numbers, the zymodeme numbering sequence also has omissions.

C. NUMERICAL ANALYSES

1. *Dendrogram*

Like that for *T. congolense* (Gashumba *et al.*, 1988), the dendrogram for *Trypanozoon* was constructed by using Ward's (1963) method. This is a phenetic approach, as relationships are established from the attributes as now observed, with no implications of evolutionary relationships.

The profile for each zymodeme was regarded as an operational taxonomic unit (OTU). Dissimilarities between all possible pairs of OTUs were calculated as Euclidean distances, which are the sums of the squared character differences. Only consistent isoenzyme bands were used (Fig. 1), although the inconsistent bands are also recorded for information. For each pattern in every profile, the presence of a consistent band is entered as "1", and its absence as "0". This is equivalent to taking the distance between profiles to be the number of non-matching bands. The computer formed a distance matrix which was used to group progressively pairs of OTUs, and to determine which pairs of groups should be clustered at any stage. Gibson *et al.* (1980) used a similar, but not identical, method to construct a dendrogram for *Trypanozoon* enzyme profiles, as did Young and Godfrey (1983) for *T. congolense*. Another dendrogram was produced by Paindavoine *et al.* (1986), based on DNA digests of *Trypanozoon* after electrophoresis.

2. *Cladogram*

With the same raw data used for the dendrogram, the computer was used to construct a phylogenetic tree, or cladogram (Gashumba *et al.*, 1988), of developmental pathways that involved the minimal amount of genetic alteration. The dissimilarity between two zymodemes was the "patristic distance" (PD) between them through the cladogram. The measure of distance between OTUs was again the number of non-matching bands (Section II C 1). Since the dendrogram had nine obvious sections (Fig. 2), the cladogram was broken into the same number of clusters, by progressively cutting it to produce on each occasion the two best-resolved groupings of zymodemes.

When constructing the initial cladogram, the characters, or bands, were

weighted by using an iterative technique. Characters that changed many times during the traverse of the phylogenetic tree were given least weighting, while those seldom changing were considered potentially significant whenever they did alter. However, with so many characters available, this refinement did not appreciably modify the form of the cladogram.

The program was devised by R.D.B.

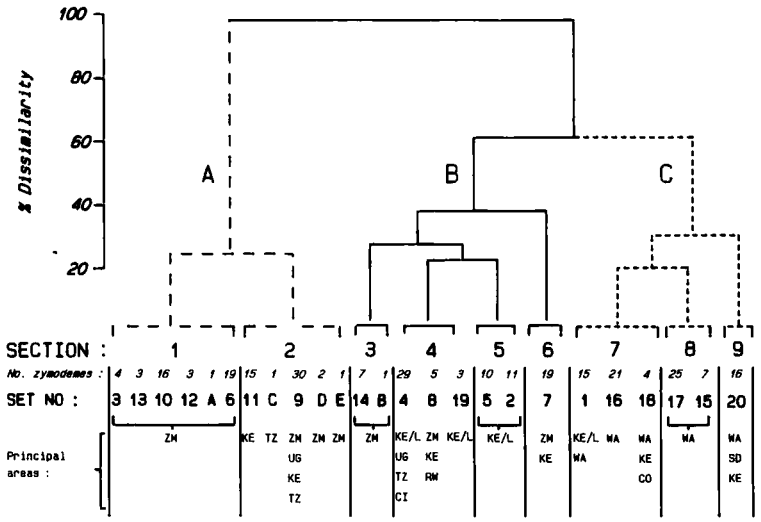

FIG. 2. Diagram representing the dendrogram of relationships between 268 *Trypanozoon* zymodemes. Three main divisions (A, B, C) are seen, and nine sections. See Table A1 for explanation of sets. CI, Ivory Coast; CO, Colombia, KE, Kenya; KE/L, Lambwe Valley, Kenya; RW, Rwanda; SD, Sudan; TZ, Tanzania; UG, Uganda; WA, West Africa; ZM, Zambia.

III. OBSERVATIONS AND COMMENTS

A. GENERAL

Altogether, 945 populations were examined, which were contained in the 268 zymodemes used in the analyses.

The enzyme patterns found are shown in Fig. 1, together with their frequency among the 268 zymodemes. The dendrogram is expressed diagrammatically in Fig. 2 and the cladogram in Fig. 3. Table A1 shows the zymodemes that remained together in both the dendrogram and the cladogram. These are numbered as "sets", and their positions can be compared in

Figs 2 and 3. Reference to the set number allows information to be found from Tables A2 and A3 on the profile of each zymodeme, as well as the number and provenance of the constituent populations.

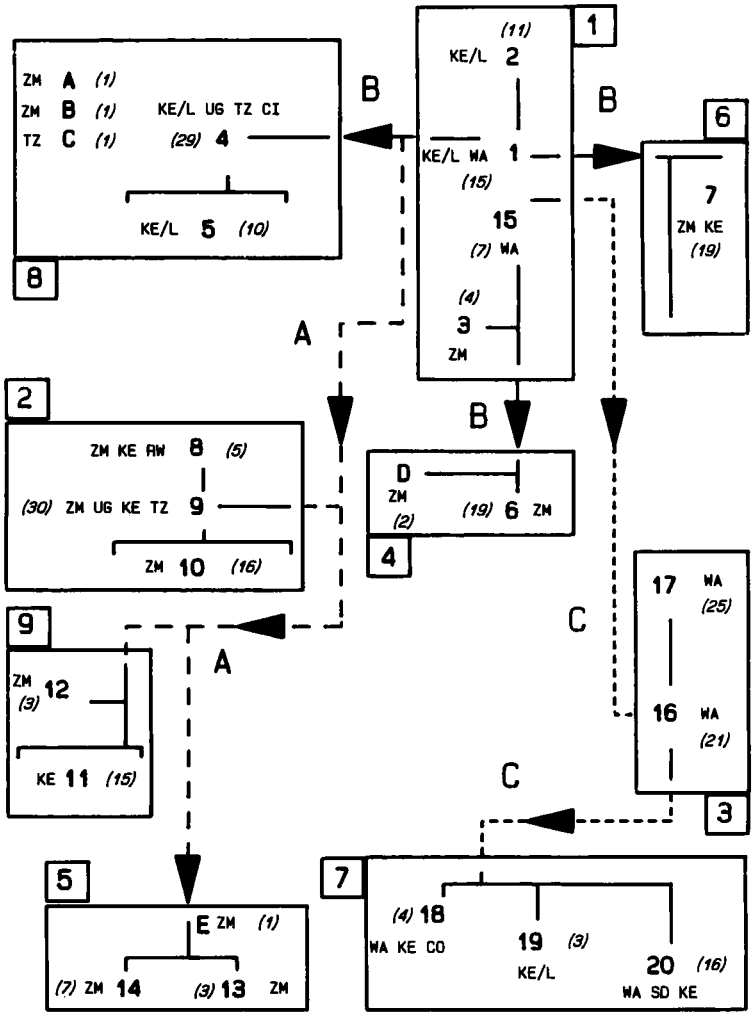

FIG. 3. Diagram representing the cladogram of relationships between 268 *Trypanozoon* zymodemes. Three main developmental pathways (A, B, C) are seen, with nine separate clusters. See Table A1 for explanation of sets, and Fig. 2 for country abbreviations. Large figures in each cluster are set numbers; figures in parentheses refer to the number of zymodemes in a set.

The dendrogram (Fig. 2) was divided into three main divisions (A, B and C). Division A consisted mostly of zymodemes associated with East Africa, and was quite distinct from the predominantly West African division, C. The third division, B, contained many zymodemes linked with the Lake Victoria region. Further subdivision, at the level of 20% dissimilarity or more, gave nine sections of related zymodemes.

In the cladogram, the clusters separated along three principal pathways, termed pathways A, B and C to facilitate comparison with the dendrogram divisions. In fact, the pathways developed in the sequence A–C–B, with pathway B being better regarded as a residual group of clusters remaining after the other pathways had broken away.

The pathways of the cladogram correspond, albeit imperfectly, to the three dendrogram divisions. Reference to Table A1, in conjunction with the distribution of sets in Figs 2 and 3, shows that 72% of the zymodemes in dendrogram division A also separated along pathway A in the cladogram. Similarly, 82% of those in division B were in pathway B, while 75% of division C zymodemes diverged along pathway C. Reflecting the closer alliance between divisions B and C in the dendrogram, pathway C did not part from pathway B until after pathway A had branched off.

Broadly, then, three similar major groupings existed in both the dendrogram and cladogram, reflecting an East and West African separation within *Trypanozoon*, together with a grouping particularly associated with the more central Lake Victoria region. Gibson *et al.* (1980) reached a similar conclusion.

There was little exact correspondence between the nine dendrogram sections and the nine cladogram clusters (Table A1); only section 6 and cluster 6 matched perfectly to produce set 7. Sections and clusters usually split into two or three large sets when the results of both analytical methods were combined. Some of the 20 principal sets so formed appeared valid, but, for a number of related sets, regrouping produced the most sensible collections of zymodemes. These aspects are considered in detail in Section IV.

B. DENDROGRAM

Because of the difficulty of visually reproducing in a small figure the relationships between many zymodemes below the 20% level of dissimilarity, the horizontal disposition of the various sets in the dendrogram (Fig. 2) is shown only approximately. Tables A2 and A3 give the details of enzyme profiles and origins of populations in the sets of zymodemes (Table A1).

1. *Division A*

Division A was separated from the other divisions at a dissimilarity approaching 100%. Within this division, sections 2 and 3 differed from each other at more than the 20% level. The original isolates in both sections were almost entirely from East Africa, principally Zambia.

(*a*) *Section 1.* Every zymodeme was associated with Zambia. The majority (76%) belonged to sets 6 and 10, which, however, separated along different pathways in the cladogram into clusters 2 and 4. Set 10 had a wider geographical affinity within East Africa than set 6, which was exclusively associated with Zambia.

(*b*) *Section 2.* The zymodemes were linked with different parts of East Africa, although no Kenyan population originated from the Lambwe Valley, near Lake Victoria. Of the zymodemes 92% were in sets 9 and 11, with strong Zambian and central Kenyan affinities, respectively. The two sets appeared in cladogram clusters 2 and 9, along pathway A.

2. *Division B*

Division B differed from division C at about 60% dissimilarity. Section 6 was the most dissimilar within the division. Most populations were East African in origin, including many from the Lake Victoria region; a few were from West Africa.

(*a*) *Section 3.* This section was about 30% dissimilar from sections 4 and 5; all populations were from Zambia. It corresponded mostly to set 14, which formed a substantial part of cladogram cluster 5 in pathway A.

(*b*) *Section 4.* A dissimilarity of over 20% separated this section from section 5. A number of zymodemes had affinities with the Lake Victoria region in both Kenya and Uganda, although there was also some association with other parts of East as well as West Africa. Of the zymodemes 78% occurred in set 4, occurring also in pathway B of the cladogram; the rest were in the small sets 8 and 19, which separated along the two other pathways.

(*c*) *Section 5.* The association with the Lake Victoria area was perhaps more striking than in the previous section. Sets 2 and 5, of similar size, formed the section, but separated into the clusters 1 and 8 in pathway B of the cladogram.

(*d*) *Section 6.* This distinct section was of the order of 37% dissimilar from the other sections within division B. It formed set 7, and corresponded exactly with cluster 6 in pathway B of the cladogram. The constituent populations were principally from the Kenyan coastal area and Zambia.

3. *Division C*

Division C was 60% dissimilar from division B. Within it, the dissimilarity between sections 7 and 8 was only 20%, which is on the lowest limit of the subjective and arbitrary partition of the dendrogram; these two sections were about 30% dissimilar from section 9. The component populations were principally West African in origin, although a substantial number came not only from elsewhere in Africa, but also, as *T. evansi*, from other parts of the world.

(*a*) *Section 7.* The large sets 1 and 16 formed 90% of this section; in addition, the small set 18 was included. Only sets 16 and 18 remained near each other along pathway C in the cladogram, albeit separately in the adjoining clusters 3 and 7. Although both sets had marked West African affinities, set 18 was more cosmopolitan and corresponded to *T. evansi*. Set 1 appeared in cluster 1 of pathway B; numerous populations came from near Lake Victoria in Kenya, although many others originated elsewhere in East and West Africa.

(*b*) *Section 8.* Sets 15 and 17, each with strong West African links, comprised this section; however, they were in different cladogram clusters and pathways, clusters 1 (pathway B) and 3 (pathway C), respectively.

(*c*) *Section 9.* The zymodemes in this distinct section conformed mostly to the concept of *T. b. gambiense*; all were contained in set 20, which was placed in cluster 7 at the end of pathway C in the cladogram. Most populations were isolated in gambian trypanosomiasis areas.

C. CLADOGRAM

The full cladogram with every zymodeme linked proportionally to the patristic distance (PD) in the phylogenetic tree is impossible to present clearly on a small scale in two dimensions. A highly stylized representation of the spatial diagram produced by the computer after the breakdown into nine clusters is shown in Fig. 3; Table A2 includes the PD of each zymodeme. However, zymodemes with similar PDs are not necessarily

closely related because they may occur along a different pathway stemming from an ancestral hypothetical taxonomic unit (HTU).

Initially, two large groups of zymodemes broke off from the others at different points before separating further into clusters along the pathways A and C. Other clusters separated individually from the residual pathway B, leaving cluster 1 as a somewhat heterogeneous collection of zymodemes around the HTU. The numbers allocated to the clusters in Fig. 3 are in the same sequence as that in which the computer established a new group of clusters, or a single one; the order was not sequential along any one pathway.

Each of the nine clusters had several internal branches, represented very simply in Fig 3; the positions of the sets (Table A1) in each cluster are given only approximately. Details of the sets are listed in Tables A2 and A3.

1. *Pathway A*

The first split in the program separated pathway A from pathways B and C. This early divergence implied that the two groups of clusters contained zymodemes with the greatest differences; it broadly corresponded with the gross dissimilarity between dendrogram division A and the other two divisions. Pathway A was almost exclusively associated with East Africa.

The pathway later divided into three clusters. Cluster 5 was the first to diverge, followed by cluster 9, leaving cluster 2 closest to the HTU.

(*a*) *Cluster 2.* A substantial number of zymodemes was spread along two principal branches. In contrast to their separation in the dendrogram, sets 8, 9 and 10, with their wide East African associations, were brought together. Although many populations were from Zambia, some were from other East African countries.

(*b*) *Cluster 9.* The final, or eighth, separation in the whole cladogram occurred along pathway A to split off cluster 9 from cluster 2, thus indicating a close relationship between the two. Cluster 9 was formed of sets 11 and 12, which were placed in adjoining dendrogram sections. The large set 11 had strong links with central Kenya, while the small set 12 was associated with Zambia.

(*c*) *Cluster 5.* The first break along pathway A, the fourth in the whole cladogram, resulted in cluster 5, making it the most distinct cluster in the pathway, as illustrated by the high PD values. Principally, sets 13 and 14 were included, but these were in separate divisions in the dendrogram. However, both were connected only with Zambia, as was the minor set E,

which was also in cluster 5. Set 13 was small; set 14 was larger, and included zymodemes with the highest PDs recorded (e.g., Z11 with PD = 289).

2. *Pathway B*

Pathway B remained when pathway C diverged at the second break in the cladogram; it resembled division B of the dendrogram. In this instance, however, "pathway" may be a misleading term; it was more a collection of zymodemes with clusters separating at intervals to leave the final residue as cluster 1, which contained the zymodemes nearest to the HTU. The four clusters in pathway B had mostly East African affiliations, especially with the region near Lake Victoria, but nonetheless there were West African links as well.

(*a*) *Cluster 1.* This cluster remained after cluster 8 had diverged at the seventh, or last, split in the cladogram. Sets 1, 2, 3 and 15 comprised the cluster, but occupied quite separate positions within the dendrogram; sets 1 and 15, however, were separated only into the marginally dissimilar sections 7 and 8. Sets 1 and 2, associated with the Lambwe Valley in Kenya, were brought close together in cluster 1.

Sets 1 and 15, with low PDs, were adjacent and central. One branch led to set 2 which, like set 1, had Lambwe Valley affinities; another branch went in another direction to the small Zambian set 3, lying near the junction with the characteristically Zambian cluster 4. A further junction led to the substantially West African pathway C.

(*b*) *Cluster 8.* This cluster remained with cluster 1 until the last separation along pathway B, and hence was closely related to it. The essentially East African sets 4 and 5 were brought together; both sets were in division B of the dendrogram, but in separate sections. A strong association existed with the epidemics around Lake Victoria, although in addition some populations in set 4 originated in a more ubiquitous fashion throughout East Africa. Moreover, this set contained a few populations from West Africa.

(*c*) *Cluster 6.* The fifth split in the cladogram formed this cluster directly from cluster 1. It contained only the zymodemes of set 7, and matched section 6 of the dendrogram. Most populations originated in Zambia and the coastal part of Kenya. The most distant zymodemes in pathway B were included in cluster 6 (e.g., Z94 with PD = 210).

(*d*) *Cluster 4.* This cluster was the first to be detached along pathway B,

by the third separation in the overall program, and was thus the most distinct from cluster 1 in that pathway. It consisted mostly of set 6, which formed part of dendrogram section 1. The constituent populations were almost entirely from Zambia. The nearest zymodemes in cluster 1, those in set 3, were also associated with Zambia.

3. *Pathway C*

Pathway C began at an early stage in the program by breaking away from pathway B at the second split; the partial relationship between the two pathways resembled that between dendrogram divisions B and C. Although the clusters were predominantly West African in character, *T. b. gambiense* from East Africa was also included, in addition to *T. evansi* with its considerably wider geographic affinities.

(*a*) *Cluster 3.* This cluster was the nearest to the HTU along pathway C. Sets 16 and 17 were brought together; they were located in the marginally different sections 7 and 8 of the dendrogram. The whole cluster was almost exclusively West African.

(*b*) *Cluster 7.* The sixth cladogram separation detached this cluster from cluster 3, with sets 18, 19 and 20 occurring along separate branches. Sets 18 and 20 were brought together, although they were in different dendrogram sections, albeit both in division C; set 19 was in division B. The origins, and other attributes, of the populations in the three sets were so distinct from each other that perhaps they should not be closely joined; the dendrogram may present the realistic view of their relationships.

Set 18 included populations corresponding to the accepted view of *T. evansi*. Set 19 was small and exclusively associated with the Kenyan Lambwe Valley epidemic. Set 20 was essentially *T. b. gambiense*, and contained zymodemes that were the most distant from the HTU along pathway C (e.g. Z268, PD = 217).

D. DISTRIBUTION

1. *Pan-African distribution*

The subgenus *Trypanozoon* is patently a genetically complex collection of organisms; many mutations must have occurred to have produced the numerous alleles. Genetic recombination may have created an even greater variety not only of the heterozygous states of an individual enzyme, but also novel combinations of the homozygous and heterozygous forms of different enzymes.

Experimental hybridization of *T. brucei* has been recently described (Jenni *et al.*, 1986; Sternberg *et al.*, 1988). However, Cibulskis (1988) considered that genetic exchange may not happen frequently in nature, and that recurrent mutation could account for much of the variety seen, including the apparently hybrid forms.

It is, of course, important to determine how the organisms alter, because this may lead to an understanding of the way in which major natural changes, such as those leading to epidemics, occur. However, notwithstanding the potential for change, our observations show that the uneven distribution of genetic characteristics throughout Africa has remained relatively constant. This does not imply that genetic changes are not taking place, but it does suggest that, if they are occurring, only certain types are normally perpetuated as the locally breeding populations.

Any advantage of a particular trait would be reinforced by the two long phases of asexual reproduction in both the mammal and the tsetse fly (*Glossina* spp.). During each phase, frequent opportunities must occur for the most successful populations to be transferred to the alternate phase. However, strict limits on the successful establishment of new genetic forms in nature will be dictated by the constraints for survival in the entirely different environments of the two phases.

As Gibson *et al.* (1980) concluded from their dendrogram, trypanosomes of the subgenus *Trypanozoon* in West Africa were different from those in East Africa, with *T. b. gambiense* and *T. evansi* existing as separate entities according to their origin. A similar phenetic analysis on an enlarged sample in the present survey, as well as our cladistic method, produced much the same overall result. The pan-African dichotomy was also confirmed by observations that the length of the variable region in the kinetoplast DNA (kDNA) maxi-circles increased as the origin of the samples moved progressively from West to East Africa, and that certain maxi-circle polymorphisms were restricted to trypanosomes of each zone (Borst *et al.*, 1981; Gibson *et al.*, 1985). More recently, using DNA probes for detecting restriction fragment length polymorphisms, Paindavoine *et al.* (1989) described a major East and West African dichotomy.

The frequencies of the most common enzyme patterns in the three main regions sampled—the Luangwa Valley of Zambia, the Lake Victoria region (Lambwe Valley and Busoga), and West Africa—are summarized in Table A4; 231 zymodemes of the 268 recorded are included. The three regions corresponded approximately to the dendrogram divisions and the cladistic pathways. Although generalizations on the distribution of enzyme patterns can be made, no single characteristic listed in Table 4 is exclusive.

Particular features of the Zambian zymodemes were the high frequencies of ICD-1, PGM-1, ASAT-1 and ALAT-2 (Fig. 1).

ICD-1 and PGM-2 were absent from the Lambwe Valley isolates, while PGM-2 was also missing from the Ugandan zymodemes. ASAT-7 was found only in the areas near Lake Victoria, and ICD-3 and PGM-3 were considerably more frequent there than elsewhere. The presence of the multiple-banded ICD-3 and PGM-3 has been regarded as evidence of genetic hybridization (Gibson *et al.*, 1980; Tait, 1980, 1983), but Gibson and Wellde (1985) pointed out that two of the appropriate homozygote forms were missing from the Lambwe Valley isolates.

ICD-2 and PGM-2 were frequent in West Africa, while other variants of these enzymes which were common elsewhere were either absent or infrequent. ASAT-2 and ALAT-3 were seen only in this region.

2. *Distribution in time*

The results (Table A3) show that certain zymodemes have existed in a particular area over a number of years. For instance, trypanosomes of Z25 (set 1) were first found in the Lambwe Valley in a reedbuck* in 1970 and in man in 1974, but were still being isolated in 1983; Z31 (set 4) populations were isolated over a period of 21 years in the Busia area of Kenya, and over 18 years at Busoga in Uganda. Within the Luangwa Valley in Zambia, trypanosomes of Z1 (set 9) persisted in man from 1972 to 1982, but had also been isolated in nearby Tanzania as early as 1959; similarly, Z3 (set 10) organisms were seen over 12 years in the same valley. Other zymodemes in East Africa also persisted over a number of years.

The evidence is not strong for the long-term existence of characterized populations in West Africa; usually only periods of 2 or 3 years are recorded. The longest period recorded was for a population of Z39 (set 16), first isolated in Liberia in 1975 and again found in the neighbouring Ivory Coast during the intervening years until 1982. The lack of evidence for long-term persistence was probably due to the absence of extensive sampling before the mid-1970s.

E. MIXED POPULATIONS

1. *Frequency*

Twenty-five populations were examined as clones, but if the enzyme profiles

* Scientific names of wild mammals mentioned in the text: bushbuck, *Tragelaphus scriptus*; capybara, *Hydrochoerus hydrochoerus*; giraffe, *Giraffa camelopardalis*; hartebeest, *Alcelaphus buselaphus*; hippopotamus, *Hippopotamus amphibius*; hyena, *Crocuta crocuta*; impala, *Aepyceros melampus*; kob, *Adenota kob*; lion, *Felis leo*; oribi, *Ourebia ouribi*; reedbuck, *Redunca* sp.; rhinoceros, *Diceros bicornis*; warthog, *Phacochoerus aethiopicus*; waterbuck, *Kobus defassa*.

did not differ from those of the parent isolate they were not recorded as separate results in Table A3. In some instances, the parent isolate of a clone was not available.

Twenty-eight isolates, mostly of Zambian origin, were found to have mixed populations, each of which was recorded separately and annotated accordingly in Table A3; the profiles are compared in Table A5. The different populations in two of the stocks were discovered by cloning. The remaining 26 stocks gave different profiles on separate occasions when the organisms were harvested for analysis. Probably one population overgrew another during the course of subpassages in mice. The results were confirmed in all cases.

2. *Triple-population mixtures*

In one instance, cloning revealed three populations (Z2, Z3 and Z263) in a Zambian isolate from a patient. Table A5 shows that Z2 and Z3 differed only in their PEP1 and ME patterns, but Z263 was markedly different in ICD, MDH, ASAT, PEP1, and ME. Z2 and Z3 were placed together in set 10, containing other Zambian zymodemes. Z263 was separated into set 7, where this one population of human origin contrasted with the general association of the set with animals and tsetse.

Three populations were also found in a stock originally isolated from a Zambian tsetse; each appeared at different times during the course of subpassaging. They were the only representatives of Z245, Z246, and Z260. The differences in ICD, MDH and ME were sufficient to place each zymodeme in different sets. Z245 was unusual because its sole constituent population was one of only two from Zambia in set 4. In contrast, Z246 was placed in set 9, with marked Zambian associations; Z260 was the only member of the minor set A.

3. *Double population mixtures*

Of the 26 stocks found to have two enzymically different populations, only one was revealed by cloning. The different profiles are listed in Table A5. The various pairs of populations in a host could differ in as many as six enzymes, or as few as one. The most frequent difference was ALAT-2 in one population and ALAT-10 in the other. Most of these primary isolates had been made in Zambia, and were usually virulent to rodents; such isolates were rapidly characterized in the laboratory, probably before one population become permanently dominant.

With six mixtures, the two populations fell into the same set. In most

other instances, although the populations were in different sets, these sets nonetheless had marked associations with the same country.

An interesting observation was the isolation of two pairs of populations from a waterbuck and a warthog in Zambia, with each animal yielding a population in the same two zymodemes, Z7 (set 8) and Z189 (set 9).

F. INFREQUENT ENZYME PATTERNS

1. *GPI*

GPI-2 was the sole variant seen from GPI-1; it was found only in the one population of Z275 (set 20).

2. *TDH*

Most of the rare variants of TDH occurred in set 11, with some in set 14. Both sets are included in strain groups with animal associations, particularly with wild animals. Two further populations with TDH-4, in sets 4 and C, also came from wild animals, although another in set 12 was from a cow.

3. *NH*

Set 11, with its unusual TDH patterns and populations from wild animals, also included zymodemes with the rare NH-2. Another population with the same variant occurred in the minor set C; again it was from a wild animal. The only other variant was NH-3, which was found in some zymodemes in set 20.

4. *MDH*

Most of the infrequent MDH patterns were usually in populations isolated from animals, especially MDH-2 in set 14; some profiles also had rare forms of TDH. However, four populations from patients in sets 7, 11 and 12 had MDH-2.

5. *ICD*

ICD-5 occurred in Z234 and Z235 only (set 7); both were represented by populations isolated from the same tsetse (Table A5).

6. *PGM*

Most of the rare PGM patterns occurred in set 4. Although many profiles in

the set had the common PGM-1, most of the other kinds of PGM also occurred.

7. *ASAT*

ASAT-3 appeared only in set 20. However, this pattern was also shared with Z63, which contained the unusual *T. evansi* from camels (see Section IV C). Perhaps the ASAT pattern was critical in placing the zymodeme, apparently erroneously, into the set. The rare ASAT-5 and ASAT-6 were seen only in populations from patients in set 16, which had otherwise little association with human infectivity.

8. *ALAT*

The rare ALAT-4, ALAT-5 and ALAT-6 were seen only in set 17, together with most examples of the less infrequent ALAT-3. This set, in fact, appeared to be characterized by the absence of the most frequent pattern, ALAT-2. Rare variants in several other sets seemed to have no particular significance. ALAT-5 has also been reported by Otieno and Darji (1985) in isolates from tsetse in the Lambwe Valley.

9. *PEP2*

The infrequent PEP2-2 occurred, along with the somewhat more frequent PEP2-3, in the specialized set 14; other rare PEP2 variants appeared in set 20. These patterns probably contributed to the separation of these particular sets. The remaining instances of the rare forms of PEP2 in other sets did not seem to be significant.

10. *PEP1*

Each zymodeme in set 14, with its high prevalence of unusual patterns of other enzymes, also had the infrequent forms of PEP1.

11. *ME*

Many of the numerous ME variants that were not often encountered appeared in sets 4, 9 and 11, but since the common forms also occurred in these sets, little significance can be attached to their occurrence, or indeed to their presence in other sets.

G. LIMITATIONS

1. *Sampling*

For whatever reasons, the results confirm that the various genetic kinds of *Trypanozoon* are not randomly distributed throughout their range. In relation to the enormous number of separate infections that must exist, the sample studied was small, but nevertheless the pan-African distribution has remained stable over the years, much as first described by Gibson *et al.* in 1980.

Sampling has been concentrated in three widely separated regions, in each of which, apart from some host-associated differences, there appeared to be characteristic enzyme pattern combinations. However, more extensive sampling may well reveal additional genetic groupings, particularly in those areas that have not been investigated. In this context, with the exception of collections from the Ivory Coast and Burkina Faso, the sampling was strongly biased towards the anglophone countries in Africa; the lack of samples from francophone countries in the southerly parts of West Africa was particularly important.

At least some sampling bias will have arisen from the concentration on the most readily accessible host, man; nearly half the populations described were from patients, while they comprised 77% of the Zambian collection. Nonetheless, many from a wide variety of animals genetically resembled those found in man. Even those sets of zymodemes principally associated with animal infectivity retained the general enzymic characteristics of a region.

Other factors that may have influenced the reliability of sampling were the failure to isolate all the natural infections in rodents, and the fact that adequate parasitaemias did not always develop in laboratory hosts. Thus, other as yet unknown genetic forms may exist. Such failures occurred frequently in gambian trypanosomiasis areas; this was reported for 25 of 122 instances in Nigeria (Gray, 1972), 48 of 136 in the Ivory Coast (Mehlitz *et al.*, 1982) and, recently, in all of 11 instances in Cameroon (P. Dukes, personal communication). It is also possible that other kinds of *Trypanozoon* have failed to infect rodents, and hence have not been analysed. However, such trypanosomes are likely to become available through a new approach to isolation and amplification, by infecting tsetse followed by culture *in vitro* of procyclic forms from the midguts. In this manner, *T. gambiense* stocks that are not infective to rodents have been isolated recently (Dukes *et al.*, 1989).

2. *Trypanosome populations*

When investigating a large number of different populations, a risk exists that the wrong one may be examined. The causes of error include contaminated equipment, poor labelling, and misplaced rodents. In a well-organized laboratory, the risk is minimal, but nevertheless evidence exists that cross-contamination has occurred in the past. Gibson *et al.* (1980) considered it probable that four populations, also included in this survey, had been replaced by others. Three were in Z32 (set 1) (see Section IV A 1), Z33 (set 8) and Z55 (set 9) (see Section IV A 2), the positions of which appeared to be wrong in our analyses.

The placing of the fourth population, Z56, in set 16 can now perhaps be seen to be not entirely anomalous. The single population (Gboko) from a patient was examined by Gibson *et al.* (1980), who pointed out its resemblance to Nigerian animal isolates; indeed later an Ivory Coast pig population was included in the same zymodeme (Table A3). Most populations in set 16 originated from West African animals. However, a few came originally from man, and it may be that the Gboko isolate fell into the special category of such isolates from patients, as discussed in Section IV B 1.

Despite the evidence for cross-contamination in the past, the great majority of results were in a reasonable order, and we believe that any such errors have had a minimal overall effect.

Some primary isolates contained at least two genetically distinct populations, and others probably remained undetected. Some primary isolates may have lost all but one population during the highly selective laboratory process of frequent transfer at peak parasitaemia between rodents. Rapid

Some primary isolates contained at least two genetically distinct populations, while others probably remained undetected. Some primary isolates concentration, only the predominant one could be characterized by its enzyme polymorphisms (Scott, 1981; Schütt and Mehlitz, 1982; Letch, 1984).

However, notwithstanding the obvious proviso that any stock examined may have been mixed, the populations characterized certainly existed in the hosts described.

3. *Isoenzymes*

Electrophoresis of an enzyme may not separate all its molecularly different forms, because not every amino acid substitution in a polypeptide results in an alteration of charge. However, enzyme polymorphism, as revealed by

electrophoresis, has yielded valuable genetic information on many species, including man (e.g., Harris and Hopkinson, 1976) and various parasites (see Godfrey, 1984). It is thought that our zymodeme groupings are valid, because a general correspondence occurred between the numerical methods, and the groupings could be also characterized by the geographical or hostal origins of the constituent populations.

A further problem is presented by the rarely occurring variants of the highly polymorphic enzymes. From a practical point of view, it is difficult to determine the validity of such patterns. When the occurrence of numerous positions and large numbers of bands is possible, as with PEP1 (Fig. 1) for example, many comparative electrophoretic analyses including known stocks are required to determine the precise mobilities of individual bands.

A low concentration of enzymes in the extract may affect the resolution of certain bands, for example the narrow ones in ME (Fig. 1), which may not be detected. Such errors might have happened over the years, but the groupings appear to be unaffected by the presence or absence of the rare, possibly spurious, variants of the highly polymorphic enzymes.

A practical need exists for clear banding with a minimal number of enzymes, without resort to numerous comparative electrophoretic determinations. This is particularly important for material that is difficult to obtain in large amounts. Consequently, recommendations are put forward in Section IV for practical identification.

IV. Strain Groups

The overall similarity between the dendrogram and cladogram demonstrated that genuine relationships existed between groups of zymodemes and the distribution of their constituent populations. Moreover, substantial numbers of zymodemes remained together as sets in both systems, which indicated close relationships within each set. At the same time, certain sets appeared to be especially affiliated to others.

The sets shown in Table A1 were numbered 1 to 20 in sequence in an attempt to follow the three cladogram pathways and the internal branches within clusters; this system also approximated to the three dendrogram divisions.

Five other sets (A, B, C, D and E) were regarded as of minor significance because each contained less than 1% of the total number of both zymodemes and populations. The sets 3, 8, 12, 13, 14, 18 and 19 also had few zymodemes, but in certain instances appeared to be of consequence. Only dendrogram section 6 and cladogram cluster 6 contained exactly the same zymodemes, those in set 7.

The positioning of the sets relative to each other within each system was sometimes disparate. For instance, set 20, equivalent to *T. b. gambiense*, was alone in section 9 of the dendrogram, whereas in the cladogram it was alongside others in cluster 7. On the other hand, sets 8, 9 and 10, with their widespread East African affiliations, were widely separated in the dendrogram, but were brought together in cluster 2. There were several other similar examples. Although the widest overall relationships and those within any one set appeared definite, the affinities between sets were less consistent in the two approaches.

Attempts were made, using the computer, to define each set with a minimal number of enzyme pattern combinations for easy identification. Such combinations, however, were generally complex. Nevertheless, during the attempts it was noticed that combinations of a reduced number of patterns recognized several sets that were closely related in one or both of the numerical analyses. These groups of sets resembled a number of the infraspecific working groups proposed for *Trypanozoon* by Gibson *et al.* (1980).

These groups form the basis of our proposals, although modifications were required because of the more extensive findings in our survey. The groupings below the level of subspecies were termed "strain groups"; they were genetically uniform, with particular geographical or hostal attributes.

In the following descriptions, *T. evansi*, *T. b. gambiense*, *T. b. rhodesiense* and *T. b. brucei* are viewed in the accepted manner, as species and subspecies. *T. evansi* and *T. b. gambiense* appeared to be distinct entities, each possibly being further subdivided into strain groups. The validity of all taxa remained uncertain, since a number of isoenzyme bands were common to every category. The generally accepted taxonomic positions were convenient as higher categories than strain groups. The status of *T. b. rhodesiense* and *T. b. brucei* was particularly uncertain, because some strain groups in each "subspecies" differed more from each other than they did from other strain groups in the other subspecies (see Section VI).

The minimal number of enzymes necessary for the identification for each category are given in Table A6, together with the limits of the definitions. The proposals will probably require adjustment as more information is gathered, particularly when the full rôle of genetic modification in nature is elucidated.

A. *T. B. RHODESIENSE*

Two kinds of *T. b. rhodesiense* appeared to exist in East Africa, both corresponding to groups previously described by Gibson *et al.* (1980).

1. *Busoga**

All except one of the 39 zymodemes containing the 222 populations from the Kenyan epidemic in the Lambwe Valley, near Lake Victoria, were recognized by the busoga definition in Table A6; the exception was Z106 in set 7, with one population from a waterbuck and one from a tsetse. All populations originating from patients in the valley were identified, together with others in Uganda and elsewhere. As pointed out by Gibson and Wellde (1985), strong evidence existed that animals in the Lambwe Valley were reservoirs of man-infective organisms: for example, the nine cattle with Z74 (set 1) trypanosomes. It should be noted that Sindo, where some populations within the sindo strain group originated (see Section IV B 2), is near the Lambwe Valley, towards the shore of Lake Victoria.

Only one zymodeme in each of sets 3 and 14 was included in the definition; the two populations were from Zambia, from a patient and a tsetse.

The bulk of the zymodemes recognized fell into the four large sets 1, 2, 4, and 5, which were linked in the two cladogram clusters 1 and 8 in the residual pathway B. A fifth small set, set 19, was distinctly separated in the cladogram, in cluster 7 along pathway C, but was placed in the same dendrogram section as three of the large sets. Its enzyme characteristics and geographical affinity, held in common with other nearby sets, suggest that the dendrogram, but not the cladogram, positioning is correct; set 19 is thus justifiably in busoga.

In the dendrogram, set 1 was placed apart in section C. Its zymodemes recognized by the busoga definition were mostly placed near each other with a dissimilarity of about 2%. The unidentified zymodemes, Z40, Z49 and Z50, were more distinct from these at about 6% dissimilarity, and were notable in containing populations from West Africa. Further breaking down by the computer may well yield additional clusters within set 1.

Four populations from patients in the Lambwe Valley grouped together in only one zymodeme (Z98) in set 5, which otherwise was strongly linked with animal infections; perhaps the set is a separate genetic kind.

Five of the eight zymodemes (19/42 populations) from man in Busoga in Uganda, again near Lake Victoria, were recognized by the strain group definition; most populations (16/19) were in four zymodemes of set 4. A further 23 populations from Busoga patients were in three zymodemes identified by the zambezi strain group characteristics (see Section IV A 2). One zymodeme (Z73) in set 4 contained a population from a dog in western

* These strain group names, busoga, etc. (see Table A6), are not intended to be formal taxonomic names, nor geographic names; hence they are normally printed in roman type with lower case initial letters (except when in headings, as here, or when starting a sentence).

Uganda, but was eliminated by the busoga definition (see Section IV E 4).

The sole population allegedly from a Ugandan patient in Z32 (set 1) may well have an incorrectly recorded history, since Gibson *et al.* (1980) reckoned it to be the subject of a laboratory error. This may be confirmed by its position in set 1, which contained only one other population (Z71) from Uganda. A superficial view suggests another anomaly, namely the population from a camel in Z62; this, however, cannot be *T. evansi*, since it is transmissible by tsetse (Gibson *et al.*, 1983).

Besides the many populations from man in the Lake Victoria region, a number in the same zymodemes came from patients elsewhere in Africa (e.g., Z5 in set 9 and Z2 in set 10). Z31, in set 4 with its widespread East African character, was of particular interest; populations persisted in Busia and Busoga over similar periods of 18 years. Busia is in Kenya near the border with Uganda, almost midway between the Lambwe Valley and Busoga.

The busoga definition eliminated the only two zymodemes (Z228, Z245) associated with Zambia in set 4. Nevertheless, two zymodemes linked with that country were identified; the single population in one (Z38, set 1) was from an area far from the Luangwa Valley, where most Zambian specimens were isolated.

A feature was the inclusion of isolates from West Africa into busoga, a strain group which was otherwise predominantly allied with the opposite side of the continent. The only two populations from patients in the region were in set 4. One (TH 1/78E [037]) in Z35 was not classed as *T. b. gambiense* by the DNA hybridization tests of Paindavoine *et al.* (1986); moreover, it was, together with the only other population (Z145; TH 1 [032]) of similar origin, sensitive to human serum (Mehlitz *et al.*, 1982).

Three of the zymodemes (Z18, Z31, Z37) placed in busoga by Gibson *et al.* (1980) are still so placed by our system. Only one isolate described by Otieno and Darji (1985) from the Lambwe Valley conformed exactly to one of our zymodemes (Z74, set 1); populations of this were the most frequent in both surveys. However, three others also conformed to the busoga definition given in Table A6. Another corresponded with the sindo definition (Section IV B 2). Two more of the zymodemes described by Otieno and Darji (1985) possessed ALAT-5, which we encountered only once (Fig. 1), in West Africa (Z44, set 17).

2. *Zambezi*

The enzyme combinations for this strain group identified zymodemes in a number of sets (Table A6), which were linked by one or both of the numerical methods. All contained populations originally isolated from

patients in and around the Luangwa Valley in Zambia. A strong link with human infectivity existed, since the zymodemes contained 97% (185/191) of the populations originating from patients in Zambia; similar populations were isolated from animals in the same collecting area. Altogether, 71% (75/106) of all zymodemes associated with Zambia were characterized by the zambezi definition; these included some represented by numerous populations (e.g., 33 in Z2, 22 in Z204, and 17 in Z3). Twenty of the unrecognized zymodemes from Zambia were in the sindo (Section IV B 2) or kakumbi (Section IV B 4) strain groups.

Most zymodemes were in sets 6, 9 and 10. Sets 6 and 10 formed the major part of section 1 in the dendrogram, with a maximal dissimilarity of about 14%; set 9, in section 2, differed by over 20%. The cladogram grouped sets 9 and 10 together in cluster 2, but separated set 6 in cluster 4. There appeared to be some rationality in this separation, since set 6 was associated exclusively with Zambia, while the other two had wider affinities.

Set 8 was probably better placed in the cladogram alongside sets 9 and 10 in cluster 6 than at its position in the dendrogram; all three sets had associations with different parts of East Africa. The small set 13, together with the minor sets D and E, all with Zambian links, appeared to be reasonably placed in zambezi.

Populations from patients in countries other than Zambia occurred in this strain group. None was from the Lake Victoria region in Kenya, but set 9 included two zymodemes (Z5, Z66) with populations from the Ugandan part of that region. This set also had two populations originating from patients in Busia in Kenya, near the border with Uganda. The collection of isolates examined by Tait *et al.* (1985) also came from this border area. Although the numbering of enzyme patterns used by Tait *et al.* does not correspond with ours, the patterns given by those enzymes used in common by both them and us suggest that their isolates should be included in zambezi. Kenyan populations in Z2 (set 10) originated from animals in the south of the country.

The only population of purportedly West African origin belonged to Z55 (set 9); this has previously been regarded as a result of laboratory cross-contamination (Kilgour and Godfrey, 1976; Gibson *et al.*, 1980), a view supported by the present findings. The one Ugandan population (Z33) in set 8 reputedly originated from a patient in the Busoga area. However, Gibson *et al.* (1980) considered it (118 clone Y) to be of doubtful origin.

The separation of zambezi and busoga is supported by the differences in the size of the variable region of the kDNA maxi-circles between representative zymodemes; stock 1196 was a member of Z31 (set 4; busoga) and stock 34 belonged to Z1 (set 9; zambezi) (Borst *et al.*, 1981).

Zambezi was first described by Gibson *et al.* (1980). Two of the zymo-

demes included by them, Z13 and Z14, have now been discarded because they were represented by two stocks of unknown history which had been maintained in the laboratory for over 60 years, mostly by frequent passages in rodents.

B. *T. B. BRUCEI*

T. b. brucei was subdivided into four strain groups, which did not correspond entirely with the groups proposed by Gibson *et al.* (1980). Their flompa group has been incorporated into *T. evansi* (Section IV C), while their kiboko group has been retained. Three new strain groups were created, one being based on later recommendations for a sindo group (Gibson *et al.*, 1985).

1. *Bouaflé*

Every zymodeme in set 16, most of those in sets 15 and 17, and eight in set 1, were identified by the enzyme combinations for the bouaflé strain group given in Table A6.

In the dendrogram, the zymodemes within each of the sets 15, 16 and 17 had a maximal dissimilarity of about 6%. Sets 15 and 17 were close together to form section 8 of the dendrogram, and were separated from set 16 in section 7. However, we have already stressed the marginal separation of sections 7 and 8. In the cladogram, the relationships stayed somewhat close along pathway C, cluster 3 being made up of sets 16 and 17, while the junction of this cluster arose from near set 15 in cluster 1.

The zymodemes identified in set 1 by the bouaflé definition were principally associated with West Africa, although five were also recognized by the enzyme combinations for busoga (see Section IV A 1). These are the only examples of zymodemes being identified by two of the definitions in Table A6.

Bouaflé was essentially a form of West African *T. b. brucei*; 141 (92%) of the 154 populations came mainly from West African animals, with some from tsetse. Three populations originated from animals or tsetse from elsewhere; ten more were from patients, eight in West Africa, one in Zambia and one in Ethiopia. One population (Z38, set 1) came from a tsetse in Ethiopia and another (Z237, set 15), from a fly in Zambia. Z71 (set 1) contained a population originally isolated from a cow in Uganda. A population from a domestic fowl belonged to Z170 (set 17) (Zillmann and Mehlitz, 1979).

The ten populations from man were in nine zymodemes. One, from a Zambian patient in Z38 (set 1), was isolated at Solwezi in the north-western

part of the country, near the border with Zaire, and far from the main sampling area in the Luangwa Valley. It is perhaps not surprising that this was distinctly different from the other Zambian populations, and was classified also in busoga (Section IV A 1). A further population from man in East Africa was in Z39 (set 16) and originated in Ethiopia.

Seven zymodemes in sets 16 and 17 were linked with human infectivity in West Africa. Populations from man in five of these zymodemes were tested by Mehlitz *et al.* (1982) for sensitivity to human serum (Rickman and Robson, 1970). Only one in Z143 (set 16) was fully serum resistant (Hawking, 1976); however, this was classed, by Paindavoine *et al.* (1986) on DNA characteristics, as not *T. b. gambiense*. Two other populations in Z142 and Z149 (sets 16 and 17) were sub-resistant, while the remaining three, in Z147 and Z148 (set 16), and in Z149 (set 17), were all serum sensitive. This was confirmed for the Z147 population with the *in vitro* test (Richner *et al.*, 1988). Thus it seems that serum-sensitive organisms may originate from man in West Africa, and genetically these differ from the true *T. b. gambiense* (Mehlitz *et al.*, 1982; Paindavoine *et al.*, 1986, 1989; Godfrey *et al.*, 1987).

We do not believe these few populations from man present a serious anomaly in regarding bouaflé as the West African form of *T. b. brucei*. Moreover, a transient and symptomless experimental infection with *Trypanozoon* in man (Hoof, 1947) does not support the accepted view that *T. b. brucei* never infects man. Serious consideration should be given to revising the rigid host-restriction definition.

Apart from the general recognition of a West African form of *T. b. brucei*, no equivalent group was proposed by Gibson *et al.* (1980), although a number of the zymodemes studied by them are now included in bouaflé.

2. *Sindo*

Sindo was recognized by the simple enzyme combination of ASAT-4 and ME-8 (Table A6), and separated out distinctly on its own as set 7 in both the dendrogram and the cladogram. The greatest dissimilarity between zymodemes was about 4% in the dendrogram, although they spread along several branches within cluster 6.

Populations from Kenya and Zambia were included in the 19 zymodemes, the majority originating from animals or tsetse. Most Kenyan populations came from the coastal areas. Two populations in Z106 were, however, isolated in the Lambwe Valley, while those in Z29 came from Sindo nearby; Otieno and Darji (1985) also recorded four isolates of Z29, corresponding to their zymodeme 4, from tsetse in the Lambwe Valley.

Predominantly, the populations from Zambia originated in tsetse or animals; many were from Kakumbi, in a part of the Luangwa Valley where game animals were plentiful and sleeping sickness rare.

The two populations from man were also from Zambia. At this stage no judgement can be made as to whether these were wrongly positioned by our numerical methods, or were *T. b. brucei* infections, perhaps temporary, in man (see Section IV B 1). Notwithstanding these discrepancies, the trypanosomes were considered as basically animal-infective, rather than man-infective, organisms.

Nothing similar was designated by Gibson *et al.* (1980), but Z29 in this category was included in their gambiense group; the other zymodemes were discovered only after 1980. Later, analysis of restriction site polymorphisms in kDNA maxi-circles demonstrated the distinct nature of sindo (Gibson *et al.*, 1985). Two isolates (1342 in Z29, and 1902 in Z106) used by these authors were also included in our survey; both analyses agreed in assigning them to sindo.

However, another isolate (LF 1; Z89, set 5), with the characteristic sindo kDNA and used in both studies, was placed in busoga by its enzyme characteristics; it did not have the consistently typical ASAT and ME patterns of the sindo strain group. No suggestion can be made regarding the apparent lack of correspondence, except of course the possibility of laboratory error.

3. *Kiboko*

One of two enzyme combinations (Table A6) recognized all but Z223 in set 11, as well as the sole zymodeme in the minor set C. The profiles were notable in possessing rare variants of either NH or TDH, or both.

Set C was adjacent to set 11 in the dendrogram. Although most zymodemes had a maximal dissimilarity of 5%, two (Z223 and Z95) were distinct at about the 17% level. In cluster 9, set 11 was joined with the small set 12 from the adjoining dendrogram section, and was dispersed along several branches; the unrecognized Z223 was on a separate branch. Set C was positioned in cluster 8 at some distance from set 11 in cluster 9.

Eighteen populations were recorded within the 15 kiboko zymodemes. All populations except two were from Kenyan animals or tsetse, in central areas away from the Lambwe Valley and the coast. The exceptions were from a lion (Z20, set 11) and a hyena (Z19, set C) in the nearby Serengeti wild-life reserve of Tanzania.

Five of the zymodemes in the kiboko group of Gibson *et al.* (1980) are included in our definition, but several others have now been split off into the kakumbi strain group (Section IV B 4). The separation appears rational because, besides the clustering differences found by our numerical analyses, kiboko has strong Kenyan affinities, in contrast to the Zambian links of kakumbi.

Two isolates (1738, Z110, set 11; and 1591, Z19, set C) placed in the kiboko group by Gibson *et al.* (1985) on kDNA maxi-circle polymorphisms, were also classified as such in our investigation. Evidence is presented in Section IV B 4 for a third isolate, used in both investigations, to be placed in kakumbi.

It is thought that kiboko is a form of East African *T. b. brucei.*

4. *Kakumbi*

The enzyme combinations shown in Table A6 identified every zymodeme except one in set 14; the minor sets A and B were also recognized. Z248 in set 14 was recognized by the enzyme combinations for busoga (see Section IV A 1); the MDH-1 in its profile was atypical, the other zymodemes in kakumbi having the relatively infrequent MDH-2.

Together with the small set B, set 14 constituted the whole of section 3 of the dendrogram, with a maximal dissimilarity of less than 5%; set A was more than 20% different. Set 14 also formed the major portion of cluster 5, alongside the small set 13 and the minor set E; the cluster was at the end of pathway A, at a great distance from the hypothetical taxonomic unit (HTU).

All populations in kakumbi were from Zambia, and each one was isolated from a wild animal or a tsetse. The population from a lion in Z10 was mixed with another in the original isolate; the other profile (Z189) differed in six enzymes, thus placing the zymodeme in set 9 in Zambezi (Section IV A 2). Interestingly, the two other populations in Z189 were also from Zambian wild animals.

Five of the zymodemes in the kiboko group of Gibson *et al.* (1980) are included in this strain group; their dendrogram revealed a marked degree of separation between these zymodemes and those now placed in kiboko (see Section IV B 3). More recently, Gibson *et al.* (1985) placed a Z11 isolate (J 10), on the evidence of its kDNA maxi-circle polymorphisms, into kiboko. This isolate, however, differed in possessing an extra, unique, restriction site, which may support its removal from kiboko into kakumbi.

Since the mammalian hosts in kakumbi were all wild animals, it may be that it is an East African form of *T. b. brucei* adapted to a specialized wild animal and tsetse cycle.

C. *T. EVANSI*

Z58 and Z64 in set 18 corresponded to the concept of *T. evansi*, because many of the constituent populations were isolated in tsetse-free regions, particularly from camels. Some populations in the other two zymodemes in the set also came from camels and, therefore, probably the whole set, with its

distinguishing enzyme combinations (Table A6), was equivalent to the species.

Z63, with the kDNA characteristics of *T. evansi* (Borst *et al.*, 1987), but unique enzymic features, was included in the definition, but was apparently misplaced in set 20 (see Section IV D). Its profile may represent a second strain group in the species; in particular, the PGM, ASAT and ME differed from other profiles in set 18. Furthermore, Stevens *et al.* (1989) showed that a Z63 isolate had a different superoxide dismutase pattern.

The four zymodemes in set 18 had less than 1% dissimilarity in the dendrogram, and were placed near set 16 in both the dendrogram and the cladogram; they had high patristic distances (PDs) of 135 or more. Z63 also had a high PD of 177, and from its position in the dendrogram was about 30% different from set 18. As part of set 20, however, it was placed near set 18 in cluster 7.

When ME-2 was omitted from the *T. evansi* definition in Table A6, then much of the related set 16 was also identified, as well as two zymodemes in set 1. Despite this close enzymic similarity, *T. evansi* is distinguished from tsetse-transmitted *Trypanozoon* by the absence of maxi-circles and low mini-circle sequence heterogeneity in its kDNA (Borst *et al.*, 1987). Three isolates examined by Borst *et al.* were in Z64 and Z58 (set 18), and in Z63 (set 20); all of them were included in our analysis (Gibson *et al.*, 1983). The Z63 isolate had mini-circles of type B in contrast to the type A of the others. The existence of two kinds of *T. evansi* is thus supported by the enzymic differences and the separation in both numerical analyses.

Although there were only five zymodemes in *T. evansi*, these embraced 99 populations. Populations in Z58 occurred in several parts of Africa, in Colombia and in the Middle East; recently, isolates in the same zymodeme have been described in Brazil (Stevens *et al.*, 1989). Most populations in Z64 were from camels in northern Kenya, but closely related populations in Z42 and Z58 were found in the south-east (Galana and Mombasa), in both camels and cattle.

In The Gambia, which is tsetse-infested, populations (Z42) from cattle were identical to those from Kenyan camels. Since The Gambia is close to the edge of the northern limit of savannah tsetse distribution, the cattle may have been in direct contact with the vectors of the non-cyclically transmitted *T. evansi*.

The question arises whether or not Z41, with its populations of Liberian origin, is truly *T. evansi*, because Liberia is well within the tsetse zones. The possibilities of significant non-cyclical transmission in such zones should be contemplated, if its kDNA is characteristic of *T. evansi*.

A population originating from a camel belonged to Z62 in set 1, but it is probably not *T. evansi*; the original isolate was KETRI 2502, which developed cyclically in tsetse (Gibson *et al.*, 1983).

A further enzyme profile described by Gibson *et al.* (1983) was, in error, not included in our analyses. However, it differed from Z58 and Z64 in PEP1 only, and so would probably have been placed alongside these in set 18.

Enzyme profiles of isolates from camels in Sudan displayed similar combinations of the variant enzymes used in common with this study (Boid, 1988), while further enzymes revealed additional polymorphisms. Although a precise comparison between the two studies is not easy, because the patterns for ALAT and PEP1 do not entirely correspond, Boid's finding of several forms of ALAT and ME indicates further genetic variation in *T. evansi*. This variation may not indicate another major form, since both enzymes are markedly polymorphic.

Although diversity in *T. evansi* is restricted in comparison with other members of *Trypanozoon*, its extent will remain uncertain until the different collections are compared directly. The significance must be explored of the polymorphisms described in hexokinase, peptidase-4 and glucose-6-phosphate dehydrogenase (Boid, 1988), together with that described in superoxide dismutase (Stevens *et al.*, 1989). Since Boid's dendrogram showed a diversity of 25% among his isolates, we have hesitated to define strain groups within *T. evansi*.

Gibson *et al.* (1980) placed Z41 and Z42 in the flompa group. Their definition, however, conforms to ours for *T. evansi*, and so those zymodemes are now placed in this taxon.

D. *T. B. GAMBIENSE*

Set 20, at the end of pathway C and distinctly separated in the dendrogram, was a set of zymodemes predominantly associated with man. It corresponded to the subspecies *T. b. gambiense*, the causative organism of chronic gambian trypanosomiasis. The identifying enzyme combinations are given in Table A6, although the two main alternatives may indicate a further subdivision into strain groups.

The definitions excluded Z63. This zymodeme was placed in set 20 in both the dendrogram and cladogram, but its profile was quite different from those for the remainder of the set, particularly in PGM and ME. The reasons for classifying Z63 as another form of *T. evansi* have been given in Section IV C.

In the dendrogram, the zymodemes in set 20 were clearly segregated as the whole of section 9, at a maximal dissimilarity of less than 8%. The set occurred along two branches in the cladogram cluster 7, separately from the branches containing sets 18 and 19. The highest PDs in pathway C were included, some exceeding 200.

Fifteen zymodemes in the subspecies were represented by 43 populations,

mostly from man, although some came from animals; the subspecies corresponded to the gambiense group of Gibson *et al.* (1980). The populations derived from man were recently considered in detail by Godfrey *et al.* (1987). Besides the enzymic characteristics, the initial low virulence to rodents, the consistent resistance to normal human serum, and particular DNA characteristics all confirm the subspecies as a taxonomic entity (Mehlitz *et al.*, 1982; Paindavoine *et al.*, 1986, 1989; Richner *et al.*, 1988). It differed from the other trypanosomes of man found at times in West Africa. The positioning within the subspecies of several populations from animals supports previous evidence of their potential rôle as reservoirs of gambian trypanosomiasis (Mehlitz *et al.*, 1982; Scott *et al.*, 1983; Paindavoine *et al.*, 1986).

E. UNIDENTIFIED ZYMODEMES

Thirteen zymodemes were not identified by any of the enzyme combinations shown in Table A6. In most cases, their classification is probably best put aside until further samples have been examined.

1. *Z235, Z236, Z240 (set 3)*

Set 3 was composed of just four zymodemes, each with a population from different hosts in Zambia; it was adjacent to sets of similar origin in the dendrogram. The three zymodemes that were not identified in Table A6 had a dissimilarity of only 1% in the dendrogram. The fourth (Z220) differed from these at about the 9% level, but was included in busoga. Its profile was quite different, especially in the absence of MDH-5. Zymodeme Z220 was also located alone on a separate branch in cluster 1.

The set was close to set 6 (zambezi) in both the dendrogram and cladogram, but differed primarily in the three unrecognized zymodemes having ICD-3 and MDH-5. All the populations were mixed with others in the primary isolates (Table A5).

2. *Set 12*

This small set of three zymodemes had an overall dissimilarity of about 2% in the dendrogram, lying in section 1 with other sets linked to Zambia. The cladistic analysis joined it to set 11, with its wide East African connections, in cluster 9. Two of the three populations were from patients; their enzyme profiles included the infrequent MDH-2. The third population, from a cow, had rare variants of both TDH and MDH.

3. *Z40 (set 1)*

Z40 was not recognized by either the busoga or the bouaflé combinations, as was the rest of set 1. If the profile had shown ME-2 instead of ME-1, then the zymodeme would have fitted into the category of *T. b. gambiense*. Any association with human infectivity is not necessarily excluded, since this property is linked with several other zymodemes in the same set.

Gibson *et al.* (1980) discussed the populations in this zymodeme at length; variations in serum sensitivity were recorded, while some stocks had low initial virulence to rodents (see Section IV D). These attributes, together with the enzymic features, suggest human infectivity, but the absence of ME-2 raises some doubt. It should be noted that the profile was first listed as possessing ME-2 (Gibson *et al.*, 1978), but the pattern seen was later recoded as ME-1 (Gibson *et al.*, 1980) in an attempt at rationalization.

4. *Z73, Z228, Z245 (set 4)*

Z228 and Z245 appeared to be logically eliminated by the busoga definition, which otherwise included most of set 4. Both profiles were unusual in not having MDH-1. Unlike those in the other zymodemes of set 4, the constituent populations were from Zambia. Zymodeme Z245 was one of three populations in an original isolate from a tsetse, the other two being included in sets 9 and A.

Z73 was not recognized by the busoga definition because of its PGM-2; the single population was the only one examined from western Uganda, some distance away from the main sampling area of Busoga. Perhaps this zymodeme was part of a group with different geographical associations.

5. *Z223 (set 11)*

Z223, with its single population from a Zambian patient, was the one in set 11 to be eliminated from kiboko. It differed in having both NH-1 and TDH-1, and was probably wrongly placed in the set which was mostly associated with Kenyan animals and tsetse. This zymodeme also had a rare MDH variant; the only other zymodemes linked with human infectivity and with unusual MDH were three in sets 7 and 12.

6. *Z169 (set 15)*

The PGM-1 in this zymodeme was unique in set 15. Its one population came from a primary isolate with several populations (Table A5); the original host was a pig from Ivory Coast.

7. *Z59 (set 17)*

Z59 differed from the rest of set 17 in having ME-2 and in not being identified by the busoga definition. However, since the only population was labelled *T. equiperdum*, but with a most uncertain history, the reasons for its failure to be recognized can be set aside until such time as authentic isolates of *T. equiperdum* have been characterized.

V. The Dispersal of The Subgenus *Trypanozoon*

From the point of view of human trypanosomiasis in Africa, the general belief has been that the disease spread from the southern parts of East Africa towards Lake Victoria and then westwards from there (see Gibson *et al.*, 1980). In contrast, the cladistic analysis, which determined the minimal lengths of pathways along which change could have occurred, indicated that the contemporary nearest relatives to the ancestors of *Trypanozoon* are mostly in the Lake Victoria region; they include isolates from both man and animals. Therefore, a reasonable assumption is that the ancestral organisms developed in the area, with close relatives developing further and spreading in several directions.

The new concept does not necessarily conflict with the earlier one. This concerned observations on the incidence of human disease, which are of course limited to relatively modern times. Busoga may have travelled in animals in southerly and westerly directions from the Lake Victoria region. It then occupied much of East Africa as infections in animals, while bouaflé developed from it to spread throughout the West African fauna. Ecological changes in East Africa could have caused an increase in the transfer of the same organisms from animals to man, in the sequence recorded historically. Outbreaks or endemic foci of human disease may have occurred after further genetic alterations.

The main body of the busoga trypanosomes retained their ability to infect a range of hosts; this may be reflected in the previous observation that *T. b. rhodesiense* is but a set of variants of *T. b. brucei* (Tait *et al.*, 1985). Further specialization was possibly towards adaptation to man, particularly in Zambia, but, more strikingly, several forms developed which were apparently confined to specialized animal/tsetse cycles.

In West Africa, bouaflé spread as a strain group that was almost exclusively limited to animals. Some trypanosomes became more specialized in West Africa as *T. b. gambiense*, involving mostly man, or as *T. evansi*, losing the ability for cyclical transmission.

This hypothesis is, of course, speculative, but it is interesting to pursue the view further by considering the details.

A. EAST AFRICA

Although the majority of populations in busoga were from the Lake Victoria region, others came from as far afield as Zambia, Ethiopia and West Africa. In the cladogram, a tendency existed for those zymodemes with the highest patristic distances (PDs) to be linked with Zambia; this was especially true for those in set 10. Generally, PDs were relatively low as compared with the other East African strain groups. However, the small set 19 included in busoga had high PDs. In the dendrogram, set 19 was positioned more sensibly and we have already argued that the cladistic analysis was at fault (see Section IV A 1).

The core of busoga was cluster 1, from which zambezi diverged. The principal association of zambezi was with man in the Luangwa Valley in Zambia, although numerous primary isolates were not only from Zambian animals but from other parts of East Africa as well. It may be that zambezi is a collection of trypanosomes adapted to a transmission cycle in Zambia, and nearby countries, that mostly involved man; such a feature may be particularly characteristic of the organisms in set 6 which separated independently from cluster 1. However, a strong sampling bias would have arisen because so few of the many Zambian isolates were from animals; domestic animals were uncommon in the Luangwa Valley and sampling from wild animals was difficult.

There is evidence that the human disease can be less acute in the Luangwa Valley endemic area (Buyst, 1974; Rickman, 1974; Wurapa *et al.*, 1984), which, if not a manifestation of acquired immunity, may be another attribute of the zambezi trypanosomes. Perhaps this further distinguishes them from the busoga organisms that cause acute disease in the Lake Victoria region.

Three strain groups, with high PDs, also existed in East Africa and were principally linked with animal infections; they may, therefore, be versions of *T. b. brucei*. Kiboko was the nearest to the hypothetical ancestral organism, both in PD and by virtue of its primary location in central and southern Kenya. It was probably a specialization of the zambezi strain group, since it broke off from this late during cluster formation.

Sindo was a more distant offshoot than kiboko, and was associated with coastal Kenya and places as far afield as Zambia. The phylogenetic tree based on kDNA maxi-circle types, constructed by Gibson *et al.* (1985), showed a similar relationship between the two groups, diverging at a point near to the ancestral position.

Our new kakumbi strain group had the largest PDs of all isolates from East Africa, and all its populations originated in Zambia, far to the south of Lake Victoria. Kakumbi developed along the same line as kiboko, which may be supported by its kDNA maxi-circle composition (see Section IV B 4).

The three groups may reflect the phenotypes adapted for optimal, but not necessarily exclusive, existence in particular animal/tsetse cycles; Maudlin and Welburn (1988) suggested that the genetic nature of the trypanosome influences its ability to mature in tsetse. Many of the trypanosomes, particularly those in the sindo and kakumbi groups, were originally isolated from wild animals; the eponymous area for kiboko is also associated with a rich wild fauna (Goedbloed *et al.*, 1971).

Goedbloed *et al.* (1971) also reported that isolates from flies caught at Kiboko were generally more virulent to mice and less infective to *G. pallidipes* than those caught at Sindo. This suggestion of behavioural differences requires experimental confirmation with defined isolates. Furthermore, any particular vector/parasite association perhaps depends not only on the infraspecific genetic structure of the trypanosome but also on that of the tsetse.

B. WEST AFRICA

An early cleavage in the cladogram separated most of the West African zymodemes, together with *T. evansi* and *T. b. gambiense*. Again, as in East Africa, cluster 1 contained the zymodemes nearest to the hypothetical ancestor. Otherwise most primary isolations were made throughout West Africa, but relatively few came from man (see Section IV B 1), making this group the widely dispersed West African form of *T. b. brucei*. Some of the zymodemes near the ancestral one in set 1 were recognized not only by the bouaflé definition but also by the busoga one (see Section IV A 1); this illustrates the close relationship between the genetic forms at the beginning of both developmental lines.

Presumably the populations in bouaflé spread from the Lake Victoria region, becoming best adapted to animal/tsetse transmission cycles in West Africa. Two important adaptations originated from this line. One was *T. evansi*, with loss of tsetse-infectivity, kDNA maxi-circles, and mini-circle heterogeneity (Borst *et al.*, 1987). Nonetheless, the enzyme polymorphisms and high PDs still demonstrated its relationship to, and its evolution from, bouaflé. Another was the causative organism of chronic human trypanosomiasis, *T. b. gambiense*. The small kDNA maxi-circles (Borst *et al.*, 1981) may also reflect the distinctly separate nature of this basically West African member of *Trypanozoon*. Paindavoine *et al.* (1989) considered that *T. b.*

gambiense may have evolved from the man-infective 'non-gambiense' trypanosomes found in West Africa (Mehlitz *et al.*, 1982; Paindavoine *et al.*, 1986; Godfrey *et al.*, 1987).

A cladogram constructed for *T. congolense* (Gashumba *et al.*, 1988) appeared to show a similar developmental sequence in relation to geographical distribution, and possible adaptation to particular transmission cycles. A basic, widely dispersed savannah strain group gave rise to an East African genetic type found near the Kenya coast, and another riverine/forest type in West Africa. The use of DNA hybridizing probes confirmed the three divisions (Gibson *et al.*, 1988).

It is of course difficult to know how much movement of trypanosomes occurs between different places. Obviously, natural disasters, modern transport, military invasion, and political upheaval would encourage such transfer, but the traditional or natural movements of both man and animals could well have played an important rôle in the past. The comments of Nelson (see Willett, 1965), concerning the association of tsetse and trypanosomes for over 600 000 years with the vast East African herds of wild animals, may well explain the spread of the busoga strain group, particularly when one considers what must have been the extensive prehistoric migrations.

T. evansi has probably moved from West to East Africa and to other continents (Hoare, 1972), and *T. b. gambiense* to parts of East Africa. Examples such as these could, however, be the result of convergent evolution arising from selection for similar circumstances in different areas. Boid (1988) suggested that *T. evansi* may be developing from *T. b. brucei* in the Sudan and northern Kenya, although its affinities appear to be more with the genetically distant forms of bouaflé from West Africa.

VI. Conclusions

This survey has shown that most isolates of the subgenus *Trypanozoon* can be identified and grouped by a minimal amount of isoenzyme electrophoresis; the findings supported observations on DNA characteristics. In addition, evolutionary pathways are proposed that resemble to some extent those based on polymorphisms in kDNA maxi-circles (Gibson *et al.*, 1985).

The present forms of *Trypanozoon* may have evolved in the Lake Victoria region as the busoga strain group, associated with one kind of rhodesian trypanosomiasis. The group spread mainly in a southerly direction, but also to a lesser extent westwards. The zambezi strain group developed from the busoga strain group in East Africa, and was again associated with a rhodesian type of disease, perhaps of a reduced virulence.

Three more forms evolved in East Africa. These strain groups (sindo, kiboko and kakumbi) seemed to be associated only with animals, and thus are kinds of East African *T. b. brucei*, possibly adapted to specialized transmission cycles.

In West Africa, there appeared to be another type of *T. b. brucei*, bouaflé, evolving from busoga. From this arose *T. b. gambiense*, the causative organism of chronic gambian trypanosomiasis, and the non-cyclically transmitted *T. evansi*. Again, these last two taxa have idiosyncratic DNA characteristics.

It is not possible to speculate on the time scale of the evolution described. Indeed, new genetic forms may be developing constantly, by whatever process, in every area, and selection pressures will continue to establish the successful forms in the particular circumstances encountered. In addition, phenotypes may be transferred from one zone to the other, especially in modern times, and be capable of survival.

These conclusions will probably need to be modified whenever collections of trypanosomes from new areas are examined; for instance, few samples came from the southernmost parts of West Africa. It is hoped, however, that the strain groups, subspecies and species as defined in this review will be a basis for future comparisons.

Finally, a complete reappraisal of taxonomy may soon be necessary, based on the observed genetic characteristics; speciation should become more apparent whenever the limits of genetic exchange in nature are established. Generally, however, many features are shared throughout the range of organisms, which suggests the existence of just a single species. Perhaps the kDNA features of *T. evansi* warrant specific status, although its enzymic similarity to the bouaflé strain group indicates otherwise.

The main problem is the categorization of the trypanosomes causing rhodesian sleeping sickness in man. These appear to be mere variants within the busoga and zambezi strain groups, not deserving the distinctive epithet of *T. b. rhodesiense*. This subspecific term could perhaps be applied to all five East African strain groups, while recognizing that sindo, kiboko and kakumbi are not normally infective to man.

Hoare (1972) pointed out the few experiments in the past when numerous unsuccessful attempts were made to infect volunteers with one or a few isolates of an East African *Trypanozoon* population isolated from animals. These isolates, of course, could have been members of the strain groups that appear to have little or no association with human infectivity. On the other hand, perhaps a minor alteration is all that is required to establish infectivity to man, maybe brought about by genetic exchange. Alternatively, considering the wide antigenic repertoires of the East African trypanosomes, some variable antigenic types in an isolate could be more infective to man than

others; such types may be associated with changes in sensitivity to human serum.

The suggestion that the name *T. b. rhodesiense* should be used for the East African strain groups raises the question of priority concerning nomenclature. The trypanosome first isolated and named as *T. brucei* (Bruce, 1895; Plimmer and Bradford, 1899) came from Zululand, and thus belonged in all probability to one of the East African strain groups. The name, of course, was a specific one, with *T. rhodesiense* being given to the human pathogen of East Africa (Stephens and Fantham, 1910); again the original trypanosomes described would have been in one of our East African strain groups. Thus, *T. b. rhodesiense* may be a reasonable term for the East African strain groups.

T. b. brucei, implying non-infectivity to man, is a suitable term for the essentially West African bouaflé strain group, although human infectivity is not entirely excluded. The distinctive genetic and behavioural characteristics of the human pathogen causing chronic disease in West Africa justifies the retention of the subspecific name *T. b. gambiense*, for a trypanosome which of course extends into parts of East Africa.

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Appendix

TABLE A1 *Sets of zymodemes remaining together in both dendrogram and cladogram*

Cladogram cluster	*Dendrogram section*								
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
1	SET 3 4 (2%)[a] 4 (<1%)[b]				SET 2 11 (4%) 28 (3%)		SET 1 15 (6%) 151 (16%)	SET 15 7 (3%) 9 (1%)	
2	SET 10 16 (6%) 97 (10%)	SET 9 30 (11%) 98 (10%)		SET 8 5 (2%) 8 (1%)					
3							SET 16 21 (8%) 57 (6%)	SET 17 25 (9%) 59 (6%)	
4	SET 6 19 (7%) 55 (6%)	SET D 2 (<1%) 2 (<1%)							
5	SET 13 3 (1%) 3 (<1%)	SET E 1 (<1%) 2 (<1%)	SET 14 7 (3%) 8 (1%)						
6						SET 7 19 (7%) 40 (4%)			
7				SET 19 3 (1%) 28 (3%)			SET 18 4 (2%) 96 (10%)		SET 20 16 (6%) 46 (5%)
8	SET A 1 (<1%) 1 (<1%)	SET C 1 (<1%) 1 (<1%)	SET B 1 (<1%) 1 (<1%)	SET 4 29 (11%) 101 (11%)	SET 5 10 (4%) 29 (3%)				
9	SET 12 3 (1%) 3 (<1%)	SET 11 15 (6%) 18 (2%)							

[a] Number of zymodemes in set and percentage of total found. [b] Number of populations in set and percentage of total examined.

TABLE A2 *Zymodeme sets and profiles*

SET[a]	*DND*[b] *SCT*	*CLD*[c] *CLS*	*ZYM*[d]	*PAT*[e] *DIS*	*FRQ*[f]	*GPI*	*NH*	*TDH*	*ICD*	*MDH*	*PGM*	*ASAT*	*ALAT*	*PEP2*	*PEP1*	*ME*	*Ref.*[g]
1	7	1	25	0	24	1	1	1	2	1	1	1	2	1	2	1	—[h]
			62	6	1	1	1	1	2	1	1	1	2	1	6	1	—[i]
			75	28	19	1	1	1	2	1	3	1	2	1	2	7	—[j]
			163	21	1	1	1	1	2	1	3	1	2	1	6	1	W[k]
			71	11	12	1	1	1	2	1	3	1	2	1	1	1	L[k]
			38	15	4	1	1	1	2	1	3	1	2	1	2	1	—[h]
			32	21	1	1	1	1	2	1	1	1	1	1	2	10	—[h]
			120	32	2	1	1	1	2	1	1	1	1	1	2	1	—[j]
			46	26	9	1	1	1	2	1	3	1	2	1	2	12	—[h]
			153	30	2	1	1	1	2	1	3	1	2	1	1	12	M[k]
			281	32	1	1	1	1	2	1	3	1	2	1	6	12	
			50	36	1	1	1	1	2	1	2	1	2	1	2	12	—[h]
			49	42	1	1	1	1	2	1	2	1	2	1	6	12	—[h]
			74	26	58	1	1	1	2	1	3	1	1	1	2	1	—[j]
			40	32	15	1	1	1	2	1	2	1	1	1	1	1	—[h]
2	5	1	299	44	1	1	1	1	2	1	1	7	12	1	2	1	
			107	56	3	1	1	1	2	1	3	1	1	1	2	14	—[j]
			119	81	2	1	1	1	2	1	3	7	1	1	2	14	—[j]
			88	86	3	1	1	1	2	1	3	7	1	1	6	14	—[j]
			140	91	1	1	1	1	2	1	1	4	1	1	2	14	—[j]
			96	88	6	1	1	1	2	1	1	7	1	1	2	14	—[j]
			296	92	1	1	1	1	2	1	1	7	1	4	1	14	
			127	94	3	1	1	1	2	1	1	7	1	1	6	14	—[j]
			294	99	1	1	1	1	2	1	1	7	2	1	2	14	
			77	103	6	1	1	1	2	1	1	7	2	1	1	14	—[j]
			301	110	1	1	1	1	2	1	3	7	12	1	2	14	

TABLE A2 (*continued*)

SET[a]	DND[b] SCT	CLD[c] CLS	ZYM[d]	PAT[e] DIS	FRQ[f]	GPI	NH	TDH	ICD	MDH	PGM	ASAT	ALAT	PEP2	PEP1	ME	Ref.[g]
3	1	1	220	45	1	1	1	1	2	1	1	1	2	14	2	4	
			236	134	1	1	1	1	3	5	1	1	10	3	9	4	
			240	154	1	1	1	1	3	5	1	1	10	3	14	4	
			235	160	1	1	1	1	3	5	1	1	2	3	9	4	
4	4	8	22	24	5	1	1	1	3	1	1	1	2	1	2	1	—[h]
			68	28	2	1	1	1	3	1	1	1	2	1	1	1	—[j]
			78	29	13	1	1	1	3	1	1	1	2	1	6	1	—[j]
			31	32	19	1	1	1	3	1	3	1	2	1	2	1	—[h]
			67	36	18	1	1	1	3	1	3	1	2	1	1	1	—[l]
			24	38	1	1	1	1	3	1	1	1	2	4	6	1	—[h]
			73	42	1	1	1	1	3	1	2	1	2	1	2	1	
			37	34	2	1	1	1	3	1	1	1	2	1	2	11	—[h]
			245	41	1	1	1	1	3	4	1	1	2	1	1	1	
			228	62	1	1	1	1	3	5	1	1	2	1	2	1	
			18	52	3	1	1	1	3	1	1	1	2	1	2	2	—[h]
			145	53	1	1	1	1	3	1	1	1	2	1	1	15	E[k]
			17	60	1	1	1	1	3	1	3	1	2	1	2	2	—[h]
			21	104	1	1	1	1	3	1	1	1	2	1	6	7	—[h]
			87	81	6	1	1	1	3	1	1	1	2	1	2	14	—[j]
			36	119	2	1	1	1	3	1	4	1	2	1	6	7	—[h]
			287	120	1	1	1	1	3	1	4	1	2	1	6	28	
			34	125	1	1	1	1	3	1	4	1	2	1	2	7	—[h]
			35	150	2	1	1	1	3	1	4	1	2	1	1	2	—[h]
			105	151	1	1	1	1	3	1	6	1	2	1	6	1	—[j]
			133	152	1	1	1	4	3	1	3	1	2	1	2	1	
			72	119	1	1	1	1	3	1	1	4	1	1	6	23	
			84	134	1	1	1	1	3	1	1	7	1	1	6	2	—[j]

			108	150	5	1	1	1	3	1	1	7	2	1	2	2	—j
			70	152	4	1	1	1	2	1	4	7	1	1	6	7	—l
			86	176	1	1	1	1	2	1	3	7	2	1	6	2	—j
			80	181	4	1	1	1	2	1	3	7	1	1	2	23	—j
			69	182	1	1	1	1	2	1	1	7	1	1	1	2	—l
			83	184	1	1	1	1	2	1	1	7	2	1	6	2	—j
5	5	8	79	84	7	1	1	1	3	1	1	7	2	1	6	14	—j
			295	93	1	1	1	1	3	1	1	7	1	1	1	14	
			81	95	11	1	1	1	3	1	1	7	1	1	6	14	—j
			91	118	1	1	1	1	3	1	3	7	2	8	1	14	
			92	143	1	1	1	1	3	1	3	4	2	8	1	14	
			85	137	1	1	1	1	3	1	1	7	1	1	7	1	—j
			101	159	1	1	1	1	3	1	1	7	1	1	6	6	
			118	165	1	1	1	1	2	1	3	7	1	1	6	1	—j
			89	175	1	1	1	1	2	1	3	7	1	1	1	1	—j
			98	186	4	1	1	1	2	1	1	7	1	4	2	1	—j
6	1	4	252	59	1	1	1	1	1	1	1	1	10	1	1	1	
			216	70	2	1	1	1	1	1	3	1	10	1	2	1	
			218	76	1	1	1	1	1	1	3	1	10	10	1	1	
			232	76	1	1	1	1	1	1	1	1	10	4	6	1	
			210	77	1	1	1	1	1	1	1	1	10	12	1	1	
			253	89	1	1	1	1	1	1	1	1	10	4	1	3	
			181	96	2	1	1	1	1	1	1	1	10	1	1	3	
			186	100	2	1	1	1	1	1	1	1	10	1	2	3	
			199	89	1	1	1	1	1	1	1	1	10	4	1	4	
			239	94	1	1	1	1	1	1	1	1	10	4	7	4	
			205	98	15	1	1	1	1	1	1	1	10	4	6	4	Zm
			188	100	11	1	1	1	1	1	1	1	10	1	2	4	
			182	104	5	1	1	1	1	1	1	1	10	1	1	4	
			212	108	1	1	1	1	1	1	1	1	10	12	6	4	
			209	111	4	1	1	1	1	1	1	1	10	10	2	4	

TABLE A2 (*continued*)

SET[a]	DND[b] SCT	CLD[c] CLS	ZYM[d]	PAT[e] DIS	FRQ[f]	GPI	NH	TDH	ICD	MDH	PGM	ASAT	ALAT	PEP2	PEP1	ME	Ref.[g]
			203	103	2	1	1	1	1	1	1	1	10	4	2	20	
			206	109	1	1	1	1	1	1	1	1	10	4	6	20	
			195	111	1	1	1	1	1	1	1	1	10	1	2	20	
			211	114	2	1	1	1	1	1	1	1	10	12	2	20	
7	6	6	234	133	1	1	1	1	5	1	1	4	10	3	16	8	
			254	140	1	1	1	1	2	1	1	4	10	1	7	8	
			221	146	3	1	1	1	2	1	1	4	10	1	1	8	
			265	151	1	1	1	1	2	1	1	4	10	10	7	8	
			90	167	11	1	1	1	2	1	1	4	2	1	7	8	
			115	171	2	1	1	1	2	1	1	4	2	1	6	8	
			262	173	1	1	1	1	2	1	1	4	2	1	1	8	
			266	177	1	1	1	1	2	1	1	4	2	10	7	8	
			117	175	1	1	1	1	2	1	3	4	2	1	7	8	
			233	186	1	1	1	1	5	1	1	4	2	1	7	8	
			29	178	5	1	1	1	2	1	1	4	1	1	7	8	—[h]
			93	183	2	1	1	1	2	1	4	4	2	1	7	8	
			259	186	1	1	1	1	2	1	1	4	1	4	7	8	
			263	195	1	1	1	1	2	2	1	4	2	1	7	8	
			106	201	2	1	1	1	2	1	1	4	11	1	6	8	
			116	203	1	1	1	1	2	1	3	4	2	8	7	8	
			94	210	2	1	1	1	2	1	1	4	2	8	7	8	
			225	185	1	1	1	1	2	3	1	4	10	10	7	8	
			226	202	2	1	1	1	2	3	1	4	2	1	7	8	
8	4	2	7	48	3	1	1	1	1	1	1	1	2	1	2	2	—[h]
			30	52	1	1	1	1	1	1	1	1	2	1	1	2	—[h]
			112	64	1	1	1	1	1	1	4	1	2	1	2	2	

			16	60	2	1	1	1	1	1	3	1	2	1	1	2	—[h]
			33	64	1	1	1	1	1	1	3	1	2	1	2	2	—[h]
9	2	2	4	28	6	1	1	1	1	1	1	1	2	1	2	1	—[h]
			5	32	28	1	1	1	1	1	1	1	2	1	1	1	—[h]
			249	36	1	1	1	1	1	1	1	1	2	4	2	1	
			102	40	1	1	1	1	1	1	1	1	2	4	1	1	
			66	39	15	1	1	1	1	1	3	1	2	1	1	1	—[l]
			215	43	1	1	1	1	1	1	3	1	2	1	2	1	
			184	43	2	1	1	1	1	1	1	1	2	1	1	21	
			196	47	1	1	1	1	1	1	1	1	2	1	2	21	
			217	50	1	1	1	1	1	1	3	1	2	1	1	12	
			197	63	1	1	1	1	1	1	1	1	2	1	13	21	
			243	76	1	1	1	1	1	1	3	1	2	1	12	1	
			255	45	1	1	1	1	1	4	1	1	2	1	1	1	
			227	49	1	1	1	1	1	4	1	1	2	1	2	1	
			246	56	1	1	1	1	1	4	1	1	2	1	1	12	
			15	52	1	1	1	1	1	1	1	1	2	1	1	7	—[h]
			189	53	3	1	1	1	1	1	1	1	2	1	2	15	
			200	65	1	1	1	1	1	1	1	1	2	4	1	6	
			300	50	4	1	1	1	1	1	1	1	2	1	2	3	
			1	54	16	1	1	1	1	1	1	1	2	1	1	3	—[h]
			257	62	1	1	1	1	1	1	1	1	2	4	1	3	
			244	69	1	1	1	1	1	1	1	1	2	4	2	19	
			213	75	1	1	1	1	1	1	1	1	2	13	1	3	
			8	79	1	1	1	1	1	1	1	1	2	1	1	5	—[h]
			185	79	2	1	1	1	1	1	1	1	2	1	1	26	
			187	83	1	1	1	1	1	1	1	1	2	1	2	26	
			55	90	1	1	1	1	1	1	3	1	2	4	1	13	—[h]
			229	91	1	1	1	1	1	1	3	1	2	4	1	1	
			114	105	1	1	1	1	1	1	1	1	2	1	1	13	
			219	115	1	1	1	1	1	1	3	1	2	4	1	27	
			191	134	1	1	1	1	1	1	1	1	2	1	2	27	

TABLE A2 (*continued*)

SET[a]	DND[b] SCT	CLD[c] CLS	ZYM[d]	PAT[e] DIS	FRQ[f]	GPI	NH	TDH	ICD	MDH	PGM	ASAT	ALAT	PEP2	PEP1	ME	Ref.[g]
10	1	2	3	50	17	1	1	1	1	1	1	1	2	1	2	4	—[h]
			2	54	33	1	1	1	1	1	1	1	2	1	1	4	—[h]
			192	55	4	1	1	1	1	1	1	1	2	1	6	4	
			208	64	1	1	1	1	1	1	1	1	2	10	1	4	
			250	68	2	1	1	1	1	1	1	1	2	1	12	4	
			231	69	1	1	1	1	1	1	4	1	2	1	1	4	
			183	65	1	1	1	1	1	1	1	1	2	1	1	20	
			190	69	4	1	1	1	1	1	1	1	2	1	2	20	
			242	73	1	1	1	1	1	1	1	1	2	4	1	20	
			193	74	1	1	1	1	1	1	1	1	2	1	6	20	
			214	81	1	1	1	1	1	1	1	1	2	14	2	4	
			204	102	22	1	1	1	1	1	1	1	2	4	6	4	K[m]
			251	106	1	1	1	1	1	1	1	1	2	4	7	4	
			267	105	1	1	1	1	1	1	2	1	2	4	6	4	
			198	112	6	1	1	1	1	1	1	1	2	4	1	4	
			201	116	1	1	1	1	1	1	1	1	2	4	2	4	
11	2	9	26	96	1	1	2	2	1	1	1	1	2	1	2	3	—[h]
			95	88	3	1	1	4	1	1	1	1	2	1	1	3	
			125	122	1	1	1	4	1	1	2	1	2	1	2	9	
			113	123	1	1	1	4	1	1	3	1	2	1	6	5	
			223	123	1	1	1	1	1	2	1	1	2	1	1	19	
			110	129	1	1	2	2	1	1	1	1	2	3	2	9	
			111	139	1	1	2	3	1	1	1	1	2	1	6	9	
			28	168	1	1	2	3	1	3	1	1	2	1	2	9	—[h]
			121	137	2	1	2	1	1	1	1	1	2	1	1	1	
			122	139	1	1	2	1	1	1	1	1	2	1	1	4	
			298	143	1	1	2	1	1	1	1	1	2	1	2	4	

			27	159	1	1	2	4	1	1	1	1	2	1	2	3	—[h]
			109	171	1	1	2	4	1	1	1	1	2	1	2	18	
			20	168	1	1	2	4	1	1	1	1	2	1	6	6	—[h]
			297	169	1	1	2	4	1	1	1	1	2	1	11	25	
12	1	9	247	141	1	1	1	3	1	5	1	1	2	1	1	4	
			222	141	1	1	1	1	1	2	1	1	2	1	2	4	
			224	152	1	1	1	1	1	2	1	1	2	1	2	20	
13	1	5	241	154	1	1	1	1	1	1	1	1	2	4	4	4	
			207	157	1	1	1	1	1	1	1	1	2	1	4	20	
			256	181	1	1	1	1	1	1	1	1	2	6	4	4	
14	3	5	248	198	1	1	1	1	2	1	1	1	2	2	15	1	
			9	215	1	1	1	1	2	2	1	1	2	2	4	1	—[h]
			12	216	1	1	1	1	3	2	1	1	2	2	4	1	—[h]
			261	226	1	1	1	1	2	2	1	1	2	2	15	1	
			6	242	1	1	1	3	2	2	1	1	2	2	3	1	—[h]
			10	263	2	1	1	2	2	2	1	1	2	3	5	1	—[h]
			11	289	1	1	1	2	2	2	1	1	2	3	5	6	—[h]
15	8	1	237	46	1	1	1	1	2	1	3	1	10	1	1	1	
			51	54	2	1	1	1	2	1	3	1	3	1	2	1	—[h]
			168	58	2	1	1	1	2	1	3	1	3	1	1	1	AB[k]
			48	60	1	1	1	1	2	1	3	1	3	1	6	1	—[h]
			169	57	1	1	1	1	2	1	1	1	3	1	2	11	AC[k]
			278	53	1	1	1	1	2	1	3	1	10	1	2	12	
			274	63	1	1	1	1	2	1	2	1	10	1	2	12	
16	7	3	39	22	22	1	1	1	2	1	2	1	2	1	1	1	—[h]
			142	26	6	1	1	1	2	1	2	1	2	1	2	1	B[k]
			160	31	3	1	1	1	2	1	2	1	2	1	6	1	T[k]
			279	32	1	1	1	1	2	1	2	1	2	10	1	1	
			155	36	1	1	1	1	2	1	2	1	2	1	2	11	O[k]
			56	39	2	1	1	1	2	1	2	1	2	4	6	1	—[h]

TABLE A2 (*continued*)

SET[a]	DND[b] SCT	CLD[c] CLS	ZYM[d]	PAT[e] DIS	FRQ[f]	GPI	NH	TDH	ICD	MDH	PGM	ASAT	ALAT	PEP2	PEP1	ME	Ref.[g]
			282	47	1	1	1	1	2	1	2	1	2	10	12	1	
			147	43	1	1	1	1	2	1	2	5	2	1	1	1	G[k]
			148	57	1	1	1	1	2	1	2	6	2	1	1	1	H[k]
			162	48	1	1	1	1	2	1	2	1	2	1	6	22	V[k]
			302	53	1	1	1	1	2	1	2	1	2	3	1	1	
			43	57	1	1	1	1	2	1	2	1	2	3	9	1	—[h]
			303	78	1	1	1	1	2	1	2	1	2	4	9	1	
			143	50	3	1	1	1	2	1	2	1	2	1	2	15	C[k]
			151	54	3	1	1	1	2	1	2	1	2	1	1	15	K[k]
			161	56	4	1	1	1	2	1	2	1	2	1	6	15	U[k]
			156	61	1	1	1	1	2	1	2	1	2	1	2	16	P[k]
			157	61	1	1	1	1	2	1	2	1	2	1	2	17	Q[k]
			290	88	1	1	1	1	2	1	2	1	13	1	2	12	
			285	48	1	1	1	1	2	1	2	2	2	1	1	1	
			175	140	1	1	1	1	2	1	2	2	7	4	1	10	AI[k]
17	8	3	44	68	1	1	1	1	2	1	2	1	5	4	1	1	—[h]
			174	52	2	1	1	1	2	1	2	1	6	1	1	1	AH[k]
			176	47	2	1	1	1	2	1	2	1	4	1	1	1	AJ[k]
			45	55	1	1	1	1	2	1	2	1	4	4	1	1	—[h]
			177	57	1	1	1	1	2	1	3	1	4	1	1	1	AK[k]
			178	69	2	1	1	1	2	1	5	1	4	1	1	1	AL[k]
			166	61	14	1	1	1	2	1	2	1	3	1	1	1	Z[k]
			170	65	4	1	1	1	2	1	2	1	3	1	2	1	AD[k]
			271	71	1	1	1	1	2	1	2	1	3	1	1	12	
			47	75	1	1	1	1	2	1	2	1	3	1	2	12	—[h]
			173	81	4	1	1	1	2	1	2	1	3	1	6	12	AG[k]
			286	73	5	1	1	1	2	1	2	1	10	1	1	1	

			149	77	2	1	1	1	2	1	2	1	10	1	2	1	I[k]
			284	81	1	1	1	1	2	1	2	1	10	4	1	1	
			180	82	1	1	1	1	2	1	2	1	10	1	11	1	AN[k]
			277	87	1	1	1	1	2	1	2	1	10	10	2	1	
			276	91	1	1	1	1	2	1	2	1	3	10	6	12	
			59	98	1	1	1	1	2	1	2	1	3	4	6	2	—[h]
			280	85	4	1	1	1	2	1	2	1	3	1	1	15	
			171	89	2	1	1	1	2	1	2	1	3	1	2	15	AE[k]
			283	95	4	1	1	1	2	1	2	1	3	1	6	15	
			167	96	1	1	1	1	2	1	2	1	3	1	1	16	AA[k]
			179	107	1	1	1	1	2	1	2	1	10	1	6	15	AM[k]
			288	123	1	1	1	1	2	1	2	2	3	1	6	12	
			291	127	1	1	1	1	2	1	2	2	3	1	1	15	
18	7	7	41	135	30	1	1	1	2	1	2	1	2	1	2	2	—[h]
			42	141	9	1	1	1	2	1	2	1	2	1	6	2	—[h]
			58	149	21	1	1	1	2	1	2	1	2	4	6	2	—[h]
			64	154	36	1	1	1	2	1	2	1	2	4	2	2	—[i]
19	4	7	128	148	1	1	1	1	2	1	1	1	1	1	6	2	—[j]
			23	153	24	1	1	1	2	1	1	1	2	1	2	2	—[h]
			104	172	3	1	1	1	3	1	1	1	1	1	6	2	—[j]
20	9	7	54	76	1	1	1	1	2	1	2	2	2	4	1	2	—[h]
			53	87	2	1	1	1	2	1	2	2	1	4	1	2	—[h]
			134	104	1	1	1	1	2	1	2	2	2	1	13	2	—[n]
			132	133	2	1	1	1	2	1	2	2	11	1	13	2	—[n]
			275	123	1	2	1	1	2	1	2	2	1	12	2	2	
			272	123	1	1	1	1	2	1	2	2	1	8	1	2	—[n]
			270	158	1	1	3	1	2	1	2	2	1	8	1	2	—[n]
			141	156	6	1	1	1	2	1	2	2	1	6	1	2	A[k]
			131	181	14	1	1	1	2	1	2	2	1	9	13	2	—[n]
			269	209	1	1	3	1	2	1	2	2	1	9	9	2	—[n]
			268	217	5	1	3	1	2	1	2	2	1	6	1	2	—[n]

TABLE A2 (*continued*)

SET[a]	*DND*[b] *SCT*	*CLD*[c] *CLS*	*ZYM*[d]	*PAT*[e] *DIS*	*FRQ*[f]	*GPI*	*NH*	*TDH*	*ICD*	*MDH*	*PGM*	*ASAT*	*ALAT*	*PEP2*	*PEP1*	*ME*	*Ref.*[g]
			144	215	3	1	1	1	2	1	2	3	1	6	1	2	D[k]
			52	113	1	1	1	1	2	1	2	1	1	4	1	2	—[h]
			293	159	1	1	1	1	2	1	2	3	1	7	1	2	—[n]
			292	172	3	1	1	1	2	1	2	3	1	7	9	2	—[o]
			63	177	3	1	1	1	2	1	3	3	2	4	1	24	—[i]
A	1	8	260	78	1	1	1	1	3	2	1	1	2	1	1	4	
B	3	8	238	88	1	1	1	1	3	2	1	1	2	3	16	1	
C	2	8	19	146	1	1	2	4	3	1	1	1	2	1	6	8	—[h]
D	2	4	202	95	1	1	1	1	1	1	1	1	10	4	2	15	
			258	102	1	1	1	1	1	1	1	1	10	4	1	16	
E	2	5	264	96	2	1	1	1	1	1	1	1	2	1	15	1	

[a] Group, as defined in Table 1. [b] Dendrogram section. [c] Cladogram cluster. [d] Zymodeme number. [e] Patristic distance. [f] Number of populations with same profile. [g] Reference first describing profile, together with original code if different from current zymodeme number. [h] Gibson *et al.* (1980). [i] Gibson *et al.* (1983). [j] Gibson and Wellde (1985). [k] Mehlitz *et al.* (1982). [l] Gibson and Gashumba (1983). [m] Dukes *et al.* (1983). [n] Godfrey *et al.* (1987). [o] Scott *et al.* (1983).

TABLE A3 *Summary of the origins and trypanosome populations in each set and zymodeme*

Set	*Zymodeme*	*Total no. of populations*[a]	*Country*	*Location*	*No. of populations/ host species*[b]	*Host*	*Years*[c]
1	25	24	Kenya	Lambwe Valley	19	Man	1974–83
				Lambwe Valley	3	Reedbuck	1970–80
				Lambwe Valley	1	Cow	1980
			Ivory Coast	Bouaflé	1	Pig[d]	1978
	62	1	Kenya	Rumuruti	1	Camel	1980
	75	19	Kenya	Lambwe Valley	18	Man	1980–83
				?	1	*G. pallidipes*	?
	163	1	Ivory Coast	Bouaflé	1	Pig	1978
	71	12	Ivory Coast	Bouaflé	11	Pig	1978–83
			Uganda	Busoga	1	Cow	1977
	38	4	Ethiopia	Illubabor	1	*G. tachinoides*	1970
			The Gambia	Dankunku	1	Horse	1977
			Ivory Coast	Bouaflé	1	Pig[d]	1978
			Zambia	Solwezi	1	Man	1983
	32	1	Uganda	Busoga	1	Man	1959
	120	2	Kenya	Lambwe Valley	2	Cow	1981
	46	9	Ivory Coast	Bouaflé	6	Pig	1978–82
				Bouaflé	2	Goat	1982
			Nigeria	Benin State	1	Cow	1977
	153	2	Ivory Coast	Bouaflé	2	Pig	1978–82
	281	1	Ivory Coast	Bouaflé	1	Pig	1982
	50	1	Nigeria	Benin State	1	Cow	1977
	49	1	Nigeria	Benin State	1	Cow	1977
	74	58	Kenya	Lambwe Valley	45	Man	1980–83
				Lambwe Valley	9	Cow	1980–81
				Lambwe Valley	1	*G. pallidipes*	1980
				?	3	*G. pallidipes*	?

TABLE A3 (*continued*)

Set	*Zymodeme*	*Total no. of populations*[a]	*Country*	*Location*	*No. of populations/ host species*[b]	*Host*	*Years*[c]
	40	15	Liberia	Bindin, etc.	14	Pig	1975–78
				Bindin	1	Dog	1975
2	299	1	Kenya	Near Lambwe Valley	1	Man	1981
	107	3	Kenya	Lambwe Valley	2	Man	1974–75
				Lambwe Valley	1	Cow	1975
	119	2	Kenya	Lambwe Valley	1	Cow	1980
				Lambwe Valley	1	Goat	1980
	88	3	Kenya	Lambwe Valley	2	Cow	1976–80
				Lambwe Valley	1	*G. pallidipes*	1980
	140	1	Kenya	Lambwe Valley	1	*G. pallidipes*	1983
	96	6	Kenya	Lambwe Valley	6	Man	1976–81
	296	1	Kenya	Lambwe Valley	1	Man	1978
	127	3	Kenya	Lambwe Valley	2	Cow	1978–80
				Lambwe Valley	1	*G. pallidipes*	1983
	294	1	Kenya	Lambwe Valley	1	Cow	1980
	77	6	Kenya	Lambwe Valley	5	Man	1980–83
				Lambwe Valley	1	Cow	1980
	301	1	Kenya	Lambwe Valley	1	Goat	1981
3	220	1	Zambia	Luangwa Valley	1	Man[d]	1981
	236	1	Zambia	Luangwa Valley	1	*G. m. morsitans*[d]	1983
	240	1	Zambia	Luangwa Valley	1	Goat[d]	1983
	235	1	Zambia	Luangwa Valley	1	*G. m. morsitans*[d]	1983
4	22	5	Kenya	Lambwe Valley	4	Man	1981–83

		Tanzania	Serengeti	1	Hartebeest	1971
68	2	Kenya	Lambwe Valley	1	Man	1980
		Uganda	Busoga	1	Man	1981
78	13	Kenya	Lambwe Valley	9	Cow	1976–80
			Lambwe Valley	4	*G. pallidipes*	1981–83
31	19	Kenya	Busia	7	Man	1961–79
			Busia	4	Cow	1964–77
			Busia	1	Bushbuck	1958
		Uganda	Busoga	5	Man	1959–77
			Busoga	1	Cow	1971
			Busoga	1	*G. pallidipes*	1960
67	18	Uganda	Busoga	11	Man	1976–79
			Busoga	5	Cow	1971–76
		Kenya	Nairobi Hospital	1	Man	1982
			Lambwe Valley	1	Dog	1980
24	1	Kenya	Lambwe Valley	1	Reedbuck	1970
73	1	Uganda	Western Uganda	1	Dog	1968
37	2	Ethiopia	Illubabor	2	Man	1968–69
245	1	Zambia	Luangwa Valley	1	*G. pallidipes*[d]	1982
228	1	Zambia	Luangwa Valley	1	*G. m. morsitans*	1982
18	3	Tanzania	Serengeti	1	Hartebeest	1971
			Serengeti	1	Lion	1971
		Kenya	Lambwe Valley	1	Goat	1970
145	1	Ivory Coast	Bouaflé	1	Man	1978
17	1	Tanzania	Serengeti	1	Waterbuck	1966
21	1	Tanzania	Serengeti	1	Hartebeest	1971
87	6	Kenya	Lambwe Valley	5	Man	1974–82
			Lambwe Valley	1	*G. pallidipes*	1980
36	2	Uganda	?	1	Sheep	1960
		Ivory Coast	Bouaflé	1	Pig	1982
287	1	Ivory Coast	Bouaflé	1	Pig	1982
34	1	Uganda	?	1	Cow	?

TABLE A3 (*continued*)

Set	*Zymodeme*	*Total no. of populations*[a]	*Country*	*Location*	*No. of populations/ host species*[b]	*Host*	*Years*[c]
	35	2	Uganda	Busoga	1	*G. pallidipes*	1955
			Ivory Coast	Bouaflé	1	Man	1978
	105	1	Kenya	Lambwe Valley	1	Hyena	1971
	133	1	Tanzania	Serengeti	1	Waterbuck	1966
	72	1	Uganda	Busoga	1	*G. pallidipes*	1976
	84	1	Kenya	Lambwe Valley	1	*G. pallidipes*	1980
	108	5	Kenya	Lambwe Valley	4	Cow	1981
				Lambwe Valley	1	Oribi	1981
	70	4	Uganda	Busoga	4	*G. f. fuscipes*	1971–72
	86	1	Kenya	Lambwe Valley	1	Waterbuck	1970
	80	4	Kenya	Lambwe Valley	4	Man	1970–76
	69	1	Uganda	Gondokera	1	Man	1977
	83	1	Kenya	Lambwe Valley	1	Cow	1980
5	79	7	Kenya	Lambwe Valley	6	Cow	1980
				Lambwe Valley	1	Dog	1980
	295	1	Kenya	Lambwe Valley	1	Cow	1980
	81	11	Kenya	Lambwe Valley	8	Cow	1970–81
				Lambwe Valley	1	Hyena	1971
				Lambwe Valley	2	*G. pallidipes*	1980
	91	1	Kenya	Coast Province	1	Sheep	1981
	92	1	Kenya	Coast Province	1	Sheep	1981
	85	1	Kenya	Lambwe Valley	1	Cow	1980
	101	1	Uganda	Busoga	1	Man	1977
	118	1	Kenya	Lambwe Valley	1	Cow	1980
	89	1	Kenya	Lambwe Valley	1	*G. pallidipes*	1980
	98	4	Kenya	Lambwe Valley	4	Man	1975–78

6	252	1	Zambia	Luangwa Valley	1	*G. pallidipes*[d]	1983
	216	2	Zambia	Luangwa Valley	2	Man[d]	1982
	218	1	Zambia	Luangwa Valley	1	Man[d]	1982
	232	1	Zambia	Luangwa Valley	1	Man	1983
	210	1	Zambia	Luangwa Valley	1	Man	1982
	253	1	Zambia	Luangwa Valley	1	Man	1983
	181	2	Zambia	Luangwa Valley	2	Man	1982–84
	186	2	Zambia	Luangwa Valley	2	Man	1982
	199	1	Zambia	Luangwa Valley	1	Man	1982
	239	1	Zambia	Luangwa Valley	1	Man	1983
	205	15	Zambia	Luangwa Valley	15	Man	1982–83
	188	11	Zambia	Luangwa Valley	11	Man[d]	1981–84
	182	5	Zambia	Luangwa Valley	5	Man[d]	1982–83
	212	1	Zambia	Luangwa Valley	1	Man	1982
	209	4	Zambia	Luangwa Valley	4	Man	1982
	203	2	Zambia	Luangwa Valley	2	Man[d]	1972–74
	206	1	Zambia	Luangwa Valley	1	Man	1981
	195	1	Zambia	Luangwa Valley	1	Man	1981
	211	2	Zambia	Luangwa Valley	2	Man[d]	1981
7	234	1	Zambia	Luangwa Valley	1	*G. pallidipes*[d]	1983
	254	1	Zambia	Luangwa Valley	1	*G. pallidipes*	1983
	221	3	Zambia	Luangwa Valley	1	Impala	1977
				Luangwa Valley	1	*G. pallidipes*	1983
				Luangwa Valley	1	*G. m. morsitans*	1983
	265	1	Zambia	Luangwa Valley	1	*G. m. morsitans*	1983
	90	11	Kenya	Coast Province	6	Goat	1981
				Coast Province	5	Sheep	1981
	115	2	Kenya	Coast Province	2	Sheep	1981
	262	1	Zambia	Luangwa Valley	1	Man	1980
	266	1	Zambia	Luangwa Valley	1	*G. m. morsitans*	1983
	117	1	Kenya	Coast Province	1	Goat	1981
	233	1	Zambia	Luangwa Valley	1	*G. pallidipes*[d]	1983

TABLE A3 (*continued*)

Set	*Zymodeme*	*Total no. of populations*[a]	*Country*	*Location*	*No. of populations/ host species*[b]	*Host*	*Years*[c]
	29	5	Kenya	Sindo, S. Nyanza	3	*G. pallidipes*	1969–70
				?	2	*G. pallidipes*	1970 & ?
	93	2	Kenya	Coast Province	1	Goat	1981
				Coast Province	1	Sheep	1981
	259	1	Zambia	Luangwa Valley	1	*G. m. morsitans*	1983
	263	1	Zambia	Luangwa Valley	1	Man[d]	1980
	106	2	Kenya	Lambwe Valley	1	Waterbuck	1971
				Lambwe Valley	1	*G. pallidipes*	1980
	116	1	Kenya	Coast Province	1	Goat	1981
	94	2	Kenya	Coast Province	1	Sheep	1981
				Coast Province	1	Goat	1981
	225	1	Zambia	Luangwa Valley	1	Giraffe	1982
	226	2	Zambia	Luangwa Valley	1	Warthog	1982
				Luangwa Valley	1	*G. m. morsitans*	1981
8	7	3	Zambia	Luangwa Valley	1	Waterbuck[d]	1974
				Luangwa Valley	1	Warthog[d]	1974
			Rwanda	Kagera	1	Man	1971
	30	1	Rwanda	Kagera	1	Man	1970
	112	1	Kenya	Kibwezi	1	*Glossina* sp.?	1979
	16	2	Tanzania	Serengeti	1	Warthog	1966
			Kenya	Kiboko	1	*G. pallidipes*	1969
	33	1	Uganda	Busoga	1	Man	1959
9	4	6	Zambia	Luangwa Valley	4	Man	1971–82
				Luangwa Valley	1	*G. m. morsitans*[d]	1981

		Kenya	Coast Province	1	Sheep	1974
5	28	Uganda	Busoga	9	Man	1976–79
			Busoga	1	Dog	1979
		Zambia	Luangwa Valley, etc.	7	Man	1972–83
			Luangwa Valley	3	*G. pallidipes*[d]	1980–83
		Tanzania	Kigoma & Serengeti	2	Cow	1970–74
			Kigoma	1	Reedbuck	1974
			?	1	Warthog	1966
		Botswana	Maun	1	Man	1960
		Ethiopia	Illubabor	1	Bushbuck	1970
		Kenya[e]	?	1	Man	1982
		UK[f]	?	1	Man	1977
249	1	Zambia	Luangwa Valley	1	Man	1983
102	1	Kenya	Busia	1	Man	1960
66	15	Uganda	Busoga	13	Man	1976–79
		Zambia	Luangwa Valley	2	Man[a]	1981–82
215	1	Zambia	Luangwa Valley	1	Man[a]	1982
184	2	Zambia	Luangwa Valley	2	Man	1981
196	1	Zambia	Luangwa Valley	1	Man	1981
217	1	Zambia	Luangwa Valley	1	Man	1981
197	1	Zambia	Luangwa Valley	1	Man	1981
243	1	Zambia	Luangwa Valley	1	Man	1981
255	1	Zambia	Luangwa Valley	1	Cow	1982
227	1	Zambia	Luangwa Valley	1	*G. m. morsitans*[d]	1981
246	1	Zambia	Luangwa Valley	1	*G. pallidipes*[d]	1982
15	1	Tanzania	Tabora	1	Hartebeest	1959
189	3	Zambia	Luangwa Valley	1	Waterbuck[d]	1974
			Luangwa Valley	1	Warthog[d]	1974
			Luangwa Valley	1	Lion[d]	1974
200	1	Zambia	Luangwa Valley	1	Man[d]	1982
300	4	Zambia	Luangwa Valley, etc.	3	Man	1980–84
			Luangwa Valley	1	*G. m. morsitans*	1983

TABLE A3 (*continued*)

Set	*Zymodeme*	*Total no. of populations*[a]	*Country*	*Location*	*No. of populations/ host species*[b]	*Host*	*Years*[c]
	1	16	Zambia	Luangwa Valley	13	Man[d]	1972–82
			Tanzania	Tabora & Kasulu	2	Man	1959–60
				Kigoma	1	Cow	1974
	257	1	Zambia	Luangwa Valley	1	Man	1978
	244	1	Zambia	Luangwa Valley	1	Bushbuck[d]	1981
	213	1	Zambia	Luangwa Valley	1	*G. m. morsitans*	1982
	8	1	Zambia	Luangwa Valley	1	Waterbuck	1974
	185	2	Zambia	Luangwa Valley	2	Man	1980–81
	187	1	Zambia	Luangwa Valley	1	Man	1981
	55	1	Nigeria	Kwang	1	Man	1967
	229	1	Kenya	Busia	1	Man	1982
	114	1	Tanzania	Katanga	1	Man	1974
	219	1	Zambia	Luangwa Valley	1	Man	1982
	191	1	Zambia	Luangwa Valley	1	Cow	1982
10	3	17	Zambia	Luangwa Valley	15	Man[d]	1972–84
				Luangwa Valley	1	Giraffe[d]	1974
				Luangwa Valley	1	Bushbuck[d]	1981
	2	33	Zambia	Luangwa Valley	26	Man[d]	1972–82
				Luangwa Valley	1	Hippopotamus	1971
				Luangwa Valley	1	Giraffe[d]	1974
				Luangwa Valley	1	*G. pallidipes*	1983
			Kenya	Meru & Galana	2	Cow	1974 & ?
			Tanzania	Kigoma	1	Goat	1974
			Mozambique	Tete	1	Man	1983
	192	4	Zambia	Luangwa Valley	4	Man	1980–83
	208	1	Zambia	Luangwa Valley	1	Man	1982

	250	2	Zambia	Luangwa Valley	1	Man[d]	1982
				Luangwa Valley	1	Cow	1983
	231	1	Zambia	Luangwa Valley	1	Man	1983
	183	1	Zambia	Luangwa Valley	1	Man	1980
	190	4	Zambia	Luangwa Valley	4	Man	1981
	242	1	Zambia	Luangwa Valley	1	Man	1981
	193	1	Zambia	Luangwa Valley	1	Man	1981
	214	1	Zambia	Luangwa Valley	1	Man[d]	1983
	204	22	Zambia	Luangwa Valley	21	Man	1981–84
				Luangwa Valley	1	*G. m. morsitans*	1983
	251	1	Zambia	Luangwa Valley	1	Goat	1983
	267	1	Zambia	Luangwa Valley	1	Man	1983
	198	6	Zambia	Luangwa Valley	6	Man[d]	1980–83
	201	1	Zambia	Luangwa Valley	1	Man	1981
11	26	1	Kenya	Kiboko	1	*G. pallidipes*	1969
	95	3	Kenya	Kibwezi	2	*G. pallidipes*	1979
				Kibwezi	1	*Glossina* spp.[g]	1978
	125	1	Kenya	Meru	1	Cow	1973
	113	1	Kenya	Masai Mara	1	*G. pallidipes*	1970
	223	1	Zambia	Luangwa Valley	1	Man	1980
	110	1	Kenya	Kiboko	1	Sheep	1970
	111	1	Kenya	Kibwezi	1	*G. pallidipes*	1979
	28	1	Kenya	Kiboko	1	*G. pallidipes*	1969
	121	2	Kenya	Meru	2	Cow	1972
	122	1	Kenya	Meru	1	Cow	1972
	298	1	Kenya	Meru	1	Cow	1972
	27	1	Kenya	Kiboko	1	*G. pallidipes*	1969
	109	1	Kenya	Kiboko	1	Rhinoceros	1970
	20	1	Tanzania	Serengeti	1	Lion	1971
	297	1	Kenya	Meru	1	Cow	1973

TABLE A3 (*continued*)

Set	*Zymodeme*	*Total no. of populations*[a]	*Country*	*Location*	*No. of populations/ host species*[b]	*Host*	*Years*[c]
12	247	1	Zambia	Luangwa Valley	1	Cow	1982
	222	1	Zambia	Luangwa Valley	1	Man	1980
	224	1	Zambia	Luangwa Valley	1	Man	1981
13	241	1	Zambia	Luangwa Valley	1	Goat[d]	1983
	207	1	Zambia	Luangwa Valley	1	Man	1981
	256	1	Zambia	Luangwa Valley	1	Man	1982
14	248	1	Zambia	Luangwa Valley	1	*G. pallidipes*[d]	1983
	9	1	Zambia	Luangwa Valley	1	Hippopotamus	1971
	12	1	Zambia	Luangwa Valley	1	Hyena	1973
	261	1	Zambia	Luangwa Valley	1	*G. pallidipes*[d]	1983
	6	1	Zambia	Luangwa Valley	1	Hippopotamus	1971
	10	2	Zambia	Luangwa Valley	1	Hippopotamus	1971
				Luangwa Valley	1	Lion[d]	1974
	11	1	Zambia	Luangwa Valley	1	Hyena	1973
15	237	1	Zambia	Luangwa Valley	1	*G. pallidipes*[d]	1983
	51	2	Nigeria	Benin State	1	Cow	1977
			Ivory Coast	Bouaflé	1	Pig	1978
	168	2	Ivory Coast	Bouaflé	2	Pig	1978
	48	1	Nigeria	Benin State	1	Cow	1977
	169	1	Ivory Coast	Bouaflé	1	Pig[d]	1978
	278	1	Ivory Coast	Bouaflé	1	Pig	1982
	274	1	Ivory Coast	Bouaflé	1	*G. p. palpalis*	1982

16	39	22	Ivory Coast	Bouaflé	7	Pig	1978–82
				Bouaflé	1	Dog	1978
				Bouaflé	3	*G. p. palpalis*	1982
				Bouaflé	1	Dog	1978
			Liberia	Wenten	8	Pig	1975–77
			Ethiopia	?	1	Man	1973
			Burkina Faso	?	1	Kob	1980
	142	6	Ivory Coast	Bouaflé	1	Man	1978
				Bouaflé	3	Pig[d]	1978–82
			Burkina Faso	?	1	Cow	1980
				?	1	Hartebeest	1980
	160	3	Ivory Coast	Bouaflé	1	Man	1979
			Burkina Faso	?	1	Kob	1980
				?	1	Waterbuck	1980
	279	1	Ivory Coast	Bouaflé	1	Pig	1982
	155	1	Burkina Faso	?	1	Kob	1980
	56	2	Nigeria	Benue State	1	Man	1968
			Ivory Coast	Bouaflé	1	Pig	1978
	282	1	Ivory Coast	Bouaflé	1	Pig	1982
	147	1	Ivory Coast	Bouaflé	1	Man	1978
	148	1	Ivory Coast	Bouaflé	1	Man	1978
	162	1	Burkina Faso	?	1	Hartebeest	1980
	302	1	Liberia	?	1	Pig	1978
	43	1	Nigeria	Nsukka	1	Pig	1962
	303	1	Liberia	?	1	Pig	1978
	143	3	Ivory Coast	Bouaflé	1	Man	1978
				Bouaflé	1	Pig	1978
			Burkina Faso	?	1	Kob	1980
	151	3	Ivory Coast	Bouaflé	3	Pig	1978
	161	4	Burkina Faso	?	2	Kob	1980
				?	1	Bushbuck	1980
				?	1	Cow	1980

TABLE A3 (*continued*)

Set	*Zymodeme*	*Total no. of populations*[a]	*Country*	*Location*	*No. of populations/ host species*[b]	*Host*	*Years*[c]
	156	1	Ivory Coast	Bouaflé	1	Pig	1978
	157	1	Ivory Coast	Bouaflé	1	Pig	1978
	290	1	Ivory Coast	Bouaflé	1	Sheep	1982
	285	1	Ivory Coast	Bouaflé	1	Pig	1983
	175	1	Ivory Coast	Bouaflé	1	Cow	1978
17	44	1	Nigeria	Ibadan	1	Horse	1968
	174	2	Ivory Coast	Bouaflé	2	Dog	1978
	176	2	Ivory Coast	Bouaflé	1	Pig	1978
			Burkina Faso	?	1	Kob	1980
	45	1	Nigeria	Ibadan	1	Cow	1974
	177	1	Ivory Coast	Bouaflé	1	Pig	1978
	178	2	Burkina Faso	?	2	Kob	1980
	166	14	Ivory Coast	Bouaflé	13	Pig	1978–82
				Bouaflé	1	Dog	1978
	170	4	Ivory Coast	Bouaflé	3	Pig	1978–82
				Bouaflé	1	Chicken	1978
	271	1	Ivory Coast	Vavoua	1	Pig	1979
	47	1	Nigeria	Benin State	1	Cow	1977
	173	4	Ivory Coast	Bouaflé	4	Pig	1978–82
	286	5	Ivory Coast	Bouaflé	5	Pig	1982
	149	2	Ivory Coast	Bouaflé	2	Man	1978
	284	1	Ivory Coast	Bouaflé	1	Pig	1983
	180	1	Burkina Faso	?	1	Kob	1980
	277	1	Ivory Coast	Bouaflé	1	Pig	1982
	276	1	Ivory Coast	Bouaflé	1	Pig	1982

	59	1	?	?	1	Horse	1920
	280	4	Ivory Coast	Bouaflé	4	Pig	1982
	171	2	Ivory Coast	Bouaflé	2	Pig	1978–82
	283	4	Ivory Coast	Bouaflé	4	Pig	1982
	167	1	Ivory Coast	Bouaflé	1	Pig	1978
	179	1	Burkina Faso	?	1	Kob	1980
	288	1	Ivory Coast	Bouaflé	1	Pig	1982
	291	1	Ivory Coast	Bouaflé	1	Dog	1982
18	41	30	Liberia	Flompa, etc.	26	Pig	1975–77
				Flompa	3	Dog	1975
			Kenya	Marsabit	1	Camel	1980
	42	9	The Gambia	Kantong Kunda, etc.	5	Cow	1977
			Kenya	Galana	4	Camel	1979–80
	58	21	Kenya	Galana, etc.	10	Camel	1979–80
				Galana	1	Cow	1981
			Nigeria	Mongonu	2	Camel	1974
			Sudan	Kassala	1	Camel	1977
			Colombia	Carimagua	3	Capybara	1973
				Carimagua	2	Horse	1973
				Carimagua	1	Dog	1973
			Kuwait	?	1	Dog	1980
	64	36	Kenya	Marsabit, Isiolo	34	Camel	1979–81
				Mombasa	2	Cow	1978
19	128	1	Kenya	Lambwe Valley	1	Cow	1980
	23	24	Kenya	Lambwe Valley	22	Man	1970–82
				Lambwe Valley	2	Cow	1970
	104	3	Kenya	Lambwe Valley	3	Cow	1980
20	54	1	Nigeria	Bida	1	Man	1968

TABLE A3 (*continued*)

Set	*Zymodeme*	*Total no. of populations*[a]	*Country*	*Location*	*No. of populations/ host species*[b]	*Host*	*Years*[c]
	53	2	Nigeria	Bida	1	Man	1968
			Zaire	Kinshasa	1	Man	1971
	134	1	Sudan	Yambio	1	Man	1982
	132	2	Kenya	Busia	1	Man	1958
			Zaire	?	1	Man	
	275	1	Ivory Coast	Bouaflé	1	Pig	1982
	272	1	Burkina Faso	?	1	Man	1977
	270	1	Ivory Coast	Bouaflé	1	Man	1979
	141	6	Ivory Coast	Bouaflé	4	Man	1978
				Bouaflé	1	Pig	1978
			Congo	Niari	1	Man	1975
	131	14	Sudan	Yambio	12	Man	1982
			Zaire	?	2	Man	
	269	1	Ivory Coast	Vavoua	1	Man	
	268	5	Ivory Coast	Vavoua	3	Man	1979–82
			Burkina Faso	?	2	Man	1980
	144	3	Ivory Coast	Bouaflé	3	Man	1978
	52	1	Nigeria	Tsuaa	1	Man	1968
	293	1	Liberia	Layie	1	Man	1981
	292	3	Congo	Niari, Makoua	2	Man	1973–74
				?	1	Sheep	
	63	3	Kenya	Ngurunit.	3	Camel	1979–80

Minor groups							
A	260	1	Zambia	Luangwa Valley	1	*G. pallidipes*[d]	1982
B	238	1	Zambia	Luangwa Valley	1	*G. pallidipes*[d]	1983
C	19	1	Tanzania	Serengeti	1	Hyena	1971
D	202	1	Zambia	Luangwa Valley	1	Man	1981
	258	1	Zambia	Luangwa Valley	1	Man	1983
E	264	2	Zambia	Luangwa Valley	2	Man	1984

[a] Total number of populations seen. [b] Number of populations found in each host species. [c] Period when primary isolates made. [d] Several populations in original isolate. [e] Patient normally lived in Zambia but had recently visited southern Sudan; primary isolate made in Kenyan hospital. [f] Primary isolate made in UK hospital after patient's visit to Zambia. [g] Original isolation made from trituration of several tsetse species.

TABLE A4 *Regional distribution of the most common variants of four enzymes*

	Total no. zymodemes[a]	*ICD*[b]			*PGM*[b]			*ASAT*[b]				*ALAT*[b]			
		1	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*4*	*7*	*1*	*2*	*3*	*10*
Zambia (Luangwa Valley)	98	72	17	8	90	1	8	89	0	11	0	1	70	0	30
Kenya (Lambwe Valley)	39	0	56	36	62	0	28	33	0	5	54	44	41	0	0
Uganda (Busoga)	12	25	25	50	42	0	42	75	0	8	17	33	67	0	0
West Africa[c]	82	0	95	5	4	80	12	77	23	0	0	16	43	20	10

[a] Total number of zymodemes from the region. [b] Percentage of zymodemes in the region with each pattern: ICD, isocitrate dehydrogenase; PGM, phosphoglucomutase; ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase. [c] Excludes Z55 (set 9); see text.

TABLE A5 *Profiles of mixed populations*

Host	*Country*[a]	*Set*	*Zymodeme No.*	*GPI*	*NH*	*TDH*	*ICD*	*MDH*	*PGM*	*ASAT*	*ALAT*	*PEP2*	*PEP1*	*ME*
Triple-population mixtures														
G. pallidipes	ZM	4	245	1	1	1	3	4	1	1	2	1	1	1
		9	246	1	1	1	1	4	1	1	2	1	1	12
		A	260	1	1	1	3	2	1	1	2	1	1	4
Man[b]	ZM	7	263	1	1	1	2	2	1	4	2	1	7	8
		10	2	1	1	1	1	1	1	1	2	1	1	4
		10	3	1	1	1	1	1	1	1	2	1	2	3
Double population mixtures														
Lion	ZM	9	189	1	1	1	1	1	1	1	2	1	2	15
		14	10	1	1	2	2	2	1	1	2	3	5	1
G. pallidipes	ZM	15	237	1	1	1	2	1	3	1	10	1	1	1
		B	238	1	1	1	3	2	1	1	2	3	16	1
Goat	ZM	3	240	1	1	1	3	5	1	1	10	3	14	4
		13	241	1	1	1	1	1	1	1	2	4	4	4
Man	ZM	6	216	1	1	1	1	1	3	1	10	1	2	1
		10	250	1	1	1	1	1	1	1	2	1	12	4
Man	ZM	3	220	1	1	1	2	1	1	1	2	14	2	4
		6	188	1	1	1	1	1	1	1	10	1	2	4
Man	ZM	6	203	1	1	1	1	1	1	1	10	4	2	20
		10	3	1	1	1	1	1	1	1	2	1	2	4
Man	ZM	6	203	1	1	1	1	1	1	1	10	4	2	20
		10	3	1	1	1	1	1	1	1	2	1	2	4
Man	ZM	6	211	1	1	1	1	1	1	1	10	12	2	20
		10	3	1	1	1	1	1	1	1	2	1	2	4
G. pallidipes	ZM	7	233	1	1	1	5	1	1	4	2	1	7	8
		7	234	1	1	1	5	1	1	4	10	3	16	8
Pig[b]	CI	1	25	1	1	1	2	1	1	1	2	1	2	1

TABLE A5 (*continued*)

Host	*Country*[a]	*Set*	*Zymodeme No.*	*GPI*	*NH*	*TDH*	*ICD*	*MDH*	*PGM*	*ASAT*	*ALAT*	*PEP2*	*PEP1*	*ME*
		15	169	1	1	1	2	1	1	1	3	1	2	11
Man	ZM	6	218	1	1	1	1	1	3	1	10	10	1	1
		9	66	1	1	1	1	1	3	1	2	1	1	1
Man	ZM	6	182	1	1	1	1	1	1	1	10	1	1	4
		10	198	1	1	1	1	1	1	1	2	4	1	4
Man	ZM	9	1	1	1	1	1	1	1	1	2	1	1	3
		10	198	1	1	1	1	1	1	1	2	4	1	4
Man	ZM	9	200	1	1	1	1	1	1	1	2	4	1	6
		10	2	1	1	1	1	1	1	1	2	1	1	4
Bushbuck	ZM	9	244	1	1	1	1	1	1	1	2	4	2	19
		10	3	1	1	1	1	1	1	1	2	1	2	4
Man	ZM	10	198	1	1	1	1	1	1	1	2	4	1	4
		10	214	1	1	1	1	1	1	1	2	14	2	4
Pig	CI	1	38	1	1	1	2	1	3	1	2	1	2	1
		16	142	1	1	1	2	1	2	1	2	1	2	1
G. m. morsitans	ZM	3	235	1	1	1	3	5	1	1	2	3	10	4
		3	236	1	1	1	3	5	1	1	10	3	10	4
Man	ZM	6	216	1	1	1	1	1	3	1	10	1	2	1
		9	215	1	1	1	1	1	3	1	2	1	2	1
G. pallidipes	ZM	6	252	1	1	1	1	1	1	1	10	1	1	1
		9	5	1	1	1	1	1	1	1	2	1	1	1
Man	ZM	6	188	1	1	1	1	1	1	1	10	1	2	4
		10	3	1	1	1	1	1	1	1	2	1	2	4
Warthog	ZM	8	7	1	1	1	1	1	1	1	2	1	2	2
		9	189	1	1	1	1	1	1	1	2	1	2	15
Waterbuck	ZM	8	7	1	1	1	1	1	1	1	2	1	2	2
		9	189	1	1	1	1	1	1	1	2	1	2	15

G. m. morsitans	ZM	9	4	1	1	1	1	1	1	1	2	1	2	1
		9	227	1	1	1	1	4	1	1	2	1	2	1
Giraffe	ZM	10	2	1	1	1	1	1	1	1	2	1	1	4
		10	3	1	1	1	1	1	1	1	2	1	2	4
G. pallidipes	ZM	14	248	1	1	1	2	1	1	1	2	2	15	1
		14	261	1	1	1	2	2	1	1	2	2	15	1

[a] ZM, Zambia; CI, Ivory Coast. [b] Different populations found after cloning.

TABLE A6 *Enzyme combinations for identification*

Organism	*Set*	*Limits*[a]	*NH*	*TDH*	*ICD*	*MDH*	*PGM*	*ASAT*	*ALAT*	*ME*	*Areas*[b]
T. b. rhodesiense											
busoga	1, 2, 3, 4, 5, 14, 19	Not 40, 49, 50; Only 220; Not 73, 228, 245; Only 248	1		Not 1	1	Not 2	1, 4, 7	1, 2, 12	Not 8	KE/L, UG
zambezi	6, 8, 9, 10, 13, D, E		1	1	1	1, 4		1	2, 10		ZM, UG
T. b. brucei											
bouaflé	1, 15, 16, 17	Only 38, 46, 49, 50, 71, 153, 163, 281; Not 169; Not 59			2		2, 3, (5)[d]	Not 4	Not 1	Not 2, 7, (14, 24)[d]	WA
sindo	7							4		8	ZM, KE
kiboko	11, C	Not 223	1[c]	4	1						KE
			2[c]		1, 3						
kakumbi	14, A, B	Not 248			2, 3	2		1			ZM
T. evansi	18				2[c]		2	1	2	2	All
	20	Only 63					3[c]	3			KE
T. b. gambiense	20	Not 63			2[c]		2		1, 11	2	WA, EA
					2[c]		2	2	2	2	

[a] Zymodemes included or excluded by identifying profile. [b] Areas in which most populations were isolated: KE/L, Lambwe Valley, Kenya; UG, Uganda; ZM, Zambia; WA, West Africa; KE, Kenya; EA, East Africa. [c] Alternative enzyme combinations. [d] Rare variants.

Genetic Diversity in *Plasmodium falciparum*

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I. Introduction 76
II. Antigenic Diversity in *Plasmodium* 78
A. Nomenclature 78
B. Secreted antigens 78
C. The circumsporozoite protein 83
D. Merozoite surface antigens 85
E. Erythrocyte-associated antigens 89
F. Rhoptry proteins 97
G. Other repetitive antigens 97
H. Cytoplasmic antigens 98
I. Antigens of sexual stages 98
J. Antigens of liver stages 98
K. Conservation of housekeeping genes 99
L. Generation of diversity in repetitive regions and antigenic diversity 99
M. Repetitive antigens and immune evasion 100
III. Chromosome Size Polymorphisms 101
A. The molecular karyotype of *P. falciparum* 101
B. Generation of chromosome size polymorphisms in *P. falciparum* by homologous recombination within sub-telomeric repeat sequences 103
C. Deletions involving coding sequences 106
IV. Meiosis and Genetic Recombination 108
A. Recombination and the parasite life cycle 108
B. Genetic crosses between malaria parasites 108

ADVANCES IN PARASITOLOGY VOL 29
ISBN 0-12-031729-X

C. Implications of crossing studies 116
V. The Genetic Basis of Drug Resistance 116
A. The genetic basis of pyrimethamine resistance 117
B. Chloroquine resistance 122
VI. Outlook 126
Acknowledgements 128
References 128

I. Introduction

Over the past five years, the application of recombinant deoxyribonucleic acid (DNA) technology to the study of *Plasmodium falciparum* and, to a lesser extent, other species of *Plasmodium* has resulted in rapid advances in our understanding of genetic diversity in malaria. Many genes have been cloned and a substantial amount of sequence information has been accumulated. Because the driving force behind most studies is the eventual development of a vaccine, most of the genes studied encode polypeptides that are antigens during natural infection. Diversity in these genes presumably will be of major importance in the design of effective vaccines. A major unexpected finding is that most of these antigens contain tandem repeats of oligopeptide sequences and a large proportion of the host immune response is directed against these repeats. Hence variation in repeat sequences from one isolate to another forms a major component of our current knowledge of genetic diversity in *Plasmodium*.

However, this is by no means the only form of genetic diversity. The introduction of pulsed field gradient (PFG) electrophoresis has allowed us to study *Plasmodium* chromosomes, and it has become clear that there is considerable variation in chromosome size from one parasite clone to another. Intriguingly, a major component of this variation appears to be recombinational expansion and contraction of repetitive sub-telomeric sequences. As many of the repetitive antigens are also encoded by sub-telomeric genes, there is a possible connection between antigenic diversity and chromosome size variation. Furthermore, the results of the first genetic cross between two defined clones of *P. falciparum* together with determination of the chromosomal location of many of the genes clearly demonstrate that, during transmission of mixed infections, novel genotypes are generated. Not only do the chromosomes segregate and recombine as expected, it now seems likely that intragenic recombination during meiosis can generate novel antigenic forms of at least one major antigen. Finally, the underlying molecular basis for another important form of genetic diversity, namely resistance to an important antimalarial drug, pyrimethamine, has now been determined. A candidate gene that may underlie resistance to the

other major antimalarial drug, chloroquine, has now been cloned.

Before studies at the DNA sequence level, studies using a wide variety of approaches had clearly identified a number of different types of genetic diversity in *Plasmodium*. Because of the difficulties of genetic studies on *P. falciparum*, rodent malaria models were particularly important (Beale *et al.*, 1978; Walliker, 1983). The most useful markers for these studies were electrophoretic mobility variants of a number of enzymes, particularly in *P. chabaudi*. A major conclusion from this work is that the blood forms of *Plasmodium* are haploid (reviewed by Walliker, 1983). Another important class of markers were drug-resistant forms; these are discussed below. Two-dimensional gel electrophoresis of polypeptides provided another approach that revealed substantial differences between isolates (Tait, 1981; Fenton *et al.*, 1985). When coupled with immunoprecipitation using serum from individuals with a history of exposure to malaria, this procedure revealed considerable antigenic differences between isolates (Brown *et al.*, 1982). Most notably, the extensive diversity of the S-antigen system of *P. falciparum* was recognized serologically by Wilson and his colleagues (Wilson *et al.*, 1969, 1975; Wilson, 1980) and the S-antigen of several isolates was defined by immunoblotting (Anders *et al.*, 1983). The development of procedures for cloning many antigens (Kemp *et al.*, 1983; Anders *et al.*, 1984; Ardeshir *et al.*, 1985) has expedited these studies.

In the present review, we will concentrate on those forms of genetic diversity that are clearly understood at the DNA level. It should be recognized, however, that some forms of genetic variation remain to be analysed at this level. Perhaps the most obvious and important example is the schizont-infected cell agglutinin (SICA) antigen system of *P. knowlesi*, a system that appears to undergo antigenic variation in a manner reminiscent of the variable surface glycoprotein system of trypanosomes. Equivalent systems seem to exist in *P. chabaudi* and, as manifested by the "cytoadherence molecule", perhaps in *P. falciparum* and *P. fragile*. Where relevant to our understanding of diversity, we will consider data from other malaria species—this is particularly relevant for the circumsporozoite (CS) protein system. However, there is no attempt to review exhaustively the data for species other than *P. falciparum*. A number of recent reviews have covered similar areas (Nussenzweig and Nussenzweig, 1985; Kemp *et al.*, 1986, 1987a; Anders, 1986a; Anders *et al.*, 1986, 1987a,b; Campbell *et al.*, 1987; Howard, 1987; Good *et al.*, 1988a; Perlmann and Wigzell, 1988).

In this paper we use the definitions adopted previously (Walliker, 1983) to describe parasites derived from natural infections.

Isolate: a sample of parasites, not necessarily genetically homogeneous, collected from a naturally infected host on a single occasion.

Line: parasites which have undergone a particular series of passages in the laboratory.
Clone: genetically identical organisms derived from a single cell by asexual division.

II. Antigenic Diversity in *Plasmodium*

A. NOMENCLATURE

The earliest malaria antigens identified were named according to their structure or function, and referred to by the corresponding set of initials, e.g. the schizont-infected cell agglutination (SICA) antigen (Brown and Brown, 1965), and the histidine-rich protein (HRP) (Kilejian, 1974). More recently, antigens have been designated by their apparent molecular weights. However, the apparent molecular weights are usually grossly in error (by 10–30%) because of anomalous migration on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Furthermore, many of the polypeptides vary in size from isolate to isolate and many exist as a series of processed fragments, often overlapping in size with other antigens. For these reasons we believe it to be less confusing to use a system of intial letters.

B. SECRETED ANTIGENS

1. *The S-antigen*

The S-antigen of *P. falciparum* is secreted into the parasitophorous vacuole and released upon schizont rupture. It exhibits a remarkable degree of size and serological diversity (Wilson *et al.*, 1969, 1975; Anders *et al.*, 1983; Howard *et al.*, 1986a). The sequences of clones encoding five antigenically different S-antigens (Fig. 1) from eight different isolates of *P. falciparum* have been determined and they clearly explain this diversity (Coppel *et al.*, 1983; Cowman *et al.*, 1985; Saint *et al.*, 1987; Brown *et al.*, 1987; Mattei *et al.*, 1987; Nicholls *et al.*, 1988; Corrick *et al.*, unpublished observations). The S-antigen gene consists of a single exon. There is a central block of tandem repeats (Fig. 1) flanked by non-repeat sequences containing regions rich in charged amino acids that show considerable homology. The S-antigen of *P. falciparum* isolate FC27 contains about 100 repeats of the 11-mer PAK-ASQGGLED*, whereas that of NF7 contains 40 repeats of an octomer with

* Amino acids are denoted throughout this chapter by the conventional single letter code: A, alanine; B, asparagine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine; Z, glutamic acid. [Editors' note.]

two variants, ARKSDEAE and ALKSDEAE. In addition, there are two repeats of a 15-mer SDAGTEGPKGTGGPG near the C-terminus. The FC27 and NF7 repeats are so different that they do not cross hybridize

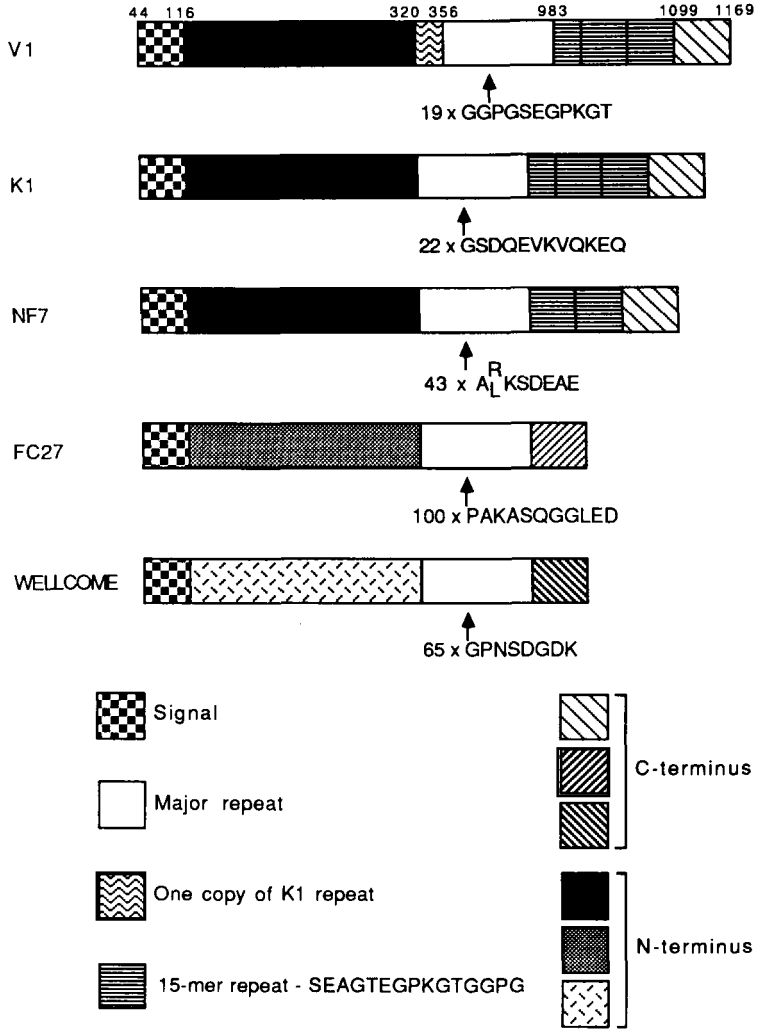

FIG. 1. Structures of S-antigen genes from five isolates of *P. falciparum*. The different shadings represent the signal sequence, N- and C-terminal non-repetitive sequences and different repeats as indicated at the bottom. The number and sequences of repeats are shown beneath each molecule. From Brown *et al.* (1987). [Single-letter codes for amino acids are explained in the footnote on p. 78.]

(Coppel *et al.*, 1983; Cowman *et al.*, 1985) and natural antibody responses directed against epitopes encoded by the repeats do not cross react immunologically (Anders *et al.*, 1985, 1986).

The major repeat from isolate V1 was an 11-mer different again from FC27 and NF7 (Brown *et al.*, 1987). However, the S-antigens of isolates V1 and K1 contained copies of the 15 amino acid sequence located near the C-terminus as in NF7 and the N- and C-terminal portions were almost identical to those of NF7. The 15-mer itself contains two internally homologous regions, and recombination within these would explain the origin of the major V1 11-mer. The major repeats of K1 were also related in sequence to the 15-mer, but differed in reading frame (Saint *et al.*, 1987). Thus, the serological diversity of S-antigens reflects different repeats that may vary in sequence, size, number or reading frame. Recently, the complete sequence of the S-antigen of the Wellcome isolate (Nicholls *et al.*, 1988) and sequences of partial complementary (c) DNA clones from the Palo Alto (Mattei *et al.*, 1987) and IT (Corrick *et al.*, unpublished observations) isolates have been determined. All encode the same conserved eight amino acid sequence GPNSDGDK, encoded by a partially degenerate 24 base-pair (bp) sequence.

It is now clear that the non-repetitive 5′ and 3′ regions of the S-antigen gene exist in several distinct forms (Fig. 1). The first two sequences determined, namely FC27 and NF7 (Cowman *et al.*, 1985), were 70% homologous in these regions, whilst that of the Wellcome isolate was about equally divergent from both of these (Nicholls *et al.*, 1988). Surprisingly, K1 (Coppel *et al.*, 1985b) and V1 (Brown *et al.*, 1987) are identical at the amino acid level and they vary at only two amino acids from NF7, rather than being intermediate between the original sequences as might have been expected. Hence, there are at least three distinct forms of the flanking sequences, and it remains to be seen how many further types will be found. When compared with the virtual lack of variation in different alleles of some *P. falciparum* housekeeping genes (see below), this variation in the non-repeat regions is quite remarkable.

The S-antigens behave as an allelic series of variant genes at a single locus (Cowman *et al.*, 1985; Brown *et al.*, 1987; Saint *et al.*, 1987) and, because of the extensive variation, they provide useful restriction fragment length polymorphism (RFLP) markers. There is no evidence for unique or repeat S-antigen sequences other than those at the single S-antigen gene of the haploid *P. falciparum* genome. Thus the generation of S-antigen diversity does not involve any sequence rearrangements like those of the trypanosome variant surface glycoprotein system.

The function of the S-antigen remains unknown. The extreme serological diversity may result from strong immunological selection and so the S-

antigen may be involved somehow in immune evasion. However, as frame changes can be tolerated it is unlikely that the exact sequences matter at all: it seems that repetitiveness *per se* is the only important feature. However, there is an unexplained paradox: the sizes of S-antigen polypeptides of the FC27 serotype are identical in each of the many natural isolates examined (Bazarga *et al.*, unpublished observations; Forsyth *et al.*, 1988) with the single exception of clone E12, derived *in vitro* from FC27. How the size could be conserved in this particular serotype but the sequence be irrelevant for S-antigens in general is a mystery. The generation of repeat diversity is considered below.

Although the S-antigen does not appear to be attached to merozoites, it is located in the parasitophorous vacuole and during schizont rupture escaping merozoites are bathed in S-antigen (Coppel *et al.*, 1983). The S-antigen may become transiently associated with the merozoite as a peripheral membrane protein. Although an anti-S-antigen monoclonal antibody inhibited merozoite invasion *in vitro* (Saul *et al.*, 1985), other evidence that host-protective strain-specific immune reponses are directed against S-antigens is lacking. The extreme diversity of the repeats in these antigens suggests that they would have little value as vaccine components; however, this is not necessarily true for the flanking sequences.

2. *The glycophorin-binding protein*

It is likely that the binding of merozoites to the red cell involves glycophorin (Miller *et al.*, 1977; Perkins, 1981; Pasvol *et al.*, 1982; Perkins and Ravetch, 1986). Perkins (1984) identified a putative "glycophorin-binding" protein (GBP) of *P. falciparum* on the basis that it bound to an aminoethyl acrylamide column to which glycophorin was attached, but this has proved controversial (van Schravendijk *et al.*, 1987). Evidence for a merozoite surface location has been obtained by electron microscopy (Pirson and Perkins, 1985; Perkins and Ravetch, 1986), but studies by two other groups have localized the antigen predominantly within the red cell cytoplasm (Bianco *et al.*, 1987; Bonnefoy *et al.*, 1988). Traces of many secreted antigens described here have indeed been found in association with the merozoite surface, but the significance of this is uncertain.

The putative GBP is a soluble polypeptide of relative molecular mass (M_r) 105 000–130 000, secreted from schizonts and released into culture supernatant (Perkins, 1984; Ravetch *et al.*, 1985; Bianco *et al.*, 1987). All isolates tested were immunologically indistinguishable, although there were slight differences in the sizes of the polypeptide. The GBP from isolate FCR3 contained a 50 amino acid sequence that was repeated 11 times with a low degree of variation (Kochan *et al.*, 1986). In the GBP from isolate FC27 the

single repeat studied lay within the consensus found for isolate FCR3 (Bianco *et al.*, 1987). The GBP gene (Kochan *et al.*, 1986) had a single exon, containing a 13 amino acid hydrophobic core of a signal sequence commencing at amino acid 51. Following this was a highly charged region of 155 amino acids, of which 35% were basic, followed by the 50 amino acid repeats, continuing to the C-terminus.

3. *The serine-rich antigen*

Experiments with monoclonal antibodies (Delplace *et al.*, 1985, 1987) and with human antibodies (Stahl *et al.*, 1986a; Coppel *et al.*, 1988a) identified a polypeptide first termed p126 as an antigen of mature stage parasites. It is present as a precursor of M_r 126 000 and processed at about the time of schizont rupture to products of M_r 73 000 and M_r 45 000. The M_r 73 000 product is apparently composed of disulphide bonded smaller components of M_r 47 000 and M_r 18 000 (Delplace *et al.*, 1987). The molecule is present in the parasitophorous vacuole (Delplace *et al.*, 1987; Chulay *et al.*, 1987; Coppel *et al.*, 1988a). It has also been detected in association with the merozoite surface using a monoclonal antibody (Chulay *et al.*, 1987), but this was not evident using human antibodies (Coppel *et al.*, 1988a). The polypeptide varied slightly in size in different isolates (Bhatia *et al.*, 1987).

The complete sequence deduced from overlapping cDNA clones from isolate FCR3 predicts a polypeptide of 989 amino acid residues with a predicted molecular mass of 111 kDA, including a signal peptide but not a membrane anchor domain (Bzik *et al.*, 1988). The protein is highly acidic and rich in serine. A hexanucleotide motif AGXTCY, where X is either C or T and Y is either A or T, encodes a polyserine stretch, 35 residues long, in FCR3. This is the only repetitive region in the molecule. Hence the name serine-rich antigen (SERA) was suggested by Bzik *et al.* (1988). The sequence of SERA from NF7 (Coppel *et al.*, 1988a) was identical to that of FCR3 over the region that could be compared. However, the short region (48 amino acid residues) of the Camp isolate that can be compared (Weber *et al.*, 1987) reveals short alternating blocks of conserved and variant regions. Hence diversity in this molecule may be of a similar pattern to that documented much more extensively for the PMMSA (see below).

4. *The acidic–basic repeat antigen*

Another secreted antigen has been designated the acidic–basic repeat antigen (ABRA) because it has a C-terminal region containing primarily KE and KEE repeats (Stahl *et al.*, 1986b; Weber *et al.*, 1988). The sequence of a

genomic DNA clone from the Camp isolate encoded a protein of 743 amino acids as a single exon with a leader sequence located at the N-terminus (Weber *et al.*, 1988). As well as the polypurine region encoding the KE and KEE 3′ repeats, an additional 5′ set of repeats was present, composed of eight hexapeptide repeats of consensus TNDEED, where positions 1, 4 and 5 were replaced by V, D and D, respectively. The protein encoded has a theoretical M_r of 86 595 (83 931 for the mature protein), considerably less than the apparent M_r of 101 000–102 000 estimated by SDS-PAGE. As with the other schizont antigens secreted into the parasitophorous vacuole, traces of ABRA can be detected in association with the merozoite surface (Chulay *et al.*, 1987).

ABRA is notable for its lack of diversity—there is little difference in size of the polypeptide detected in seven different isolates (Stahl *et al.*, 1986a,b; Weber *et al.*, 1988). The sequence of a 1.8 kilobase (kb) cDNA from isolate FCR3 revealed only three differences from the Camp sequence, each resulting in an amino acid substitution. The partial FC27 sequence (Stahl *et al.*, 1986b) was identical except for some minor rearrangements within the 3′ repeats. Extreme caution is necessary in deciding whether these minor rearrangements are real or artefacts of recombination in *Escherichia coli.*

C. THE CIRCUMSPOROZOITE PROTEIN

The circumsporozoite (CS) protein located on the surface of mature sporozoites is the only sporozoite-specific molecule identified so far. It varies dramatically in M_r and differs in immunological reactivity in different species (Potocnjak *et al.*, 1980; Yoshida *et al.*, 1980; Nardin *et al.*, 1982). CS proteins from all species studied possess a typical signal sequence at the N-terminus and a hydrophobic sequence at the C-terminus (Ellis *et al.*, 1983; Godson *et al.*, 1983, 1984; Ozaki *et al.*, 1983; Dame *et al.*, 1984; Arnot *et al.*, 1985; Eichinger *et al.*, 1986; Sharma *et al.*, 1985; Enea *et al.*, 1984a,b). The CS precursor is cleaved before insertion in the membrane (Nardin *et al.*, 1982; Yoshida *et al.*, 1981).

All CS proteins possess a central region of tandem repeats (Fig. 2) that are relatively conserved within any one molecule, a feature strikingly similar to the S-antigen system. The repeats differ considerably in length, sequence and number of repeats among species in the same manner as the S-antigens differ from isolate to isolate. The *P. falciparum* CS protein (clone 7G8) contains 37 copies of NANP and four copies of a variant repeat NVDP (Dame *et al.*, 1984). As with S-antigens, conservation of the repeats is less pronounced at the nucleotide level as the 37 copies of the 12-mer encoding NANP are coded by 11 different nucleotide sequences. In *P. cynomolgi* and *P. yoelii* the repetitive region of the CS protein is composed of two different blocks

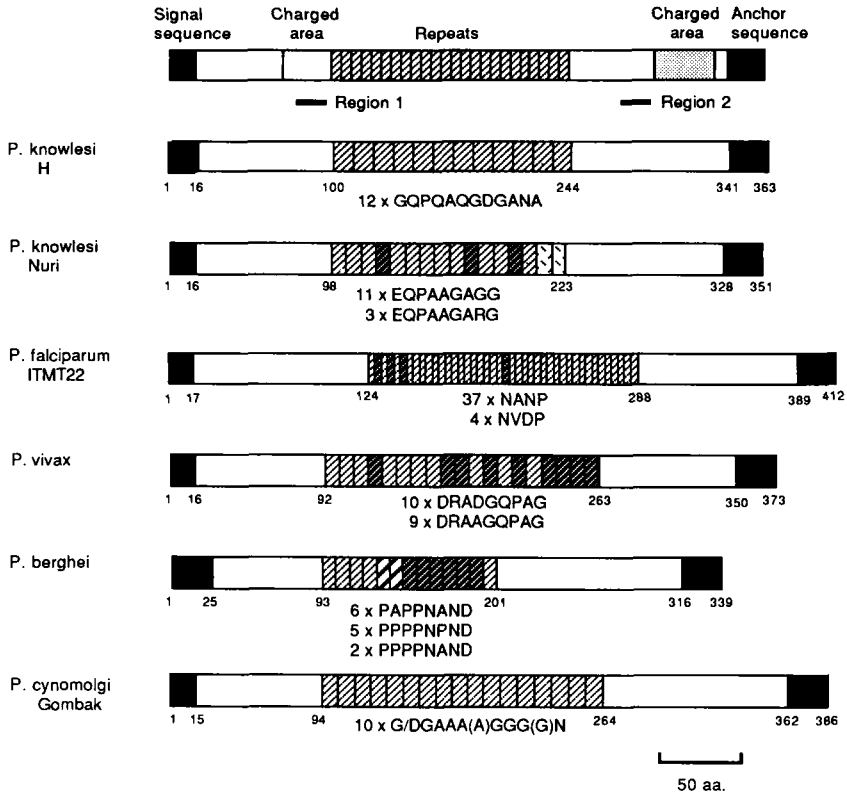

FIG. 2. Structures of CS proteins from different species of *Plasmodium*. At the top the general structure is shown, with the different shadings representing the signal sequence, charged areas, repeats and anchor sequence. Regions 1 and 2 are indicated. The next five molecules are from the species indicated at the left. The number and sequence of repeats are shown below each molecule. The small numbers indicate amino acid positions; aa, amino acids. From Kemp *et al.* (1987a). [Single-letter codes for amino acids are explained in the footnote on p. 78.]

(Galinski *et al.*, 1986; Eichinger *et al.*, 1986). Monoclonal antibody studies of the CS protein detected a single repetitive immunodominant epitope (Zavala *et al.*, 1983, 1985). Variation in the repeats of the CS protein within a species occurs, but the extent differs. In *P. knowlesi*, the CS protein of the Nuri isolate contains 11 repeats of EQPAAGAGG and three repeats of the closely related sequence EQPAAGARG (Sharma *et al.*, 1985), a sequence that differs from that of the H isolate (GQPQAQGDGANA). Five of the six *P. cynomolgi* isolates have CS proteins with different repetitive regions (Galinski *et al.*, 1986). As yet no *P. falciparum* isolate has been detected that

lacks the NANP repeat sequence, but the number of repeats varies (Weber and Hockmeyer, 1985) and there is variation outside the repeats (Lockyer and Schwartz, 1987; de la Cruz and McCutchan, 1986; del Portillo *et al.*, 1987). In some *P. falciparum* isolates the 5′ flanking region contains two to four copies of a five amino acid sequence rich in asparagine (de la Cruz and McCutchan, 1986; Lockyer and Schwartz, 1987). At the 5′ boundary of the repeats there is a region with 27 charged amino acids among 48 in *P. knowlesi* and 27 among 53 in *P. falciparum* (Fig. 2). A second less extensive charged area occurs near the anchor sequence.

The sequences of *P. falciparum* and *P. knowlesi* CS proteins show little homology other than two short sequences, region 1 where nine of 15 amino acids are identical and region 2 where 12 of 13 amino acids are identical (Dame *et al.*, 1984). The CS protein of *P. vivax* is closely related to those of *P. knowlesi* and *P. cynomolgi* in the regions outside the repeat (Arnot *et al.*, 1985), confirming that *P. vivax* is more closely related to the simian malarias than to *P. falciparum* (McCutchan *et al.*, 1985).

It is likely that regions of the CS protein outside the repeats are important for hepatocyte recognition (Aley *et al.*, 1986a). The CS protein may simultaneously serve as an immune defence mechanism (Godson *et al.*, 1984) as it is readily shed from the sporozoite surface and presents multiple copies of the repetitive epitope per molecule to the immune system. Recently, Good *et al.* (1988a,b,c) have defined two T-cell recognition sites in the CS protein of *P. falciparum*. These two sites overlap one another and correlate with the polymorphic regions located outside the repeats. As there is considerable evidence that cell-mediated immunity is more important than antibodies in protective immunity to sporozoites (reviewed by Good *et al.*, 1988a), this result suggests that the polymorphisms located outside the repeats may have considerably more to do with immune selection than repeat variation.

D. MEROZOITE SURFACE ANTIGENS

1. *Precursor to the major merozoite surface antigens*

A potentially important vaccine candidate is the precursor to the major merozoite surface antigens (MSA1) otherwise known as the 195K protein (Holder and Freeman, 1982, 1984), P190 (Hall *et al.*, 1983, 1984b), the 200 'Kd' [sic] protein (Perrin *et al.*, 1984), the polymorphic schizont antigen (McBride *et al.*, 1985) or gp185 (Reese *et al.*, 1985). Protective immune responses directed against the MSA1 have been demonstrated in several systems (Holder and Freeman, 1981; Epstein *et al.*, 1981; Hall *et al.*, 1984a; Perrin *et al.*, 1984, 1985; Siddiqui *et al.*, 1986; Cheung *et al.*, 1986). The MSA1 is synthesized at the schizont stage and has an M_r of 180 000–220 000

(Holder and Freeman, 1984; Perkins, 1982; Heidrich *et al.*, 1983; Howard and Reese, 1984; Braun-Breton *et al.*, 1986; Howard, R. S. *et al.*, 1986). Proteolytic cleavage of the MSA1 generates the majority of the antigens on the surface of the mature merozoites. These include polypeptides of M_r 83 000, 42 000 and 19 000 (Holder and Freeman, 1984; Heidrich *et al.*, 1983). The M_r 83 000 and 19 000 components are apparently shed during invasion but the M_r 42 000 antigen remains associated with the ring-stage parasites (Holder and Freeman, 1984) that form a complex on the merozoite surface (McBride and Heidrich, 1987).

A panel of antibodies has provided evidence that the protein has variable and constant epitopes (Hall *et al.*, 1984a; McBride *et al.*, 1982, 1985; Lyon *et al.*, 1987; Gentz *et al.*, 1988). The complete sequences of the MSA1 gene from four isolates of *P. falciparum* (Holder *et al.*, 1985; Mackay *et al.*, 1985; Tanabe *et al.*, 1987; Peterson, M. G. *et al.*, 1988a) and partial sequences from four other isolates (Cheung *et al.*, 1985; Weber *et al.*, 1986; Certa *et al.*, 1987; Peterson, M. G. *et al.*, 1988b) have now been reported. These include representatives of the two major serotypes defined by monoclonal antibodies. The single exon (Fig. 3) commences with an N-terminal signal peptide followed near the N-terminus by a relatively short region of variable tripeptide repeats. Near the C-terminus there is a hydrophobic sequence and a cluster of cysteine residues. Detailed comparison of the complete sequence and a partial sequence from the Camp isolate revealed that diversity in these allelic genes is confined to specific parts of the polypeptide chain (Fig. 3). The gene could be divided into 17 discrete blocks, ranging in homology from as low as 10% to as high as 87% at the amino acid level (Tanabe *et al.*, 1987). The two most highly conserved regions, blocks 1 and 17, encode the N- and C-terminal sequences, respectively.

The outstanding feature of the variable blocks is that each has only two versions among the first four complete sequences compared. There are almost no differences between homologous versions within these blocks but there are many differences between the two versions and so the antigen is dimorphic. If isolates MAD20 and K1 are considered to be the two dimorphic prototypes, then the Camp and Wellcome types could be readily generated by intragenic recombination (Tanabe *et al.*, 1987). As the MSA1 gene is unique in the haploid genome, this could occur only during meiosis.

The only substantial exception to the dimorphic rule is in the tripeptide repeat region. It contains varying numbers of repeats of general type S–X–X and, from the first six sequences that can be compared, up to four different amino acids were found in some positions. Hence this region is reminiscent of the S-antigen. More recently, a third form of variable block 2 (Fig. 3) has been found in two independent isolates, namely RO-33 from Ghana (Certa *et al.*, 1987) and CSL2 from Thailand (Peterson, M. G. *et al.*, 1988b). This

third sequence occurs in different recombinational forms in these two isolates of widely disparate geographical origin. It remains to be seen how many other forms exist in nature.

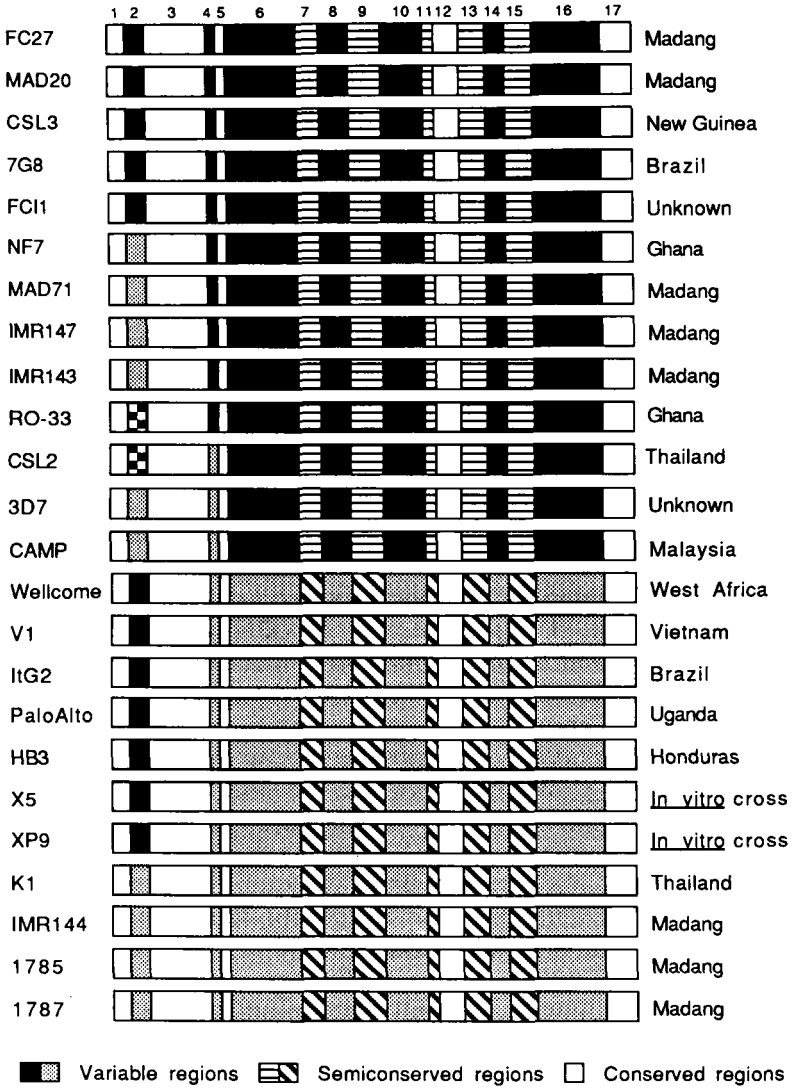

FIG. 3. Structure of MSA1 genes from 24 isolates of *P. falciparum*. The 17 blocks indicated by the numbers at the top correspond to regions that differ in homology, as indicated by the shading. Revised after Peterson, M. G. *et al.*, (1988b).

The M_r 83 000 fragment from the Wellcome strain has been localized to the N-terminal region by amino acid sequencing and commences at residue 25 (Holder *et al.*, 1985). The M_r 42 000 fragment is most probably the C-terminus (Schwarz *et al.*, 1986) and is acylated, presumably with a glycosyl phosphatidyl inositol group (Haldar *et al.*, 1985) that may function as a membrane anchor. The locations of several epitopes recognized by monoclonal antibodies have been defined within a few hundred base pairs (Holder *et al.*, 1985; Lyon *et al.*, 1986) and a processing pathway has been proposed (Lyon *et al.*, 1986). The relationships of the observed repetitive and non-repetitive variable regions to the immunologically variable regions should soon be known.

2. *The M_r 45 000 merozoite surface antigen*

The sequence of a clone encoding this polypeptide (MSA2) from isolate FC27 (Smythe *et al.*, 1988) revealed that it is a distinct integral membrane protein, rather than a fragment of the MSA1. Immunoelectron microscopy of sections has clearly shown that it is located on the merozoite surface (Miettinen-Baumann *et al.*, 1988). A number of studies with monoclonal antibodies are now known to have identified this same molecule, although the observed size varied considerably in different laboratories (Stanley *et al.*, 1985; Ramasamy, 1987; Epping *et al.*, 1988; Fenton *et al.*, 1989). This size variation probably reflects both the artefactual variation from laboratory to laboratory typically observed for malaria antigens and real variation in a polymorphic molecule. The sequence of the cDNA clone predicted a polypeptide of 264 amino acids, containing an N-terminal signal sequence of 15 hydrophobic amino acids flanked by charged residues (Smythe *et al.*, 1988). The sequence encodes two identical copies of a 32 amino acid sequence, arranged in tandem, beginning at residue 60 and continuing to residue 124. Preceding the C-terminus is a sequence of 17 hydrophobic amino acids. This is believed to be the signal for addition of a glycosyl phosphatidyl inositol anchor as it is homologous with the corresponding region of the variant surface glycoprotein of *Trypanosoma brucei* (Smythe *et al.*, 1988). The M_r 45 000 MSA2 can be labelled with myristic acid and glucosamine, consistent with this conclusion.

The gene for the MSA2 was present in all isolates examined and RFLPs have been observed (J. Smythe, personal communication). Furthermore, it is clear from studies with monoclonal antibodies that there are considerable antigenic differences between different isolates (Clark *et al.*, 1988; Fenton *et al.*, 1989). The epitope in the MSA2 from isolate FC27 recognized by a monoclonal antibody has been identified and contains the sequence STNS (Epping *et al.*, 1988). As this occurs within the repeat, it is represented twice

in the molecule. Because the merozoite surface location of this antigen and the inhibition of invasion of merozoites observed with antibodies to it (Miettinen-Baumann *et al.*, 1988) make it a prime candidate vaccine molecule, diversity in this molecule is currently of great interest.

The antigenic polymorphism of this molecule is currently under investigation using polymerase chain reaction amplification of genomic DNA. This procedure is simplified by the absence of introns in the MSA2 genomic sequence. Isolates were selected which did not react with the inhibitory monoclonal antibody mapped to the STNS epitope of the FC27 repeat. So far, two negative isolates, 3D7 and Indochina 1 (IC), have been cloned and sequenced.

The most noticeable difference between 3D7, IC and the FC27 isolate sequence is in the repetitive region of the molecule. Whereas FC27 has two copies of a 32 amino acid repeat, both 3D7 and IC have multiple copies (5 and 12, respectively) of a four amino acid repeat GGSA. The first 43 and last 74 amino acids (including the hydrophobic stretches) are comon to all three isolates. Although 3D7 and IC continue to show homology beyond these regions, their sequences are markedly different from those of the FC27 clone. Dot blot hybridization analysis of genomic DNA using probes to both repeat types has shown the existence of a third variant of this molecule which encodes neither repeat sequence; this form appears to contain an octapeptide repeat (J. Smythe, personal communication).

E. ERYTHROCYTE-ASSOCIATED ANTIGENS

It has become clear over the past few years that a number of parasite-encoded proteins become associated with the host erythrocyte, in particular with the erythrocyte membrane skeleton. While this is not surprising, it was certainly not expected that many of these have a similar two-exon structure, where exon 1 is a "mini-exon" encoding the signal sequence and exon 2 contains repetitive sequences (Fig. 4). Although little diversity has yet been detected in the limited studies on some of these genes, it is very important to note that most of them do not hybridize detectably to *P. chabaudi* DNA (M. Sheppard *et al.*, unpublished observations). It is therefore clear that they belong to a new class of rapidly evolving genes that without doubt represent the quantitatively most significant source of species-specific diversity among *Plasmodium* species. The functions of these molecules are still largely a mystery.

1. *The ring-infected erythrocyte surface antigen*

This antigen (RESA, designated Pf 155) is associated with the membrane of

erythrocytes containing ring-stage parasites (Coppel *et al.*, 1984; Perlmann *et al.*, 1984). Antibodies to RESA have been reported to inhibit merozoite invasion *in vitro* (Wahlin *et al.*, 1984; Berzins *et al.*, 1985), although this has proved controversial (Coleman and Jensen, 1987). RESA is transferred to the erythrocyte surface from within merozoites at or shortly after invasion and is assumed not to be involved in the interaction between the merozoite surface and the erythrocyte. Immunization with fused polypeptides containing RESA fragments apparently protected *Aotus* monkeys against overwhelming infection (Collins *et al.*, 1986), suggesting that it may be a candidate vaccine molecule, but technical problems have made this experiment difficult to reproduce. In merozoites and in ring-stages, RESA has an M_r of 155 000 (Coppel *et al.*, 1984; Brown *et al.*, 1985). RESA interacts with the erythrocyte membrane skeleton and is phosphorylated (Anders *et al.*, 1987b). It seems unlikely that RESA penetrates the erythrocyte membrane as the sequence lacks a typical membrane-spanning hydrophobic sequence.

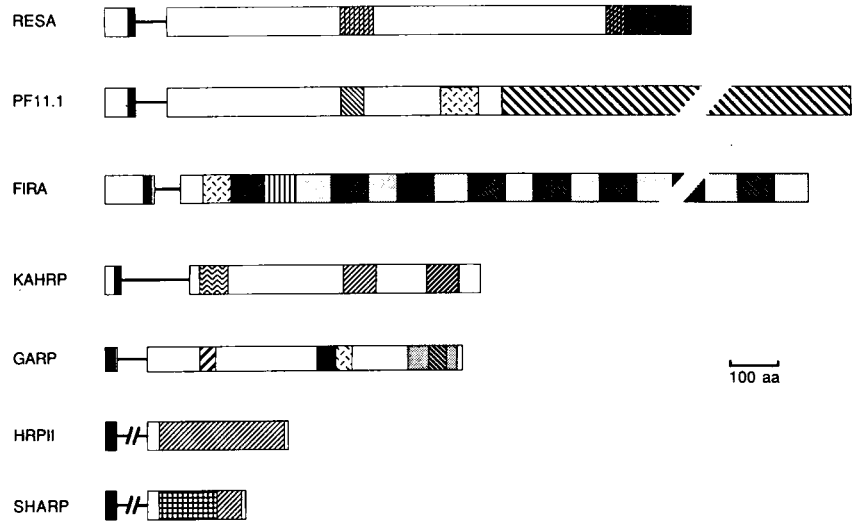

FIG. 4. Structures of genes for erythrocyte-associated antigens. The genes indicated at the left are taken from the relevant sequences referred to in the text. The boxed regions represent exons and the lines represent introns. Filled segments represent hydrophobic segments of signal sequences. Shaded areas represent repetitive regions. Minor variants are not shown and it should be realized that in some molecules, e.g. FIRA, most repeats are variants; aa, amino acids. [Antigen codes are explained in the text.]

The RESA gene is typical of these mini-exon genes (Fig. 4). It contains two exons separated by a short intervening sequence (Favaloro *et al.*, 1986).

Exon 1 commences with a hydrophilic sequence followed by a hydrophobic stretch of 13 residues commencing at amino acid 52, and terminates with an intervening sequence that follows this signal core. The major open reading frame of 1008 amino acids is encoded by exon 2, commencing 203 bases downstream. RESA contains two blocks of tandem repeats within exon 2 (Cowman *et al.*, 1984; Favaloro *et al.*, 1986). A 3′ block of repeats contains closely related acidic sequences of eight amino acids (EENVEHDA), four amino acids (EENV) and a minor component (EEYD), and three amino acids (EEV). Approximately 600 bases 5′ to this is a second block of repeats encoding degenerate sequences related to the acidic sequence DDEH-VEEPTVA. The two blocks of repeats encode cross-reacting antigenic epitopes (Cowman *et al.*, 1984; Anders *et al.*, 1986). A natural immunogenic epitope in a non-repeat region of the RESA polypeptide has recently been identified (Corcoran *et al.*, unpublished observations).

The pattern of sequence variation in the 5′ repeats is strikingly different from that of the S-antigens, because the RESA 5′ repeats are degenerate along the molecule but highly conserved between two different isolates. RFLPs defining two alleles of RESA were observed at both the 5′ and 3′ ends of RESA (Favaloro *et al.*, 1986), and all five isolates studied belonged to one or other of these two alleles. The sequences of representatives of these two alleles (isolates FC27 and NF7) were almost identical over the region that could be compared (Cowman *et al.*, 1984; Favaloro *et al.*, 1986).

2. *The mature parasite-infected erythrocyte surface antigen*

A high molecular weight mature stage antigen localized on the inner surface of erythrocytes infected with trophozoites and schizonts has been termed the mature parasite-infected erythrocyte surface antigen (MESA) (Coppel *et al.*, 1986). It has been shown that this is the same antigen as that termed Pf EMP2 (Howard, 1987; Howard *et al.*, 1987). The gene shows dramatic RFLPs, but in contrast to S-antigen genes all isolates tested so far cross-hybridize strongly (Coppel *et al.*, 1986). The polypeptide ranges in size between about M_r 250 000 and 280 000 and exhibits antigenic diversity between different isolates. It is a phosphoprotein associated with the membrane protein band 4.1 of erythrocytes infected with trophozoites and schizonts, although the degree of association with the membrane skeleton appears to differ between isolates (Coppel *et al.*, 1988b). Although the properties of MESA are similar to those of the strain-specific molecule (Leech *et al.*, 1984b) believed to be responsible for sequestration there is no evidence that MESA is involved in cytoadherence. The cDNA clone from FC27 encoded the hexapeptide repeat GESKET (Coppel *et al.*, 1986), but attempts to define this molecule further have been frustrated by a propensity

of chromosomal segments to delete in *E. coli*. However, a clone that appears to encode the C-terminus of MESA has recently been isolated (Howard *et al.*, 1988).

3. *Falciparum interspersed repeat antigen*

Falciparum interspersed repeat antigen (FIRA) is a high molecular weight antigen localized within newly parasitized erythrocytes (Stahl *et al.*, 1985a). All isolates of *P. falciparum* tested so far express FIRA. As antibodies to FIRA are abundant in the majority of individuals living in endemic areas, it is unlikely that it is a target of protective antibody responses. FIRA contains blocks of 13 tandemly repeated hexapeptide sequences (Fig. 4) related to the consensus sequence PVTTQE. These blocks of hexapeptide repeats alternate with an 81 amino acid sequence, and the entire unit forms a higher order repeat, which is present about ten times in the FIRA molecule. The gene shows marked RFLPs in different isolates, suggesting that the higher order repeat number varies. The hexapeptide repeats encode naturally immunogenic epitopes, but it is not yet known whether the 81 amino acid repeat, the longest so far found in a malaria antigen, encodes epitopes recognized by antibodies from individuals exposed to malaria.

The FIRA gene (Stahl *et al.*, 1987) is a typical mini-exon gene with a single intervening sequence located after the signal sequence (Fig. 4). So far, 31 different varieties of the hexapeptide repeat have been found in the FC27 sequence, but this is almost certainly an underestimate because of deletions that occur in *E. coli*. The high degree of variability along the molecule makes FIRA a prime candidate as an immunological "smokescreen" molecule (see below).

4. *The histidine-rich protein of* P. lophurae

Kilejian (1974) isolated a protein of M_r 35 000–40 000 from cytoplasmic granules of the avian malaria parasite *P. lophurae* that was remarkably rich in histidine residues (*c.* 73%), which was therefore named the histidine-rich protein (HRP). Though its function remains unknown, and it is out of context in this section because it is not an erythrocyte-associated antigen, the HRP is historically important as it was the first malaria protein known to have an unusual structure. Studies in which ducklings immunized with HRP were protected against fatal infections with *P. lophurae* have proved controversial (McDonald *et al.*, 1981; Kilejian, 1981; Sherman, 1981). The HRP gene (Ravetch *et al.*, 1984; Irving *et al.*, 1986) was the first known typical mini-exon gene with a single intervening sequence located after the signal sequence (Fig. 4). After a short stretch of hydrophilic amino acids, the

remainder of the polypeptide is composed primarily of repeats, of which the predominant varieties are the decamers APH_8 and DAH_8 (Ravetch *et al.*, 1984; Irving *et al.*, 1986).

Because of the early interest in this protein, there were attempts to find the corresponding gene product by labelling *P. falciparum* with [^{3}H]-histidine. Some polypeptides relatively rich in histidine were found (see below), but sequence data have shown no other relationship of these genes to the HRP of *P. lophurae*.

5. *The knob-associated histidine rich protein*

The presence of "knobs" (Trager *et al.*, 1966), electron-dense protruberances of the plasma membrane of red cells infected with mature parasites, is implicated in cytoadherence of infected cells (Langreth *et al.*, 1979; Udeinya *et al.*, 1983). A protein of M_r 85 000–105 000, designated the knob-associated histidine-rich protein (KAHRP) by Kilejian (1979), has been found in knobby but not in knobless lines (Kilejian, 1979, 1980; Hadley *et al.*, 1983; Leech *et al.*, 1984a). Isolation of cDNA clone SD17 encoding KAHRP has allowed an unambiguous demonstration by immunoelectron microscopy that the product of the KAHRP gene is localized in the knobs (Culvenor *et al.*, 1986; Taylor *et al.*, 1987; Pologe *et al.*, 1987).

The complete structure (Fig. 4) of KAHRP from isolates NF7 (Triglia *et al.*, 1987) and FCR3 (Pologe *et al.*, 1987; Sharma and Kilejian, 1987) has recently been determined, as well as partial sequences for isolate FCR-3 (Kilejian *et al.*, 1986; Ardeshir *et al.*, 1987). As the predicted sequence includes only *c.* 8% histidine, the term KAHRP is actually a misnomer. The histidine content is contributed mainly by strings near the 5′ end of exon 2, containing 11, 6 and 10 histidine residues in NF7, and 7, 6 and 9 histidine residues in FCR-3. The sequences of KAHRP from NF7 and FCR-3 are remarkably similar, with a number of single amino acid substitutions. The molecule is a typical mini-exon gene where exon 2 contains two blocks of repeats (Triglia *et al.*, 1987). The 5′ repeats consist of five relatively degenerate copies of a 13–16 amino acid sequence related to the 14-mer SKKHKDNEDAESVK. The 3′ repeats in NF7 contain seven copies, and in FCR3 five copies, of the ten amino acid sequence SKGATKEAST, with substitutions of G by E at position 3 and vice versa at position 7 being most common. Studies with precisely defined expression clones and with synthetic peptides have demonstrated that the histidine-rich region and the 5′ and 3′ repeats each encode non-cross-reacting immunological epitopes recognized during natural infection of man (Triglia *et al.*, 1987). There are minor polypeptide size polymorphisms and RFLPs between isolates.

6. *Histidine- and alanine-rich proteins*

A small histidine- and alanine-rich protein (SHARP) was identified by screening clones with human serum (Stahl *et al.*, 1985b). This protein was polymorphic amongst different strains ($M_r \simeq 28\,000$ to 33 000). The complete sequence of the cDNA clone from FC27 (Stahl *et al.*, 1985b) revealed two blocks of repeats, composed of the hexamer AHHAAN and the pentamer HHDGA, respectively. A cDNA clone from SGE2 was 98% homologous, differing only in the number of repeats and in single base changes within the repeat blocks (Knapp *et al.*, 1988). Antibodies purified on the cloned antigen also cross reacted with a second, larger polypeptide ($M_r \simeq 60\,000$), although these two polypeptides were separate gene products. Both genes show RFLPs in different isolates, suggesting differences in repeat numbers. Recently, the genes for both SHARP (designated HRP-III) and the larger protein (designated HRP-II) were cloned from isolate 7G8 (Wellems and Howard, 1986). The two genes (Fig. 4) both have the typical mini-exon structure and show high levels of homology, suggesting that they have originated from a common ancestral sequence. The repeat of the larger polypeptide is most commonly AHHAAD, differing only in the sixth position from that in SHARP (AHHAAN). The flanking regions are less divergent than the repeat. The HRP-II gene from SGE2 was identical to that of isolate 7G8 in the 3′ non-coding region (Knapp *et al.*, 1988). Variation between these sequences occurred only in the number and arrangement of the tripeptide repeat units and in single base changes. Again, it is important to note the possibility of recombination in *E. coli* of these clones.

Studies with a monoclonal antibody revealed that about 50% of the HRP-II molecules were secreted from intact parasitized erythrocytes between 2 and 24 h of culture (Howard, R. S. *et al.*, 1986). Immunoelectron microscopy has been used to identify HRP-II in several cell compartments, including the parasite cytoplasm, as concentrated "packets" in the host erythrocyte cytoplasm and at the infected red blood cell membrane. Immunization studies in *Aotus* monkeys suggest that HRP-II may be a target of protective immunity (Knapp *et al.*, 1988).

7. *Pf11-1*

A genomic DNA clone expressing a repetitive portion of a *P. falciparum* antigen was identified by its reaction with human sera from African adults and designated Pf11-1 (Koenen *et al.*, 1984). The structure of this gene has now been determined (Kahane *et al.*, 1987; Scherf *et al.*, 1988): it commences with a mini-exon coding for a signal sequence (Fig. 4). Exon 2 contains three sets of repeats, each embedded in unique DNA. The repetitive region

contains 12 repeats of three amino acids (most commonly EKD), 14 repeats of six amino acids (most commonly EEEKLT) and a large block of highly degenerate nine amino acid repeats, of consensus PEELVEEVI with the leucine (L) often replaced by valine or isoleucine, and the isoleucine (I) by valine. Hybridization studies with synthetic oligonucleotides demonstrated that the six amino acid repeat is absent from, or drastically different in, two of seven isolates investigated, while the three and nine amino acid repeats appear to be present in all seven isolates. Most interestingly, the gene appears to have a tandem duplicate which has not yet been fully characterized (Kahane *et al.*, 1987; Scherf *et al.*, 1988). This raises interesting possibilities of generation of diversity by mitotic recombination. The corresponding *P. falciparum* antigen is believed to be a schizont antigen associated with the membrane of the infected erythrocyte (Koenen *et al.*, 1984). The protein ranges from M_r 260 000 to 350 000 in different isolates. The nine amino acid repeats of Pf11-1 are quite similar in sequence to repeat regions at the C-terminus of RESA (EENVEENV and EENVEHDA) (Coppel *et al.*, 1984). Not surprisingly, polyclonal antibodies to the amino acid repeats of Pf11-1 reacted with RESA, and vice versa (Wahlgren *et al.*, 1986a), and this is but one of a large number of cross reactivities among *P. falciparum* antigens (Anders, 1986b).

8. *The glutamic acid-rich protein*

Another gene with the typical mini-exon structure has been identified by screening an expression library (Triglia *et al.*, 1988). The sequence predicts a remarkable protein with four sets of repeats plus two strings of glutamic acid, of 29 and 10 residues, respectively (Fig. 4), and so it has been termed the glutamic acid-rich protein (GARP). There is no evidence from the sequence that it is a pseudogene and cDNA hybridization suggests that it is expressed as messenger ribonucleic acid (mRNA). However, attempts to identify the corresponding polypeptide have failed. No RFLPs have been found so far.

9. *The circumsporozoite protein-related antigen*

A monoclonal antibody designated 5.1, prepared against the cultured erythrocytic stages of isolate K1, was shown by indirect immunofluorescence to react with *P. falciparum* sporozoites. Surprisingly, the target antigen in sporozoites was the CS protein (Hope *et al.*, 1984). In the blood stages, however, the target antigen was an M_r 23 000 polypeptide named Exp-1 or circumsporozoite related antigen (CRA). Human antibodies to the blood-stage antigen also reacted with the CS protein (Coppel *et al.*, 1985a). The

corresponding cDNA clone was isolated using antibodies prepared against the purified polypeptide (Hope *et al.*, 1985) or by screening with serum from adults resident in Papua New Guinea (Coppel *et al.*, 1985a). The sequence is now available for six different isolates (Hope *et al.*, 1985; Coppel *et al.*, 1985a; Simmons *et al.*, 1987), and predicts a polypeptide of 162 amino acids, commencing with a signal sequence on a mini-exon. Unlike the other mini-exon genes described above, however, the intervening sequence is not located immediately 3′ to the signal sequence, but corresponds to a site 23 amino acids after the signal hydrophobic core (Simmons *et al.*, 1987). The gene has a second intervening sequence towards the 3′ end (Simmons *et al.*, 1987) and apparently there is a third intervening sequence in the 5′ untranslated sequence (S. Foote, personal communication). The predicted polypeptide contains an integral stretch of 28 amino acids that presumably functions as a membrane anchor sequence.

The epitope recognized by monoclonal antibody 5.1 is located within a 22 amino acid stretch that is homologous to the NANP repeats of the CS protein but does not itself contain any exact repeats. All the naturally occurring mutants of this region that do not bind monoclonal antibody 5.1 studied so far have the same A → G transition abolishing the target for the antibody (Simmons *et al.*, 1987). This alters residue 136 (aspartic acid, D) within the sequence NADP to glycine, thereby precisely defining the epitope. Apart from this change, the polypeptide is very highly conserved, the only other variable position being amino acid 160 (proline or threonine). No RFLPs have been observed (Coppel *et al.*, 1985a).

Immunoelectron microscopy with monoclonal antibody 5.1 revealed that the antigen is located within the parasitophorous vacuole and is also associated with membranous structures in the erythrocyte cytoplasm (Simmons *et al.*, 1987), as observed for MESA, KAHRP and HRP-II.

10. *The schizont-infected cell agglutinin antigen of* P. knowlesi

Brown and Brown (1965) showed that an antigen on the surface of cells infected with schizonts of *P. knowlesi* was immunologically variable during the course of infections in monkeys. The antigen was denoted the schizont-infected cell agglutinin (SICA) antigen because the schizonts underwent agglutination when incubated in homologous antiserum. It has recently been shown that changes in SICA antigen specificity could occur in a cloned *P. knowlesi* line (Howard, R. J. and Barnwell, 1984). The SICA antigen was identified as a set of high molecular weight polypeptides on the surface of cells infected with schizonts that differed in size and antigenicity in the cloned progeny of a single cell (Howard, R. F. *et al.*, 1984). It is possible that a similar phenomenon occurs in *P. falciparum* (Hommel *et al.*, 1983;

Sherwood *et al.*, 1985; Aley *et al.*, 1986b). Although this phenomenon appears to be analogous to antigenic variation in trypanosomes, the underlying molecular mechanisms are not necessarily the same. A number of possible mechanisms involving recombination in single or multiple genes can readily be envisaged.

F. RHOPTRY PROTEINS

The rhoptries, secretory organelles believed to be involved in merozoite invasion, contain a considerable number of polypeptides (Howard, R. F. *et al.*, 1984). Some of these are organized into distinct complexes (Campbell *et al.*, 1984; Schofield *et al.*, 1986; Lustigman *et al.*, 1988). They will not be considered further here as the polypeptides seem notable for their conservation rather than diversity as far as can be discerned from the limited data currently available, except that a monoclonal antibody may fail to react with some isolates (Schofield *et al.*, 1986).

G. OTHER REPETITIVE ANTIGENS

Clones encoding asparagine-rich proteins (ARPs) with the asparagines in the repeats of the tetramer NNNM and octamer NNNMNHNM or alternatively clustered in strings (CARP) have been described (Stahl *et al.*, 1986b; Wahlgren *et al.*, 1986b). A number of cDNA expression clones that react with anti-ARP antibodies but do not cross hybridize with ARP have been isolated (Kemp *et al.*, 1986), as well as a cDNA clone containing a different tetramer-based asparagine-rich repeat that does not hybridize to the above classes (G. Woodrow, personal communication). Fluorescent antibody studies suggest that the ARP(s) is or are located within the blood-stage parasite. The antibodies also react with sporozoites (Stahl *et al.*, 1986b), but probably not with the NANP repeat, even though the repeat region of the CS protein of *P. falciparum* is itself rich in asparagine. cDNA clone Ag394 predicts a polypeptide with six repeats of an octapeptide, based on KSGSKDDI and located at the 3′ end of the fragmentary sequence (Favaloro *et al.*, 1989). As the cloned polypeptide reacts with human antibodies, the polypeptide has been termed the octapeptide repeat antigen (ORA). However, studies with antibodies have failed to define the relevant *P. falciparum* polypeptide. The ORA gene shows marked RFLPs and provides a polymorphic marker for chromosome 12.

Fragmentary information on a number of other antigens has been published (McGarvey *et al.*, 1984; Stahl *et al.*, 1986a; Langsley *et al.*, 1985).

H. CYTOPLASMIC ANTIGENS

A set of antigens that appear to be internal components of the parasite are now known to be heat shock proteins. The first of these to be discovered, termed Pfhsp70-1, contained repeats of the tetrapeptide GGMP inserted into a poorly conserved region of hsp70 (Bianco *et al.*, 1986; Yang *et al.*, 1987). It is closely related to the "cognate hsp70", a non-inducible member of the same sequence family (O'Malley *et al.*, 1985). An antigen that was thought to be a merozoite surface protein is in fact the same gene product (Flint *et al.*, 1986; Ardeshir *et al.*, 1987; Rickman and Reese, 1988). More recently, a second of these proteins, Pfhsp70-2, has been defined and it is clear that there are at least five different hsp70 genes (or genes plus pseudogenes) distributed on at least four chromosomes (Peterson, M. G. *et al.*, 1988c). At present, no evidence for strain diversity exists.

I. ANTIGENS OF SEXUAL STAGES

Monoclonal antibodies have been used to define a number of antigens of the sexual stages (Carter *et al.*, 1984, 1988). The reactions of these antibodies with a number of isolates indicate that there is little diversity. Bianco *et al.* (1988) found that affinity-purified antibodies to eight cloned blood-stage antigens reacted with gametocytes by indirect immunofluorescence, but antibodies to another four did not. Recently, a gene encoding the M_r 26 000 polypeptide of zygotes has been cloned (Kaslow *et al.*, 1988). Sequences of this gene from eight different isolates revealed only a single base substitution (D. Kaslow, personal communication).

J. ANTIGENS OF LIVER STAGES

A clone encoding a fragment of a liver specific antigen (LSA) was identified by using serum from a missionary who had never had malaria because of chemoprophylaxis but had a long history of exposure to sporozoites (Guerin-Marchand *et al.*, 1987). The clone did not react with sera from transfused malaria patients. The LSA contains a 17 amino acid repeat that is highly conserved at the amino acid level, although there are numerous silent substitutions. The repeat sequence is EQQSDLEQERLAKEKLQ. RFLPs were observed with *Rsa*1 and *Alu*1 and the gene is located on one of the large chromosomes.

Studies with monoclonal antibodies provided clearcut evidence that the MSA1 gene expressed in the blood stages is also expressed in liver stages (Szarfman *et al.*, 1988b).

K. CONSERVATION OF HOUSEKEEPING GENES

In contrast to the diversity seen in some of the antigens described above, the limited data available for housekeeping genes suggest a high level of conservation. By far the most well studied is the DHFR-TS gene (Bzik *et al.*, 1987; Cowman *et al.*, 1988; Peterson, D. *et al.*, 1988). Apart from mutations in the active site that mediated pyrimethamine resistance, the gene is identical in most isolates (see below). The only other sequence data available are for the hypoxanthine phosphoribosyl transferase gene from isolates FC27 (R. Coppel, personal communication) and 7G8 (Sullivan *et al.*, 1987), which were almost identical. Even with this limited baseline, it can be inferred that variation such as that seen in the non-repetitive regions of the S-antigen is dramatic. Although the two actin genes of *P. falciparum* are very different from each other and from those of other organisms, different isolates have not yet been compared (Wesseling *et al.*, 1988).

L. GENERATION OF DIVERSITY IN REPETITIVE REGIONS AND ANTIGENIC DIVERSITY

The molecular basis of diversity in *P. falciparum* antigens of different isolates or species has now been studied in some detail in the S-antigen, CS protein, MSA1 and MSA2 systems. In each case, diversity results from differences in the repeats and in the flanking regions. In the MSA1 a variant repeat system similar to, but more limited than, that of the S-antigen is found; however, the repeat region is a minor segment of the molecule and diversity outside the repeats is much more significant (Tanabe *et al.*, 1987; Peterson, M. G. *et al.*, 1988a,b).

The different repeats in these genes could have arisen by a variety of mechanisms. The two extreme possibilities are that the different repetitive genes are a series of diverse alleles at a single locus, or that there is a multigene family in the genome that can be activated by DNA rearrangement or transcriptional mechanisms. However, all the available data support the belief that the S-antigen genes, the MSA1 and MSA2 genes, and the CS protein genes are a series of alleles at single loci.

The most remarkable property of the S-antigen repeats is that, even though they can differ so drastically from isolate to isolate, they can be precisely conserved along the molecule. Unequal crossing over or gene conversion can readily explain how a mutation, deletion or insertion can be spread along a repeat array; this subject has been studied extensively in the case of transcriptionally inert repetitive "satellite" DNA in eukaroytes (Smith, 1976). The data suggest that, as repeats mutate, the mutations

spread through the repeat array by unequal crossing over. As a result, parasite populations emerge with S-antigens that differ in sequence and in antigenic properties. However, many of these populations co-exist at any one time and within a restricted geographical area. The same principle presumably applies to all the repetitive antigens, except that the outcome depends on the balance of selective forces for each gene. Similarly, for the CSP, it has been argued that the patterns of diversity are similar to those observed in satellite DNA (Enea and Arnot, 1988).

M. REPETITIVE ANTIGENS AND IMMUNE EVASION

Although many polypeptides of many organisms contain extensive sequence repeats, the *P. falciparum* repetitive polypeptides are of special interest because these antigens are dominant natural immunogens. FIRA in particular is a dominant specificity in the sera of many individuals exposed to malaria (Stahl *et al.*, 1985a) and the repeats are the immunodominant parts of the molecule. It is probable that a large proportion of the protein diversity that exists amongst different isolates of *P. falciparum* (Tait, 1981; Fenton *et al.*, 1985) reflects, at least in part, the repeat structure of these antigens.

The genes for some antigens may have evolved from the grafting of a common ancestral 5′ mini-exon to the repetitive regions. The repetitive antigens have presumably evolved to serve a number of purposes using widely employed evolutionary strategies. As the repeats are often highly immunogenic, it is likely that they compete against host-protective immune responses. It is interesting to note that those genes with the 5′ mini-exon structure so far identified, all of which encode products that become associated with the erythrocyte, include the genes that can be deleted without effect on growth of the parasite *in vitro* (see below). Deletion of a gene for an antigen of *P. knowlesi* has also been observed (Hudson *et al.*, 1988). In this latter case the lines with the deletion arose as recrudescences after a vaccination trial, and so there is direct evidence for immune selection. In such a situation of immunological selection, gene duplication and variation by spreading of mutations in repetitive sequences would increase immune competition against protective responses and allow elaboration of the resulting gene products into an immunological "smokescreen". Hence the repeats in some of these gene products, and indeed a number of the gene products themselves, may now have no function other than in immune evasion, whereas other repetitive genes may retain critical functions. The finding of multiple cross reactivities amongst many of these antigens has suggested an additional more subtle mechanism, mediated by the effects of these repeats on B-cell proliferation and antibody affinity maturation (Anders, 1986b). It is likely that, together, these mechanisms are responsible

for distinctive features of the immune response in malaria such as marked hypergammaglobulinaemia, slow development of unstable immunity and autoantibody production (Anders, 1986b).

III. Chromosome Size Polymorphisms

A. The Molecular Karyotype of *P. falciparum*

Until recently, the number of chromosomes in *P. falciparum* and other species of *Plasmodium* was unknown. This was so because the chromosomes do not condense into discrete entities during metaphase and the complexities of the life cycle have hindered the development of conventional genetics and prevented the determination of linkage groups. Electron microscopic observation of the number of kinetochores (Sinden, 1978) had provided the only approach to this question, and Prensier and Slomianny (1986) concluded that there were 14 kinetochores. As there is clear evidence that the blood-stage parasite is haploid, from genetic crosses of rodent malarias (Walliker, 1983), and more recently of *P. falciparum* (Walliker *et al.*, 1987), it can be assumed that there are 14 chromosomes.

Pulsed field gradient (PFG) electrophoresis (Schwartz and Cantor 1984) has revolutionized the study of chromosomes of *Plasmodium*. The technique is capable of fractionating DNA molecules up to at least 10^7 bases (10 megabases) long, a size range that encompasses the size of intact *Plasmodium* chromosomes. Intact chromosomal DNA molecules can be simply prepared in the absence of shear forces by embedding the living organisms in low melting agarose, and then lysing them and extracting the components in a mixture containing a high concentration of ethylenediaminetetraacetic acid (EDTA) to inhibit nucleases, sarkosyl detergent, and proteinase K. The intact molecules are then size-fractionated by PFG electrophoresis using pulses of appropriate duration. Not only does this allow determination of the sizes and numbers of chromosomes, but cloned DNA molecules can be hybridized to blots of the chromosomes, thereby assigning the corresponding genes to the chromosomes. Furthermore, the chromosomes embedded in agarose can be digested with restriction enzymes before and/or after electrophoresis, thereby allowing the construction of genetic maps.

The earliest studies of *P. falciparum* by PFG electrophoresis resolved at least seven chromosomes (Kemp *et al.*, 1985; Van der Ploeg *et al.*, 1985). Subsequently, the estimate was increased to 11 (Langsley *et al.*, 1987) and finally to 14 chromosomes (Kemp *et al.*, 1987b; Wellems *et al.*, 1987), consistent with the results from electron microscopy. The introduction of the contour-clamped homogeneous electric field (CHEF) apparatus of Chu *et al.*

(1986) allows fractionation of the chromosomes in straight tracks (Peterson *et al.*, 1988b), a very useful technical improvement.

The 14 chromosomes range from *c.* 600 to *c.* 3000 kilobases in size, and are polymorphic: homologous chromosomes in independent isolates vary frequently and dramatically in size (Kemp *et al.*, 1985; Van der Ploeg *et al.*, 1985). These polymorphisms were observed in cloned lines of *P. falciparum* in culture and in isolates taken directly from infected individuals under conditions that precluded selection *in vitro* (Corcoran *et al.*, 1986). Furthermore, in a cloned line, the chromosomes were of identical size in rings, trophozoites and schizonts (Corcoran *et al.*, 1986). Although the karyotype of a clone is relatively stable during culture and so provides a useful marker for different clones, some size variation does occur *in vitro*, most commonly from deletions (Corcoran *et al.*, 1988). Studies on progeny from a genetic cross show that polymorphisms can arise during the cross (see below).

By hybridizing cloned DNA molecules to blots of the PFG gels, the corresponding genes can be assigned to chromosomes. The assignment of 25 markers to 12 of the 14 chromosomes in these cloned parasite lines demonstrated that chromosomal size variation can greatly change the relative migration of genetically equivalent chromosomes (Fig. 5). Hence it was necessary arbitrarily to choose a standard clone for numbering the chromosomes. Clone 3D7 was taken as the standard (Kemp *et al.*, 1987b); this clone can undergo the entire life cycle (Walliker *et al.*, 1987), and its chromosomes are much more easily resolved than those of other suitable cloned lines such as HB3. At present, it appears that the use of the three clones D10, E12 and 3D7 together allows unambiguous assignment of any gene. This is so because different size polymorphisms in each clone in the poorly resolved region between chromosomes 4 and 9 produce different patterns. For example, chromosome 9 in D10 and E12 is much smaller than that in 3D7, while chromosome 8 is much larger in E12 and 3D7 than in D10 (Kemp *et al.*, 1987b). A marker has now been found for chromosome 6 and so this interpretation has been confirmed. The assignment of 14 further markers (Peterson, M. G. *et al.*, 1988c; Cowman *et al.*, 1988; and our unpublished results) has produced no evidence that any of these polymorphisms result from interchromosomal translocation events. Rather, they all seem to be intrachromosomal events, usually deletions if the changes have occurred *in vitro*.

The antigen markers are scattered among chromosomes and so an important, if obvious, conclusion can be drawn from the linkage maps: meiotic segregation will generate novel antigenic combinations in the progeny. That this is indeed true is demonstrated below.

The chromosomes of *P. chabaudi* have been less intensively studied than those of *P. falciparum*. There are 14 chromosomes in *P. chabaudi*, *P. berghei*

and *P. vinckei* (Sheppard *et al.*, 1989). Size variations are found in different isolates of *P. chabaudi* and several chromosome-specific DNA markers are available (Langsley *et al.*, 1987; Sharkey *et al.*, 1988).

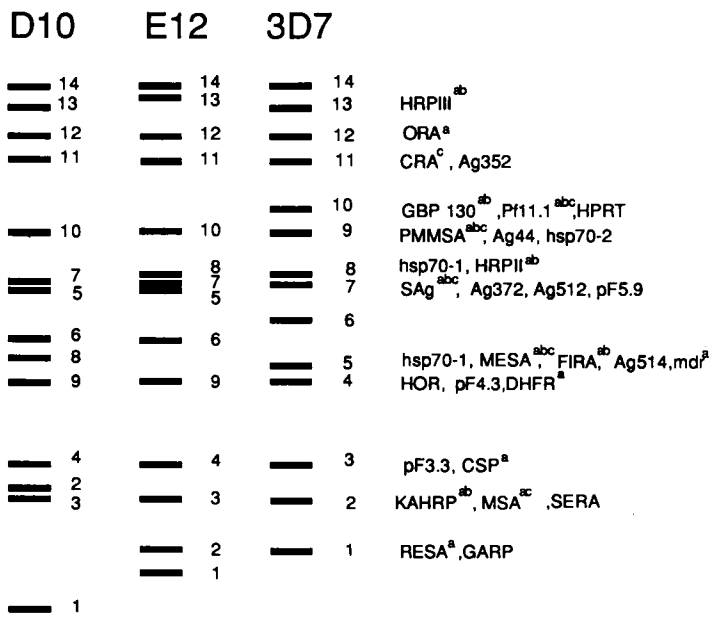

FIG. 5. Karyotypes of three clones of *P. falciparum* (D10, E12, and 3D7). The chromosomes are represented in increasing order of size from the bottom. The numbers are aligned with the chromosomes of clone 3D7, chosen as standard because it can undergo the entire life cycle. The relationships were established by hybridization. [a] Restriction fragment length polymorphism; [b] electrophoretic variants; [c] antigenic variants. Note that FIRA is assigned to chromosome 5, and not 7 as previously thought. Revised after Kemp *et al.* (1987b). [Antigen codes are explained in the text.]

B. GENERATION OF CHROMOSOME SIZE POLYMORPHISMS IN *P. FALCIPARUM* BY HOMOLOGOUS RECOMBINATION WITHIN SUB-TELOMERIC REPEAT SEQUENCES

Restriction maps for chromosomes 1 and 2 of six cloned lines of *P. falciparum* have recently been produced (Corcoran *et al.*, 1988). A map of chromosome 1 from Palo Alto has been produced independently (Patarapotikul and Langsley, 1988). Two of the clones were D10 and E12 which differ in the size of chromosomes 1 and 2 even though they both derive from isolate FC27 and share the same S-antigen specificity (Anders *et al.*, 1983).

The other clones were 3D7 and HB3, the parental clones used for the genetic cross (Walliker *et al.*, 1987), and two of the progeny. These maps, defined with seven different enzymes that cut the adenine + thymine-rich genome rarely, delineate the locations of eight genetic markers including genes for five antigens (Fig. 6).

In all the parasites, chromosome structure is conserved in central regions, but is polymorphic both in length and sequence near the telomeres (Corcoran *et al.*, 1988). A telomere probe from *P. berghei* (Ponzi *et al.*, 1985) was known to hybridize to the telomeres of *P. falciparum* (Dore *et al.*, 1986). The telomeric repeats are restricted to an area less than 15 kilobases from the end of the chromosome, and are associated with a microheterogeneity in fragment size that is characteristic of telomeric sequences (see Blackburn, 1984). An *Apa*I site occurs frequently in a conserved position 12–15 kilobases from the end of the chromosome in many cases, even in non-homologous chromosomes.

A *P. falciparum* specific repetitive DNA element has been described by several independent investigators (Bhasin *et al.*, 1985; Guntaka *et al.*, 1985; Aslund *et al.*, 1985; Oquendo *et al.*, 1986; Zolg *et al.*, 1987). The element (referred to here as rep20; Oquendo *et al.*, 1986) is based on repetition of a 21 base-pair unit with a consensus sequence 5′-ATA/CTAGGTCTTAAT/GG/ATTACTA-3′ (Oquendo *et al.*, 1986; Aslund *et al.*, 1986; Zolg *et al.*, 1987). Clusters of this 21 base-pair unit occur in blocks containing at least 13 repeats which can contain more than 14 kilobases. Rep20 sequences were reported to reside on all chromosomes (Oquendo *et al.*, 1986). However, chromosome 1 in D10 and chromosome 2 in E12 lacked detectable rep20 sequences (Corcoran *et al.*, 1988). Significantly, these two represent the smallest versions of chromosome 1 and 2 found. Furthermore, rep20 sequences were associated only with the ends of chromosomes, just adjacent to the telomeres (see Fig. 6). In its position, species-specificity and structure, the rep20 domain bears a striking resemblance to the sub-telomeric repeats recently cloned from *P. berghei* (Pace *et al.*, 1987; Ferreria *et al.*, 1986; see below). The structure consisting of telomere-*Apa*I site-rep20 domain was called a telomere complex (TC) (Corcoran *et al.*, 1988).

Certain chromosomes of intermediate length (E12 and Palo Alto chromosome 1 and D10 chromosome 2) have a rep20 cluster at only one end. It was proposed (Corcoran *et al.*, 1988; Patarapotikul and Langsley, 1988) that generation of chromosome length polymorphism occurs by recombination within, and deletion of, expendable rep20 repeats. The maps for chromosomes 1 and 2 of the six cloned lines indicate strong structural conservation in central regions. The length polymorphisms coincide with fragments bearing rep20 repeats and it is evident that blocks of rep20 can vary in copy number.

Small telomeric *Apa*I fragments (15 or fewer kilobases) characteristic of

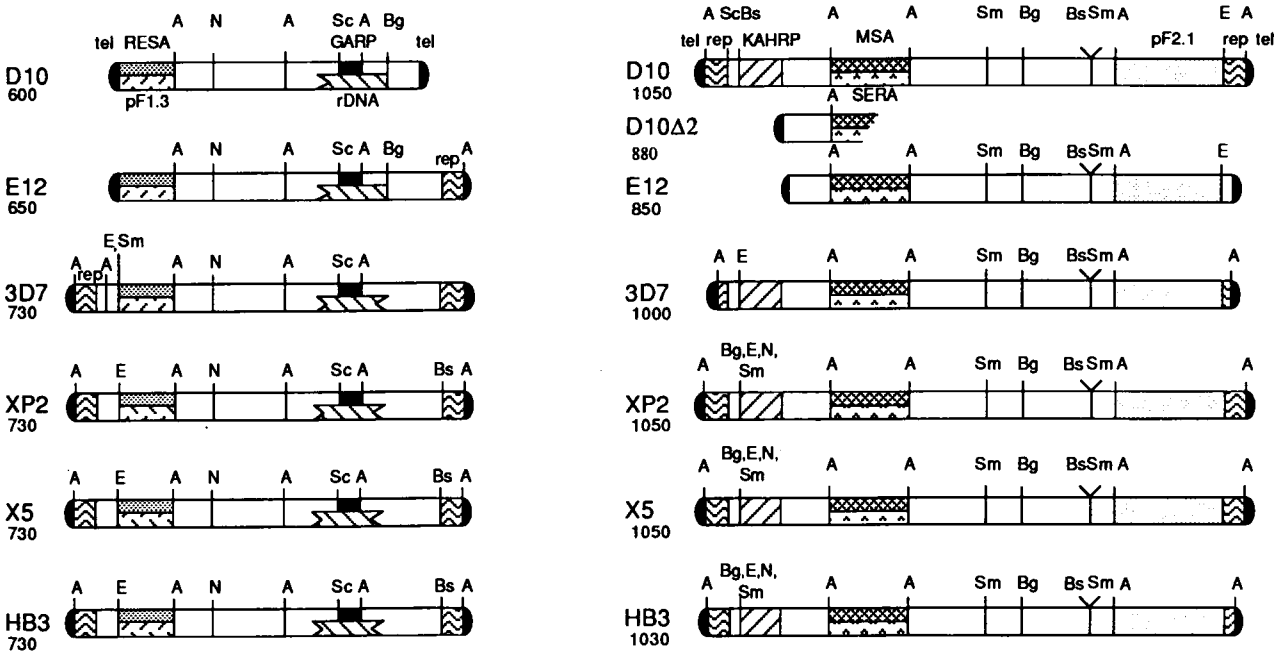

FIG. 6. Restriction maps of chromosomes 1 and 2 for six clones of *P. falciparum*. A, *Apa*I; B, *Bgl*1; Bs, *Bssh*II; E, *Eag*1; N, *Nar*1; Sc, *Sac*II; Sm, *Sma*1. The different shadings represent different markers, as indicated the first time they appear, reading from the top down. Revised after Corcoran *et al.* (1988). [Antigen codes are explained in the text.]

TCs were found on all chromosomes (Corcoran *et al.*, 1988). However, a number of chromosomes also have one larger telomeric fragment. The rep20 probe hybridized to two fragments on all chromosomes except those that had one large telomeric *Apa*I fragment. Hence, absence of the small *Apa*I telomeric fragment correlates with the lack of the rep20 domain from the TC, most probably because of deletion. It can be concluded that, with the exception of such deletions that vary from isolate to isolate, the general structure of each end of every chromosome is the same.

An important recent finding is that rep20 deletions can occur in natural parasites that have not been cultured *in vitro* (Biggs *et al.*, unpublished observations). One such isolate had typical rep20 deletions on both ends of chromosome 2. Hence, these deletions are not simply laboratory artefacts.

C. DELETIONS INVOLVING CODING SEQUENCES

In several cases, size differences between homologous chromosomes of the order of 100 kilobases or more are known to result in a concomitant loss of genes encoding antigens. *P. falciparum* cultured *in vitro* often loses the ability to produce knobs on the infected red cells. It is now clear that in many knobless isolates the gene encoding KAHRP, normally located on chromosome 2, is completely (Corcoran *et al.*, 1986; Culvenor *et al.*, 1986; Ellis *et al.*, 1987) or partially (Pologe and Ravetch, 1986) deleted from the genome. Where part of the gene remained following the deletion, it was possible to show that the KAHRP gene remnant was located near a telomere (Pologe and Ravetch, 1986). Mapping studies demonstrated that in clones E12 and D10Δ2 the deletions had removed all detectable rep20 sequences and the *Apa*I site from the TC normally adjacent to the KAHRP gene (Corcoran *et al.*, 1988). This is also true for a number of other independent KAHRP deletions (Biggs *et al.*, unpublished observations). So far, all known cases of loss of expression of KAHRP following such deletions have a knobless phenotype. As there is no doubt that KAHRP is localized in the knobs we conclude that KAHRP is essential for knob formation.

The histidine-rich and alanine-rich protein genes are also subject to deletions. The SHARP/HRP-III gene is not expressed in isolate HB3 because the gene is deleted from the terminus of chromosome 13 (Wellems *et al.*, 1987; Kemp *et al.*, 1987b). Furthermore, HRP-II expression has been lost from clone D10 (Stahl *et al.*, 1985b). It appears that most, but not all, of the HRP-II gene has been lost concomitant with a large deletion from D10 chromosome 8 (Kemp *et al.*, 1987b). It was predicted that the gene for HRP-II lies near a chromosome 8 telomere, and is subject to deletions similar to those described for other members of the HRP gene family (Kemp *et al.*, 1987b).

It was proposed that the mechanism of rearrangement was similar to the homologous recombination between rep20 repeats but involved telomeric rep20 repeats and sequences related to rep20 that lie in the vicinity of the HRP genes. Alternatively, it could have involved non-homologous recombination. A third possibility is that these deletions arose from chromosome breakages followed by addition of telomeric repeats to the broken ends by the telomere terminal transferase. Deletions of large stretches of sequences adjacent to telomeres by any of these mechanisms may occur relatively frequently, but may create a selective disadvantage. HRP genes may escape this selection pressure, either because they serve no function in culture *in vitro*, or because they can functionally complement each other *in vivo*.

Deletion of the MESA gene has also been observed in clone IT (J. Leech, personal communication), but it was not clear if this involved rep20. The RESA gene is not expressed at detectable levels in isolate FCR3, as defined by immunoblotting studies with monoclonal antibodies against the 5′ or 3′ repeats (M. R. van Schravendijk and R. Anders, personal communication). Hybridization studies with a number of different segments of the RESA structural gene show that a region overlapping exon 1 of the gene is deleted. Larger scale mapping studies by PFG show that there is a typical rep20 deletion that removes the *Apa*I site and *c.* 120 kilobases of DNA between RESA and the adjacent telomere in FCR3 (Cappai *et al.*, unpublished observations). The sequence of the junction shows that a rep20-associated deletion has removed the 5′ end of the RESA gene but not the 3′ end of the gene and telomeric repeats are attached directly to the RESA intron (Cappai *et al.*, unpublished observations).

It is also fascinating to note that FCR3 may lack expression of other repetitive antigens as well as the RESA. Expression of the MESA was not detectable using antibodies against the GESKET sequence (R. Anders, personal communication). Hybridization with a MESA repeat probe showed that this region of the gene was nevertheless present. However, as little is known about the structure of the MESA gene because of its propensity to delete in *E. coli*, and chromosome 5 of *P. falciparum* (on which MESA is encoded) has not yet been mapped, it is not yet clear whether these observations have anything to do with rep20-associated deletions. In FCR3 cultures, variable numbers of cells cannot express KAHRP because they have a typical rep20 and KAHRP gene deletion on chromosome 2. Furthermore, the overall level of FIRA expression is extremely low in FCR3 as measured by reaction with human antibodies from Papua New Guinea. As FCR3 was one of the first *P. falciparum* isolates established in culture, it has had ample time to delete rep20 sequences *in vitro* and, in so doing, to provide insights into the relevance of repetitive antigens in *P. falciparum*.

IV. Meiosis and Genetic Recombination

A. Recombination and the parasite life cycle

Genetic recombination is the process by which new combinations of genes arise in the progeny of crosses between two parent organisms. Recombination occurs principally at meiosis, and involves both the random assortment of genes located on different chromosomes and crossing over events between linked genes on homologous chromosomes. In malaria parasites, meiosis occurs shortly after zygote formation in mosquitoes and crossing experiments have demonstrated that recombination is a frequent occurrence at this stage. Recombination may also occur at mitosis, but at a much lower frequency than at meiosis. Studies on chromosomes of cloned lines of *P. falciparum* imply that mitotic recombination can occur during prolonged *in vitro* culture of blood forms (see Section III).

The haploidy of blood forms and exo-erythrocytic stages of malaria parasites has been shown by the segregation of genetically determined characters in rodent parasites and in *P. falciparum* (Walliker *et al.*, 1975, 1987; Szarfman *et al.*, 1988b). Studies on DNA synthesis in *P. berghei* using microfluorometry have provided additional evidence that sporozoites, ring forms, young trophozoites and mature microgametes possess a similar quantity of DNA, assumed to be the haploid amount (Janse *et al.*, 1986). Mature macrogametocytes possess approximately 50% more DNA than this quantity, possibly due to amplification of gametocyte-specific genes. The only diploid stage is the zygote (ookinete), produced by fertilization of gametes in the mosquito midgut. Meiosis occurs within a few hours of zygote formation, as shown by the presence at this stage of synaptonemal complexes, characteristic of meiotically dividing chromosomes (Sinden and Hartley, 1985). There is synthesis of approximately four times the haploid DNA quantity within 3 h of fertilization (Janse *et al.*, 1986). This is consistent with the duplication of the diploid chromosome set which occurs at the first stage of eukaryotic meiosis. No information is yet available on subsequent chromosomal divisions in the parasite which lead to the haploid sporozoite stage.

B. Genetic crosses between malaria parasites

Crossing experiments are the traditional basis of genetic studies. Data on the recombination and segregation of genes in crosses can be used to allocate genes to particular chromosomes, and to determine their linear order on the chromosomes. Genes located on different chromosomes assort randomly in the progeny of crosses, while linked genes (located on the same chromo-

some) segregate together, unless separated by crossing over events during meiosis. Genetic crosses can also provide precise information on the extent to which variation in a given character is due to allelic variation of a gene at a single locus, or to variation between genes at different loci in the genome. For example, variation in a character such as drug resistance may involve structural alterations in a single gene, or interactions between the products of several variant genes. Recombination between such genes in crosses can be used to elucidate the genetic basis of the character under investigation.

Malaria parasites are crossed by permitting mosquitoes to feed on a mixture of gametocytes of two cloned lines differing in a number of genetic markers. This allows cross-fertilization to occur between gametes of each clone. Self-fertilization events are also expected, and, assuming that each clone produces equal numbers of male and female gametes and that fertilization occurs randomly, equal numbers of hybrid and parental zygotes will be produced. The mixed parasites are allowed to develop to sporozoites which are used to establish infections in appropriate vertebrate hosts. The resulting blood infections are examined for the appearance of forms exhibiting recombination between parental characters, resulting from cross-fertilization. Such recombinant forms are isolated from the progeny by cloning.

Early crossing experiments were done with cloned lines of *P. chabaudi* and *P. yoelii* maintained in laboratory mice (reviewed by Walliker, 1983). In this review, we shall concentrate mainly on a cross between two clones of *P. falciparum* (Walliker *et al.*, 1987). The cross was carried out by feeding mosquitoes on a mixture of cultured gametocytes of the clones and infecting a chimpanzee with the resulting sporozoites. The blood forms developing in this animal were subsequently cultured and cloned in human erythrocytes. The parent clones, denoted by 3D7 and HB3, respectively, had different geographical origins, and differed in numerous characteristics including the sizes of certain chromosomes, restriction fragment length polymorphisms, electrophoretic forms of isoenzymes and other proteins, antigens and drug sensitivity. The inheritance of some of these characteristics is summarized here.

1. *Segregation and crossing-over of chromosomes*

As described in Section III, the genome of *Plasmodium* consists of chromosomes which can be visualized by pulsed field gradient (PFG) electrophoresis. Individual chromosomes vary considerably in size in different parasite isolates. There is, therefore, considerable interest in the behaviour of the chromosomes at meiosis and its genetic consequences. Although little information is yet available on the mechanisms of chromosome pairing, crossing experiments in *P. falciparum* and *P. chabaudi* have shown that

segregation of homologous chromosomes and crossing-over events occur in the manner expected for eukaryotic cells.

In *P. falciparum*, homologous chromosomes of different cloned isolates, including clones 3D7 and HB3, exhibit several types of variation. First, the sizes of certain chromosomes vary markedly. Chromosomes 3 and 4, for example, are larger in 3D7 than in HB3 (Walliker *et al.*, 1987). Second, sequence variations occur in chromosomes examined by mapping, including chromosomes 1 and 2 (Corcoran *et al.*, 1988), chromosome 4 (Sinnis and Wellems, 1988), and chromosome 13 (Wellems *et al.*, 1987). Third, certain genes are absent from one or other parent. For example, a histidine-rich protein gene denoted SHARP/HRP-III is present on chromosome 13 of 3D7, but is absent from that of HB3. Finally, variations occur in the quantity of rep20 in different chromosomes (Patarapotikul and Langsley, 1988; Corcoran *et al.*, 1988).

(*a*) *Chromosomal segregation.* During meiosis of hybrid zygotes, each member of a given pair of chromosomes can be expected to segregate randomly into the haploid progeny. This has been demonstrated in both *P. chabaudi* and *P. falciparum*, in crosses between parasites in which homologous chromosomes differ in size and other features.

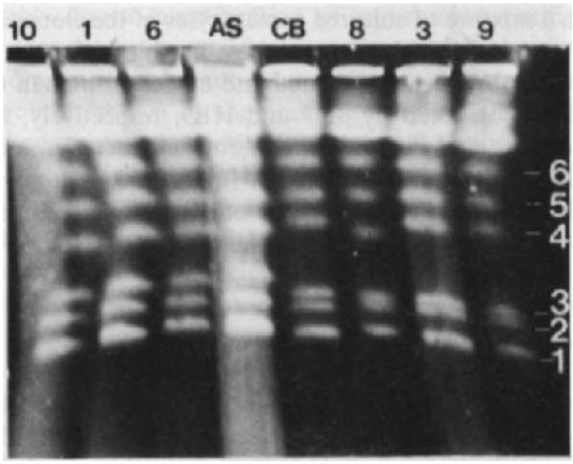

FIG. 7. Segregation of chromosomes following meiosis in *P. chabaudi*. The figure shows chromosomes 1–7 of two parent clones AS and CB, and of six progeny clones derived from a cross between AS and CB. Note that clones 10, 1 and 6 possess chromosomes of parent-type AS, and 3 and 9 of type CB. Progeny clone 8 possesses chromosome 4 of type CB and chromosome 5 of type AS.

In *P. chabaudi*, a cross was made between two cloned lines denoted AS and CB which differed in the sizes of two chromosomes (Sharkey *et al.*, 1988). Chromosome 4 in the clone AS was larger than that of clone CB, while chromosome 5 was smaller. The two clones were crossed in mosquitoes and six progeny clones examined for their chromosomes (Fig. 7). In five clones, identified as recombinants by enzyme and antigen markers, the chromosomes segregated as parental types. In the sixth clone, chromosome 4 could be identified from its size as being derived from the CB parent, while chromosome 5 was from the AS parent.

In *P. falciparum*, random chromosome segregation has been found in the progeny of the 3D7/HB3 cross. For example, in a progeny clone denoted X5, chromosome 1 appears from restriction mapping to derive from HB3 (Corcoran *et al.*, 1988), while chromosome 13 is derived from 3D7; this is shown by the presence of the SHARP/HRP-III gene which is absent from the HB3 form of this chromosome (Wellems *et al.*, 1987; Kemp *et al.*, 1987b).

(*b*) *Crossing-over of chromosomes.* A striking feature of chromosomes of natural isolates of *P. falciparum* is their size variation (Corcoran *et al.*, 1986). An important result from the *P. falciparum* crossing studies was the demonstration that novel sized chromosomes were generated at meiosis (Fig. 8). When the parent clones alone were transmitted through mosquitoes, chromosome sizes appeared to remain unchanged. However, novel forms of at least one chromosome were seen in seven of 12 progeny clones examined. Size changes of chromosomes of clones may also occur during prolonged *in vitro* culture of asexual forms (Corcoran *et al.*, 1988; Wellems *et al.*, 1988), although most probably at a lower frequency than at meiosis.

Changes in the size of chromosome 4 during the cross have been studied by Sinnis and Wellems (1988). This chromosome contained approximately 1490 kilobases in 3D7 and 1200 kilobases in HB3. In four of 12 progeny clones examined, chromosome 4 was intermediate in size. For example, in a progeny clone denoted XP5, it had about 1400 kilobases. By comparing restriction maps of parent and progeny clones, it was found that the XP5 chromosome contained restriction sites characteristic of the left end of the 3D7 chromosome and of the right end of that of HB3. This showed that the intermediate-sized chromosome had been produced by crossing over between parent-type chromosomes during the cross.

Chromosome 2 size variation has been the subject of several studies, as described above (Section III). Following the cross between 3D7 and HB3, it was found that certain progeny clones possessed a form of this chromosome which was larger than that of either parent. Two of these clones, XP2 and X5, possessed an increased quantity of the repetitive DNA sequence rep20

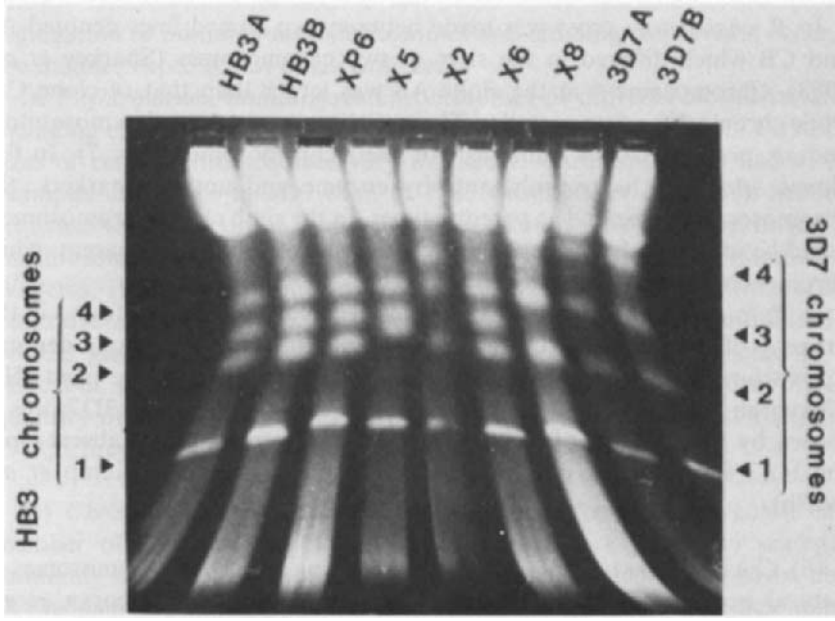

FIG. 8. Chromosomes of parent and progeny clones derived from cross between *P. falciparum* clones 3D7 and HB3. The conditions of electrophoresis show clearly only chromosomes 1–4. The two tracks on the extreme left and the two on the extreme right show, respectively, chromosomes of clone HB3 and of clone 3D7 before (A) and after (B) mosquito transmission. The remaining tracks show chromosomes of a selection of progeny clones. Note that clones XP8 and X2 possess HB3-type chromosomes, and X6 has 3D7-type chromosomes. Clone X2 possesses a chromosome 4 of size intermediate between that of each parent. In clone X2, chromosome 2 is larger than in either parent.

at a sub-telomeric site, compared to the parents (Corcoran *et al.*, 1988). An unequal crossing-over event in the rep20 sequence at meiosis of hybrid 3D7/HB3 zygotes is the probable explanation of this finding.

The sub-telomeric regions of chromosomes of several organisms have now been shown to be subject to great variability and genetic rearrangements. Vernick *et al.* (1988) have investigated the inheritance of a telomere-related sequence denoted pPftel.1 in the 3D7/HB3 cross by hybridization of this sequence to blots of restriction enzyme-digested DNA from 12 progeny clones. In many progeny, fragments of DNA which hybridized with this sequence were of novel, non-parental sizes. Some of these fragments were located at internal chromosome sites, as well as at sub-telomeric locations. Such sites thus appeared to be genetically unstable at meiosis in crosses

between different parasites, perhaps representing "hot-spots" of recombination. Similar variations in the repetitive rep20 sequence have been found following crossing between 3D7 and HB3 (P. Oquendo *et al.*, unpublished observations).

2. *Genetics of isoenzymes and other proteins*

Electrophoretic forms of enzymes and other proteins are particularly suitable for studies on the basic genetics of an organism because they are usually determined by nuclear genes which undergo a straightforward Mendelian pattern of inheritance. For example, the inheritance patterns of variant forms of two enzymes, lactate dehydrogenase and 6-phosphogluconate dehydrogenase, provided the first unequivocal evidence that the blood forms of malaria parasites are haploid (Walliker *et al.*, 1975). In *P. falciparum*, variant forms of six enzymes were reported by Sanderson *et al.* (1981), and Andrews *et al.* (unpublished observations) have demonstrated polymorphism in nine of 26 enzymes examined. Two-dimensional polyacrylamide gel (2D-PAGE) electrophoresis has revealed variation in at least 12 of 100 proteins detectable by this method (Fenton *et al.*, 1985).

In the *P. falciparum* cross, the inheritance patterns of electrophoretic forms of the enzyme adenosine deaminase (ADA) and of certain 2D-PAGE protein variants showed a Mendelian pattern of inheritance, typical of a eukaryotic haploid organism. Table 1 shows the forms of these characters present in 3D7 and HB3 and in progeny clones derived from the cross. A given progeny clone has only one or other of the parental forms of each protein, not both together or novel forms. Segregation of each form of each protein thus occurred during the cross, showing that they are determined by allelic variants of their respective genes. In addition, recombination between each of the genes occurred, as shown by the non-parental combinations of each among the progeny.

The chromosomal location of these genes is unknown at present and, therefore, it is not known whether the recombination was due to chromosomal reassortment, or to crossing over between linked genes. While recombination had clearly occurred between each gene, insufficient progeny clones have been analysed to provide information on linkage.

3. *Genetics of antigens*

Recombination studies have been made on two merozoite antigens, the MSA1 and MSA2 (see Section II D). Diversity in both these antigens has been observed in *P. falciparum*. For the MSA1 antigen, this has been demonstrated by examining cloned isolates with strain-specific monoclonal

antibodies in immunofluorescence tests (McBride *et al.*, 1985) and by DNA sequence analysis (Tanabe *et al.*, 1987). The gene appears to be present as a single copy in the genome and has been mapped in PFG blots to chromosome 9 (Kemp *et al.*, 1987b). The MSA2 antigen has been less extensively studied, but diversity has also been found in different isolates using monoclonal antibodies (Clark *et al.*, 1988) and it has been mapped to chromosome 2 (Kemp *et al.*, 1987b).

TABLE 1 *Forms of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) proteins and of adenosine deaminase (ADA) in* Plasmodium falciparum *clones 3D7 and HB3, and in progeny clones derived by crossing 3D7 and HB3*[a]

	2D-PAGE protein				*ADA*
	C	D	G	K	
Parental clones					
3D7	3	2	1	3	1
HB3	7	4	2	1	2
Progeny clones					
Parental types (7 clones)					
XP6, X10	7	4	2	1	2
X8, X14, X17, X18, X32	3	2	1	3	1
Recombinant types (19 clones)					
X2	3	4	2	1	1
X11, XP3	7	4	1	3	1
X6, X35	7	4	2	3	2
X12, XP8	7	4	2	3	1
X5, XP2	7	4	1	1	1
XP5, X34	7	4	2	1	1
XP1	7	2	2	3	2
X4	7	2	1	3	2
XP4	7	2	2	3	1
XP7, XP9	7	2	1	1	2
X13	7	4	2	3	2
X30	3	2	1	1	2
X33	3	2	1	3	2

[a] Individual progeny clones are designated with prefixes X or XP. Letters C, D, G and K refer to the positions of four proteins on 2D-PAGE gels; variant forms of each protein are denoted by numbers (Fenton *et al.*, 1985). Forms of adenosine deaminase designated 1 and 2 refer, respectively, to fast and slow forms of this enzyme detectable by starch gel electrophoresis.

The forms of these two antigens among the progeny of the 3D7/HB3 cross, detected by strain-specific monoclonal antibodies, are given in Table 2. With regard to the MSA1, the two forms characteristic of 3D7 and HB3 clearly undergo segregation from one another in the progeny clones, con-

firming that they are allelic variants of the same gene. No clones showing non-parental combinations of the monoclonal antibodies 7.3 and 9.2 were found, which could have been produced had the MSA1 gene been at a different locus in each parent clone. Six of the 26 clones examined showed recombination between the MSA1 and the MSA2, which is explained by independent assortment of chromosomes 2 and 9 during meiosis of hybrid zygotes.

TABLE 2 *Forms of the merozoite antigens MSA1 and MSA2 in* Plasmodium falciparum *clones 3D7 and HB3, and in progeny clones derived by crossing 3D7 and HB3*[a]

	MSA1		*MSA2*
	7.3	9.2	12.3
Parent clones			
3D7	−	+	+
HB3	+	−	−
Progeny clones			
Parental types			
5 clones	−	+	+
15 clones	+	−	−
Recombinant types			
3 clones	−	+	−
3 clones	+	−	+

[a] 7.3 and 9.2 are monoclonal antibodies recognizing the MSA1 antigen; 12.3 recognizes the MSA2 antigen. + and − refer to immunofluorescence reactions of each monoclonal antibody with parent and progeny clones.

Tanabe *et al.* (1987) proposed, from DNA sequence data, that diverse forms of the MSA1 found in natural isolates of *P. falciparum* could be generated by intragenic recombination during meiosis of zygotes heterozygous for this antigen. This mechanism could also be responsible for the diversity seen in natural parasite populations of S-antigens and other characters, especially of those whose genes contain a repetitive DNA sequence. Definitive evidence that intragenic recombination occurs is not yet available from crossing experiments; the process is likely to occur only rarely, and thus recombinants could be isolated only by establishing and characterizing large numbers of progeny clones.

4. *Linkage*

The crossing experiments have demonstrated that recombination readily occurs between the genes determining characters such as antigens, enzymes

and other proteins following mosquito transmission of mixed clones. The chromosomal location of many of these genes has not yet been determined. It is possible that each is located on a separate chromosome, and thus the recombination observed may simply be due to chromosomal segregation at meiosis. Crossing over between linked genes is also likely, as novel-sized chromosomes are frequently generated at meiosis.

Crossing studies are likely to be of only limited value in elucidating the linear order of genes on the chromosomes of *Plasmodium* because of the technical difficulty of isolating sufficient progeny clones to obtain meaningful recombination frequencies. Chromosome mapping can be achieved more easily by PFG electrophoresis, as discussed in Section III. The order of genes on a given chromosome can be obtained by subjecting individual chromosomes to digestion with appropriate restriction enzymes and hybridizing the resulting fragments with specific gene probes. This approach has already provided linear maps of some chromosome-specific sequences on chromosomes 1, 2, 4 and 13 (Corcoran *et al.*, 1988; Wellems *et al.*, 1987).

C. IMPLICATIONS OF CROSSING STUDIES

A finding of interest from the crossing experiments with both rodent parasites and *P. falciparum* is that recombinant-type parasites are often generated at a frequency which is higher than expected. As discussed below, 50% of the progeny of a cross can be expected to derive from self-fertilization events and thus be parental types. However, in the *P. falciparum* cross between 3D7 and HB3, only three of the 22 progeny characterized so far have proved to be parental for all markers. The reasons for this are unknown, but could involve: (i) selection favouring certain recombinant genotypes; (ii) disproportions in the numbers of micro- and macro-gametes produced by each parent clone; or (iii) cross-fertilization being favoured over self-fertilization.

These findings have clear implications for the biology of *P. falciparum* and, by implication, other species of malaria parasites. Mosquito transmission provides a highly efficient means of generating parasites with novel genotypes, both by independent assortment of unlinked genes and by crossing-over events. The capacity of *P. falciparum* to undergo genetic recombination is thus considerable, and this will need to be taken into account in future research to develop new methods of control by either chemotherapy or vaccination.

V. The Genetic Basis of Drug Resistance

The control of human malaria relies in particular on the use of antimalarial

drugs. In recent years, the problem of drug resistance has enormously complicated the use of drugs to control malaria. Chloroquine, a representative of the 4-aminoquinoline group of drugs, and antifolate drugs, such as pyrimethamine, are the most widely used and, consequently, their mechanism of action has been studied in detail. The emergence of resistance to chloroquine and pyrimethamine has necessitated a detailed analysis of the mechanisms of resistance to these two drugs and we will focus here on the genetic mechanisms.

A. THE GENETIC BASIS OF PYRIMETHAMINE RESISTANCE

Development of resistance to antifolate drugs in the field occurred very rapidly and resistance was reported soon after their first use in widely separated areas. This suggested that a single mutation could be responsible for the development of resistance to drugs such as pyrimethamine. Selection of forms of *P. gallinaceum* resistant to proguanil and pyrimethamine in the laboratory suggested that mutational events were involved (Bishop, 1962). Genetic crosses with pyrimethamine resistant and sensitive lines of *P. chabaudi* (Walliker *et al.*, 1975, 1976) and *P. yoelii* (Knowles *et al.*, 1981) showed a pattern of inheritance typical for a single gene.

1. *Action of pyrimethamine*

Pyrimethamine has been known to be an antifolate for many years (Hitchings, 1960). Studies on bacteria (Wood and Hitchings, 1959) and mammals (Hitchings *et al.*, 1952) identified the site of action of antifolate drugs such as pyrimethamine. Pyrimethamine is a potent dihydrofolate reductase (DHFR) inhibitor in *P. berghei* and the affinity of pyrimethamine for the parasite enzyme is 2000-fold greater than the host DHFR (Ferone *et al.*, 1969; Ferone, 1970). Consequently, the effectiveness of pyrimethamine relies on its more effective inhibition of the parasite DHFR than of the host enzyme. Inhibition of DHFR results in a decreased pool of the tetrahydrofolate co-factors which are used for the synthesis of purines, methionine, thymidylate and the interconversion of glycine to serine. As a result, pyrimethamine binding to DHFR should, therefore, result in inhibition of DNA synthesis (Schellenberg and Coatney, 1961; McCormick *et al.*, 1971). However, in *P. knowlesi*, inhibition of synthesis occurs only after morphological abnormalities are visible and growth has ceased (Gutteridge and Trigg, 1971). Therefore, it is likely that inhibition of DHFR by antifolate drugs not only affects DNA synthesis but also that consequent depletion of folate co-factors required for other functions plays a significant part in the lethal effect of pyrimethamine and other antifolate drugs on *Plasmodium*.

2. *Mechanisms of resistance*

Development of strains of malaria parasites resistant to antifolate drugs in the field and in the laboratory has been described in detail previously (reviewed by Peters, 1970, 1985; Walliker, 1983). However, detailed study of the mechanisms of resistance to pyrimethamine, particularly in *P. falciparum*, has begun only in the last few years. The isolation and cloning of the gene for DHFR from *P. falciparum* has enabled analysis of the basis of pyrimethamine resistance at the level of the gene. The most likely mechanisms of resistance to pyrimethamine are: (i) decreased uptake of pyrimethamine; (ii) increased levels of DHFR due to gene amplification, increased transcription, or increased stability of the enzyme; or (iii) alterations to binding affinity of the drug to DHFR as a result of mutations in the gene.

Decreased uptake of pyrimethamine has not been shown to be involved in drug resistance in *Plasmodium*. A number of *P. falciparum* clones that were induced by mutagenesis and selected by growth in medium containing pyrimethamine (Banyal and Inselburg, 1986) accumulated pyrimethamine as efficiently as the drug-sensitive parent clone (Inselburg *et al.*, 1987). One of the parasite clones exhibited a greater level of uptake of pyrimethamine but this was associated with an increased level of the DHFR-thymidylate synthase protein present in the parasite. Decreased drug uptake also could not explain the pyrimethamine resistance of isolate W2, when it was compared to the sensitive clone FCR3 (Dieckmann and Jung, 1986a). Reduced uptake of antifolates has been demonstrated in resistant lines of *Leishmania major* (Ellenberger and Beverley, 1987) but it has yet to be shown conclusively in *Plasmodium*. *P. falciparum* isolates resistant to sulfadoxine have reduced uptake of the drug (Dieckmann and Jung, 1986b), and it would be surprising if the same mechanism did not play a part in resistance to pyrimethamine in *Plasmodium*.

An increased level of DHFR activity and mutations within the gene could account for the resistance of strains of *P. berghei* (Ferone, 1970; Diggens *et al.*, 1970). The mechanism of pyrimethamine resistance in *P. chabaudi* clones (Sirawaraporn and Yuthavong, 1984) involved no increase in DHFR enzyme level, but there was a decrease in inhibitor binding to DHFR in resistant clones, indicating that changes to the DHFR enzyme were involved.

Isolation and analysis of the DHFR gene from *P. falciparum* (Bzik *et al.*, 1987; Cowman *et al.*, 1988; Peterson, D. *et al.*, 1988; Snewin *et al.*, 1989) have enabled analysis of the basis of pyrimethamine resistance at the DNA level. It had been previously shown that DHFR exists as a bifunctional protein with thymidylate synthase (TS) in protozoa (Garrett *et al.*, 1984), including *P. falciparum* and *P. lophurae*. Analysis of the DHFR-TS gene of

P. falciparum indicates that the bifunctional enzyme has a weight of approximately 71 700 dalton, and is separated into the DHFR and TSS domains by a junctional sequence of approximately 100 amino acids. The sequences of the DHFR-TS gene from the pyrimethamine-sensitive clone FCR3 (Bzik *et al.*, 1987) and from a number of pyrimethamine-resistant lines have been determined (Cowman *et al.*, 1988; Peterson, D. *et al.*, 1988; Snewin *et al.*, 1989).

Overexpression of the DHFR enzyme has been shown to occur in the Palo Alto (pyrimethamine-resistant) isolate of *P. falciparum* and this could explain its resistance (Kan and Siddiqui, 1979). There was a 50–80 fold increase in enzyme activity, but in a Palo Alto isolate maintained in another laboratory (Cowman *et al.*, 1988) there is a single DHFR-TS gene and the sequence of the DHFR portion of the gene is identical to that of the pyrimethamine-sensitive isolate FCR3 (Peterson, D. *et al.*, 1988). The Palo Alto isolate has been grown for a number of years without drug selection and it is possible that alterations have occurred that have changed the DHFR-TS gene copy number in this isolate. It is also possible that the isolates have been contaminated and are totally different in the two laboratories.

Analysis of other pyrimethamine-resistant isolates of *P. falciparum* has shown no increased expression of DHFR that could account for resistance. The only exception has been the demonstration of a five- to ten-fold increase in expression of the enzyme in a pyrimethamine-resistant clone selected by drug pressure *in vitro* (Inselburg *et al.*, 1987). Although this *P. falciparum* clone overexpresses the DHFR-TS protein, the level of DHFR enzyme activity is equal to that of its pyrimethamine-sensitive parent. Activity of the TS enzyme was ten-fold higher than in the parent clone and there were clearly changes in the DHFR enzyme of the selected clone that reduced its activity. As well as the alterations in the enzyme, duplication of the DHFR-TS gene has been reported. This cloned line was obtained by mutagenesis and selection by sequential increases in drug concentration and it is possible that a number of mutational events combined to produce the observed drug-resistant phenotype.

Pyrimethamine resistance and DHFR activity has been analysed in a number of *P. falciparum* clones (McCutchan *et al.*, 1984; Dieckmann and Jung, 1986a; Walter, 1986; Chen *et al.*, 1987) in which the mechanism of drug resistance involves altered properties of the enzyme. Sequencing of the DHFR gene of these *P. falciparum* clones and isolates has enabled identification of amino acids that are responsible for pyrimethamine resistance (Cowman *et al.*, 1988; Peterson, D. *et al.*, 1988; Snewin *et al.*, 1989). The DHFR activity of the pyrimethamine-sensitive clone 3D7 has been compared with that of resistant clones HB3 and 7G8 and the mechanism of

resistance in both cases involves a decreased affinity for pyrimethamine by a structurally altered enzyme (McCutchan *et al.*, 1984; Chen *et al.*, 1987). The pyrimethamine-resistant clones 7G8 and HB3 have levels of DHFR activity similar to that of 3D7. The DHFR-TS gene is present as a single copy in all three clones (Cowman *et al.*, 1988; Peterson, D. *et al.*, 1988) and comparison of the DHFR sequences of 3D7, 7G8 and HB3 has identified the amino acids that determine pyrimethamine resistance (Cowman *et al.*, 1988; Peterson, D. *et al.*, 1988) (Table 3). The progeny of the 3D7/HB3 cross (Walliker *et al.*, 1987) have been analysed and an RFLP has been identified that segregates with pyrimethamine resistance (Peterson, D. *et al.*, 1988). This polymorphism is tightly linked to the DHFR-TS gene (Table 3).

Further analysis of the DHFR gene in other *P. falciparum* isolates has demonstrated that the amino acid that determines pyrimethamine resistance in HB3 is an asparagine, rather than a serine at position 108. This same change is present in the DHFR gene of virtually all resistant isolates analysed so far (Cowman *et al.*, 1988; Peterson, D. *et al.*, 1988; Snewin *et al.*, 1989) (Table 3). In the pyrimethamine-resistant isolate K-1 from Thailand, there is no overexpression of DHFR and resistance is a result of a modification of DHFR (Walter, 1986). Copy number analysis has confirmed the presence of a single DHFR-TS gene in this isolate (Cowman *et al.*, 1985; Snewin *et al.*, 1989). The K-1 DHFR gene contains an asparagine at position 108, but it also has a second amino acid change, cysteine to arginine, at position 59. The K-1 gene is much more resistant to pyrimethamine than is HB3, and this second amino acid change is believed to encode the increased resistance (Cowman *et al.*, 1988; Snewin *et al.*, 1989). Two other south-east Asian isolates (CSL2 and V-1) have the same sequence as K-1, except for a conservative change at position 164, and they are equally resistant to pyrimethamine.

The same amino acid (asparagine at position 108) has been shown to be encoded by the DHFR gene of other *P. falciparum* isolates from different continents (Peterson, D. *et al.*, 1988) (Table 3). Isolates from south-east Asia (DD2), Africa (Sierra Leone) and Malaysia (M. Camp) all encoded asparagine at residue 108 instead of the threonine which is present at this position in FCR3. The only isolate that has been classified as pyrimethamine resistant that does not have asparagine as residue 108 is Palo Alto; it has threonine at this position and is identical in sequence to the pyrimethamine-sensitive clone FCR3 (Cowman *et al.*, 1988; Peterson, D. *et al.*, 1988). The Palo Alto isolate used in the study is partially resistant and is most probably equivalent in drug resistance to FCR3. These two parasite isolates have not been tested for pyrimethamine sensitivity in the same laboratory.

In summary, there is clearly a number of mechanisms involved in

TABLE 3 *Amino acids in dihydrofolate reductase (DHFR) that encode pyrimethamine resistance*

Clone/strain	*Geographical origin*	*Resistant phenotype*[a]	*DHFR genotype (amino acid)*					*Ref.*
			16	*51*	*59*	*108*[b]	*164*	
3D7	Netherlands	S	Ala	Asn	Cys	Ser	Ile	1, 2
FCR3	The Gambia	S	Val	Asn	Cys	Thr	Ile	2
Sierra Leone	Sierra Leone	S	Ala	Asn	Cys	Ser	Ile	2
Palo Alto	Uganda	—[d]	Val	Asn	Cys	Thr	Ile	1
HB3	Honduras	R	Ala	Asn	Cys	Asn	Ile	1, 2
M. Camp	Malaysia	R	Ala	Asn	Cys	Asn	Ile	2
7G8	Brazil	R	Ala	Ile	Cys	Asn	Ile	1, 2
V-1	Vietnam	R	Ala	Asn	Arg	Asn	Ile	1
K-1	Thailand	R	Ala	Asn	Arg	Asn	Ile	1, 3
Csl-2	Thailand	R	Ala	Asn	Arg	Asn	Leu	1
DD2	Indochina	R	Ala	Ile	Arg	Asn	Ile	2

[a] S, pyrimethamine sensitive; R, pyrimethamine resistant.
[b] Amino acid 108 has been identified as the key residue involved in pyrimethamine resistance. All resistant *P. falciparum* clones encode asparagine at this residue.
[c] 1, Cowman *et al.*, 1988; 2, Peterson, D. *et al.*, 1988; 3, Snewin *et al.*, 1989.
[d] Palo Alto is partially resistant (see Cowman *et al.*, 1988).

resistance to pyrimethamine in *Plasmodium*. Decreased uptake of pyrimethamine has not been demonstrated to occur in *Plasmodium*; it has been shown to be involved in antifolate drug resistance of *L. major*, as it is more than likely that it can occur in *Plasmodium*. Overexpression of the DHFR enzyme occurs in *P. berghei* but it is not known if amplification of the DHFR gene is involved. In *P. falciparum*, increased expression of the DHFR enzyme has been shown to occur in one isolate but analysis of the DHFR enzyme in other isolates indicates that alterations in the enzyme are the most common mechanism mediating drug resistance.

B. CHLOROQUINE RESISTANCE

Chloroquine resistance first arose in south-east Asia near the border between Thailand and Kampuchea in 1957 (Harinasuta *et al.*, 1962) and spread rapidly over the next decade to Malaysia and Indonesia until it was first observed in Papua New Guinea in 1975. It is now present in most of Oceania. It is believed that chloroquine resistance spread from this original focus in south-east Asia across India and Pakistan and into western and eastern Africa (Clyde, 1987). The first cases of resistance in South America were reported in 1961 (Moore and Lanier, 1961), and it has now spread over a wide area of Latin America. The striking geographical progression and the chronological order of the appearance of chloroquine-resistant *P. falciparum* across the world suggests that there were two original foci of resistance (Clyde, 1987): the first probably in south-east Asia in 1957 and a second probably in South America at the same time or very soon after. It is possible that all chloroquine resistance arose from the single focus in south-east Asia and spread to South America; however the only definite link between the emergence of resistance in these two regions is the fact that it occurred at approximately the same time.

Following the first development of chloroquine resistance in south-east Asia, an increase in expression of resistance to the drug developed from RI (parasitaemia cleared by 25 mg of chloroquine per kg of body weight, but subsequent recrudescence) to RII (parasitaemia reduced, but not cleared) and eventually RIII (not affected by the chloroquine dose). These incremental steps in expression of resistance suggest that successive mutations were necessary to obtain RIII chloroquine resistance. High level chloroquine resistance (RIII) was in some cases found to be associated with cross resistance to amodiaquine (Hall *et al.*, 1975) and quinine (Chongsuphajaisiddhi *et al.*, 1981; Smrkovski *et al.*, 1982; Lambros and Notsch, 1983). Mefloquine was developed by the Walter Reed Army Institute of Research, but it has not been widely used because of fears of resistance to it developing. Trials of mefloquine in Thailand have shown examples of failure

of the drug in the treatment of malaria, even though this drug had not been used in these regions before (Boudreau *et al.*, 1982; Lapierre *et al.*, 1982). This multidrug resistance phenotype of malaria parasites in the field has become a major problem in designing effective chemotherapeutic regimes.

Production of chloroquine-resistant strains of *Plasmodium* in the laboratory has been difficult, probably because a number of mutations is required to yield high-level resistance. This multifactorial mechanism of chloroquine resistance may explain the small number of foci involved in the emergence of resistance in the field. Some chloroquine-resistant forms of *P. berghei* have been produced (Ramakrishnan *et al.*, 1957; Sautet *et al.*, 1959; Sargent and Poncet, 1959) and highly chloroquine-resistant forms of *P. berghei* (Peters, 1965), *P. vinckei* (Powers *et al.*, 1969) and *P. chabaudi* (Rosario *et al.*, 1978; Padua, 1981) have been obtained by using increasing levels of the drug over prolonged periods.

Genetic crosses between chloroquine-sensitive and chloroquine-resistant *P. chabaudi* (Rosario, 1976; Padua, 1981) have shown that high-level resistance is due to a number of mutations in different genes. Analysis of the progeny from one genetic cross (Padua, 1981) revealed parasites with intermediate levels of resistance. Segregation of each mutant gene into different progeny could presumably result in this intermediate phenotype.

1. *Action of chloroquine*

Chloroquine has a toxic effect on a wide range of cell types. This effect is evidenced by the wide range of its therapeutic applications. However, *P. falciparum* is exquisitively sensitive to the drug and any model of chloroquine action must account for this phenomenon. At present there is no universally accepted model for chloroquine action, but evidence is accumulating to support two broadly distinct theories. Uptake of chloroquine into the acidic vacuoles (Friedman *et al.*, 1979; Mikkelsen *et al.*, 1982) is thought to occur by diffusion of the molecule, which is singly protonated at natural pH, across the membranes. Thus it is trapped, because in the acidic environment it is doubly protonated and hence is unable to cross the membrane. In order to explain the 100–1000 fold increase in sensitivity of *P. falciparum* to chloroquine, Warhurst (1986) postulated the existence of a permease that is able to pump chloroquine from the red cell cytoplasm into the parasite cytoplasm from whence it diffuses into the acidic food vacuoles. The consequences to the parasite's well-being of the concentration of chloroquine in the food vacuole is not known; however, it reduces the acidity of the vacuole (Krogstad *et al.*, 1985; Yayon *et al.*, 1985), which may inhibit protease digestion. Furthermore, chloroquine itself is a potent protease inhibitor (Yayon *et al.*, 1984; Vander-Jagt *et al.*, 1986); thus it may prevent

haemoglobin digestion and starve the parasite. In fact, Yayon *et al.* (1984) have shown that therapeutic doses of chloroquine inhibit the release of amino acids from infected erythrocytes and prevent degradation of the contents of the food vacuole.

Chloroquine binds ferriprotoporphyrin IX (FP) in the food vacuole. Normally FP is sequestered as malaria pigment or haemozoin (Yamada and Sherman, 1979) and as such is unable to bind chloroquine (Chou *et al.*, 1980; Fitch *et al.*, 1982). In the presence of chloroquine, an FP-chloroquine complex forms which is not sequestered and is toxic to the cell. FP-chloroquine lyses *P. chabaudi* cell membranes (Orjih *et al.*, 1981) and impairs the ability of the cell to maintain cation gradients (Chou and Fitch, 1981).

Chloroquine intercalates into DNA (Hahn, 1974); however, the association constant of chloroquine is much lower than its therapeutic concentration and, while this may play a part in its toxicity to mammalian cells, it is certainly not important in explaining its action against the parasite.

Chloroquine has been shown to accumulate in the phagolysosomes of both *P. gallinaceum* and *P. berghei* (Aikawa, 1972). Elements of both of these postulated modes of action may well eventually be invoked in explaining chloroquine's predilection for *P. falciparum*, but at present the matter is far from decided.

2. *Mechanisms of resistance*

Verapamil completely reverses chloroquine resistance in a number of resistant clones of *P. falciparum*, a phenomenon described in multidrug-resistant neoplastic cell lines by Fojo *et al.* (1985) and Rogan *et al.* (1984). Analysis of these cell lines suggested a rapid efflux of the drugs from the cells, preventing toxic levels from accumulating, and the authors also observed that verapamil reversed this drug resistance by inhibiting the efflux of the drugs from the cell. Martin *et al.* (1987) observed the same phenomena in multidrug resistant *P. falciparum*, and suggested that the mechanism of resistance in the parasites was the same as that in tumour cell lines. The rapid efflux of anticancer drugs from the tumour cells has been shown to be due to amplification of domains of DNA and overexpression of a gene termed the multidrug resistance (MDR) gene (Gros *et al.*, 1986a,b,c,d; Riordan and Ling, 1985; Roninson *et al.*, 1986). This gene has been isolated from rodent and human cell lines by Gros *et al.* (1986a,b,c,d) and Chen *et al.* (1986), respectively, and shown to encode a cell surface glycoprotein that has striking homology to bacterial transport proteins. This suggested a role for MDR in normal transport processes that could result in decreased accumulation of cytotoxic drugs (Skovsgaard, 1978; Fojo *et al.*, 1985), thus accounting for the multidrug resistant phenotype.

Recently, the *P. falciparum* homologue of rodent and human MDR gene has been isolated (Foote *et al.*, 1989) and it is now possible to ask if this gene is involved in chloroquine and multidrug resistance. The gene contains an internal duplication that is also seen in the mammalian MDR gene (Chen *et al.*, 1986) and it is closely homologous to both the rodent and human MDR proteins (Foote *et al.*, 1989). It is present in more copies in some chloroquine-resistant strains than it is in sensitive clones of *P. falciparum*. This increase is associated with a large increase in the size of chromosome 5 due to tandem repeats of a sequence of about 100×10^3 bases. The increase in copy number was associated with an increase in transcript levels. A number of chloroquine-resistant isolates of *P. falciparum* that were analysed did not contain any increase in copy number of the MDR gene. However, it has been shown that mutations in a transcription factor in yeast can alter the regulation of MDR and cause overexpression of the gene, resulting in expression of the multidrug resistant phenotype (Balzi *et al.*, 1987). Mutations in the human MDR gene have also been shown to increase resistance to particular chemotherapeutic drugs but not to others (Choi *et al.*, 1988). Therefore, it is possible that mutations within the *P. falciparum* MDR gene, as well as overexpression of the MDR protein, are necessary to obtain high levels of chloroquine resistance. Consequently, it will be necessary to sequence the MDR gene from a number of chloroquine-resistant *P. falciparum* strains to identify any possible mutations. There appears to be a strong link between the MDR gene and chloroquine resistance; however, it has yet to be conclusively demonstrated that this gene or a member of the MDR gene family is involved in the multidrug phenotype in *P. falciparum*.

Rapid efflux of chloroquine from the parasite is clearly a mechanism of drug resistance, but there may be more than one mechanism. Analysis of the genetics of resistance to chloroquine in *P. chabaudi* demonstrated that resistance increases gradually, possibly in a series of small steps (Padua, 1981). This stepwise nature of increasing resistance would fit the concept of amplification of an MDR or MDR-like gene and overexpression of a protein that mediates rapid efflux of the drugs from the parasite. However, in this case, the resistance was stable and its inheritance multigenic, suggesting that alterations in a number of genes were necessary for high levels of drug resistance.

As already discussed, chloroquine has a number of effects, and it is not yet clear which is the most important concerning its antimalarial properties. It is possible that all the effects contribute to some degree, and high levels of resistance are acquired by mutations in a number of genes. Nevertheless, rapid transport of chloroquine out of the cell is one clearly established mechanism and it is likely that the MDR gene or another gene with homologous functions is involved.

VI. Outlook

This review has been mainly concerned with recent findings on chromosomal polymorphism and DNA sequence variation in genes for antigens and other characters of *P. falciparum*. To conclude, we consider briefly the relevance and implications of this diversity to attempts to control the disease.

Optimism that vaccines could be developed against *P. falciparum* malaria came from the observation that people continually exposed to the disease in endemic areas eventually become clinically immune, even though they may carry significant parasite loads. Further impetus for the production of vaccines came from: (i) the development of the *in vitro* culture technique of blood forms of *P. falciparum*; (ii) the use of monoclonal antibodies and of sera from immune patients to identify and characterize parasite antigens; and (iii) the cloning and expression of antigen genes in prokaryotes by recombinant DNA technology. However, the early optimism has been tempered in recent times by the disappointing results of some vaccine trials based on synthetic peptides or recombinant fusion products (Ballou *et al.*, 1987; Herrington *et al.*, 1987), by evidence that genetic restriction in host response to such antigens may occur (Good *et al.*, 1988a,b,c), and by the finding that many candidate antigens are genetically diverse. For example, seven allelic forms of MSA1 have been demonstrated in a survey of only 37 isolates using specific monoclonal antibodies (McBride *et al.*, 1985). The significance of this variation is obscure and it may be unimportant with regard to the immune response of the host. However, variations in epitopes concerned in T- and B-cell recognition are likely to be of considerable significance in the design of vaccines. In order to be fully effective in stimulating protective immune responses, vaccines will almost certainly need to contain a "cocktail" of variant forms of many antigens.

An important finding in natural infections of *P. falciparum* is that individual patients are frequently infected with a mixture of genetically distinct parasites (Graves *et al.*, 1984; Thaithong *et al.*, 1984; Webster *et al.*, 1985). The crossing studies described above (Section IV) have demonstrated that mosquito transmission of such mixtures provides a highly efficient means for the generation of parasites with novel genotypes. The capacity of this organism to undergo genetic recombination in nature is thus considerable, and this will need to be taken into account in eventual field trials of vaccines.

Evidence is also accumulating of geographical variations in many *P. falciparum* characters. Most information on this subject has come from surveys of variant forms of enzymes and proteins characterized by two-dimensional polyacrylamide gel electrophoresis (PAGE) as well as of certain antigens. Similar forms of most of these characters occur in all countries

studied so far, but often at differing frequencies (reviewed by Walliker, 1985). For example, two electrophoretic forms of the enzyme glucose phosphate isomerase termed GPI-1 and GPI-2 occur at similar frequencies in isolates from East and West Africa (Carter and Voller, 1975). In contrast, a form of the enzyme adenosine deaminase denoted ADA-2, which occurs in only 4% of isolates in The Gambia and Thailand (Sanderson *et al.*, 1981; Thaithong *et al.*, 1981), is found in approximately 70% of isolates in Brazil (Couto *et al.*, 1983). Studies on the distribution of proteins identified by two-dimensional PAGE in Thailand have provided some evidence for differences in the frequency of some variant proteins in different regions of the country (Walker, 1985).

The geographical variation of antigens being considered as vaccine candidates has not been intensively studied. A form of MSA1, denoted serotype I, is rare in The Gambia and Brazil, but common in Thailand (J. S. McBride, unpublished work; Walliker, 1985). In long-term studies in Papua New Guinea, Forsyth *et al.* (1988) have shown that the prevalence of S-antigen serotypes varied markedly from one village to another, as did their frequency, over a 2-year period. Such local variations may mean that vaccines will need to contain different formulations of candidate antigens in different areas. Further work on this subject is necessary.

With regard to chemotherapy, the experience of the past 50 years shows that resistance is likely to develop to any new antimalarial drug. The only type of resistance which has been studied at the gene level is resistance to pyrimethamine, as discussed above (Section V A). In this instance, knowledge of the precise changes in the target enzyme of the drug, dihydrofolate reductase, in resistant organisms could lead to the design of new antifolates which would inhibit the altered enzyme.

Mixtures of drugs with different mechanisms of action (e.g. Fansidar®, containing the antifolate pyrimethamine and the sulphonamide sulphadoxine) have been advocated, and, in some instances, widely used. The rationale for the use of such mixtures is that resistance to each component most probably has a different genetic basis. Parasites simultaneously containing mutations conferring resistance to both drugs can thus be expected to be exceedingly rare in the population. Curtis and Otoo (1986) pointed out that the use of such drug mixtures is sensible provided that a large proportion of the parasite population is not exposed to the drugs, that mutant genes conferring resistance to each drug are rare in the population, and that free recombination can occur between the resistance genes. The appearance of resistance to Fansidar® in *P. falciparum* in south-east Asia (Black *et al.*, 1981) was most probably due to widespread use of this drug mixture in a region in which the prevalence of resistance to pyrimethamine was already high. An understanding of the genetic basis of drug-resistance is thus of

crucial importance if the likelihood of resistance developing to such mixtures is to be predicted and, thereby, allow alternative strategies of treatment to be planned.

The application of molecular biology and genetics to the study of *P. falciparum* has produced new insights into the nature of the disease. The parasites can now be perceived as a population of genetically diverse organisms which readily undergo mating and recombination during passage through the mosquito vector, and not as a collection of distinct "strains". The use of cloned blood forms is clearly essential for laboratory studies on, for example, antigen characterization. However, a given clone is likely to exist for only a short time in nature, and may undergo irreversible genetic changes, such as chromosome deletions, when maintained in long-term culture. Such clones are, therefore, quite untypical of natural infections. In future, model systems will be needed, for testing new antimalarial drugs and vaccines, which take account of the parasite's complete life cycle, and of its remarkable capacity for producing novel genetic forms.

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Immunoelectron Microscopy of Parasites

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I. Introduction 151
II. Methods 152
A. Fixation 152
B. Accessibility of antigenic sites 153
C. Visualization of antigenic sites 155
D. Immunocytochemical controls 158
III. Applications to Parasitology 160
A. *Plasmodium* 160
B. *Toxoplasma*, *Sarcocystis* and *Eimeria* 187
C. *Theileria* 188
D. *Trypanosoma* and other flagellates 190
E. *Trichinella* and other nematodes 190
F. *Schistosoma* and *Fasciola* 192
IV. Conclusions 194
Acknowledgements 195
References 195
Technical appendix 210
A. Fixation 210
B. Embedding and sectioning with LR White and LR Gold resins 211
C. Labeling procedures for LR White and LR Gold sections 212

I. Introduction

Immunocytochemistry includes the broad collection of techniques that use antibodies and suitable markers to localize specific antigens in cells or tissues

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by light or electron microscopy. Procedures which use fluorescein- or peroxidase-antibody conjugates to localize antigens by light microscopy have been widely used by parasitologists, but it is only recently that the more powerful ultrastructural techniques of immunoelectron microscopy have been applied to the study of protozoan and helminth parasites.

Immunoelectron microscopy was introduced by Singer (1959) with the development of ferritin–antibody conjugates. In the 30 years since then, refinements of the technique have made immunoelectron microscopy an indispensable tool for biological scientists. Recent applications of cell and molecular biology to parasitology have made immunoelectron microscopy increasingly important for localizing protective antigens for vaccine development and for characterizing the subcellular make-up and function of specific parasite organelles. In this review, we discuss the theory and methods of immunoelectron microscopy and summarize current applications to the study of protozoan and helminth parasites. We hope that the procedures and applications which are described in this chapter will encourage other parasitologists to use this technique for molecular and ultrastructural studies of host/parasite interactions.

II. Methods

A. Fixation

Fixation for immunoelectron microscopy should: (i) stabilize the antigen or antigens under investigation to prevent their diffusion or extraction from the tissue during subsequent processing; (ii) preserve the tertiary structure of the antigen or antigens to retain antigenicity and reactivity with antibodies; and (iii) preserve acceptable ultrastructure (Hayat, 1981; Bullock, 1984). In general, fixatives that preserve good ultrastructure by extensively cross-linking proteins often do so at the expense of antigenicity. The most widely used fixatives for immunoelectron microscopy are glutaraldehyde and formaldehyde. Many workers have preserved acceptable ultrastructure and antigenicity by using low concentrations of buffered glutaraldehyde (0.1–1.0%) for brief time periods (10–30 min). Higher glutaraldehyde concentrations and longer fixation times often destroy immunoreactivity, although many antigens are able to survive. Formaldehyde is superior to glutaraldehyde as a fixative for immunoelectron microscopy because its lower molecular weight allows rapid penetration of tissue and because it is able to stabilize most proteins without destroying their antigenicity (Hayat, 1981). However, when used alone, neither low (1%) nor high (up to 8%) concentrations of buffered formaldehyde preserve ultrastructure well. In addition, formaldehyde fixation is, to some extent, reversible, depending on the concen-

tration of the formaldehyde solution, length of exposure to the fixative and type of cell or tissue.

Most workers have tried to combine the best qualities of glutaraldehyde and formaldehyde by using mixtures of the two—generally 1–4% formaldehyde and 0.05–1.0% glutaraldehyde. These solutions give the best results when prepared fresh from paraformaldehyde powder and recently distilled monomeric glutaraldehyde. Commercial formalin solutions should be avoided because they often contain methanol and other impurities (Hayat, 1981).

Since some antigens are extremely sensitive to glutaraldehyde fixation, several glutaraldehyde-free fixatives have been developed. These include carbodiimidates and bifunctional diimidoesters such as diethylmalonimidate and dimethylsuberimidate (McLean and Singer, 1970; Hassell and Hand, 1974; Yamamoto and Yasuda, 1977; Hayat, 1981) and more commonly used formaldehyde-based fixatives such as PLP (periodic acid–lysine–paraformaldehyde) fixative (McLean and Nakane, 1977) and formaldehyde–picric acid combinations (Stefanini *et al.*, 1967). PLP fixatives were devised to preserve fine structure by the oxidation of carbohydrates with periodic acid to produce aldehyde groups which could then be cross-linked to each other or to amino groups of proteins by lysine and paraformaldehyde (McLean and Nakane, 1977). Picric acid has been used as an ingredient because it is believed to precipitate proteins without significant denaturation (Bullock, 1984). Both PLP and formaldehyde–picric acid fixatives have been modified to include glutaraldehyde (Dae *et al.*, 1982; Newman *et al.*, 1982; Somogyi and Takagi, 1982; Gendelman *et al.*, 1983). The incorporation of amines such as cyclohexylamine into formaldehyde-based fixatives may also improve structural preservation without destroying antigenicity (Luther and Bloch, 1989). Antigens sensitive to glutaraldehyde may also be protected from relatively harsh conditions of fixation by the use of ethylacetimidate (Tokuyasu, 1984). This compound blocks amino groups and helps to reduce the amount of cross-linking during glutaraldehyde fixation.

Secondary fixation with osmium tetroxide is not recommended for post-embedding immunoelectron microscopy because it destroys the antigenicity of most cellular antigens. Rarely, the antigenicity of material fixed with glutaraldehyde–osmium and embedded in resin may be restored if grid-mounted thin sections are etched with a strong oxidizing agent such as hydrogen peroxide or sodium metaperiodate (Bendayan and Zollinger, 1983).

B. ACCESSIBILITY OF ANTIGENIC SITES

Unlike surface antigens which can be labeled by incubation of intact living or fixed cells with immunoreagents, intracellular antigens must first be made

accessible to antibodies. A variety of pre- and post-embedding techniques has been devised to solve this problem. The choice of a particular procedure is affected by the sensitivity of the antigen to buffers, dehydrating agents and embedding media, and by the quality of structural preservation that is needed.

1. *Pre-embedding immunocytochemistry*

Pre-embedding immunocytochemical procedures include techniques that are applied to cells or tissues before they are embedded in a suitable supporting medium. These procedures avoid the loss of immunoreactivity that often accompanies exposure to harsh dehydration and embedding reagents, but require that intact cells and tissues be made permeable to immunoreagents. Both physical and chemical methods are available for doing this. Physical methods include: (i) infiltrating the sample with a suitable cryoprotectant, such as sucrose, glycerol or dimethylsulfoxide, and subjecting the cells to rapid freezing and thawing in liquid nitrogen; or (ii) passing the sample through an ascending and descending series of ethanol dilutions that were prepared with the rinsing or storage buffer (Somogyi and Takagi, 1982; Eldred *et al.*, 1983). Both methods disrupt cellular membranes sufficiently to allow penetration of immunoreagents, while still preserving acceptable ultrastructure (Eldred *et al.*, 1983; Priestley, 1984). Chemical methods include brief exposures to low concentrations of detergents such as Triton X-100 or saponin to disrupt cell membranes (Priestley, 1984).

2. *Post-embedding immunocytochemistry*

Post-embedding techniques are used when intact cells and tissues are needed to examine the intracellular distribution of antigens. Samples are embedded and sectioned before labeling with immunoreagents. Post-embedding procedures have a number of advantages over pre-embedding techniques. Cells are not damaged while being rendered permeable, a single sample can be sectioned and tested with a variety of different antibodies, and double-labeling techniques with particulate markers are simple to perform.

(*a*) *Cryo-ultramicrotomy.* Cryo-ultramicrotomy is still the best method for localizing antigens that are sensitive to organic solvents or embedding resins. In this procedure, tissues are infused with 2.3 M sucrose and then quickly frozen in liquid nitrogen. During sectioning, ice acts as the supporting material (Griffiths *et al.*, 1984; Tokuyasu, 1986). While this is currently the most sensitive post-embedding technique, cryo-ultramicrotomy requires specialized equipment, is technically difficult, and yields sections that have low contrast.

(*b*) *Resins.* During the past 10 years several popular hydrophilic acrylic and methacrylate resins have been developed. These include methacrylate/acrylate mixtures such as Lowicryl K4M, HM20, K11M and HM23 (Armbruster *et al.*, 1982; Carlemalm *et al.*, 1982, 1985; Roth, 1989), acrylic resins such as LR White and LR Gold (Timms, 1986; Bendayan *et al.*, 1987; Newman and Hobot, 1987), the older methacrylates such as glycol methacrylate (Leduc and Bernhard, 1967; Cole, 1984), and mixtures of methyl methacrylate and newer acrylic resins (Escolar *et al.*, 1988). These resins are attractive because they can be used at low temperatures where the denaturing effects of alcohols and other organic solvents are reduced (Armbruster *et al.*, 1983). Their polar, hydrophilic nature allows them to polymerize without complete dehydration of the sample, thereby preserving hydration shells around proteins and allowing closer interaction of aqueous solutions of antibodies with the resin surface (Armbruster *et al.*, 1982; Newman and Hobot, 1987). With glycol methacrylate and methyl methacrylate–acrylic mixtures, dehydration of the tissue is possible without exposure to alcohols (Leduc and Bernhard, 1967; Escolar *et al.*, 1988).

The choice of a post-embedding procedure will ultimately depend on the sensitivity of the antigen or antigens of interest. Bendayan *et al.* (1987) found that embedding media had a significant effect on the localization of amylase in pancreatic tissue. Intense labeling was obtained with cryosectioned material and material embedded in glycol methacrylate, while epoxy and acrylic resins were less efficient.

C. VISUALIZATION OF ANTIGENIC SITES

Since ferritin–antibody conjugates were first introduced as electron-dense markers for immunoelectron microscopy (Singer, 1959), several different probes have been developed for the visualization of antigen–antibody reaction sites. These include particulate markers conjugated to a suitable ligand by covalent or non-covalent bonds, e.g. ferritin, colloidal gold and iron–dextran complexes, and covalently conjugated enzymatic markers that produce an electron-dense reaction product when incubated with an appropriate substrate, such as horseradish peroxidase (Horisberger, 1984). Both enzymatic and particulate marking systems have inherent advantages. Final choice of a marker will depend on the degree of resolution that is desired, on whether quantitative or qualitative information is needed, on the accessibility of the antigen to antibodies, and on the sensitivity of the antigen to fixation and embedding.

1. *Enzymatic markers*

Horseradish peroxidase is the most popular enzymatic marker for immuno-

electron microscopy. Tissue-bound antibodies are visualized by exposure of the peroxidase-antibody conjugate to 3,3′-diaminobenzidene to form an osmiophilic, electron-dense reaction product (Horisberger, 1984; Sternberger, 1986). This enzyme can be coupled directly to the primary antibody and used as a one-step, direct procedure, or it can be coupled to secondary antibodies or other ligands and used as a multistep, indirect technique that significantly increases the sensitivity of the procedure. These indirect "sandwich" approaches include the peroxidase–antiperoxidase (PAP) technique and its many modifications (Ordronneau, 1982; Sternberger, 1986), the avidin–biotin–peroxidase complex (ABC) technique (Childs and Unabia, 1982; Childs, 1983a), the protein A-peroxidase technique (Dubois-Dalcq *et al.*, 1977), the dinitrophenyl (DNP) hapten sandwich staining (DHSS) procedure (Newman and Jasani, 1984), and the streptavidin–peroxidase technique (Shi *et al.*, 1988).

Immunoenzyme techniques have a number of advantages over procedures which use particulate markers. The relatively small size of most of the reagents, as compared with ferritin and colloidal gold markers, allows them to penetrate cells and some hydrophilic resins in pre- and post-embedding procedures (Zafrani *et al.*, 1983; Newman and Hobot, 1987). In pre-embedding protocols, this allows the immunoreaction to be completed before dehydration and embedding steps that may destroy antigenicity. Relatively harsh fixations with osmium tetroxide may then be carried out to improve ultrastructure and contrast the reaction product. Immunoenzyme techniques are especially useful when antigen concentration is low or when only small numbers of immunoreactive sites have survived fixation and tissue processing (Pelletier and Morel, 1984). In addition, the electron-dense reaction product is often easy to spot at low magnifications. Unfortunately, immunoenzyme techniques have much lower resolution than procedures that use particulate markers. Reaction product may be artifactually deposited on structures surrounding the antigenic targets or the structures associated with specific antigens may be obscured (Courtoy *et al.*, 1983). In addition, unstained sections must be viewed in order to distinguish clearly the reaction product from surrounding structures (Pelletier and Morel, 1984; Priestley, 1984).

2. *Particulate markers*

Antibody–ferritin conjugates and colloidal gold probes which are bound to a variety of different ligands are the most popular particulate markers in current use. Iron–dextran complexes and hemocyanin have also been conjugated to immunoglobulins, but have not been widely adopted for ultrastructural studies (Miller *et al.*, 1981; Horisberger, 1984). While the small size

(5.5–6.0 nm core) and high resolution of antibody–ferritin conjugates have made them popular, their net negative charge at neutral pH may cause problems with non-specific binding to cellular structures and embedding resins in pre- and post-embedding immunocytochemical techniques (Parr, 1979; Sternberger, 1986). In addition, their small size makes them difficult to distinguish at low magnifications.

The application of colloidal gold probes to electron microscopy was a major advance (Faulk and Taylor, 1971). Colloidal gold offers much greater versatility than other markers because: (i) it can be prepared in a variety of sizes for use in double- and triple-labeling experiments on the same tissue section (Slot and Geuze, 1981, 1984, 1985); (ii) it is suitable for use in both scanning and transmission electron microscopy (Hodges *et al.*, 1987); (iii) it can be conjugated easily to a variety of ligands (Horisberger and Rosset, 1977; Hodges *et al.*, 1984; Horisberger and Clerc, 1985; Lucocq and Baschong, 1986); and (iv) its particulate nature allows precise quantification of labeling in carefully controlled studies (Gagne and Miller, 1987; Howell *et al.*, 1987; Kehle and Herzog, 1987; Kellenberger *et al.*, 1987; Posthuma *et al.*, 1987a).

A variety of different ligand–gold conjugates is available for immunoelectron microscopy, including protein A–gold, avidin–gold, streptavidin–gold, and immunoglobulin–gold (Tolson *et al.*, 1981; Bendayan, 1984; Roth, 1986). Protein A is a cell-wall constituent of most strains of *Staphylococcus aureus* that binds with high affinity to the Fc portion of immunoglobulin (Ig) (Goding, 1978; Langone, 1982). Protein A binds particularly well to IgG from hosts such as rabbits and guinea-pigs, but relatively poorly to IgG from sheep or goats or to any of the subclasses of IgG from mice (Richman *et al.*, 1982). When mouse monoclonal antibodies are used for immunolabeling, this problem can be circumvented by conjugating the monoclonal antibody directly to colloidal gold. To avoid the preparation of separate probes for each antibody, most workers label with a secondary antibody, e.g. rabbit anti-mouse IgG, followed by incubation with protein A–gold or immunoglobulin–gold. This approach intensifies the immunolabeling and allows the use of a single protein A–gold or immunoglobulin–gold probe for a variety of different monoclonal and polyclonal antibodies. Protein G, a cell-wall protein of a human group G streptococcal strain, has been purified and used to prepare gold probes. Like protein A, it binds specifically to the Fc fragment of immunoglobulin molecules (Bendayan, 1987). Björck (1988) recently described protein L, an Ig-binding protein which has been isolated from the surface of *Peptococcus magnus*. This protein shows considerable promise for immunocytochemistry because it binds κ and λ light chains from all classes of immunoglobulins.

Avidin, a protein isolated from egg whites, has been used for over 40 years

as a ligand for immunocytochemical studies because of its high affinity for the coenzyme biotin. Avidin–biotin marker systems depend on the specific binding of avidin-marker conjugates to biotinylated antibodies or tissue antigens. In recent years, streptavidin has been isolated from cultures of *Streptomyces avidini* and used in place of avidin (Bonnard *et al.*, 1984). Streptavidin is similar to avidin in its affinity for biotin, but is superior as a ligand because it is non-glycosylated, has a neutral pI, and is consequently less likely to bind non-specifically to tissues (Hofmann *et al.*, 1980; Bonnard *et al.*, 1984). Consequently, streptavidin has been used more widely than avidin in preparation of gold conjugates, although neither ligand has been as popular as protein A or immunoglobulin.

D. IMMUNOCYTOCHEMICAL CONTROLS

1. *Method specificity*

A number of non-immunological factors can cause inaccurate labeling of tissues. These factors include interaction of antibodies or probes with unquenched aldehyde groups, hydrophobic or ionic interactions of reagents with tissue components or embedding media, and pseudoperoxidase or endogenous peroxidase activity in some cells or organelles (Van Leeuwen, 1986). In addition, naturally occurring antibodies or contaminating antibodies that were raised to a poorly purified immunogen may bind to tissues to give a deceptively specific, but inaccurate, immunolocalization (Petrusz, 1983; Van Leeuwen, 1986). The following controls should be included in every labeling procedure to test the specificity of the method.

(i) Incubation with secondary antibodies and markers, or with markers alone, to establish that an immunoreaction is dependent on a complete series of sequentially added reagents.

(ii) Substitution of primary antibody with preimmune serum or an irrelevant monoclonal antibody of the same class and subtype and use of a dilution series with both primary and control sera to test for tissue binding by mechanisms unrelated to antigen–antibody reactions (Buffa *et al.*, 1979; Grube, 1980). This control will also help to determine optimal antibody concentrations and whether polyclonal sera contain naturally occurring antibodies to tissue antigens other than the one(s) under study.

(iii) Whenever available, use of positive controls that were fixed and embedded by the same procedures. These help to prevent false-negative

results and confirm that antigenic sites survived fixation and are accessible to antibodies.

2. *Serum specificity*

Confirming the specificity of polyclonal or monoclonal antibodies is the most important part of an immunocytochemical study. Since antibodies recognize only relatively small regions or sites on the surface of antigens, they cannot distinguish between unrelated polypeptides that have shared or cross-reacting epitopes. Polyclonal antisera may also contain contaminating antibodies which recognize antigens other than the intended one. In addition, cross-reactions are affected by: (i) the affinity of the antibodies, e.g. antibodies with low affinity for a specific antigen may be capable of binding totally dissimilar antigens; (ii) the class of the antibody, e.g. IgM antibodies have a high avidity and are "stickier" than IgG antibodies; and (iii) the structure of the antigenic target, e.g. antigens with repeating amino acid sequences seem to be more susceptible to cross reactions (Ghosh and Campbell, 1986). Another complication is the observation that the type and characteristics of the blocking agent, e.g. pH, ionic strength, detergent and protein carrier, may enhance or inhibit cross reactions (Ghosh and Campbell, 1986). Pool *et al.* (1983), Van Leeuwen (1982, 1986) and Van der Sluis and Boer (1986) have described controls that should be used to establish specificity of an antibody in immunocytochemical procedures. Unfortunately, a test that can unequivocally establish the specificity of a serum or antibody on intact tissues or tissue sections has not yet been devised (Van Leeuwen, 1982, 1986). Electrophoretic transfer of proteins from sodium dodecyl sulfate polyacrylamide gels to a suitable matrix, followed by an immunoblot with the serum or antibody of interest, is commonly used to establish specificity (Cumming and Burgoyne, 1985). The results may be misleading, however, since the relationships between an antigen and surrounding tissue are destroyed during extraction and purification. Since no single test can prove antibody specificity, most authors recommend using as many independent lines of evidence as possible (Childs, 1983b). These include adsorption tests with homologous or heterologous antigen to abolish immunolabeling and use of negative control tissue that lacks the antigen under investigation. When available, a panel of monoclonal antibodies to different epitopes on the same molecule may be useful for evaluating specificity; however, results may still be misleading since fixation and processing may destroy some epitopes and create other cross-reacting epitopes on unrelated molecules.

III. Applications to Parasitology

A. *Plasmodium*

1. *Erythrocytic stages*

(*a*) *Merozoite surface coat.* The primary surface antigens of the *Plasmodium falciparum* merozoite are derived from a large precursor glycoprotein (Pf195) that is synthesized late in schizogony and proteolytically processed at the time of merozoite release into smaller molecular weight fragments (Lyon *et al.*, 1987; Holder *et al.*, 1987). Several laboratories have identified fragments of 83 kDa, 42 kDa, and 19 kDa molecular mass in extracts of free merozoites that were surface-labeled by lactoperoxidase-catalyzed iodination, as well as a variety of other processing fragments that are shed from the merozoite surface (Holder and Freeman, 1984; McBride and Heidrich, 1987; Camus *et al.*, 1987; Lyon *et al.*, 1987). Standard transmission electron microscopy and biochemical studies have shown that most of the merozoite surface coat is shed at the time of erythrocyte invasion (Aikawa *et al.*, 1978). Only a few processing fragments of low molecular weight are detectable in erythrocytes infected with ring forms (Holder *et al.*, 1985a).

Vaccination experiments with purified and synthetic fragments of Pf195 have been successful in inducing partial and complete immunity to challenge infections in *Aotus* and *Saimiri* monkeys as well as humans (Cheung *et al.*, 1986; Siddiqui *et al.*, 1987; Patarroyo *et al.*, 1988). Both pre- and post-embedding immunolabeling with immune serum or monoclonal antibodies to Pf195 and specific processing fragments have localized epitopes associated with this molecule at the surface of merozoites and mature schizonts (Fig. 1) (Langreth and Reese, 1979; Howard *et al.*, 1984, 1985; Pirson and Perkins, 1985; Atkinson *et al.*, 1987a; Chulay *et al.*, 1987), suggesting that circulating antibodies may bind to merozoites and inhibit invasion of erythrocytes or facilitate phagocytosis of the parasites by macrophages and neutrophils.

Similar high molecular weight glycoproteins have been identified in mature schizonts of *P. knowlesi* and *P. yoelii* and localized on the surface of mature schizonts and merozoites by immunoelectron microscopy (Epstein *et al.*, 1981; David *et al.*, 1984; Oka *et al.*, 1984; Aikawa *et al.*, 1986a). Aikawa *et al.* (1986a) used cryo-ultramicrotomy and protein A–gold to localize the 143/140 kDa fragment of the *P. knowlesi* merozoite surface antigen on the endoplasmic reticulum and plasma membrane of multinucleated *P. knowlesi* schizonts (Fig. 2). These observations indicated that the molecule is synthesized first in the endoplasmic reticulum before being transported to

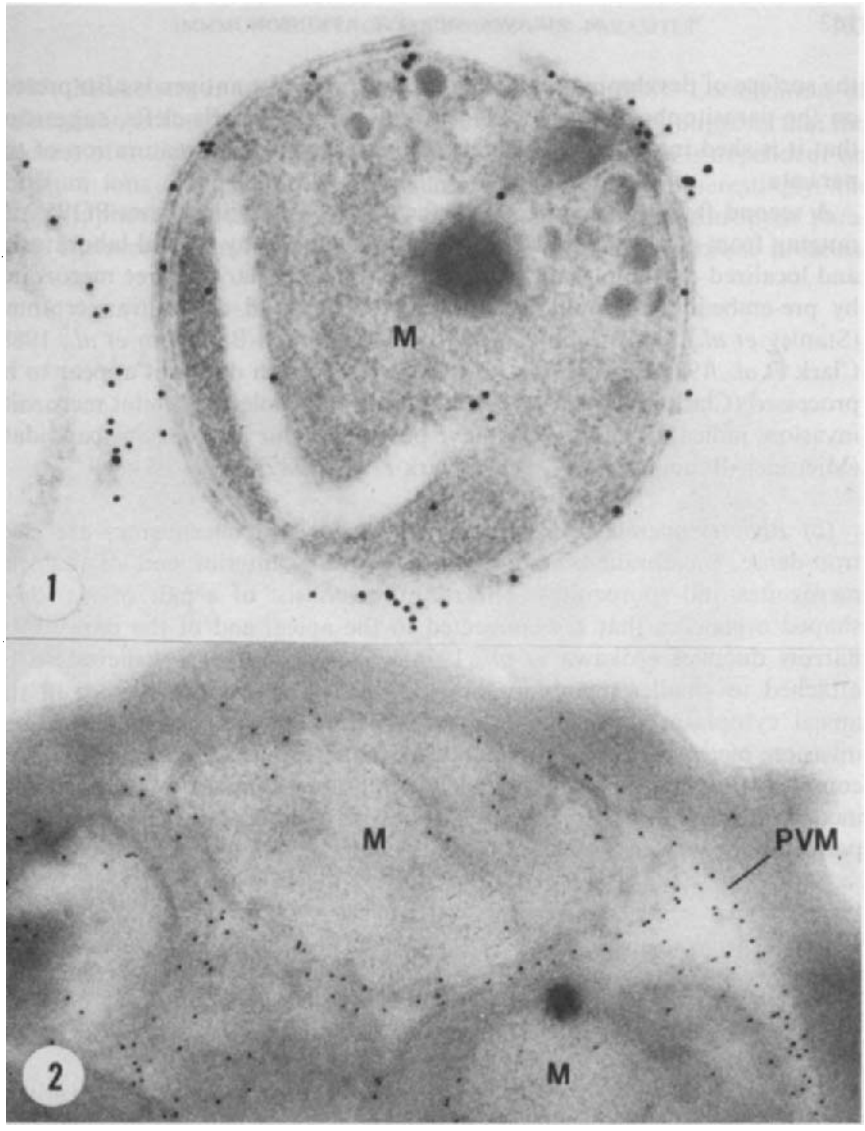

FIG. 1. LR White section of a *P. falciparum* merozoite (M) labeled with human hyperimmune serum and protein A-gold. Gold particles are found on the surface of the merozoite, indicating that potential protective epitopes are associated with the merozoite surface coat. × 43 000.

FIG. 2. Cryosection of a *P. knowlesi* schizont, labeled with a mouse monoclonal antibody to protective 142/140 kDa antigens. Label is associated with the surface of merozoites (M) and with the parasitophorous vacuole membrane (PVM). × 35 000. (Reproduced by permission from Aikawa *et al.*, 1986, *European Journal of Cell Biology*, **41**, 207–213.)

the surface of developing schizonts. Interestingly, the antigen is also present on the parasitophorous vacuole membrane and Maurer's clefts, suggesting that it is shed into the intracellular compartment during maturation of the parasite.

A second family of merozoite surface antigens, distinct from Pf195 and ranging from 45 kDa to 56 kDa, has been identified by several laboratories and localized on the plasma membrane of intracellular and free merozoites by pre-embedding immunoelectron microscopy and cryo-ultramicrotomy (Stanley *et al.*, 1985; Epping *et al.*, 1988; Miettinen-Baumann *et al.*, 1988; Clark *et al.*, 1989). In contrast to Pf195, this antigen does not appear to be processed (Clark *et al.*, 1989). Antibodies to this molecule inhibit merozoite invasion, indicating that it may have potential value as a vaccine candidate (Miettinen-Baumann *et al.*, 1988; Clark *et al.*, 1989).

(*b*) *Rhoptry-microneme complex.* Rhoptries and micronemes are electron-dense, membrane-bound organelles at the anterior end of malarial merozoites and sporozoites. The rhoptries consist of a pair of tear-drop shaped organelles that are connected to the apical end of the parasite by narrow ductules (Aikawa *et al.*, 1978). The rhoptries are believed to be attached to smaller membrane-bound, electron-dense micronemes in the apical cytoplasm to form an interconnected complex. During host-cell invasion, merozoites appear to secrete material from the rhoptry–microneme complex which perturbs the host-cell membrane/cytoskeleton and becomes incorporated into both the erythrocyte membrane and the invaginating parasitophorous vacuole membrane (Aikawa *et al.*, 1981a; Brown *et al.*,

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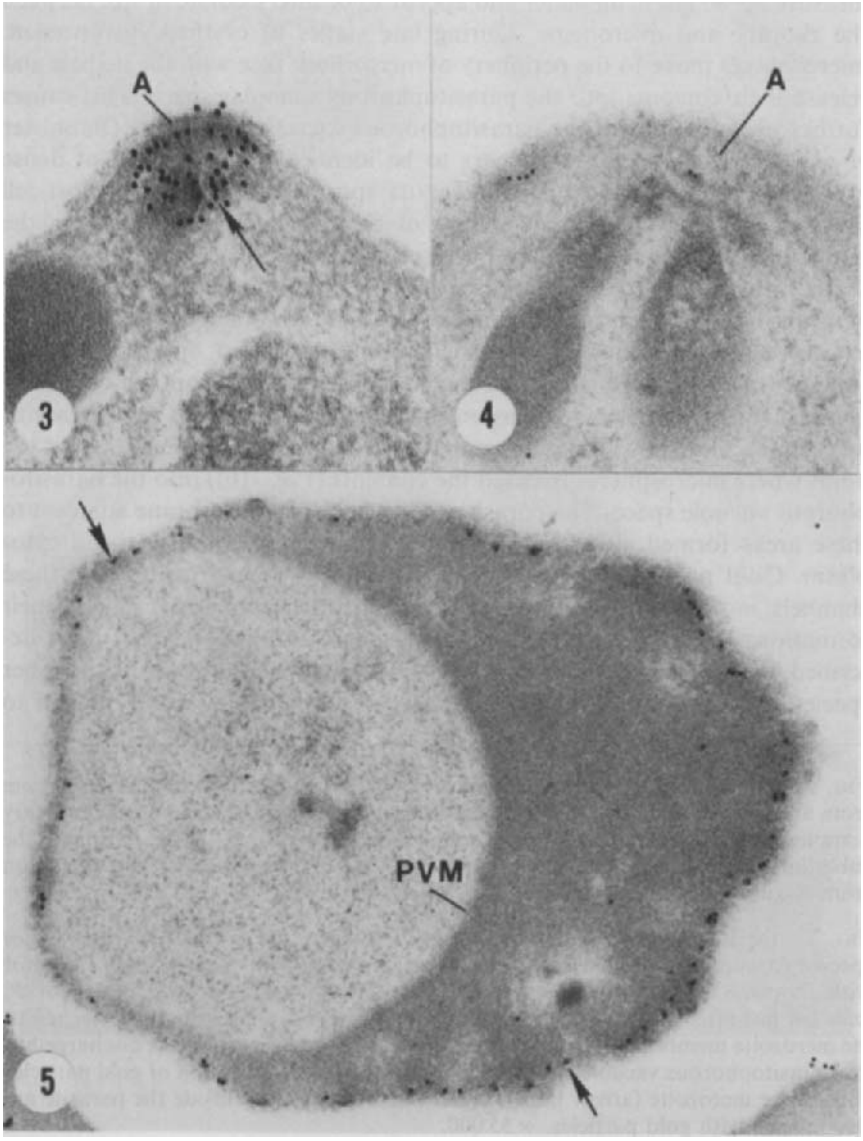
FIG. 3. LR White section of a *P. falciparum* merozoite, labeled with a sheep polyclonal antibody to calmodulin. Gold particles (arrow) are concentrated at the apical end (A) of the merozoite, demonstrating the presence of calmodulin. × 74 000. (Reproduced by permission from Matsumoto *et al.*, 1987, *European Journal of Cell Biology*, **45**, 36–43.)

FIG. 4. LR White section of a *P. falciparum* merozoite that was treated with a calmodulin inhibitor; section labeled with sheep polyclonal antibody to calmodulin. Note absence of gold particles at the apical end (A) of the merozoite. × 56 000. (Reproduced by permission from Matsumoto *et al.*, 1987, *European Journal of Cell Biology*, **45**, 36–43.)

FIG. 5. LR White section of an erythrocyte infected with *P. falciparum*, labeled with a mouse monoclonal antibody to glycophorin A. Note the absence of this erythrocyte integral membrane protein from around the parasitophorous vacuole membrane (PVM), indicating that the protein is not carried into the erythrocyte by the invading merozoite. Label is present on the erythrocyte membrane (arrows). × 26 000. (Reproduced by permission from Atkinson *et al.*, 1987, *European Journal of Cell Biology*, **45**, 192–199.)

1985; Mikkelsen *et al.*, 1988; Sam-Yellowe *et al.*, 1988). Localization of calmodulin in the rhoptries by immunoelectron microscopy suggests that the process of attachment and invasion of the host erythrocyte is dependent on calcium ions (Figs 3 and 4) (Matsumoto *et al.*, 1987). Interestingly, the parasitophorous vacuole membrane that surrounds intraerythrocytic parasites is devoid of erythrocyte cytoskeletal and integral membrane proteins,



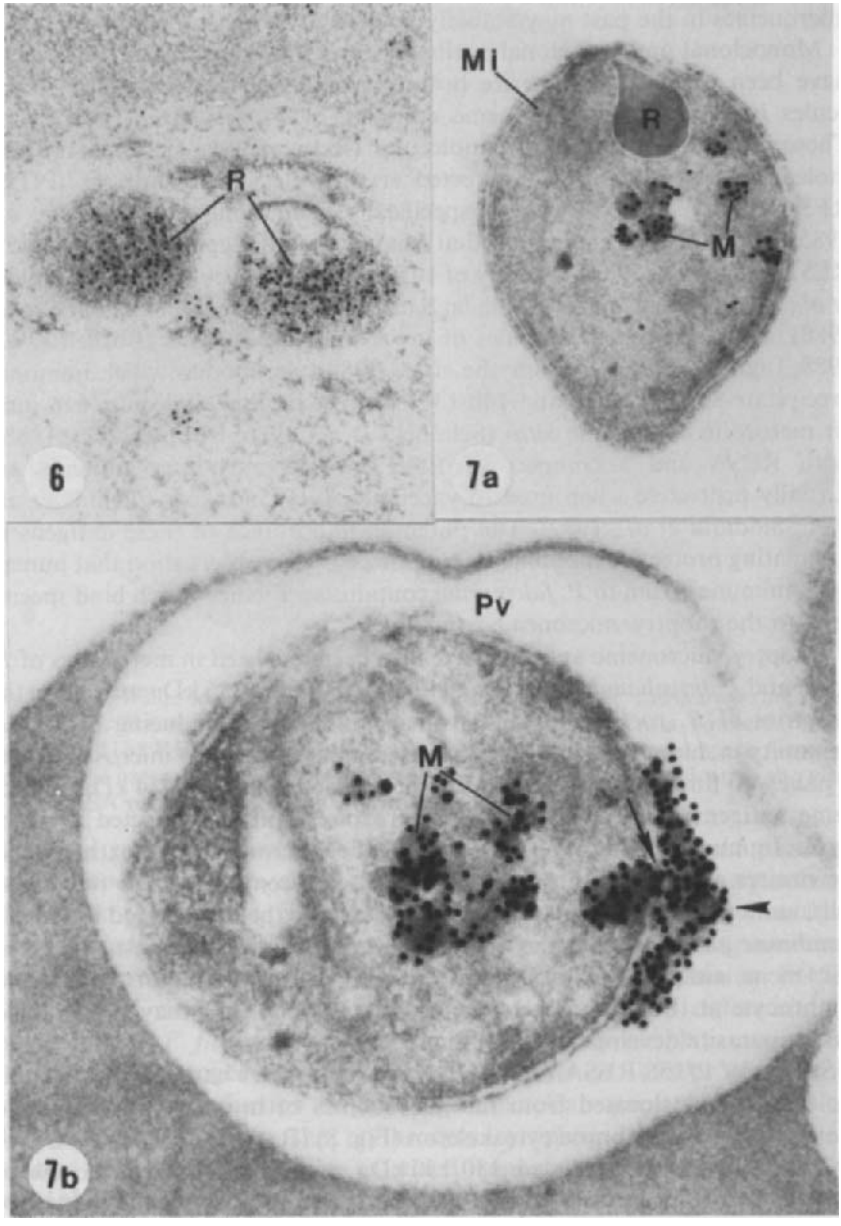
suggesting that it either originates from rhoptry-microneme secretions or develops from an extensively modified erythrocyte plasma membrane (Fig. 5) (Aikawa *et al.*, 1978, 1981a; Atkinson *et al.*, 1987b; Dluzewski *et al.*, 1988).

In 1975, Bannister *et al.* described microspheres (also called dense granules) at the apical end of *P. knowlesi* merozoites. These organelles are round, measure 50–80 nm in diameter and appear to be intermediate in size between the rhoptry and microneme. During late stages of erythrocyte invasion, microspheres move to the periphery of merozoites, fuse with the pellicle and release their contents into the parasitophorous vacuolar space. This causes further invaginations of the parasitophorous vacuole membrane (Bannister *et al.*, 1975). This process appears to be identical to the fusion of dense granules with the pellicle of *Sarcocystis* sporozoites soon after host-cell invasion and the subsequent release of electron-dense material into the parasitophorous vacuole space (Entzeroth *et al.*, 1986).

Recently Torii *et al.* 1989b performed immunoelectron microscopy with antibodies which react specifically with *P. knowlesi* microspheres. In extracellular merozoites, gold particles were associated only with microspheres (Fig. 7(a)), while no labeling was observed on rhoptries or micronemes. During erythrocyte invasion, microspheres moved to the surface of merozoites. Gold label was aggregated at the outside of the merozoites at the point where microspheres released the contents (Fig. 7(b)) into the parasitophorous vacuole space. The parasitophorous vacuole membrane adjacent to these areas formed elongated finger-like channels into the host-cell cytoplasm. Gold particles extended from the surface of merozoites into these channels, indicating that contents of the microspheres may have caused their formation. Although discharge of microsphere contents has not been described, these organelles also appear to be present in the merozoites of other species of *Plasmodium*. Since micronemes and microspheres are difficult to

FIG. 6. LR White section of a *P. falciparum* merozoite labeled with immune serum from an *Aotus* monkey which had been vaccinated with a 102/132/143 kDa rhoptry complex. Gold particles are associated with the rhoptries (R), confirming the subcellular location of this immune target. × 46 500. (Reproduced by permission from Atkinson *et al.*, 1987a, *Journal of Parasitology* **73**, 1235–1240.)

FIG. 7. (a) LR White section of a *P. knowlesi* merozoite labeled with antibodies specific to microspheres (M). Label is associated only with microspheres (M), not with rhoptries (R) and micronemes (Mi). × 34 000. (b) LR White section of *P. knowlesi* just after host cell invasion, labeled similarly. A microsphere is attached to the merozoite membrane (arrow) and the content of the microsphere is discharged to the parasitophorous vacuole space (PV), resulting in an aggregation of gold particles outside the merozoite (arrow head). Other microspheres (M) inside the parasite are also labeled with gold particles. × 55 000.


R
6
Mi
R
M
7a
Pv
M
7b

distinguish morphologically, antigens which were thought to be present in micronemes in the past may actually be present in microspheres.

Monoclonal and polyclonal antibodies and immunoelectron microscopy have been used to characterize both low and high molecular mass molecules in the rhoptry–microneme complex of *P. falciparum* merozoites. These consist of 240/225 kDa molecules (Roger *et al.*, 1988), a 155 kDa molecule known as the ring-infected erythrocyte surface antigen (Pf155/RESA) that has been localized specifically in micronemes (Brown *et al.*, 1985), 130/150 kDa molecules that share common epitopes with Pf155/RESA (Uni *et al.*, 1987), a family of 105–145 kDa molecules (Fig. 6) (Holder *et al.*, 1985b; Atkinson *et al.*, 1987a; Sam-Yellowe *et al.*, 1988; Cooper *et al.*, 1988), and a group of molecules of lower mass, 40–80 kDa (Bushell *et al.*, 1988; Ingram *et al.*, 1988; Smythe *et al.*, 1988). Antibodies which immunoprecipitate 80/66/42 kDa and 140/130/105 kDa rhoptry molecules can inhibit merozoite invasion *in vitro* (Schofield *et al.*, 1986; Cooper *et al.*, 1988). Both RESA and a complex of 102/132/143 kDa rhoptry proteins are partially protective when used to vaccinate *Aotus* monkeys (Collins *et al.*, 1986; Siddiqui *et al.*, 1987). The potential importance of these antigens in stimulating protective immunity is supported by the observation that human hyperimmune serum to *P. falciparum* contains antibodies which bind specifically to the rhoptry–microneme complex.

Rhoptry–microneme antigens have also been localized in merozoites of *P. yoelii* and *P. brasilianum*. Oka *et al.* (1984) localized a 235 kDa antigen in the rhoptries of *P. yoelii* merozoites which is capable of inducing protective immunity in mice. Torii *et al.* (1989a) used immunoelectron microscopy and a panel of three monoclonal antibodies to localize 18/16/14 kDa microneme antigens of *P. brasilianum* in merozoites and ring-infected erythrocytes. Immunolabeling was detected in the micronemes of extracellular merozoites, on the erythrocyte membrane of recently invaded red blood cells, and on knobs and caveolae that developed in the infected red blood cell membrane as the parasites matured. These observations suggest that some microneme antigens are inserted into the plasma membrane of the infected erythrocyte at the time of host-cell invasion, where they may be modified during parasite development into knobs and caveolae.

Studies of Pf155/RESA of *P. falciparum* merozoites have shown that this molecule is translocated from the micronemes of invading merozoites to the erythrocyte membrane/cytoskeleton (Fig. 8) (Brown *et al.*, 1985). Uni *et al.* (1987) localized a related 130/150 kDa molecule of *P. falciparum* in micronemes of merozoites and on the membrane of newly-invaded erythrocytes, suggesting that it is secreted into the membrane by merozoites at the time of invasion. Unlike the situation with *P. brasilianum*, the antigens were no longer detectable on the erythrocyte membrane after the parasites

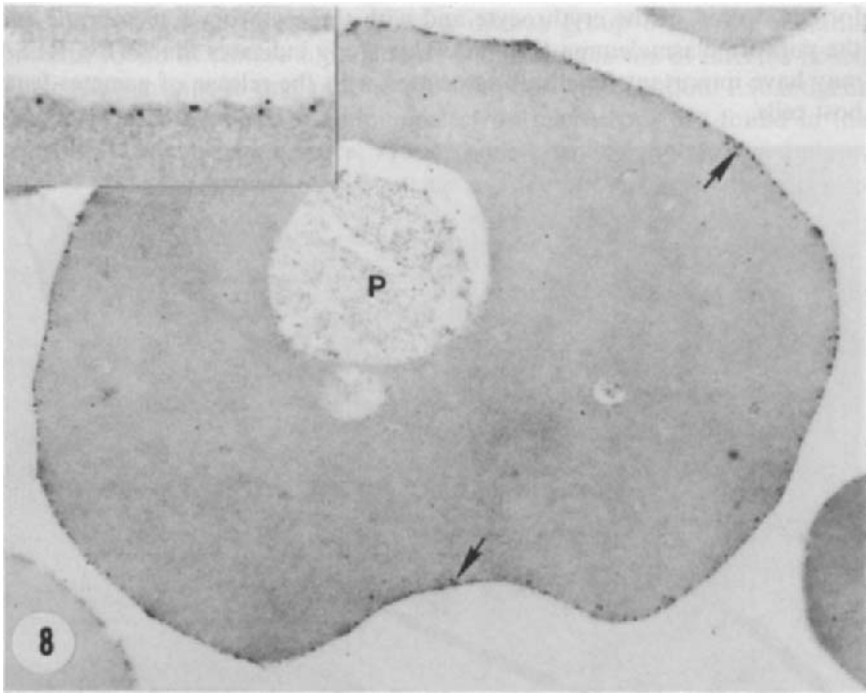


FIG. 8. LR White section of an erythrocyte infected with a *P. falciparum* ring stage parasite (P). The section was labeled with a rabbit antiserum to a synthetic repeat region of Pf155/RESA. Gold label on the infected erythrocyte membrane (arrows) indicates the presence of Pf155/RESA. × 13 000. Inset: higher magnification showing Pf155/RESA on the infected erythrocyte membrane. × 42 000.

developed into trophozoites. Uni *et al.* (1987) found that gold particles became more numerous within the parasite and the erythrocyte cytoplasm adjacent to the parasite. Significant labeling was found in the erythrocyte cytoplasm adjacent to mature gametocytes. A similar study using immunoelectron microscopy and polyclonal and monoclonal antibodies to Pf155/RESA found dense immunolabeling around mature and differentiating gametocytes of *P. falciparum*, suggesting that this molecule plays an important role in gametogenesis (Figs 9–11) (Quakyi *et al.*, 1989). Within five minutes after the induction of gametogenesis, clear spaces surrounded by Pf155/RESA formed in the erythrocyte cytoplasm around developing gametes (Fig. 10). These spaces extended from a region immediately adjacent to the parasite to the erythrocyte membrane. Fifteen minutes after the induction of gametogenesis, gametes were extracellular or within a lysed erythrocyte membrane that was densely labeled by antibody to Pf155/RESA and colloidal gold. Labeling of lysed erythrocytes was associated with the

former stroma of the erythrocyte and with the erythrocyte membrane and the parasite plasmalemma (Fig. 11). This study indicates that Pf155/RESA may have important functions associated with the release of gametes from host cells.

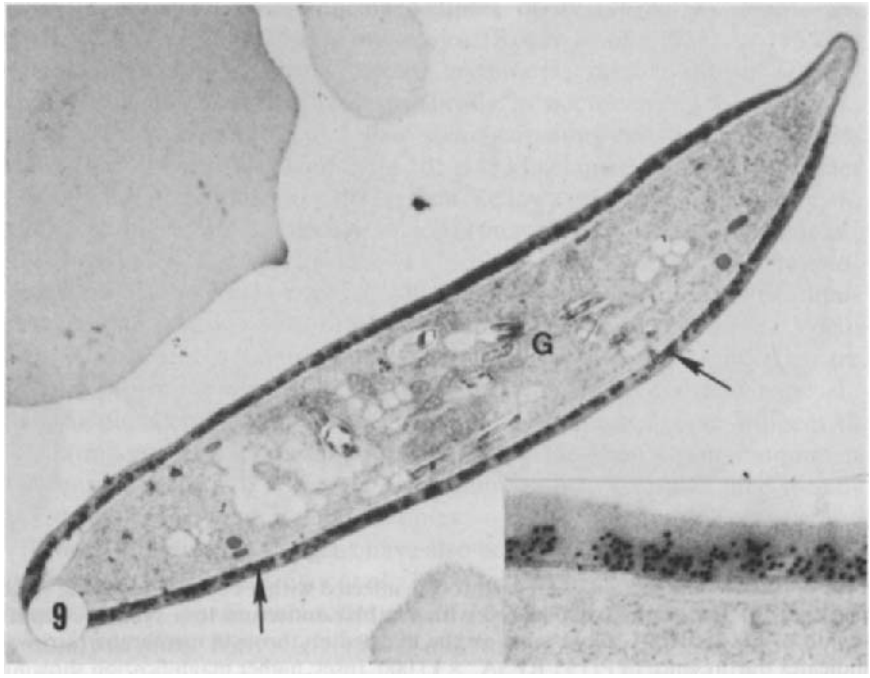
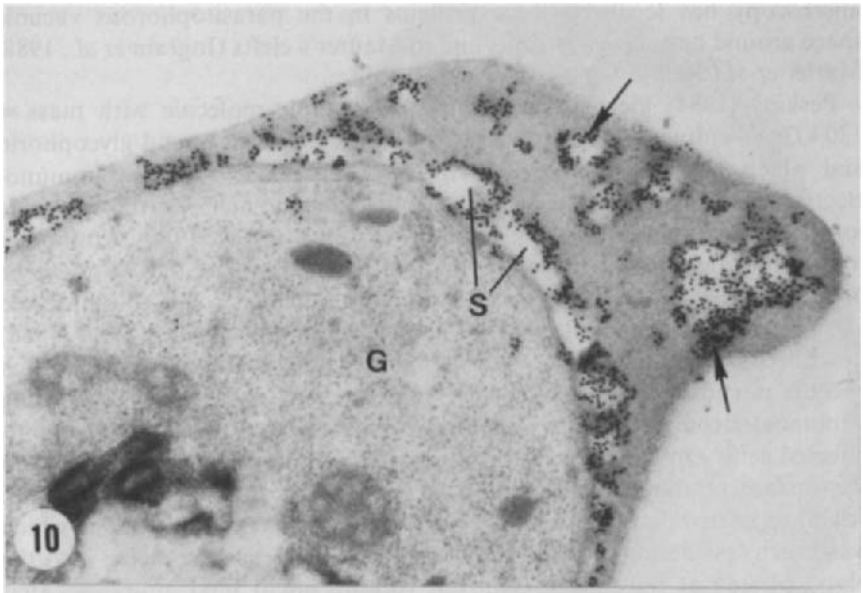


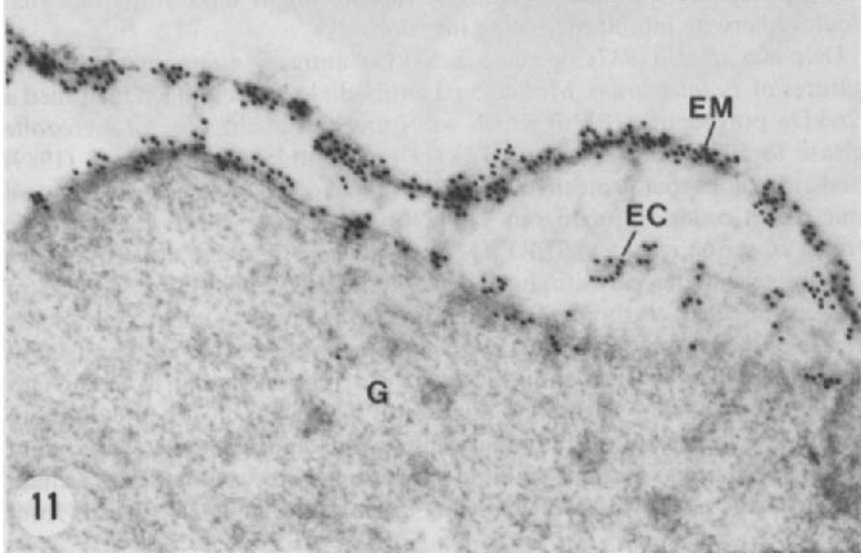
FIG. 9. LR White section of an erythrocyte infected with a *P. falciparum* gametocyte (G), labeled with a human monoclonal antibody which identifies Pf155/RESA. Note the presence of Pf155/RESA (arrows) in the erythrocyte cytoplasm adjacent to the parasite. × 8250. (Reproduced by permission of the American Society for Microbiology from Quakyi *et al.*, 1989, *Infection and Immunity*, **57**, 833–839.) Inset: higher magnification showing Pf155/RESA in the erythrocyte cytoplasm. × 38 000.

FIG. 10. LR White section of a *P. falciparum* gametocyte (G) fixed within 5 min after induction of gametogenesis. Pf155/RESA (arrows) is associated with clear spaces (S) within the erythrocyte cytoplasm. × 21 000. (Reproduced by permission of the American Society for Microbiology from Quakyi *et al.*, 1989, *Infection and Immunity*, **57**, 833–839.)

FIG. 11. As gametogenesis progresses, gold particles associated with Pf155/RESA become scattered throughout the lysed erythrocyte in association with remnants of the erythrocyte cytoplasm (EC) and the erythrocyte membrane (EM), suggesting that this protein is important in release of gametes from host erythrocytes. G, *P. falciparum* gametocyte. × 29 000. (Reproduced by permission of the American Society for Microbiology from Quakyi *et al.*, 1989, *Infection and Immunity*, **57**, 833–839.) × 29 000.

(*c*) *Exoantigens.* Exoantigens are a diverse group of soluble malarial proteins found in culture supernatants and in the plasma of infected hosts. Their relationships to one another are still poorly understood. Exoantigens that have been localized by immunoelectron microscopy are found in the parasitophorous vacuole space, Maurer's clefts and erythrocyte cytoplasm around schizonts and mature segmenters. They appear to be released into





culture supernatants or plasma when infected erythrocytes rupture and release merozoites, although at least one 72 kDa histidine-rich exoantigen (PfHRP2) is secreted by intact cells (Howard *et al.*, 1986).

S-antigens are the largest group of exoantigens which have been characterized, and consist of a family of serologically diverse, heat-stable proteins of large but variable molecular weights and poor immunogenicity (Wilson *et al.*, 1975; Anders *et al.*, 1983; Cowman *et al.* 1985). Immunoelectron microscopy has localized these proteins in the parasitophorous vacuole space around maturing schizonts and in Maurer's clefts (Ingram *et al.*, 1988; Mattei *et al.*, 1988).

Perkins (1984) identified a soluble, heat-stable molecule with mass = 130 kDa in culture supernatants of *P. falciparum* which bound glycophorin and which could be localized on the surface of merozoites by immunoelectron microscopy. A repeat sequence of 50 amino acids on this molecule formed a binding domain for glycophorin (Kochan *et al.*, 1986). Antibodies to this molecule inhibited merozoite invasion, suggesting that it might be involved in the initial recognition and binding of merozoites to uninfected erythrocytes. More recently, Bianco *et al.* (1987) and Bonnefoy *et al.* (1988) described complementary deoxyribonucleic acid (cDNA) clones which encode portions of this molecule and localized it by post-embedding immunoelectron microscopy within the erythrocyte cytoplasm of schizont-infected cells. Only a minor proportion of the protein appeared to bind to the surface of intracellular and extracellular merozoites. Bonnefoy *et al.* (1988) suggested that this protein might be a distinct type of S-antigen which was much less abundant than polymorphic S-antigens. Its release into the blood plasma at the time of schizont rupture might bind antibodies that would otherwise inhibit merozoite invasion.

Delplace *et al.* (1987) detected a 50 kDa antigen in supernatants from cultures of *P. falciparum*. Monoclonal antibodies to this antigen identified a 126 kDa polypeptide (P126) which was processed at the time of merozoite release to 50 kDa, 47 kDa, and 18 kDa polypeptides. Knapp *et al.* (1989) used antibodies to a protective 140 kDa antigen of *P. falciparum* to isolate a gene which codes an analogous 113 kDa polypeptide with a characteristic stretch of serine residues (SERP I). Both laboratories were able to localize this antigen in the parasitophorous vacuole space and Maurer's clefts of schizont-infected erythrocytes, suggesting that it plays some role in the release of merozoites. Cochrane *et al.* (1988) identified a 120 kDa antigen of *P. brasilianum* which has an intracellular distribution similar to P126. This antigen first appears as discrete aggregates within the host cell cytoplasm around late trophozoites. In immature schizonts and segmenters, abundant labeling becomes associated with the parasitophorous vacuole space around budding merozoites (Fig. 12). Labeling in mature segmenters spreads from

the parasitophorous vacuole space into the erythrocyte cytoplasm, suggesting that this antigen, like P126, may have proteolytic functions that are important in the final stages of merozoite budding and the disruption and breakdown of the erythrocyte membrane.

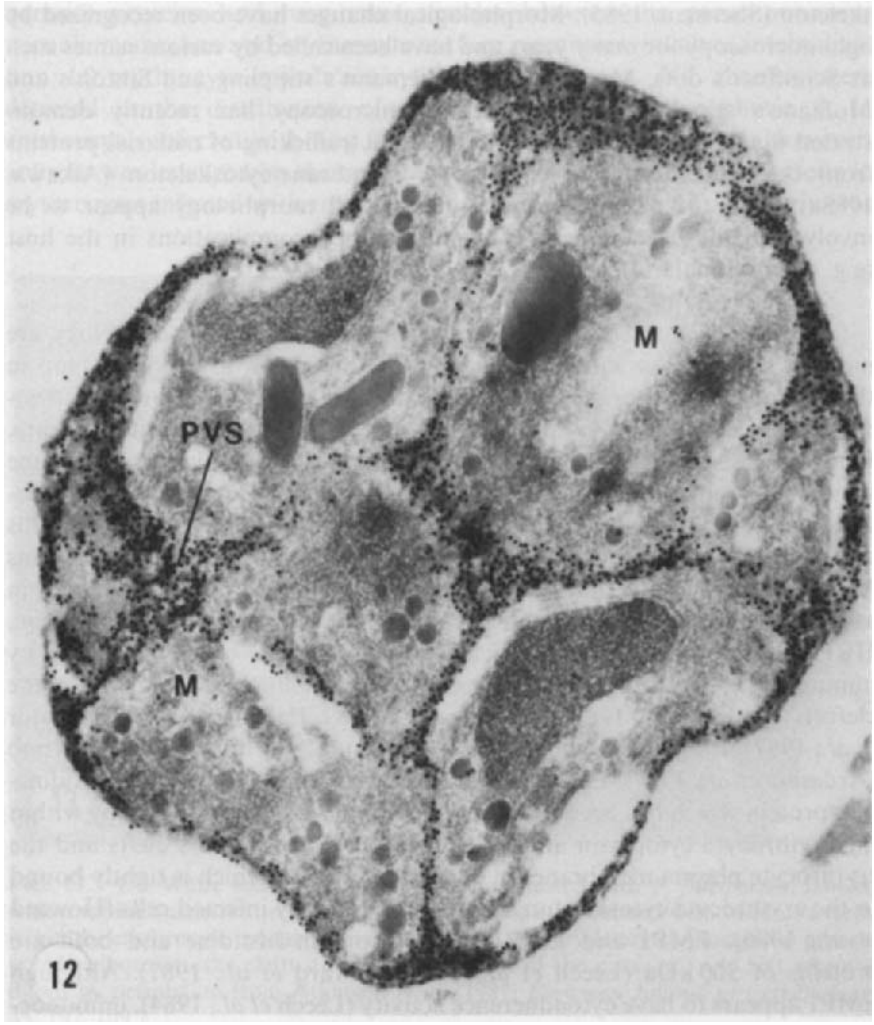


FIG. 12. LR White section of a *P. brasilianum* schizont, labeled with a mouse monoclonal antibody to a 120 kDa antigen. Gold particles are associated with the parasitophorous vacuole space (PVS) and erythrocyte cytoplasm around the mature merozoites (M). × 27 000. (Reproduced by permission of the American Society for Microbiology from Cochrane *et al.*, 1988, *Infection and Immunity*, **56**, 2080–2088.)

(*d*) *Host-cell alterations induced by* Plasmodium. The erythrocytic stages of malarial parasites induce significant morphological and functional changes in infected erythrocytes including structural alterations, i.e. knobs, clefts, caveolae, and caveolae–vesicle complexes, and changes in the composition and antigenic characteristics of the erythrocyte membrane/cytoskeleton (Sherman, 1985). Morphological changes have been recognized by light microscopy for many years and have been called by various names such as Schüffner's dots, Maurer's clefts, Ziemann's stippling and Sinton's and Mulligan's stippling. Immunoelectron microscopy has recently demonstrated that these changes are related to the trafficking of malarial proteins from the parasite to the erythrocyte membrane/cytoskeleton (Aikawa, 1988a). Some of these alterations in host-cell morphology appear to be involved in the development of malaria-related complications in the host (e.g. cerebral malaria).

(i) *Plasmodium falciparum.* Several changes in host cell morphology are induced by infection with *P. falciparum.* Knob-like protrusions develop in the erythrocyte membrane/cytoskeleton and clefts with associated electron-dense material appear in the erythrocyte cytoplasm as parasites mature. Knobs are conical 40 nm protrusions of the erythrocyte plasma membrane which are underlain by electron-dense material. Knobs possess cytoadherence activity and often form focal junctions with the endothelial cells and adjacent erythrocytes (Aikawa, 1988b). At least four malarial proteins (HRP1, HRP2, EMP1 and EMP2) have been identified on the surface or in association with the cytoskeleton of erythrocytes infected with *P. falciparum.* HRP1 is a 90 kDa histidine-rich protein that has been localized by immunoelectron microscopy within electron-dense knob material and in the clefts (Ardeshir *et al.*, 1987; Culvenor *et al.*, 1987; Pologe *et al.*, 1987; Taylor *et al.*, 1987). It appears to be related to the structural formation of the knob (Ardeshir *et al.*, 1987). HRP2 is a different, 70 kDa water-soluble histidine-rich protein which has been localized by immunoelectron microscopy within the erythrocyte cytoplasm and in association with Maurer's clefts and the erythrocyte plasma membrane. In contrast to HRP1, which is tightly bound to the erythrocyte cytoskeleton, HRP2 is secreted by infected cells (Howard *et al.*, 1986). EMP1 and EMP2 do not contain histidine and both are proteins of 300 kDa (Leech *et al.*, 1984; Howard *et al.*, 1987). Although EMP1 appears to have cytoadherence activity (Leech *et al.*, 1984), immunoelectron microscopy has so far failed to demonstrate its presence on the surface of the knobs. EMP2 has been localized in the parasitophorous vacuole of schizonts, within membrane-bound vesicles in the erythrocyte cytoplasm and in association with knobs and the cytoplasmic side of the erythrocyte membrane/cytoskeleton (Coppel *et al.*, 1986; Howard *et al.*, 1987).

Several recent studies have shown that both malarial and host-cell antigens are associated with the Maurer's clefts of erythrocytes infected with *P. falciparum*. Atkinson *et al.* (1987b) studied the distribution of ankyrin (Fig. 13), spectrin, band 3 (Fig. 14), and glycophorin A (Fig. 5) in erythrocytes infected with *P. falciparum*, to determine whether movement of parasite proteins and membrane vesicles between the parasitophorous vacuole membrane and erythrocyte surface membrane involves internalization of erythrocyte membrane/cytoskeletal proteins. No changes were detected in the distribution of spectrin, band 3 or glycophorin A in erythrocytes infected with ring forms, trophozoites, or schizonts. However, ankyrin was localized on the cytoplasmic face of flattened Maurer's clefts in erythrocytes infected with trophozoites or schizonts (Fig. 13). Ankyrin was

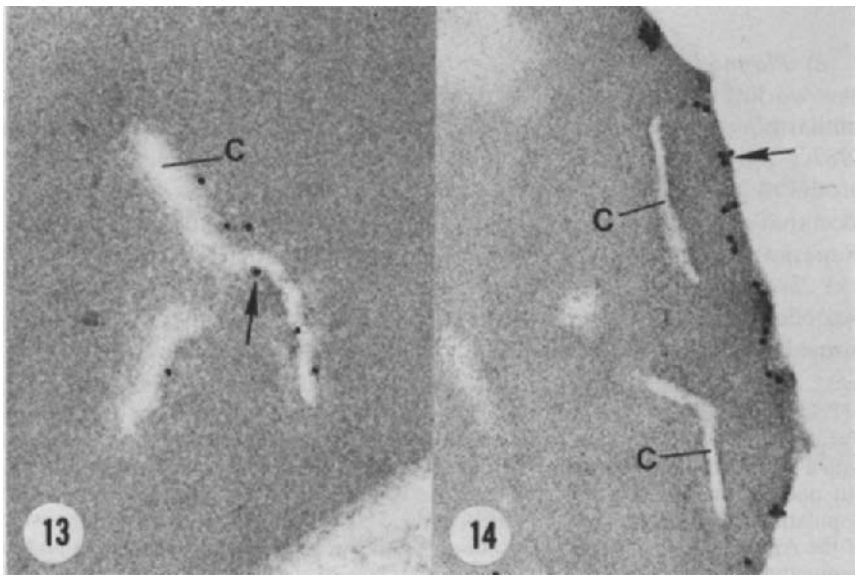


FIG. 13. LR White section of an erythrocyte infected with *P. falciparum*, labeled with a rabbit antiserum to ankyrin. Label (arrow) is associated with Maurer's clefts (C) in the erythrocyte cytoplasm, indicating that this erythrocyte cytoskeletal protein is redistributed to the clefts during development of the parasite. × 45 500. (Reproduced by permission from Atkinson *et al.*, 1987, *European Journal of Cell Biology*, **45**, 192–199.)

FIG. 14. LR White section of an erythrocyte infected with *P. falciparum*, labeled with a rabbit antiserum to band 3. In contrast to ankyrin, the distribution of this erythrocyte integral membrane protein does not change within infected red blood cells. Label (arrow) is associated with the erythrocyte membrane. No label is seen in association with clefts (C). × 38 000. (Reproduced by permission from Atkinson *et al.*, 1987, *European Journal of Cell Biology*, **45**, 192–199.)

not detected on a second population of circular or vesicular clefts that appeared to originate directly from the parasitophorous vacuole membrane. The presence of ankyrin on short, flattened Maurer's clefts supports the idea that the clefts connect to the surface of erythrocyte and may function in the transport of knob-associated electron-dense material.

Malarial proteins that have been localized on Maurer's clefts in erythrocytes infected with *P. falciparum* include exoantigens. e.g P126 and HRP2 (Howard *et al.*, 1986; Delplace *et al.*, 1987; Ingram *et al.*, 1988), knob-associated HRP1 (Culvenor *et al.*, 1987), EMP2 (=MESA) (Coppel *et al.*, 1986; Howard *et al.*, 1987), and 15/19 kDa and 46 kDa molecules which appear to be integral parts of the cleft membranes (Hui and Siddiqui, 1988; Kara *et al.*, 1988a,b). Both Hui and Siddiqui (1988) and Kara *et al.* (1988b) suggested that these molecules may play a role in transport of parasite antigens to the erythrocyte membrane/cytoskeleton.

(ii) *Plasmodium brasilianum.* This species is a quartan malarial parasite of new world monkeys which has morphological, immunological and genetic similarities to *P. malariae* of man (Sterling *et al.*, 1972; Atkinson *et al.*, 1987c; Lal *et al.*, 1988). Many monoclonal antibodies that have been produced to *P. brasilianum* cross-react with *P. malariae*, supporting the idea that these two organisms are closely related (Cochrane *et al.*, 1988; Nagasawa *et al.*, 1988).

P. brasilianum, like *P. falciparum*, causes the formation of knobs and cytoplasmic clefts within infected erythrocytes. Cytoplasmic clefts are membrane-bound and can be divided into three types, namely short clefts, long

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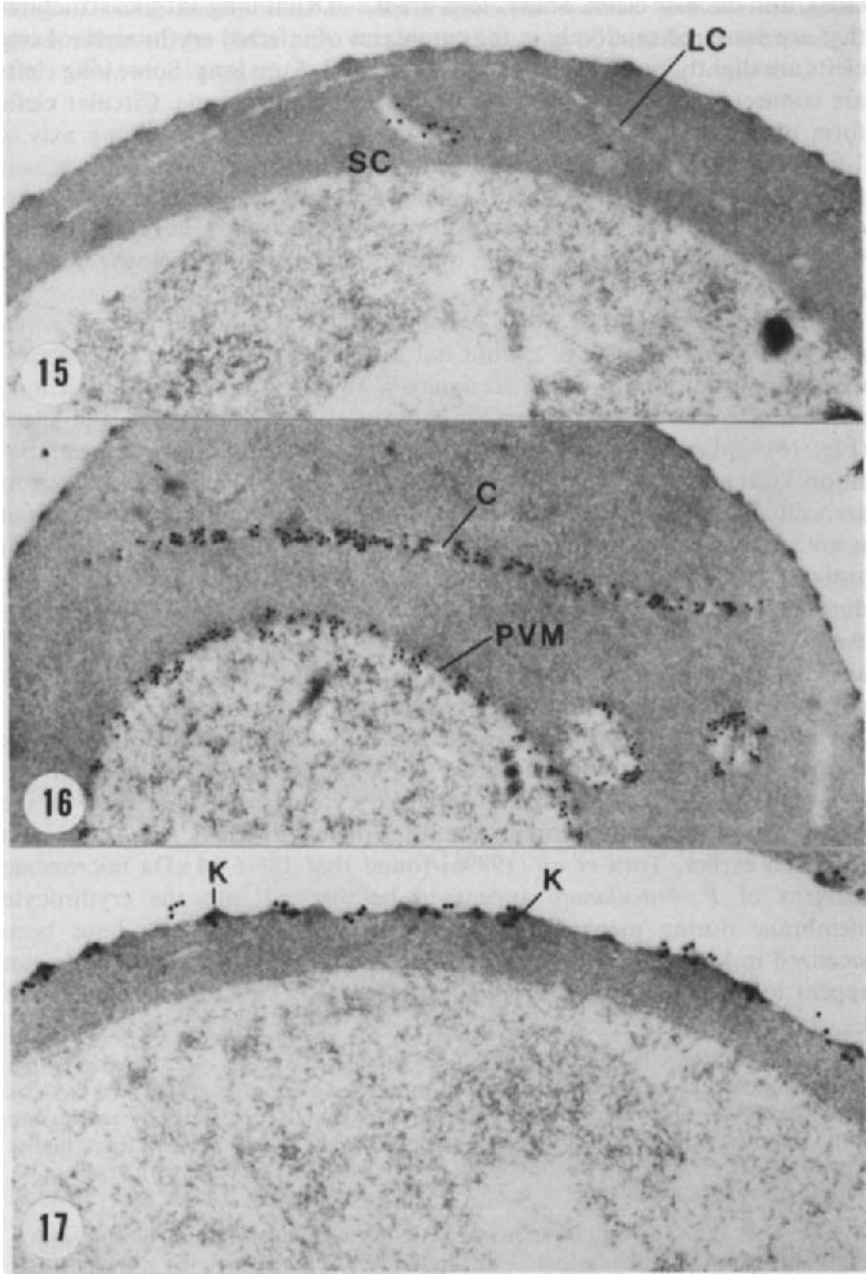
FIG. 15. LR White section of an erythrocyte infected with *P. brasilianum*, labeled with a mouse monoclonal antibody to a 38 kDa antigen. Label is on short clefts (SC), but not on long clefts (LC), indicating an antigenic difference between these two populations of membranous structures. ×31 000. (Reproduced by permission of the American Society for Microbiology from Cochrane *et al.*, 1988, *Infection and Immunity*, **56**, 2080–2088.)

FIG. 16. LR White section of an erythrocyte infected with *P. brasilianum*, labeled with a mouse monoclonal antibody to a 16 kDa antigen. The distribution of this molecule is restricted to long clefts (C) and the parasitophorous vacuole membrane (PVM), suggesting a close relationship. ×22 000. (Reproduced by permission of the American Society for Microbiology from Cochrane *et al.*, 1988, *Infection and Immunity*, **56**, 2080–2088.)

FIG. 17. LR White section of an erythrocyte infected with *P. brasilianum*, labeled with a mouse monoclonal antibody to 18/16/14 kDa antigens. Label is associated with electron-dense knobs (K) in the erythrocyte membrane. ×37 000. (Reproduced by permission of the American Society for Microbiology from Cochrane *et al.*, 1988, *Infection and Immunity*, **56**, 2080–2088.)


LC
SC
15
C
PVM
16
K
K
17

clefts, and circular clefts. Short clefts are 0.3–0.8 μm long slit-like structures that are scattered randomly in the cytoplasm of infected erythrocytes. Long clefts are slightly curved or undulating and are 3–5 μm long. Some long clefts are connected with the parasitophorous vacuole membrane. Circular clefts form oblong loops in the erythrocyte cytoplasm and have a long axis of 1.5–2.3 μm. The morphological relationships among these types of clefts is unclear, but it has generally been assumed that they develop as a single interconnected network from the parasitophorous vacuole membrane. Recent work has shown, however, that they differ in antigenic composition (Cochrane *et al.*, 1988).

Monoclonal antibodies which recognize a 38 kDa antigen of *P. brasilianum* label short clefts (Fig. 15) but not long or circular clefts. By contrast, monoclonal antibodies which recognize a 16 kDa antigen label both long and circular clefts and the parasitophorous vacuole membrane and space (Fig. 16), indicating that these structures have a similar antigenic composition (Cochrane *et al.*, 1988). The fact that the long-cleft antigen is associated with the parasitophorous vacuole, whereas the short-cleft antigen is not, suggests that there are regional differences in the distribution of cleft antigens or two or more distinct populations of cytoplasmic clefts. Three-dimensional maps of the intracellular distribution of these antigens may eventually help to answer this question. It is clear, however, that structural and functional relationships between these populations of clefts may be more complicated than previously recognized.

Maurer's clefts are believed to function in the transport of electron-dense knob material erythrocytes infected with *P. falciparum* (Aikawa *et al.*, 1986b). It is unclear, however, what role cytoplasmic clefts play in the transport of knob material in erythrocytes infected with *P. brasilianum*. As discussed earlier, Torii *et al.* (1989a) found that 18/16/14 kDa microneme antigens of *P. brasilianum* appear to be inserted into the erythrocyte membrane during merozoite invasion. These same antigens have been localized in knobs of erythrocytes infected with *P. brasilianum*, but do not appear to be present on clefts (Figs 15–17). It is possible that the nature of

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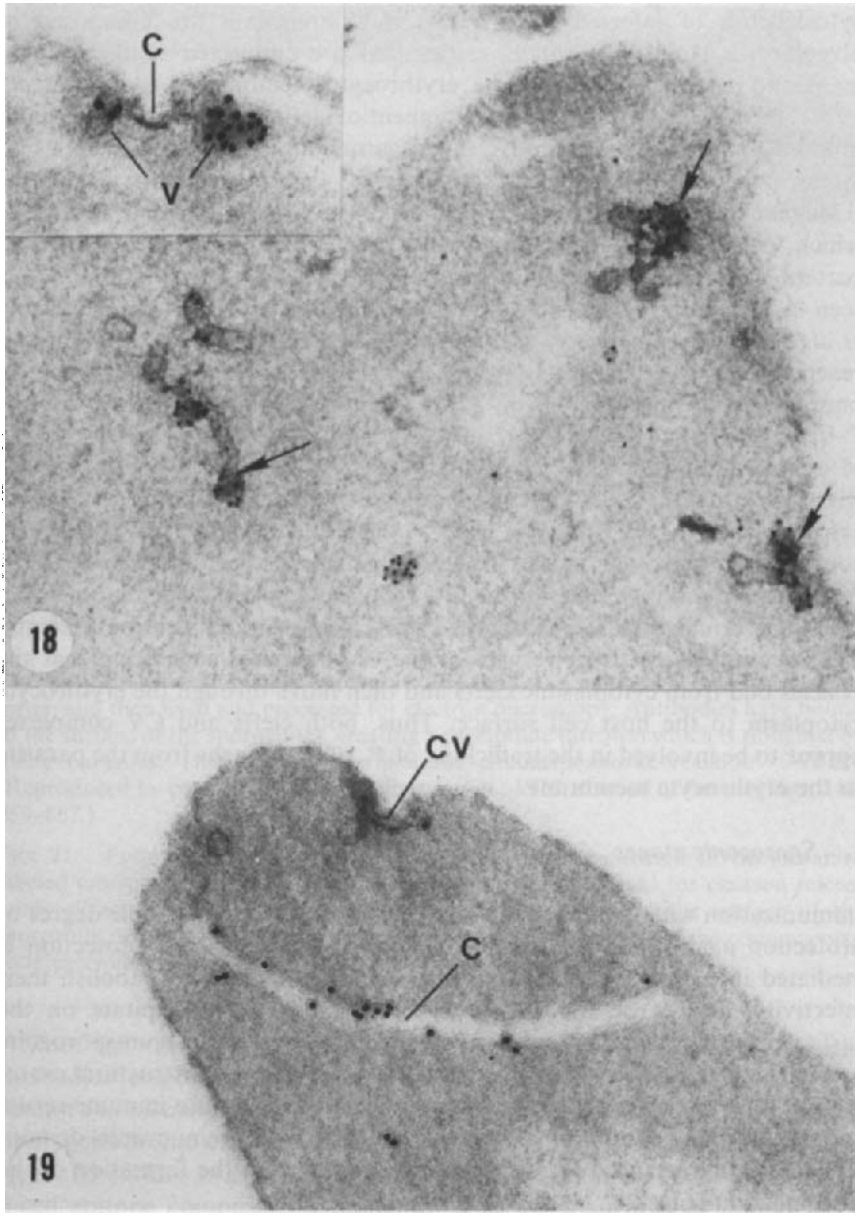
FIG. 18. LR White sections of an erythrocyte infected with *P. vivax*, labeled with a mouse monoclonal antibody to an 86 kDa antigen, which is associated with vesicles of the caveola–vesicle complexes (arrows). ×48 000. (Reproduced by permission from Udagama *et al.*, 1988, *American Journal of Pathology*, **131**, 48–52.) Inset: higher magnification of a caveola–vesicle complex. Gold label is associated with vesicles (V), but not caveola (C). ×60 000.

FIG. 19. LR White section of an erythrocyte infected with *P. vivax*, labeled with a mouse monoclonal antibody to a 28 kDa antigen which is associated with clefts (C) and caveola–vesicle complexes (CV), suggesting movement between the two. ×63 000.

antigens being transported to the erythrocyte surface and the antigenic composition of the clefts may vary with the stage of parasite development.



(iii) *Plasmodium vivax*. Erythrocytes infected with *P. vivax* exhibit a fine stippling composed of reddish dots when stained with Romanovsky stains and examined by light microscopy. Electron microscopy has shown that these dots correspond to caveolae–vesicle (CV) complexes in the membrane/cytoskeleton of infected erythrocytes. CV complexes are composed of alveolar-like clusters of small vesicles that are connected to the base of invaginations or caveolae in the erythrocyte membrane (Aikawa *et al.*, 1977). *P. vivax* also causes the development of clefts within the cytoplasm of infected erythrocytes. Recently, investigators have produced a series of monoclonal antibodies against antigens of *P. vivax* erythrocytic stages (Udagama *et al.*, 1988; Matsumoto *et al.*, 1988a). Monoclonal antibodies which identify 95 kDa and 86 kDa *P. vivax* proteins produce a stippled pattern by immunofluorescence microscopy similar to the Schüffner's dots seen in Romanovsky-stained erythrocytes infected with *P. vivax* (Udagama *et al.*, 1988; Matsumoto *et al.*, 1988a). Other monoclonal antibodies which react with a 28 kDa protein produce a linear pattern when viewed by immunofluorescence microscopy (Matsumoto *et al.*, 1988a).

Immunoelectron microscopy has been used to identify the precise location of these *P. vivax* antigens. The 95 kDa and 86 kDa proteins are associated with vesicles of the CV complex and with scattered vesicles in the erythrocyte cytoplasm (Fig. 18) (Udagama *et al.*, 1988; Matsumoto *et al.*, 1988a). By contrast, the 28 kDa protein is associated primarily with cytoplasmic clefts, but is also found in vesicles of the CV complex (Fig. 19) (Matsumoto *et al.*, 1988a). A double-labeling technique clearly demonstrated that both 28 and 95 kDa antigens occur in vesicles of the CV complex, suggesting that the vesicles bud from cytoplasmic clefts and then move through the erythrocyte cytoplasm to the host cell surface. Thus, both clefts and CV complexes appear to be involved in the trafficking of *P. vivax* antigens from the parasite to the erythrocyte membrane.

2. *Sporogonic stages*

Immunization with irradiated sporozoites produces a considerable degree of protection against rodent, simian and human malaria. This protection is mediated in part by antibodies which neutralize sporozoites, abolish their infectivity, and cause the formation of a thread-like precipitate on the surface of parasites. This reaction has been named the circumsporozoite precipitation (CSP) reaction (Vanderberg *et al.*, 1969). Ultrastructural examination of sporozoites that have been incubated with mouse immune serum and an anti-mouse immunoglobulin conjugated with hemocyanin demonstrated the importance of antisporozoite antibodies in the formation of the CSP reaction (Figs 20, 21) (Cochrane *et al.*, 1976).

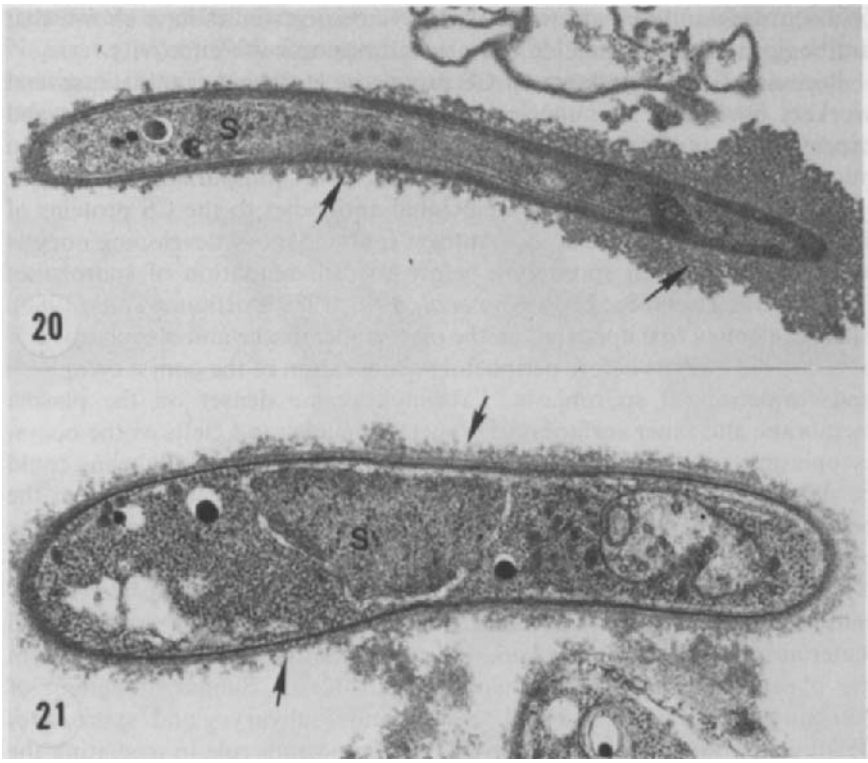


FIG. 20. Epon section of a sporozoite (S) that was incubated in hyperimmune serum and then fixed and processed for electron microscopy. Antibodies have bound to the surface of the sporozoites, creating a precipitate (arrows) which is sloughed by living parasites. This has been termed the circumsporozoite reaction. × 20 000 (Reproduced by permission from Cochrane *et al.*, 1976, *Journal of Immunology*, **116**, 859–867.)

FIG. 21. Epon section of a sporozoite (S) that was incubated in hemocyanin-labeled antisporozoite antibodies and then fixed and processed for electron microscopy. Presence of angular hemocyanin crystals (arrows) on the surface of the sporozoite demonstrates the role of antibodies in the formation of the circumsporozoite precipitate reaction. × 16 000.

Since the study by Yoshida *et al.* (1981), monoclonal and polyclonal antibodies to sporozoite antigens from several species of rodent, simian, and human *Plasmodium* have been produced. Most of these antibodies recognize a polypeptide known as the circumsporozoite (CS) protein which completely coats the surface of sporozoites. CS proteins contain an immunodominant B-cell epitope composed of tandemly repeated sequences of amino acids

(Nussenzweig and Nussenzweig, 1985). Numerous studies have shown that antibodies to this polypeptide can neutralize sporozoite infectivity.

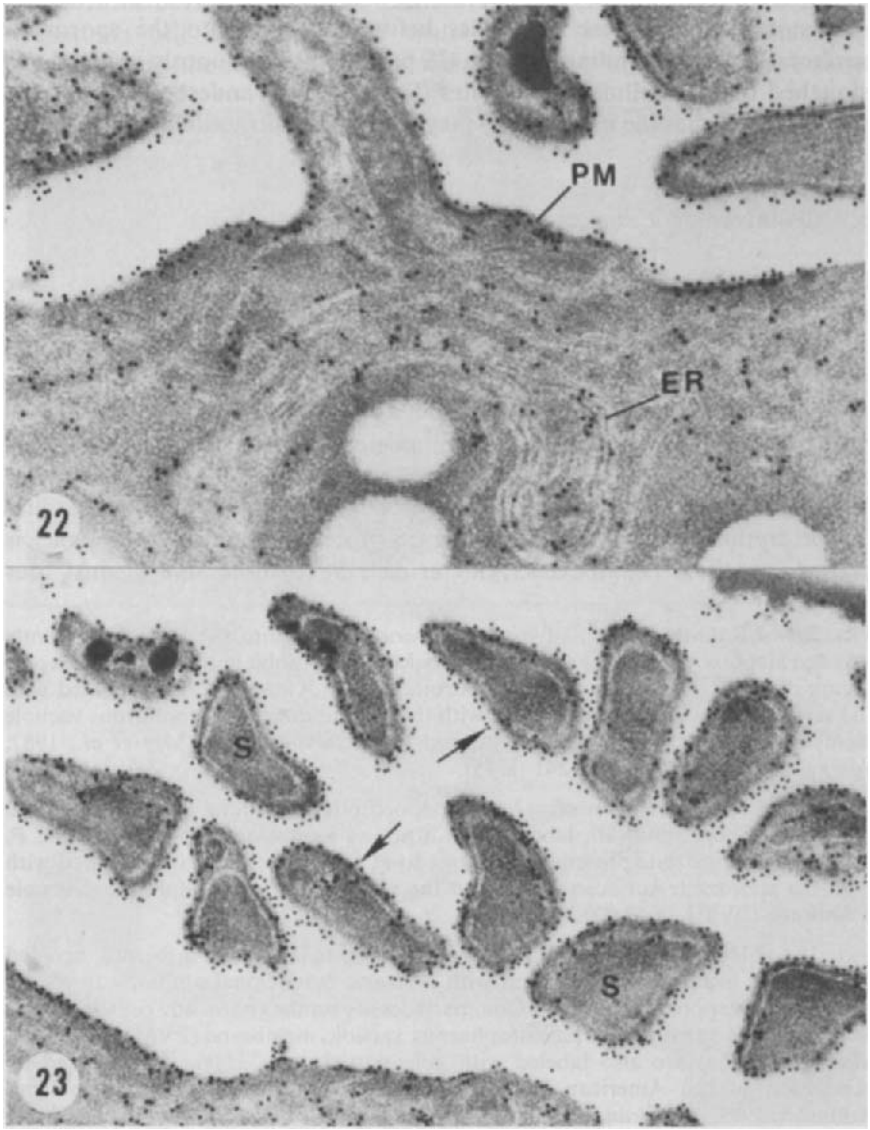
Because of the importance of CS protein as a vaccine candidate, several workers have used immunoelectron microscopy to trace its origins and expression throughout the life cycle of malarial parasites. Immunoelectron microscopy of the sporogonic development of *P. falciparum*, *P. ovale*, *P. malariae* and *P. berghei* with monoclonal antibodies to the CS proteins of each species has shown that this antigen is abundant in developing oocysts and appears early in sporogony before the differentiation of sporozoites (Hamilton *et al.*, 1988a; Nagasawa *et al.*, 1987, 1988; Posthuma *et al.*, 1988). Specific labeling first appeared on the plasma membrane and cytoplasm of 5- to 6-day old oocysts before peripheral vacuolization of the oocyst cytoplasm and formation of sporoblasts. Labeling became denser on the plasma membrane and inner surface of peripheral vacuoles and clefts as the oocyst cytoplasm was subdivided into sporoblasts. At the same time, labeling could be detected on perinuclear membranes and endoplasmic reticulum in the oocyst cytoplasm, indicating that the CS proteins are synthesized in the cytoplasm of sporoblasts before export to the plasma membrane (Fig. 22). As developing sporoblasts contracted away from the oocyst capsule, significant labeling remained associated with its inner surface as well as with material in the subcapsular space, indicating that CS antigen sloughs from the plasma membrane of developing sporoblasts. Similar sloughing of CS antigen has been observed from mature salivary gland sporozoites (Posthuma *et al*, 1987b), and may play an important role in mediating the gliding motility of sporozoites (Stewart and Vanderberg, 1988). As sporozoites bud from the surface of sporoblasts, they are uniformly covered with CS antigen on the outer surface of their plasma membranes (Fig. 23). Studies of *P. berghei* have shown that all three membranes of the sporozoite pellicle contain CS protein (Aikawa *et al.*, 1981b; Hamilton *et al.*, 1988a; Atkinson *et al.*, 1989a). Mature oocyst sporozoites as well as salivary-gland sporozoites are labeled on their surface with equal intensity by antibodies to the CS protein and also exhibit internal labeling on micronemes, perinuclear

FIG. 22. LR Gold section of a *P. ovale* oocyst labeled with a mouse monoclonal antibody to the *P. ovale* circumsporozoite protein, which is located on endoplasmic reticulum (ER) and the plasma membrane (PM) of a developing sporoblast. ×22 000. (Reproduced by permission of the American Society for Microbiology from Nagasawa *et al.*, 1987, *Infection and Immunity*, **55**, 2928–2932.)

FIG. 23. LR Gold section of a *P. ovale* oocyst labeled with a mouse monoclonal antibody to the *P. ovale* circumsporozoite protein. Gold label (arrows) is associated with the surface of the sporozoites (S). ×42 000. (Reproduced by permission of the American Society for Microbiology from Nagasawa *et al.*, 1987, *Infection and Immunity*, **55**, 2928–2932.)

membranes and Golgi apparatus (Fine *et al.*, 1984; Hamilton *et al.*, 1988a; Nagasawa *et al.*, 1988; Posthuma *et al.*, 1988). These findings suggest that continuous synthesis of CS protein by sporozoites may enhance their infectivity.

In contrast to the merozoite stages of *Plasmodium*, little is known about



the antigenic composition of the the rhoptry–microneme complex of sporozoites. Immunoelectron microscopy of *P. knowlesi*, *P. malariae* and *P. berghei* sporozoites with monoclonal antibodies to the CS protein and colloidal gold has localized this antigen within micronemes (Fine *et al.*, 1984; Nagasawa *et al.*, 1988; Atkinson *et al.*, 1989a). Fine *et al.* (1984) found labeling on the membranes of micronemes in *P. knowlesi* sporozoites and occasionally on rhoptries, and speculated that the antigen may be processed and stored within these organelles before movement to the sporozoite surface. The recent finding that the CS protein is continuously secreted and sloughed by extracellular sporozoites (Stewart and Vanderberg, 1988) supports the idea that the micronemes may serve as an intracellular reservoir for this molecule.

3. *Exoerythrocytic stages*

It is only recently that improvements in culture techniques *in vitro* have made the exoerythrocytic stages of malarial parasites available for antigenic analysis. Most studies have used immunofluorescence microscopy and antibodies to antigens from erythrocytic parasites to trace their expression in exoerythrocytic schizonts (Hollingdale *et al.*, 1983; Aley *et al.*, 1987a; Szarfman *et al.*, 1988). Studies by immunoelectron microscopy have focused on this protein.

Unlike the merozoite surface coat, which is mostly shed when merozoites invade erythrocytes, all or most of the CS protein of malarial sporozoites is carried into host hepatocytes. Aley *et al.* (1987b) used sera against four

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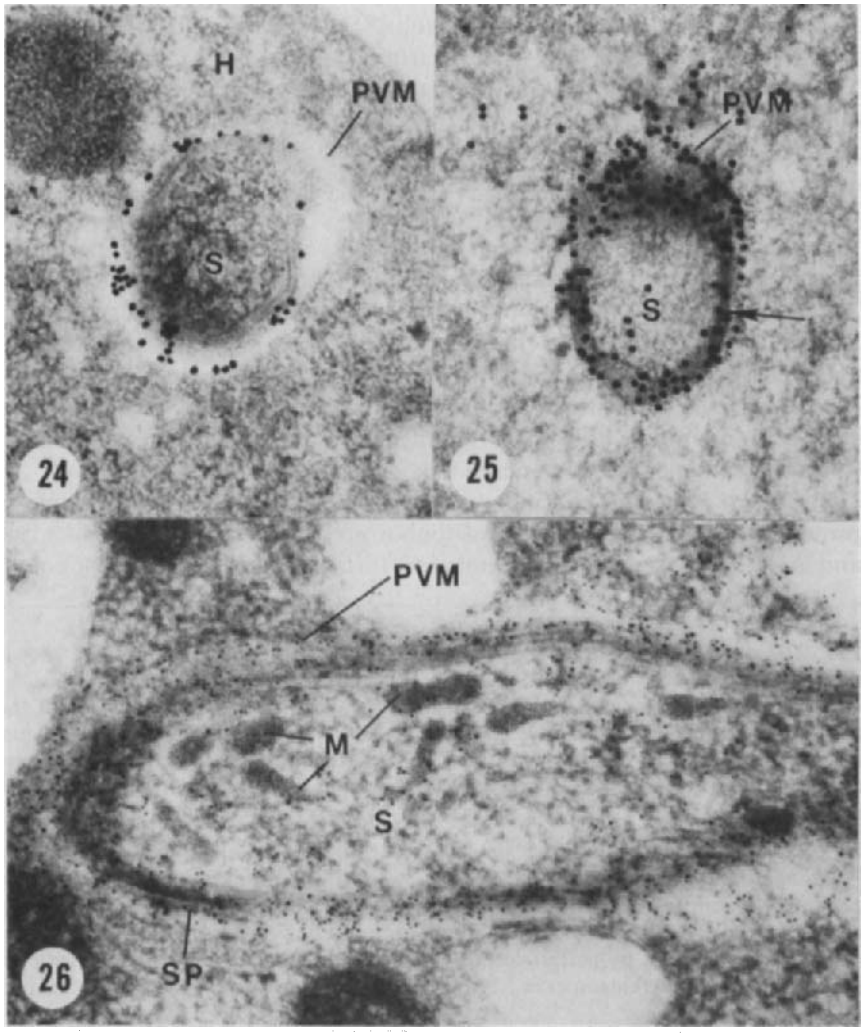
FIG. 24. LR Gold section of a *P. falciparum* sporozoite (S) within a recently invaded HepG2-A16 hepatoma cell (H), labeled with a rabbit antiserum to the repeat region of the *P. falciparum* circumsporozoite protein. Gold label is associated with the surface of the sporozoite, but not with the surrounding parasitophorous vacuole membrane (PVM). ×42 000. (Reproduced by permission from Aley *et al.*, 1987, *Journal of Parasitology*, **73**, 1241–1245).

FIG. 25. LR Gold section of a *P. vivax* sporozoite (S) within a recently invaded HepG2-A16 hepatoma cell, labeled with a mouse monoclonal antibody to the *P. vivax* circumsporozoite protein. In contrast to *P. falciparum*, label is associated with both the sporozoite surface (arrow) and the surrounding parasitophorous vacuole membrane (PVM). ×42 000.

FIG. 26. LR Gold section of a *P. berghei* sporozoite (S) within a recently invaded HepG2-A16 hepatoma cell, labeled with a mouse monoclonal antibody to the *P. berghei* circumsporozoite protein. Gold particles are on the sporozoite pellicle (SP) as well as on the surrounding parasitophorous vacuole membrane (PVM) and space. Micronemes (M) are also labeled with gold particles. ×72 000. (Reproduced by permission of the American Society of Tropical Medicine and Hygiene from Atkinson, 1989, *American Journal of Tropical Medicine and Hygiene*, **41**, 9–17.)

defined regions of the *P. falciparum* CS protein, including both repeat and non-repeat regions, to determine whether or not particular regions of the CS protein behave differently during the invasion process. Antisera to each of the four regions bound specifically to the outer surface and pellicle of sporozoites after invasion of HepG2-A16 hepatoma cells, but not to the plasma membrane or surface of infected host cells or to the parasitophorous vacuole membrane around the intracellular sporozoites (Fig. 24). Studies of hepatoma cell invasion by *P. vivax* and *P. berghei* sporozoites using antibodies to the repeat region of their respective CS proteins have shown



that CS protein is carried into host cells by these species also (Atkinson *et al.*, 1989a). In contrast to *P. falciparum*, however, CS protein could also be localized on the parasitophorous vacuole membrane (Figs 25 and 26). Interestingly, *P. falciparum* sporozoites are not able to grow in HepG2-A16 hepatoma cells, while both *P. vivax* and *P. berghei* can complete exoerythrocytic schizogony in these cells. This suggests that interaction of CS protein with the parasitophorous vacuole membrane may be important in mediating early stages of intracellular development.

After entry of sporozoites, CS protein has been detected on the surface of developing exoerythrocytic schizonts in cultures of *P. falciparum*, *P. vivax*, *P. cynomolgi*, and *P. berghei* (Figs 27 and 28) (Aley *et al.*, 1987a; Hamilton *et al.*, 1988b; Suhrbier *et al.*, 1988; Szarfman *et al.*, 1988; Atkinson *et al.*, 1989a,b). CS protein persists throughout exoerythrocytic schizogony in cultures of *P. falciparum*, *P. vivax*, and *P. berghei* (Fig. 28) and can be detected on the surface of exoerythrocytic merozoites by immunofluorescence microscopy. By contrast CS antigen appears to disappear during exoerythrocytic development of *P. cynomolgi* (Atkinson *et al.*, 1989b). Immunoelectron microscopy of *P. berghei* has confirmed the presence of CS protein on the surface of exoerythrocytic merozoites (Suhrbier *et al.*, 1988; Atkinson *et al.*, 1989a). CS protein has not been found on erythrocytic merozoites, indicating that these two populations of merozoites differ in antigenicity. Nussenzweig and Nussenzweig (1985) speculated that persistence of CS antigen during exoerythrocytic development may help maintain a state of premunition, thus allowing infected hosts to escape overwhelming infections in hyperendemic areas where exposure to sporozoites is high.

Studies of *P. berghei* by immunoelectron microscopy have shown that CS protein is associated with the parasitophorous vacuole membrane and space and with the schizont plasma membrane during early stages of development

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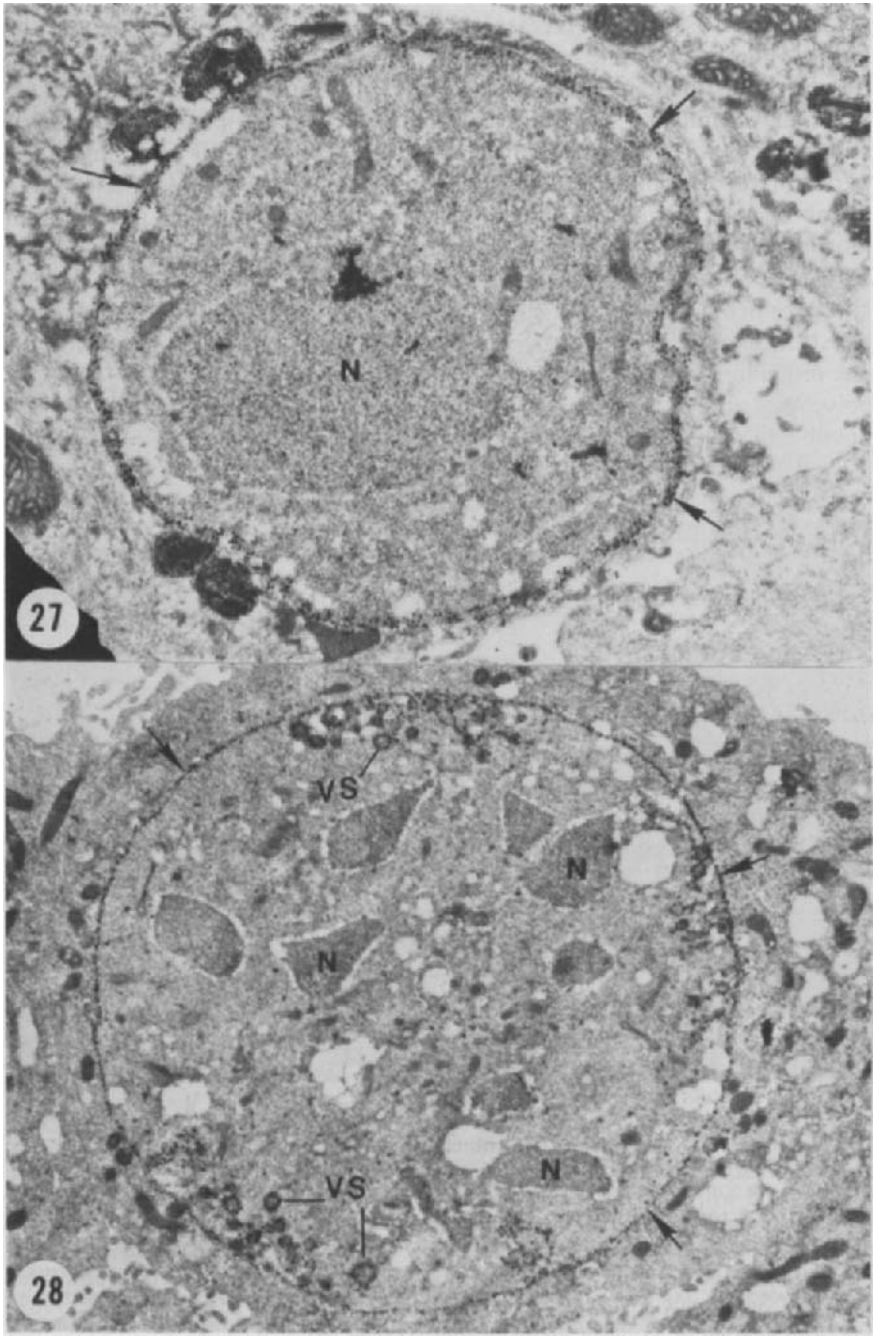
FIG. 27. LR Gold section of a *P. cynomolgi* exoerythrocytic form within a rhesus monkey hepatocyte, labeled with a mouse monoclonal antibody to the *P. cynomolgi* circumsporozoite protein. Gold label (arrows) is associated with the surface of the parasite and the surrounding parasitophorous vacuole membrane. N, nucleus. × 18 000. (Reproduced by permission of the American Society of Tropical Medicine and Hygiene from Atkinson *et al.*, 1989, *American Journal of Tropical Medicine and Hygiene*, **40**, 131–140.)

FIG. 28. LR Gold section of a *P. berghei* exoerythrocytic schizont within a HepG2-A16 hepatoma cell, labeled with a mouse monoclonal antibody to the *P. berghei* circumsporozoite protein, which is associated with the surface (arrows) and with membrane-bounded vesicles (VS) in the peripheral schizont cytoplasm. N, nucleus. × 8000. (Reproduced by permission of the American Society of Tropical Medicine and Hygiene from Atkinson *et al.*, 1989, *American Journal of Tropical Medicine and Hygiene*, **41**, 9–17.)


N
27
VS
N
N
N
VS
28

(Fig. 28). As schizonts incease in size, the density of labeling on the parasitophorous vacuole membrane decreases. Shortly before exoerythrocytic schizonts break into cytomeres between 36 and 50 h after sporozoite invasion, an unusual change in the distribution of CS protein occurs that seems to be correlated with the appearance of flocculent material in the parasitophorous vacuole space. Labeling can be detected on the limiting membranes of peripheral vesicles in the schizont cytoplasm that contain morphologically similar flocculent material (Fig. 29). The presence of CS antigen on the limiting membranes of the vesicles suggests that CS protein and flocculent material may be synthesized at the same time and exported to the surface of the parasite. Attempts to localize CS antigen in the endoplasmic reticulum and Golgi of developing schizonts with both monoclonal and polyclonal antibodies to CS protein have not been successful, however. It is possible that the vesicles may be originating from the surface of the schizont by endocytosis.

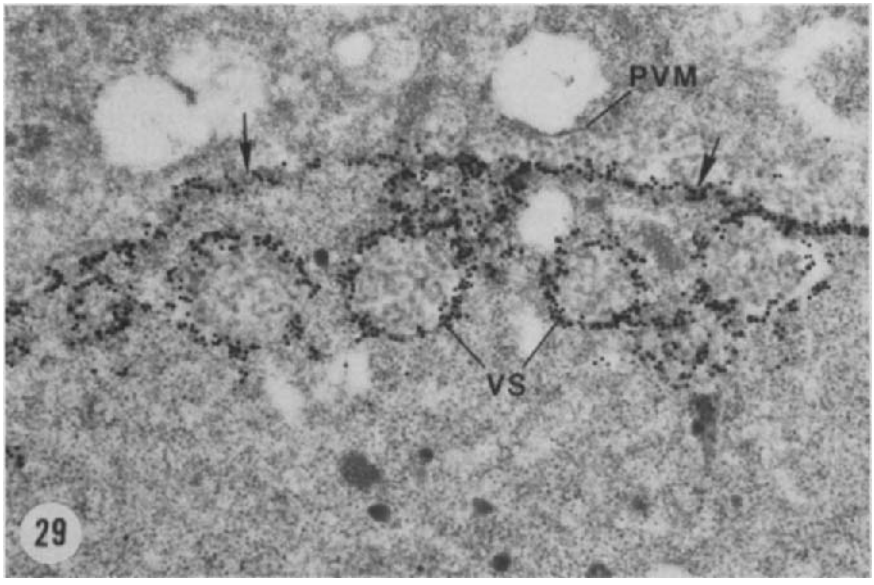


FIG. 29. LR Gold section of a *P. berghei* exoerythrocytic schizont within a HepG2-A16 hepatoma cell, labeled with a mouse monoclonal antibody to the *P. berghei* circumsporozoite protein. CS protein is associated with the schizont plasmalemma (arrows) and with membranes of peripheral vesicles (VS) which contain flocculent material. Label is absent from the parasitophorous vacuole membrane (PVM). The presence of label on these internal vesicles suggests that CS antigen is moving either into or out of the developing schizont. × 30 000. (Reproduced by permission of the American Society of Tropical Medicine and Hygiene from Atkinson *et al.*, 1989, *American Journal of Tropical Medicine and Hygiene*, **41**, 9–17.)

B. *TOXOPLASMA, SARCOCYSTIS* AND *EIMERIA*

As with *Plasmodium*, pre- and post-embedding immunoelectron microscopy of coccidian parasites has focused primarily on the surface and rhoptry–microneme antigens of merozoites and sporozoites. Sibley and Sharma (1987) reported the ultrastructural localization of *T. gondii* antigens which

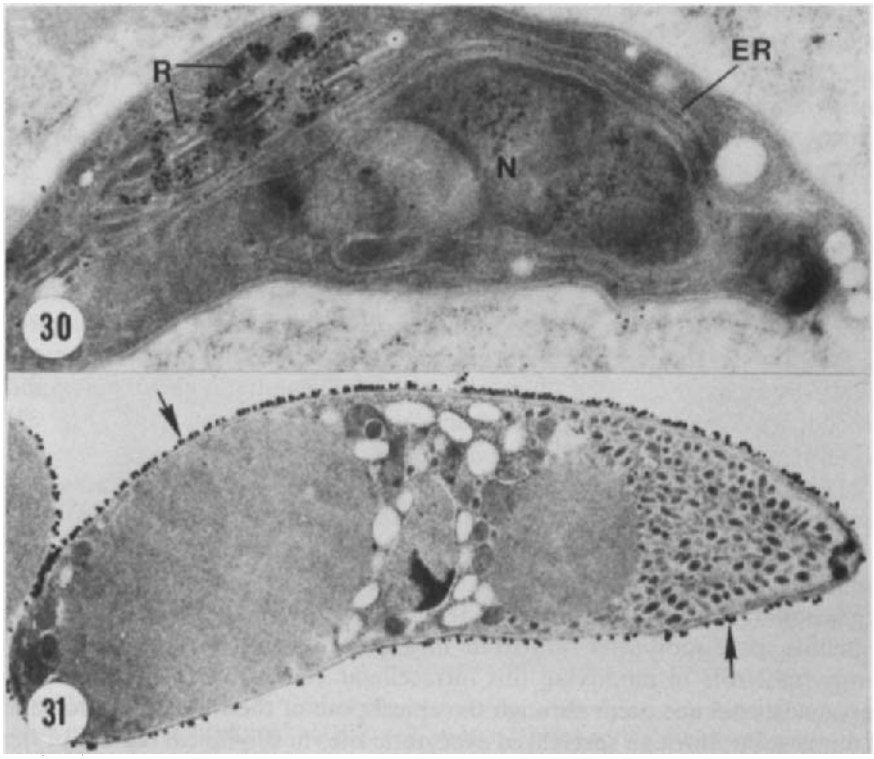


FIG. 30. Lowicryl K4M section of a *Toxoplasma gondii* tachyzoite, labeled with a mouse monoclonal antibody to a 55/60 kDa *Toxoplasma* rhoptry protein. Gold label is associated with rhoptries (R). ER, endoplasmic reticulum; N, nucleus. × 25 000. (Reproduced by permission of Elsevier Science Publishers from Sadak *et al.*, 1988, *Molecular and Biochemical Parasitology*, **29**, 203–211.)

FIG. 31. Epon embedded sporozoite of *Eimeria tenella* that had been incubated for 10 minutes with a mouse monoclonal antibody to a sporozoite surface antigen, pre-fixed with glutaraldehyde and then incubated with goat anti-mouse IgG colloidal gold. Colloidal gold (arrows) is associated with the surface of the sporozoite. Capping and sloughing of antibody occurred only when sporozoites were incubated with both antibody and colloidal gold before fixation. × 30 000. (Reproduced by permission from Speer *et al.*, 1985, *Journal of Parasitology*, **71**, 33–42.)

partially protect mice from lethal challenge. Both 58 kDa and 28 kDa proteins appear to be localized primarily beneath the cell surface membrane. The distribution is somewhat different from that in *Plasmodium*, in which protective antigens of merozoites and sporozoites are mostly exposed on the surface of the parasites. Other studies have localized surface and rhoptry–microneme antigens by immunoelectron microscopy (Fig. 30). Some of these antigens are secreted at the time of host-cell invasion and modify the parasitophorous vacuole membrane and space around intracellular parasites (Sibley *et al.*, 1986; Kimata and Tanabe, 1987; Sadak *et al.*, 1988). Yasuda *et al.* (1988) recently localized actin in the apical end of *Toxoplasma* zoites in association with microtubules, polar rings and the conoid and suggested that interaction of this cytoskeletal protein with microtubules and myosin may be important in mediating motility.

Immunoelectron microscopy has been used to localize surface and refractile body antigens in species of *Eimeria* (Speer *et al.*, 1983; Whitmire and Speer, 1986; Augustine and Danforth, 1987; Augustine *et al.*, 1988). Speer *et al.* (1983) and Whitmire and Speer (1986) used pre-embedding immunoelectron microscopy to localize antigens on the surface of *Eimeria* sporozoites and on the inner walls of sporocysts and oocysts. Immunoelectron microscopy has been used in several studies to investigate the capping and shedding of immune complexes from the surface of sporozoites (Fig. 31) (Dubremetz *et al.*, 1985; Speer *et al.*, 1985).

Only once has post-embedding immunoelectron microscopy been used to study the early stages of intracellular development of a coccidian parasite. Entzeroth *et al.* (1986) studied the exocytosis of a 21 kDa antigen from dense granules in the apical end of *Sarcocystis muris* sporozoites after host cell invasion (Fig. 32). This antigen was released into the parasitophorous vacuole space soon after sporozoite invasion, suggesting that it plays some important role in modifying this intracellular compartment. Interestingly, exocytosis did not occur through the apical pore of the rhoptry–microneme complex, but through specialized exocytotic sites in sub-apical regions of the zoite as observed in *Plasmodium* merozoites.

C. *THEILERIA*

Only a few investigators have used immunoelectron microscopy to characterize antigens and host–parasite interactions in piroplasms. Dobbelaere *et al.* (1985a) used a monoclonal antibody to a 68 kDa sporozoite antigen of *Theileria parva* to localize this molecule on the surface of sporozoites by immunoelectron microscopy. Studies of the expression of this antigen in the salivary glands of ticks demonstrated that it was synthesized by developing sporoblasts before the formation of mature sporozoites (Dobbelaere *et al.*,

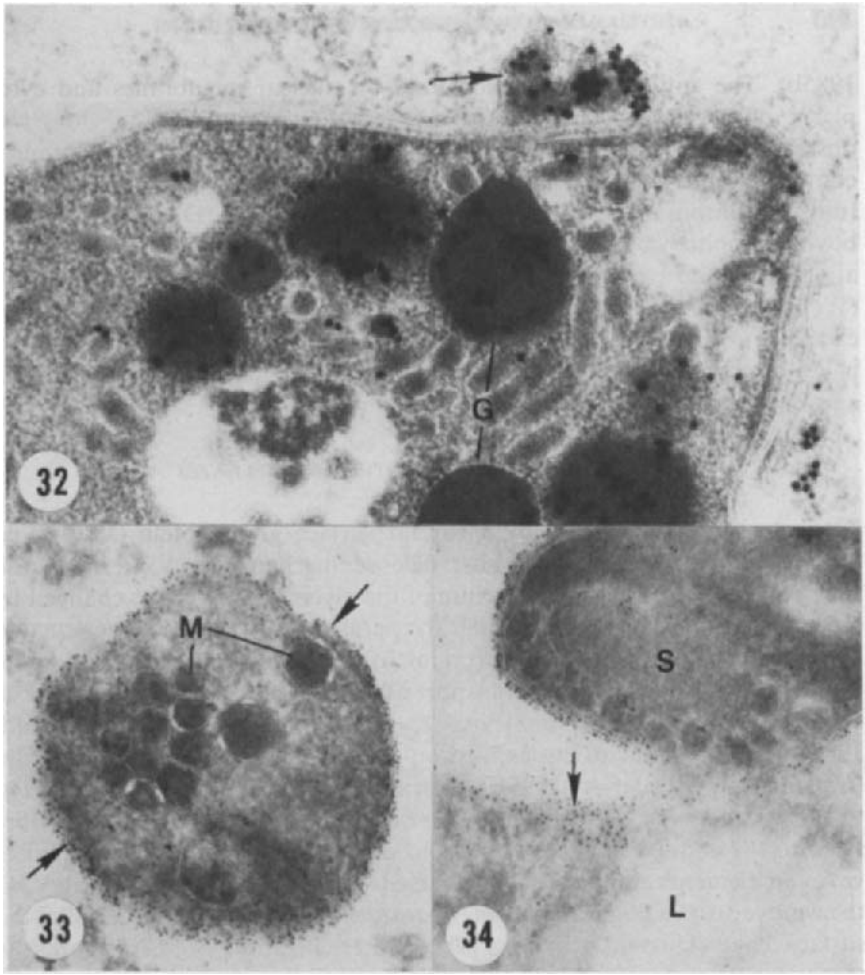


FIG. 32. Lowicryl K4M section of a *Sarcocystis muris* cystozoite fixed 30 minutes after invasion of a cultured MDCK cell; section labeled with a rabbit antiserum to a 21 kDa protein. Gold label is associated with contents of dense granules (G) at the anterior end of the cystozoite. The contents of these granules are released into the parasitophorous vacuole space (arrow) around recently invaded cystozoites. × 14 300. (Reproduced by permission from Entzeroth *et al.*, 1986, *European Journal of Cell Biology* **41**, 182–188.)

FIG. 33. Cryosection of a *Theileria parva* sporozoite that had been incubated with a mouse monoclonal antibody to the sporozoite surface coat. The surface membrane (arrows) and some micronemes (M) within the sporozoite are labeled. × 45 000. (Reproduced by permission from Webster *et al.*, 1985, *European Journal of Cell Biology*, **36**, 157–162.)

FIG. 34. Cryosection of a *Theileria parva* sporozoite (S). During invasion of bovine lymphocytes (L) the sporozoite surface antigen (arrow) is shed on to the lymphocyte membrane. × 45 000. (Reproduced by permission from Webster *et al.*, 1985, *European Journal of Cell Biology*, **36**, 157–162.)

1985b). The antigen could be localized on nuclear membranes and cytoplasm of developing sporoblasts. As sporozoites matured, labeling was observed on the sporozoite surface and on the membranes and luminal contents of micronemes, suggesting that the micronemes may be involved in transport of this antigen to the parasite surface (Fig. 33). During invasion of bovine lymphocytes, the sporozoite surface antigen was shed and became attached to the lymphocyte surface at the site of parasite entry (Fig. 34) (Webster *et al.*, 1985). Significantly, these workers detected the apparent exocytosis of surface antigen from micronemes, suggesting that these organelles discharge materials that may be important during host cell invasion and early intracellular development.

D. *TRYPANOSOMA* AND OTHER FLAGELLATES

Trypanosomes are covered by a variant surface glycoprotein (VSG) coat which protects them against host defense mechanisms (Vickerman and Barry, 1982). The antigenic structure of the glycoprotein can be changed by a gene-switching mechanism so that the parasite is able to avoid destruction by host antibodies. Immunoelectron microscopy has been used to study the intracellular distribution and transport of VSG in blood-stream forms of *T. brucei* (Duszenko *et al.*, 1988) and *T. congolense* (Frevert and Reinwald, 1988) and the onset of its expression during development in tsetse flies (*Glossina* spp.) (Tetley *et al.*, 1987). Intracellular transport of VSG is limited to an area between the flagellar pocket and the nucleus. VSGs are synthesized in the endoplasmic reticulum and move to Golgi complexes, tubular vesicular elements and flattened cisternae (Fig. 35). Vesicles containing VSG then move to the flagellar pocket where the protein is integrated into the surface coat (Duszenko *et al.*, 1988). Internalization of VSG from the trypanosome surface has also been observed. Endocytotic vesicles containing VSG may form from the flagellar pocket membrane and move to fuse with lysosomes.

Immunoelectron microscopy has also been used to study the intracellular distribution of tubulin in trichomonads and *Giardia* (Crossley *et al.*, 1986; Batista *et al.*, 1988) and to characterize specific surface antigens of *T. cruzi* (Bretana *et al.*, 1986; Tachibana *et al.*, 1986; Peyrol *et al.*, 1987).

E. *TRICHINELLA* AND OTHER NEMATODES

Most studies of nematodes in which immunoelectron microscopy has been used have employed immune sera from infected animals to identify specific antigenic targets (Prüsse *et al.*, 1983; Takahashi *et al.*, 1988, 1989) or to study expression of cuticular antigens or developmental changes in surface

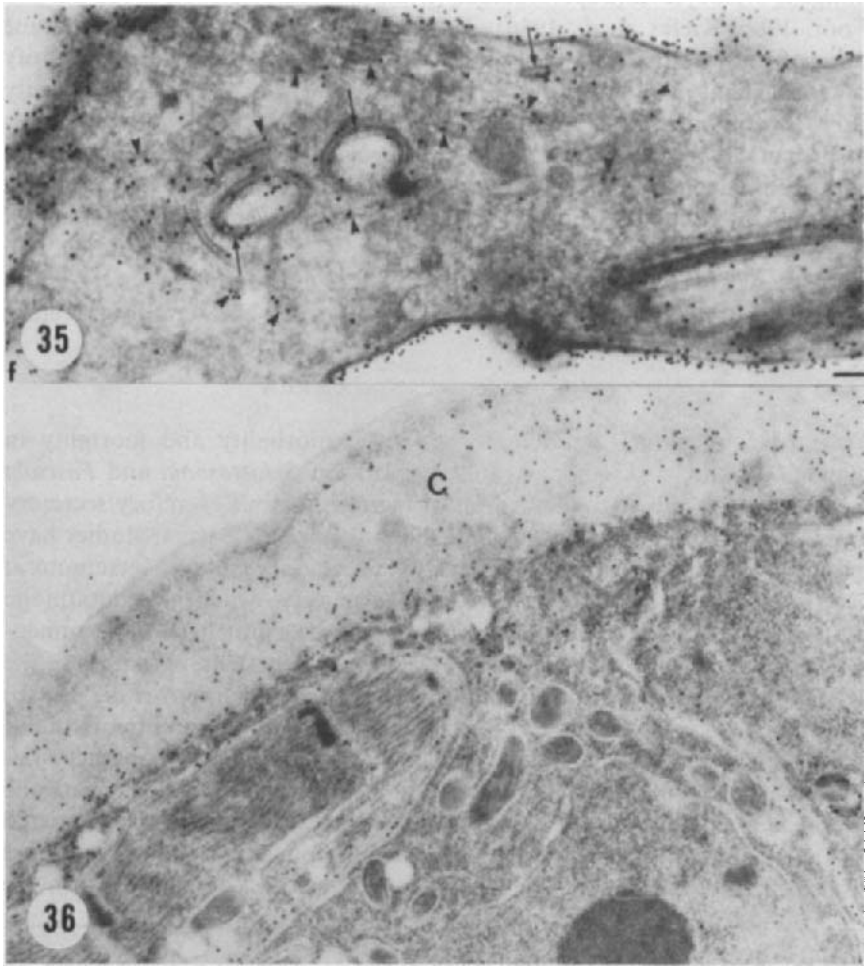


FIG. 35. Cryosection of a *Trypanosoma brucei* trypomastigote, labeled with antibody to the variant surface glycoprotein. Gold label is associated with the surface as well as portions of the Golgi apparatus, including tubulovesicular elements (arrowhead) and flattened cisternae (arrows), indicating that the trypanosome surface coat is synthesized and transported to the surface of the parasite along the classical intracellular route for glycoproteins. × 50 000. (Reproduced from Duszenko *et al.*, 1988, *Journal of Cell Biology*, **106**, 77–86, by copyright permission of the Rockefeller University Press.)

FIG. 36. LR White section of a *Trichinella spiralis* muscle larva, incubated with rat immune serum to *T. spiralis*. Gold label is associated with possible protective antigens in the inner layers of the cuticle (C). × 17 000. (Reproduced by permission from Takahashi *et al.*, 1988, *Journal of Parasitology*, **74**, 270–274.)

antigens of oocytes and spermatozoa (Bradley and Burghardt, 1976; Wu and Foor, 1980; Kiefer *et al.*, 1986). Takahashi *et al.* (1988a,b) used immune serum from rats that had been infected with *Trichinella spiralis* to identify organelles that may be involved in the development of protective immunity (Fig. 36). Immune targets were associated with the surface and hypodermis of the cuticle, material within the esophagus and midgut, antigenic components of the hemolymph, stichocyte granules, and aggregates of glycogen in a variety of cells. The authors concluded that only a limited number of parasite constituents are antigenic in rats. Most of these are associated with surfaces of the parasites that are exposed to the immune system or to materials that may be released from intact worms as excretory/secretory products.

F. *SCHISTOSOMA* AND *FASCIOLA*

Trematode infections are a major cause of morbidity and mortality in humans and domestic animals. Recent work on *Schistosoma* and *Fasciola* has focused on the characterization of tegumental and excretory/secretory antigens that may have protective or diagnostic value. Several studies have localized protective antigens in the tegument of *S. mansoni* (Matsumoto *et al.*, 1988b; Taylor *et al.*, 1988). A 28 kDa antigen which has glutathione transferase activity has been identified in excretory epithelial cells, tegumental and sub-tegumentary parenchymal cells, and tegumental granules in the head glands of adult worms (Taylor *et al.*, 1988). Matsumoto *et al.* (1988b) localized paramyosin and actin in the tegument of *S. mansoni* by immunoelectron microscopy (Figs 37 and 38). Actin was localized in the paracrystalline cores of tegumental spines (Fig. 37) and in cortical muscle beneath the tegument (Matsumoto *et al.*, 1988b). By contrast, paramyosin was restricted to the tegument where it was unexpectedly found in membrane-bound elongate bodies (Fig. 38). The presence of this cytoskeletal protein in the

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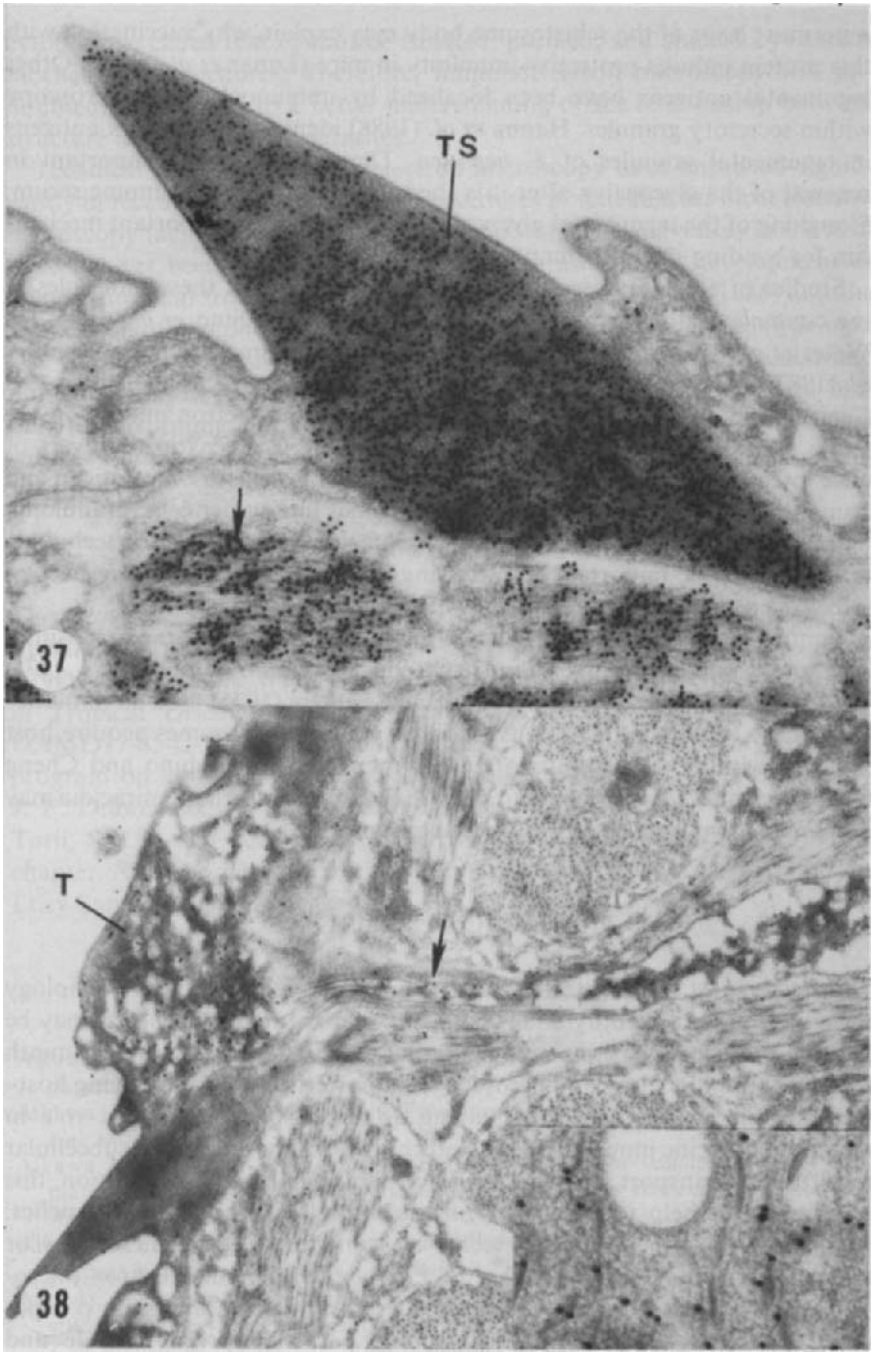
FIG. 37. LR Gold section of an adult *Schistosoma mansoni*, labeled with an antibody to chicken gizzard actin which recognizes all actin isoforms. This cytoskeletal protein is a major constituent of the tegumental spines (TS) and is also present in cortical muscle (arrow) beneath the tegument. × 30 000. (Reproduced by permission from Matsumoto *et al.*, 1988, *Nature*, **333**, 76–78. Copyright © 1988 Macmillan Magazines Ltd)

FIG. 38. LR Gold section of an adult *Schistosoma mansoni*, labeled with a rabbit antiserum to paramyosin. This invertebrate cytoskeletal protein is associated with non-filamentous, membrane-bound elongate bodies within the tegument (T), cytons and the cytoplasmic tubes (arrow) which connect the tegument to cytons. × 15 180. (Reproduced by permission from Matsumoto *et al.*, 1988, *Nature*, **333**, 76–78. Copyright © 1988 Macmillan Magazines Ltd) Inset: gold particles associated with elongate bodies of the tegument. × 54 000.


TS
37
T
38

outermost layer of the schistosome body may explain why vaccination with this protein induces protective immunity in mice (Lanar *et al.*, 1986). Other tegumental antigens have been localized by immunoelectron microscopy within secretory granules. Hanna *et al.* (1988) identified glycocalyx antigens in tegumental granules of *F. hepatica.* These granules are important in renewal of the glycocalyx after it is shed in the presence of immune serum. Sloughing of the tegumental glycocalyx appears to be an important mechanism for evading the host immune response.

Studies of excretory/secretory antigens have localized these molecules in the contents and epithelium of the intestinal tract (Fujino *et al.*, 1985; De Water *et al.*, 1986a,b), in the excretory system (i.e. flame cells) (De Water *et al.*, 1987a), and in the gland cells and epidermis of miracidia (Bogitsh and Carter, 1975). De Water *et al.* (1987b) used immunoelectron microscopy to study the fate of circulating anodic and cathodic antigens in the livers of mice infected with *S. mansoni.* These workers found schistosome antigen and immune complexes within secondary lysosomes in Kupffer cells, granuloma macrophages, and endothelial cells from infected livers. Thus, the technique may prove to be important for studying immunopathological processes in infected hosts.

Immunoelectron microscopy has also been used to study the acquisition and mimicry of host antigens by trematodes (McLaren and Smithers, 1975; Yoshino and Cheng, 1978). McLaren and Smithers (1975) used an immunoperoxidase technique to demonstrate that young schistosomes acquire host antigens within several hours after host penetration. Yoshino and Cheng (1978) used a similar procedure to show that surface antigens of miracidia may mimic hemolymph proteins of their snail hosts.

IV. Conclusions

During the past decade, techniques of immunology and molecular biology have been used to identify and isolate specific parasite proteins that may be capable of producing protective immunity against protozoan and helminth parasites. Immunoelectron microscopy is a powerful tool for studying host–parasite interactions and it is playing an increasingly important role in identifying specific immune targets and characterizing the precise subcellular localization, transport, and expression of parasite antigens. In addition, this technique can help to clarify specific functions of subcellular organelles, which may not otherwise be detected by standard electron microscopy or biochemical techniques. In studies of *Plasmodium*, immunoelectron microscopy has been especially valuable in characterizing the antigenic composition of intracellular compartments, e.g. parasitophorous vacuole and

cytoplasmic clefts, that cannot be isolated, purified, and studied by current biochemical procedures. Therefore, immunoelectron microscopy will undoubtedly contribute to a better understanding of the relationship between structure and function in parasites.

Technical aspects of immunoelectron microscopy have improved significantly in recent years, making these procedures practicable for most electron microscopy facilities. At the same time, important new advances in the field have not yet been applied to the study of parasites. The development of cryofixation and freeze-substitution techniques (Linner *et al.*, 1986) and the application of immunocytochemistry to freeze-fracture techniques (Forsman and Da Silva, 1988) show considerable promise for studying molecular and ultrastructural interactions between hosts and parasites. As technical barriers to performing immunoelectron microscopy diminish, these techniques will have many applications in the field of parasitology.

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Technical Appendix

Immunoelectron microscopy exposes fresh tissues to fixatives, buffers, organic solvents and embedding media that may extract molecules and cause denaturation and loss of antigenicity. For abundant antigens or antigens concentrated in discrete compartments, large losses of immunoreactivity during processing may still allow good ultrastructural localization. By contrast, labile or sparsely distributed antigens may be difficult to localize with any of the methods that are commonly used. We use procedures developed by Tokuyasu (1986) for preparing and labeling cryosections, but have developed our own modifications for embedding, sectioning and labeling when using LR White and LR Gold resins.

A. FIXATION

We have experimented with a variety of different fixatives, but have had best results with a mixture of 1% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. This fixative preserves relatively good ultrastructure and preserves the antigenicity of a wide variety of molecules. Prepare as follows:

(i) Mix 1.0 g of paraformaldehyde powder with 45 ml of double-distilled water, cover with aluminum foil, and heat with stirring on a hot-plate at 60°C for 20–30 min.

(ii) Add 4–6 drops of 1 N NaOH. The milky white solution should clear instantly.

(iii) Cool to room temperature and mix with 50 ml of 0.2 M phosphate buffer, pH 7.4.

(iv) Add 0.8 ml of the 25% stock glutaraldehyde solution and mix.
(v) Check pH, bring total volume to 100 ml, and cool.

The fixation time and temperature will depend on the tissue. We routinely fix *Plasmodium*-infected erythrocytes for 10 min at room temperature.

B. EMBEDDING AND SECTIONING WITH LR WHITE AND LR GOLD RESINS

LR White resin is a premixed acrylic formulation which is polymerized by heat or by addition of an initiator for a rapid "cold" cure (Newman and Hobot, 1987). LR White was originally designed for use at room temperature, but we have found that it will infiltrate tissues at temperatures as low as −20°C. At temperatures between −20°C and −30°C the resin will solidify. LR White is used to infiltrate tissues at low temperatures to protect antigens from the denaturing effects of ethanol (Armbruster *et al.*, 1983) and the resin is then polymerized at 40°C for 5 days. This temperature is lower than that recommended by the manufacturer, but still adequate to produce blocks that are easy to section. Undercuring the resin at lower temperatures seems to increase the immunoreactivity of the embedded sample, perhaps by reducing intermolecular cross-linking and allowing better penetration of immunoreagents.

LR Gold resin was designed for use at temperatures as low as −25°C and we normally use this resin for tissues that are not darkly pigmented or rich in hemoglobin. LR Gold resin preserves antigenicity of some molecules better than Lowicryl, but may not preserve ultrastructure as well (Yokota and Oda, 1985). We have had only limited success in producing well-polymerized blocks of erythrocytes with resin that are polymerized with ultraviolet light, e.g. Lowicryl and LR Gold. The high oxygen content and dark color of hemoglobin-rich erythrocytes may inhibit polymerization of the resin or limit penetration of ultraviolet light. Sectionable blocks can be produced, however, if erythrocytes are polymerized as a suspension rather than as a pellet. LR Gold can be polymerized at low temperatures after addition of appropriate initiators (benzoin methyl ether, benzoil or camphoquinone) and exposure to intense ultraviolet or blue light. We routinely use benzoin methyl ether as an initiator at concentrations of 0.5–0.75% (w/v) and polymerize the tissue at −20°C under intense ultraviolet light for 24–48 h. Higher initiator concentrations, i.e. 0.75%, require shorter polymerization times and seem to produce blocks with better sectioning qualities.

Tissues should be dehydrated as rapidly as possible to minimize exposure to alcohols. Actual times will depend on the size and density of the tissue. When tissue is embedded in LR Gold, initiator is dissolved in the resin at room temperature and the mixture is then chilled at −20°C. Ethanol solutions and resin mixtures should be made beforehand and cooled to −20°C before use.

(i) 30% Ethanol; 10–20 min; 4°C.
(ii) 50% Ethanol; 10–20 min; −20°C.
(iii) 70% Ethanol; 10–20 min; −20°C.
(iv) 1 part 95% Ethanol, 2 parts resin; 10–20 min; −20°C.

(v) 100% resin; 60 min; −20°C.
(vi) 100% resin; 60 min; −20°C.
(vii) 100% resin; 60 min; −20°C.
(viii) Fresh resin overnight. For LR White embedding, samples are left overnight at 4°C. For LR Gold embedding, samples are left overnight at −20°C.
(ix) Transfer samples to gelatin capsules and cap tightly to exclude as much oxygen as possible. LR White is polymerized at 40°C for five days. LR Gold is polymerized for from 24 h to several days at −20°C under ultraviolet light. Polyethylene Beem capsules can be used to hold the tissue, but the surface of the blocks may be tacky after polymerization.

Polymerized blocks of LR White and LR Gold resin are brittle, but may be sectioned easily with glass or diamond knives. Sections are picked up on unsupported 200–400 mesh nickel grids. Formvar-coated grids may be used, but sections tend to wrinkle during immunostaining. Since subsequent immunolabeling steps use solutions which contain detergents (Tween 20), sections often detach and float away unless they are bound firmly to the grids. We make grids "sticky" by coating the metal surfaces of the grids with formvar before they are used to pick up sections. We prepare the grids by dipping them into a 0.5% (w/v) solution of formvar in dichloroethane and then rapidly draining away all excess solution on a piece of highly absorbent filter paper. It is important that the grids be placed flat on the filter paper before the formvar solution dries; otherwise, thick deposits of plastic will obscure the spaces between grid bars.

C. LABELING PROCEDURES FOR LR WHITE AND LR GOLD SECTIONS

1. *Blocking*

Fixation with glutaraldehyde can cause the non-specific binding of ligands and ligand-marker conjugates to unquenched aldehyde groups in tissue sections (Hodges *et al.*, 1984). Most authors suggest that free aldehyde groups should be blocked before immunolabeling with solutions that contain free amino groups to reduce non-specific background binding. This step is especially important in pre-embedding immunocytochemistry and cryo-ultramicrotomy where tissues or tissue sections are incubated with antibodies and electron-dense markers before embedding. Suitable blocking agents include buffered solutions of gelatin, serum albumin, non-fat dried milk, 0.1 M glycine, 0.1 M lysine, 0.05 M ammonium chloride or 0.05 M ammonium carbonate, and solutions of 0.05–1.0% sodium borohydride (Lillie and Pizzolato, 1972; Weber *et al.*, 1978; Farr and Nakane, 1981; Van Leeuwen, 1982; Eldred *et al.*, 1983). Some authors have suggested that treatment with sodium borohydride before embedding may help to restore antigenicity to glutaraldehyde-fixed tissue (Eldred *et al.*, 1983). We use solutions of 5% non-fat dried milk to block thin sections of resin-embedded material.

2. *Etching*

Etching techniques unmask antigenic sites that may have been destroyed or altered during dehydration and embedding. Strong oxidizing agents such as sodium metaperiodate and hydrogen peroxide are often used on epoxy-embedded tissues before immunolabeling to increase the hydrophilic nature of the sections, unmask antigenic sites and improve their reactivity with aqueous solutions of antibodies (Bendayan and Zollinger, 1983). This treatment oxidizes hydrophobic alkane side chains in the resin to alcohols, aldehydes and acids, thereby making the resin surface more hydrophilic (Causton, 1984). Since this treatment may also oxidize tissue-associated antigens and affect their reactivity with antibodies, it should be used with caution. Most of the acrylic resins now in use, e.g. LR White, LR Gold and Lowicryl, are hydrophilic and do not require this treatment. We have found, however, that treatment of LR White or LR Gold sections with a saturated aqueous solution of sodium metaperiodate may improve the immunoreactivity of some resin-embedded antigens. Actual removal of resin from grid-mounted sections with solutions of sodium ethoxide may significantly enhance immunolabeling (Mar and Wight, 1988). Ingram *et al.* (1988) have successfully used this technique in combination with sodium metaperiodate to unmask antigens in erythrocytes embedded in LR White resin.

3. *Labeling*

A variety of techniques for labeling grid-mounted resin sections has been described. Many workers use individual drops of reagents on wax or parafilm sheets, porcelain plates with 6–12 individual wells, or multi-well microtiter plates for holding washing and incubation solutions. We have found that the Hiraoka staining kit (Polysciences, Inc.) offer a number of advantages over other procedures. This device consists of a flexible plate of nalgene with precisely cut rows of shallow slits. Grids are firmly mounted in a vertical position on the plate by their rims, where they can be incubated in drops of blocking solutions, antibodies and colloidal gold and "jet-washed" gently with a Pasteur pipette or dipped into beakers that contain appropriate washing solutions. This procedure allows both sides of grid-mounted resin sections to be labeled simultaneously, to increase the density of immunolabeling. In addition, the plates are much easier and faster to handle than individual grids and allow large numbers of grids to be incubated and washed at the same time. The procedure is described below.

(i) Etch grids in drops of saturated aqueous sodium metaperiodate for 30–60 min and rinse with distilled water. This step is optional and may destroy the immunoreactivity of some antigens.

(ii) Cover grids with drops of PBS–milk–Tween blocking solution (5% non-fat dried milk in 0.1 M phosphate buffer, pH 7.4, with 0.01% Tween 20 and 0.9% NaCl) and incubate for 30 min.

(iii) Remove blocking solution and cover grids with drops of the primary antibody diluted with PBS–BSA–Tween (1% bovine serum albumin, fraction V, in 0.1 M phosphate buffer, pH 7.4, with 0.01% Tween 20 and 0.9% NaCl). The primary antibody can also be diluted in the PBS–milk–Tween blocking solution to control problems with non-specific background labeling. While this works well with some antibodies, it may completely abolish labeling with others. Optimal antibody dilutions must be determined by trial and error, although we have had good results with antibody concentrations ranging from 2 to 200 $\mu g\,ml.^{-1}$ Incubate for 2 h at room temperature or overnight at 4°C in a humidity chamber.

(iv) Jet-wash grids with PBS–BSA–Tween and incubate with three 5-min changes of PBS–BSA–Tween.

(v) Incubate grids with rabbit anti-mouse IgG secondary antibody diluted to approximately 20–40 $\mu g\,ml^{-1}$ with PBS–BSA–Tween. The optimal dilution must be found by trial and error, but should remain the same for each lot of antibody. Incubate the grids for 1 h at room temperature in a humidity chamber.

(vi) Jet-wash grids with PBS–BSA–Tween and incubate with three 5-min changes of PBS–BSA–Tween.

(vii) Incubate grids with protein A-gold or goat anti-rabbit antibody-gold diluted 1/20 with PBS–BSA–Tween for 1 h at room temperature in a humidity chamber. To control background problems, immunoglobulin-gold can also be diluted in the PBS–milk–Tween blocking solution.

(viii) Jet-wash grids with 0.1 M phosphate buffer, pH 7.4, and fix grids in drops of 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 15 min to stabilize the bound gold.

(ix) Jet-wash with double-distilled water and dry with filter paper.

(x) Stain the grids for 30 min in 2% (w/v) uranyl acetate dissolved in 50% methanol. Jet-wash the grids with 50% methanol and dry.

(xi) Stain sections with Reynold's lead citrate for 5 min, wash with double-distilled water and dry.

(xii) Coat grids lightly with carbon in a vacuum evaporator to stabilize the sections.

Echinostoma and Echinostomiasis

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I. Introduction and the Genus *Echinostoma* 215
II. Biology of 37-Collar-spined Echinostomes 224
III. Infectivity Studies in First Intermediate Snail Hosts 228
IV. Infectivity Studies in Second Intermediate Hosts 232
V. Infectivity, Growth and Development in Vertebrate Hosts 238
A. Infectivity 240
B. Worm distribution 240
C. Development in vertebrate hosts 242
D. Worm crowding 246
VI. Immunology of Echinostome Infections in Vertebrate Hosts 246
VII. Clinical and Pathological Effects of Echinostomes 248
VIII. Epidemiology and Disease of Echinostomes 253
A. Source of infection 253
B. Diagnosis and treatment 254
IX. Physiology and Biochemistry of *Echinostoma* 254
A. Physiology 254
B. Biochemistry 256
X. Concluding Remarks and Future Prospects 258
References 260

I. Introduction and the Genus *Echinostoma*

This review examines studies on the biology, life history, infectivity, immunology, pathology, epidemiology, physiology and biochemistry of *Echino-*

ADVANCES IN PARASITOLOGY VOL 29
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stoma. It considers some studies on other genera of Echinostomatidae only as they relate to *Echinostoma*.

There is confusion as to what a species is in the genus *Echinostoma* and the number of reported species ranges from 61 to 114 (Bashkirova, 1979; Kanev, 1985). The systematics of the 37-collar-spined *Echinostoma* in the *E. revolutum* group has been revised based on morphological, biometrical, isoenzymatical and biological characteristics (Kanev, 1985). Recent studies (Voltz *et al.*, 1988; Odaibo *et al.*, 1988, 1989) support Kanev's revision and a synopsis of the revision is presented in Table 1. This review is concerned mainly with *E. trivolvis*, *E. caproni* and *E. revolutum* for which most of the literature is available. We use correct names after Kanev (1985) whenever possible, but when there is doubt as to the species used by an author, such uncertainty is indicated.

TABLE 1 *Correct names of 37-collar-spined* Echinostoma *used in research on echinostomes*

Correct name[a]	*Previous names used by various authors*
Echinostoma caproni (Richard, 1964)	*E. revolutum* by: Barus *et al.* (1974); Christensen (1980); Sirag *et al.* (1980); Christensen *et al.* (1981a,b, 1984, 1985, 1986); Bindseil and Christensen (1984); Simonsen and Andersen (1986) *E. liei*: by Jeyarasasingam *et al.* (1972); Christensen *et al.* (1980); Kuris (1980); Evans (1985); Thorndyke and Whitfield (1987). *E. paraensei*: by Lie and Basch (1967). *E. togoensis*: by Jourdane and Kulo (1981).
Echinostoma trivolvis (Cort, 1914)	*E. revolutum*: by Beaver (1937); Senger (1954); approximately 60 studies by Fried and co-workers, 1968–1988
Echinostoma echinatum (Zeder, 1803)	*E. barbosai*: by Jeyarasasingam *et al.* (1972). *E. lindoense*: by Sandground and Bonne (1940). *E. lindoense*: by Lie (1964).
Echinostoma revolutum (Frölich, 1802)	*E. audyi*: by Lie and Umathevy (1965).

[a] See Kanev (1985).

Although agreement on the higher taxa of platyhelminths is not universal, a convenient scheme showing classification of *Echinostoma* is given in Table 2. The species of *Echinostoma* studied most intensively are those with 37-collar-spines. However, the number of collar spines for species of *Echinostoma* is variable ranging from 27 to 51. Various species of non-37-collar-

spined *Echinostoma* are listed in Table 3 along with pertinent information on each. The validity of some of these species is not certain.

TABLE 2 *Higher taxa of* Echinostoma

Phylum	Platyhelminthes
Class	Trematoda
Subclass	Digenea
Superorder[a]	Anepitheliocystidia
Order	Echinostomata
Superfamily	Echinostomatoidea
Family	Echinostomatidae
Subfamily	Echinostomatinae

[a] Cercariae in this superorder possess thin nonepithelial excretory bladders in contrast to the cercariae in the Epitheliocystidia which have thick-walled epithelial bladders.

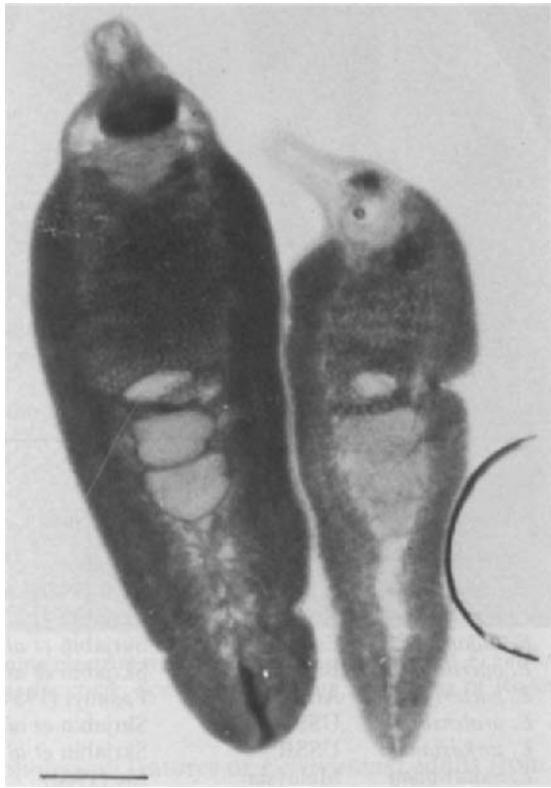

FIG. 1. Live 14-day-old adults of *E. trivolvis* (left) and *E. caproni* (right) from a concurrent infection in the golden hamster. Scale bar = 1 mm. (Reproduced with the permission of the editors of the *Journal of Parasitology* from Huffman *et al.*, 1988.)

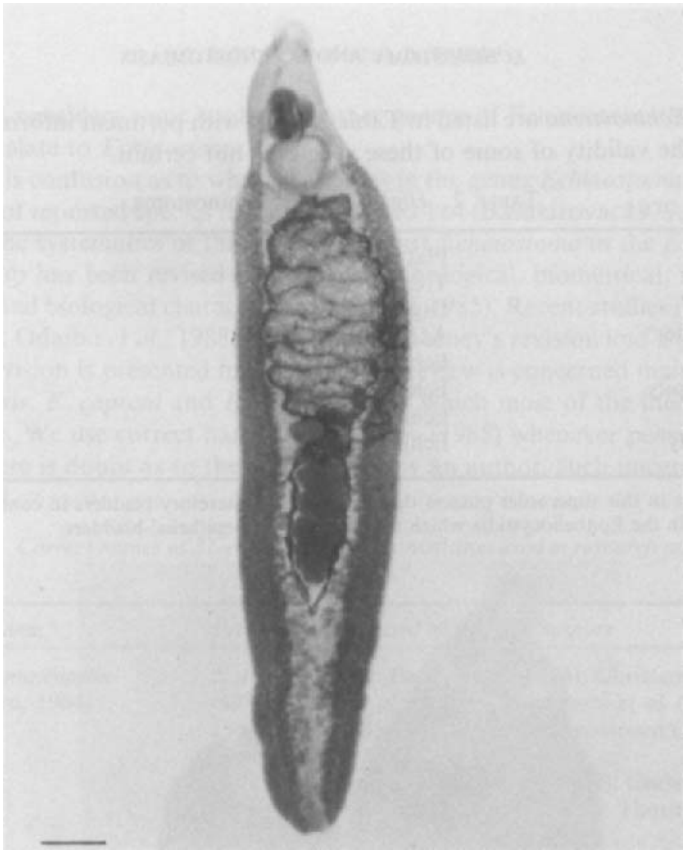

FIG. 2. *E. trivolvis* fixed in hot alcohol–formalin–acetic acid, stained in Gower's carmine, and grown for 28 days in the golden hamster. Scale bar = 1.1 mm.

TABLE 3 *Some* Echinostoma *species with collar-spine counts other than 37*

No. of collar spines	*Species*	*Location*	*Reference*
27	*E. stridulae*	Central Europe	Skrjabin *et al.* (1979)
27–28	*E. hortense*	Japan	Tani (1976a,b)
29	*E. ignavum*	Australia	Skrjabin *et al.* (1979)
31	*E. sudanense*	Egypt	Skrjabin *et al.* (1979)
33	*E. operosum*	Brazil	Skrjabin *et al.* (1979)
35	*E. porteri*	Africa	Fashuyi (1984)
39	*E. uralensis*	USSR	Skrjabin *et al.* (1979)
41	*E. turkestanum*	USSR	Skrjabin *et al.* (1979)
43	*E. malayanum*	Malaysia	Lie (1963)
45	*E. lahorensis*	Pakistan	Bhutta and Khan (1974)
47	*E. hilliferum*	Australia	Skrjabin *et al.* (1979)
49	*E. condigum*	Brazil	Skrjabin *et al.* (1979)
49–51	*E. ilocanum*	Malaysia	Lie and Nasemary (1973)

The morphology of 37-collar-spined *Echinostoma* can be seen in living or fixed specimens prepared for light or scanning electron microscopy (SEM). Live *E. caproni* and *E. trivolvis* grown concurrently in hamsters for 14 days are shown in Fig. 1, and a 28-day-old *E. trivolvis* adult grown in a hamster, fixed in hot alcohol–formalin–acetic acid (AFA) and stained in Gower's carmine is shown in Fig. 2. Figures 3 to 7 show SEMS of *E. caproni* and *E. trivolvis* adults prepared as described in Fried and Fujino (1984).

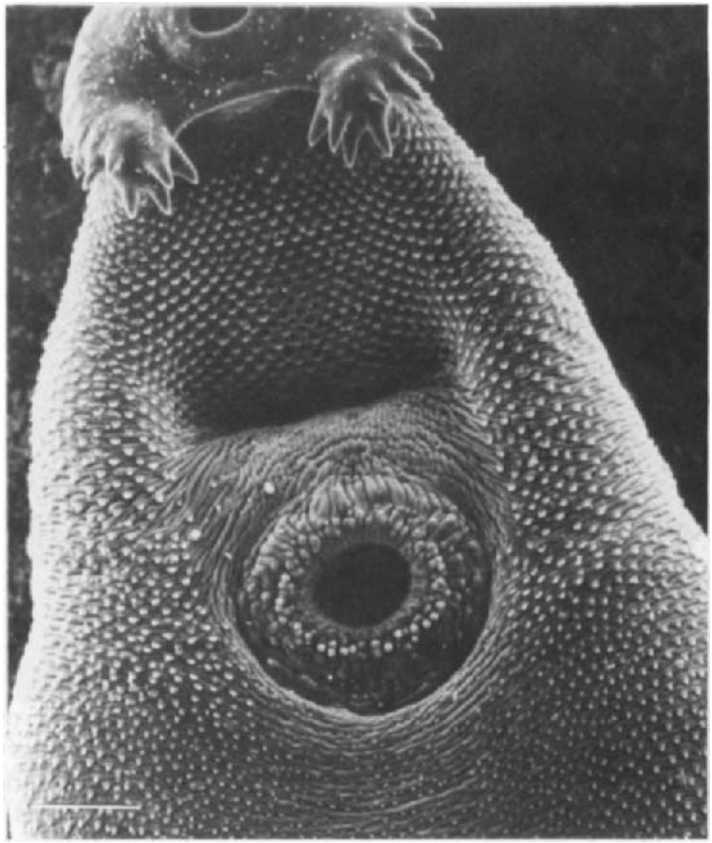

FIG. 3. Scanning electron micrograph of a seven-day-old *Echinostoma trivolvis* adult from the domestic chick. Scale bar = 135 μm. (Courtesy of Roger A. Donovick.)

The morphological features of *Echinostoma* adults from avian and mammalian hosts are as follows. The length is variable ranging from 4 to 20 mm in ovigerous adults. Considerable variation exists in size depending upon fixation, definitive host, crowding effects, and species of echinostome (Beaver, 1937; Jeyarasasingam *et al.*, 1972; Franco *et al.*, 1988). The length is

FIG. 4. SEM of the anterioventral view of a 14-day-old *E. caproni* adult from the chick. Scale bar = 100 μm. (Courtesy of Roger A. Donovick.)

about three to five times the width and the most characteristic morphological feature is the cephalic collar of spines (Figs 3–6). The spines may be arranged in one or two circles around the collar and the number varies from 27 to 51 depending upon the species. The number of spines is usually constant within the species except where spines are lost or damaged during specimen preparation. The spines in the corner are often grouped in a characteristic arrangement (Fig. 3). The collar contains numerous sensory papillae and is incomplete in the ventral aspect (Figs 3–5). The tegument contains scale-like spines on both dorsal and ventral surfaces (Figs 3, 4, 7). The number and size of tegumentary spines is reduced in the posterior half of the body (Fried and Fujino, 1984). The ventral aspect of the body anterior to the acetabulum may be indented (Fig. 3). The oral and ventral suckers are

close to each other in the anterior third of the body and the diameter of the acetabulum greater than that of the oral sucker. The rim of the suckers may contain papillae and spines.

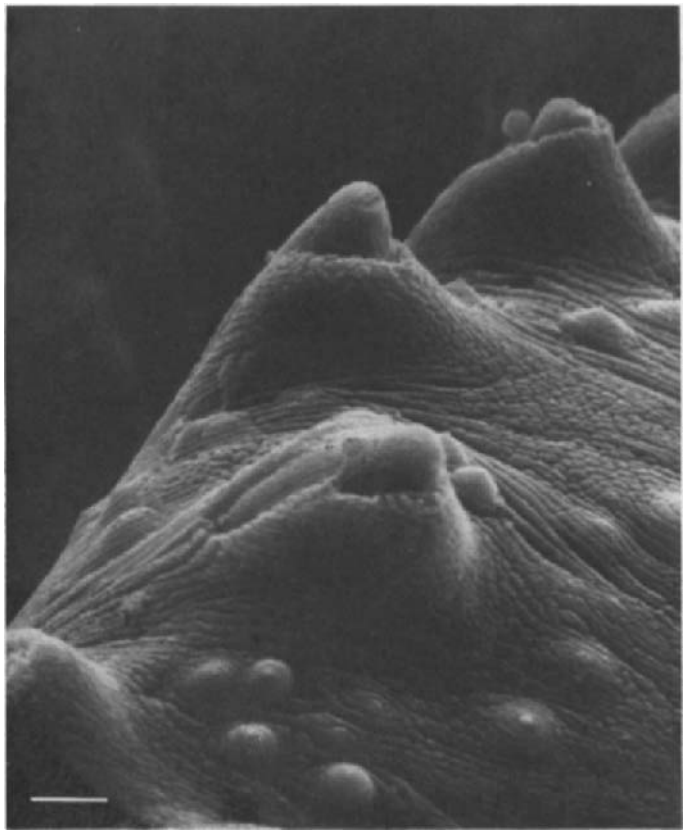

FIG. 5. SEM of the oral collar of a 14-day-old *E. trivolvis* grown in the chick showing spines and sensory papillae. P, papillae. Scale bar = 8 μm. (Courtesy of Roger A. Donovick.)

The testes are in tandem in the posterior half of the body; they may be smooth or lobate, although fixation may change a smooth testis to one that is lobate (Jeyarasasingam *et al.*, 1972). The ovary is spherical or ovoid and is located in front of the anterior testis. The ootype is between the anterior testis and the ovary and the uterus is between the ovary and acetabulum. It may be distended with eggs as in *E. trivolvis* from hamsters where the number of eggs may be in the thousands (Franco *et al.*, 1986). Most eggs *in*

FIG. 6. SEM of a single oral collar-spine from a seven-day-old *E. trivolvis* grown in the chick. Scale bar = 5 μm. (Courtesy of Roger A. Donovick.)

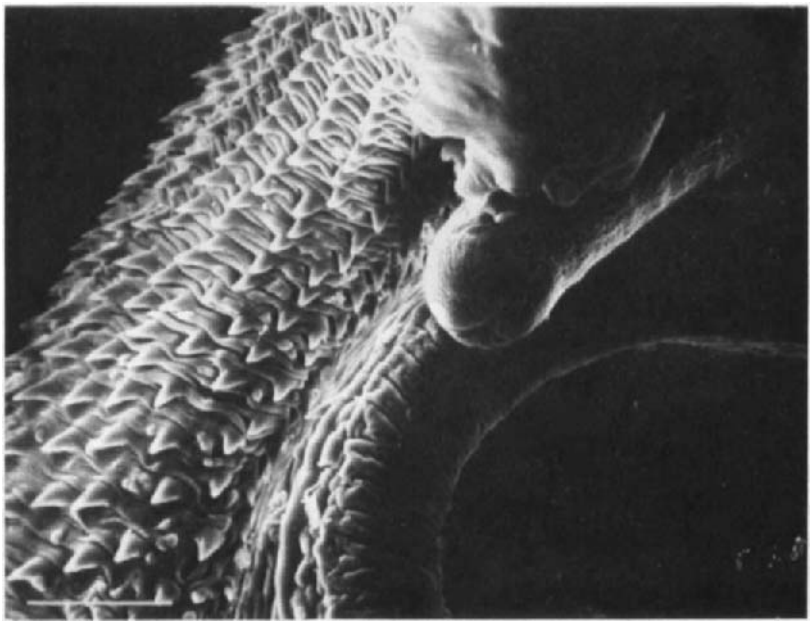

FIG. 7. SEM of the cirrus from a 14-day-old *E. caproni* grown in the chick. Scale bar = 100 μm. (Courtesy of Roger A. Donovick.)

utero are fertile but not well developed; eggs are operculate and about 90–130 μm long and 50–70 μm wide (Fig. 8). The vitellaria are extensive in both lateral margins extending from the midacetabulum to the posterior end of the body (Figs. 1 and 2). Some species have additional vitellaria just beyond the posterior testis. The genital pore is median and preacetabular. The cirrus pouch passes dorsal to the acetabulum and contains a coiled seminal vesicle, prostate gland and cirrus. The cirrus may be protruded (Fig. 7; Fried and Vates, 1984).

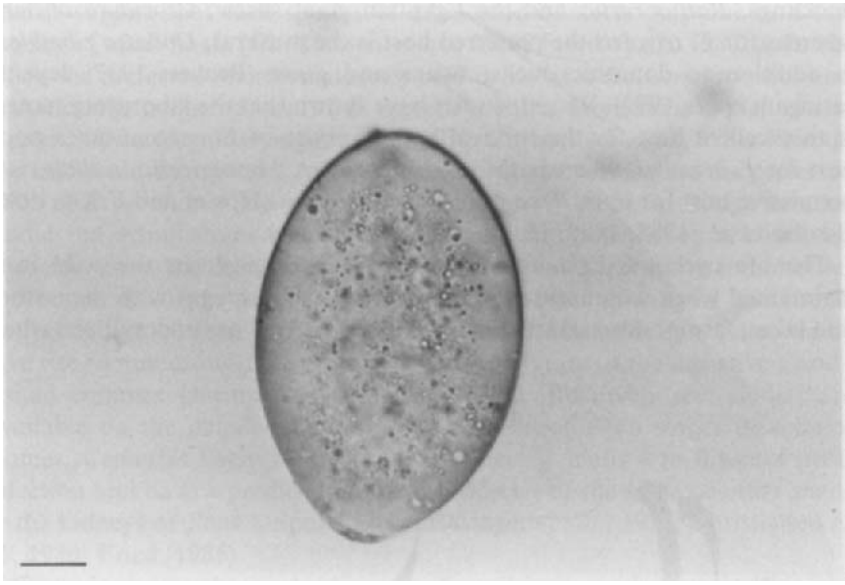

FIG. 8. Light micrograph of an egg from a foecal smear of an ICR mouse experimentally infected with 14-day-old adults of *Echinostoma caproni*. Scale bar = 25 μm.

The digestive system has a muscular pharynx posterior to the oral sucker. The short esophagus bifurcates anterior to the acetabulum to give rise to intestinal ceca that run medial to the vitellaria and end blindly in the posterior aspect of the worm. The excretory pore is terminal or subterminal at the posterior tip of the body; the pore leads to an excretory bladder that bifurcates behind the posterior testis and gives rise to both protonephridial tubes which are lateral to the gonads and uterus and medial to the intestinal ceca.

II. Biology of 37-Collar-Spined Echinostomes

Echinostome adults are cosmopolitan, hermaphroditic digeneans that live in the intestines and bile ducts of numerous vertebrate hosts, particularly aquatic or semi-aquatic birds and mammals. Adults of *Echinostoma* spp. have also been reported from reptiles (Yamaguti, 1958). Lack of definitive host specificity is a characteristic of most 37-collar-spined echinostomes. *E. trivolvis* and *E. caproni* infect numerous avian and mammalian hosts, although each species has its preferred definitive hosts both in the wild and in the laboratory. Preferred hosts for *E. caproni* in the wild are domestic ducklings, *Rattus rattus* and the Egyptian giant shrew, *Crocidura olivieri*, whereas for *E. trivolvis* the preferred host is the muskrat, *Ondatra zibethica*, in addition to domestic ducks, swans and geese (Beaver, 1937; Jeyarasasingam *et al.*, 1972). Recent studies have shown that the laboratory mouse is an excellent host for the survival and longevity of *E. caproni* but a poor host for *E. trivolvis*, whereas the golden hamster, *Mesocricetus auratus*, is a permissive host for both *E. caproni* and *E. trivolvis* (Hosier and Fried, 1986; Odaibo *et al.*, 1988, 1989; Huffman *et al.*, 1988a,b).

The life cycle of *Echinostoma* is shown in Fig. 9. In the wild it is maintained when avian and mammalian hosts release eggs with their stool into lakes, farmponds and streams. The fertilized eggs are undeveloped when

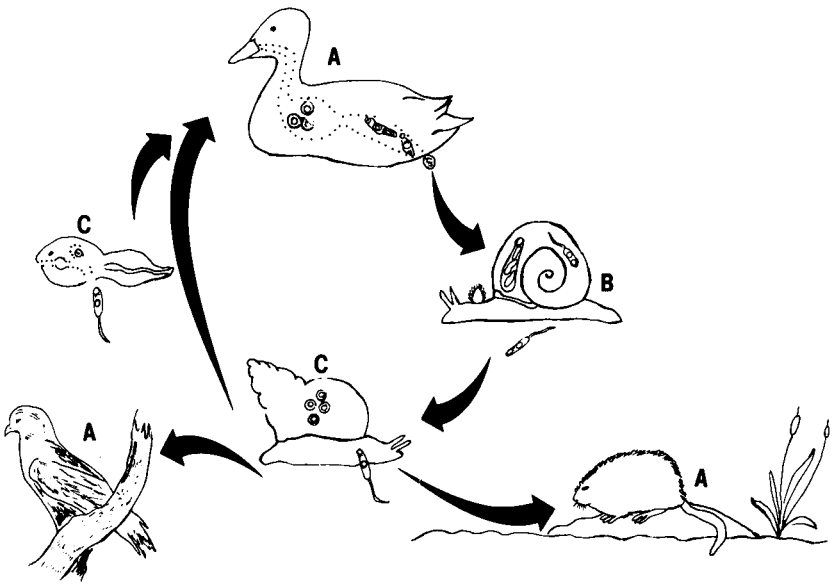

FIG. 9. Generalized life cycle of an echinostome, A, definitive; B, first intermediate host; C, second intermediate hosts.

laid and take about 2–3 weeks at 22°C to reach the fully developed miracidial stage (Fried and Weaver, 1969; Fried 1985). Development is more rapid at higher temperatures, and at 26–28°C *E. caproni* eggs develop into fully developed miracidia in 8–12 days (Jeyarasasingam *et al.*, 1972). Eggs can be maintained for at least 5 months at 4°C and still retain their ability to develop and hatch (Christensen *et al.*, 1980).

Miracidia of *E. caproni* hatch from eggs mainly in the late morning and actively penetrate *Biomphalaria glabrata* snails (Jeyarasasingam *et al.*, 1972). Although a broad spectrum of first intermediate host snails has been reported for some 37-collar-spined echinostomes (Beaver, 1937), recent studies suggest that each species of echinostome infects only one or a few species of snail hosts (Christensen *et al.*, 1980; Fried *et al.*, 1987b). In the USA, the preferred intermediate host of *E. trivolvis* is *Helisoma trivolvis* and in Africa the preferred host of *E. caproni* is either *Biomphalaria glabrata* or *B. alexandrina* (Jeyarasasingam *et al.*, 1972; Christensen *et al.*, 1980; Fried *et al.*, 1987b). In Europe and Asia several species of *Lymnaea* are the preferred hosts of *E. revolutum* (Lo and Cross, 1975; Kanev, 1985). Although detailed studies on echinostome miracidial attraction and penetration are lacking, miracidia enter the head–foot region of the snail and transform into mother sporocysts usually in the region of the heart; mother sporocysts give rise to mother rediae (at least in *E. caproni*) in the ventricle and the mother rediae give rise to numerous daughter rediae which migrate to the digestive gland–gonad complex (Jeyarasasingam *et al.*, 1972). Relatively few studies are available on the detailed biology of the intramolluscan stages of echinostomes. Cercariae begin to emerge from infected snails 4 to 6 weeks post-infection and have a predilection for the kidneys of the same or other snails or the kidneys of *Rana* tadpoles (Jeyarasasingam *et al.*, 1972; Christensen *et al.*, 1980; Fried, 1985).

Cercariae enter the nephridiopore of snail hosts and encyst within the saccular kidney about 2 h after entrance into the tubular kidney (Anderson and Fried, 1987). The second intermediate host is not specific and usually numerous species of snails, clams, frogs and even fishes serve as second intermediate hosts of *Echinostoma* (Beaver, 1937; Jeyarasasingam *et al.*, 1972; Christensen *et al.*, 1980; Fried *et al.*, 1987). However, there are differences in the susceptibility of second intermediate hosts for cercarial penetration by *E. trivolvis* and *E. caproni* (Christensen *et al.*, 1980; Anderson and Fried, 1987).

In the wild, birds and mammals that feed on second intermediate hosts containing metacercarial cysts of echinostomes become infected; in the laboratory, experimental definitive hosts, such as domestic chicks, laboratory mice and hamsters, become infected by feeding them metacercarial cysts obtained from second intermediate hosts (Beaver, 1937; Fried, 1984; Franco

et al., 1986; Hosier and Fried, 1986). Metacercarial cysts excyst usually in the ileum of the vertebrate host, and newly emerged juveniles establish in the lower intestine within 4 h post-feeding of cysts (Fried and Kletkewicz, 1987; Hosier and Fried, 1986). Presumably, conditions in the intestine, i.e. alkaline pH, trypsin-bile salts and a temperature of 37–43°C, influence the excystation process (Fried and Butler, 1978; Fried and Emili, 1988). Excystation *in vitro* in an alkaline bile salts–trypsin medium in the absence of acid–pepsin pretreatment has been reported for several species of echinostomes (Fried and Butler, 1978; Fried and Emili, 1988). *In vivo*, *Echinostoma* spp. develop into sexually mature adults within 7–9 days post-infection (Fried, 1984; Fried *et al.*, 1988a). Differences in development and growth can be influenced by the species of host used in the laboratory. Thus, *E. trivolvis* is relatively short lived in the laboratory mouse, lives longer in the domestic chick and survives for several months in the golden hamster (Fried, 1984; Hosier and Fried, 1986; Franco *et al.*, 1986). *E. caproni* survives for several months in laboratory mice and hamsters, but has a relatively short life span in the domestic chick (Odaibo *et al.*, 1989; Huffman *et al.*, 1988b; Fried *et al.*, 1988a).

In the laboratory, the life cycle can be maintained by dissecting eggs from the uteri of adult worms and incubating them in tap water for 8–12 days at 26–28°C until the miracidia hatch (Fried, 1985; Fried *et al.*, 1988a). Eggs can also be obtained from the feces of infected hosts, cleaned by sedimentation (Pritchard and Kruse, 1982) and embryonated as described above. This latter technique is more cumbersome than dissecting eggs from the uteri of worms obtained at necropsy.

Infection of first intermediate snail hosts is done using freshly emerged miracidia (those obtained within 2–4 h of emergence from eggs) and placing the miracidia 10–20 per snail in multiwell chambers, small petri dishes or Stendor dishes (Jeyarasasingam *et al.*, 1972; Fried, 1985; Fried *et al.*, 1987b). The age of the snail intermediate host may be a factor in some echinostome infectivity studies since young *B. glabrata* are more susceptible to the miracidia of *E. caproni* than are older snails (Jeyarasasingam *et al.*, 1972). This situation is not the same in miracidial infections of *E. trivolvis* in *Helisoma trivolvis* snails where older snails are as susceptible as younger ones (Fried *et al.*, 1987b).

Exposed snails can be maintained in aquaria in artificial spring water (Cohen *et al.*, 1980), about 10–20 snails per 1000 ml of water. Snails that become infected release cercariae in about 4–6 weeks at 22–24°C. Placement of other snails of the same or different species in aquaria with infected first intermediate host snails will allow for cercarial encystment in the renal tissues of the snails. Snails serving as first intermediate hosts should be marked with nail polish to distinguish them from second intermediate host

snails (Fried, 1985). Infected second intermediate host snails can be dissected and the kidneys removed to obtain encysted metacercariae (Fried and Weaver, 1969; Fried, 1985). Encysted metacercariae are infective to vertebrate hosts from about 1 day to 6 months post-encystment in the snail kidney (Anderson and Fried, 1987). In practice, it is convenient to remove cysts from snail kidneys and place them in Locke's solution (1 : 1) at 4°C where they can be stored for up to 1 year and remain viable (Fried and Perkins, 1982; Fried and Emili, 1988).

Numerous verebrates can serve as experimental definitive hosts (particularly domestic chicks, ducklings, laboratory mice and hamsters) and cysts are administered *per os* by pipet or stomach tube (Fried and Weaver, 1969; Christensen *et al.*, 1980; Franco *et al.*, 1986; Hosier and Fried, 1986). For necropsy, avian hosts can be anesthetized with Equi-thesin (Fried and Berry, 1961) and killed by decapitation; mice and hamsters can be anesthetized with ether or nembutal and then killed by cervical dislocation prior to examining the intestine and bile duct for echinostomes (Huffman *et al.*, 1988a).

III. Infectivity Studies in First Intermediate Snail Hosts

Echinostoma miracidia actively penetrate a specific snail host for the continuance of the life cycle. Miracidial development within the egg takes about 1–3 weeks in freshwater and the development is temperature dependent. Hatching is stimulated by light, but other factors are involved since some hatching occurs in the dark (Fried and Weaver, 1969). The development of the miracidium of *E. caproni* is shown in Fig. 10.

Miracidia of *Echinostoma* are typically digenean with uniform ciliation on the epidermal plates except in the suture areas; miracidia have an apical papilla at the anterior end followed by a primitive gut; the eyespots are located posterior to the gut; one pair of flame cells is present and germinal balls are located posterior to the eyespots. The tier arrangement of the epidermal cells in *Echinostoma* is typically 6, 6, 4, 2 from the anterior to the posterior aspect of the miracidium (Beaver, 1937; Jeyarasasingam *et al.*, 1972). Studies on site finding or ultrastructure of *Echinostoma* miracidia are not available.

Echinostome miracidia are host specific for one or a few closely related snail species. Miracidia of *E. trivolvis* infect a Pennsylvania strain of *Helisoma trivolvis*, but not a Colorado strain; *Physa heterostropha* and *Biomphalaria glabrata* are not susceptible to miracidial infection with this echinostome (Fried *et al.*, 1987b). Older snails may be more refractory to infection with some species of *Echinostoma* miracidia (Jeyarasasingam *et al.*, 1972), but this is not the case with *E. trivolvis* where older *H. trivolvis* are as

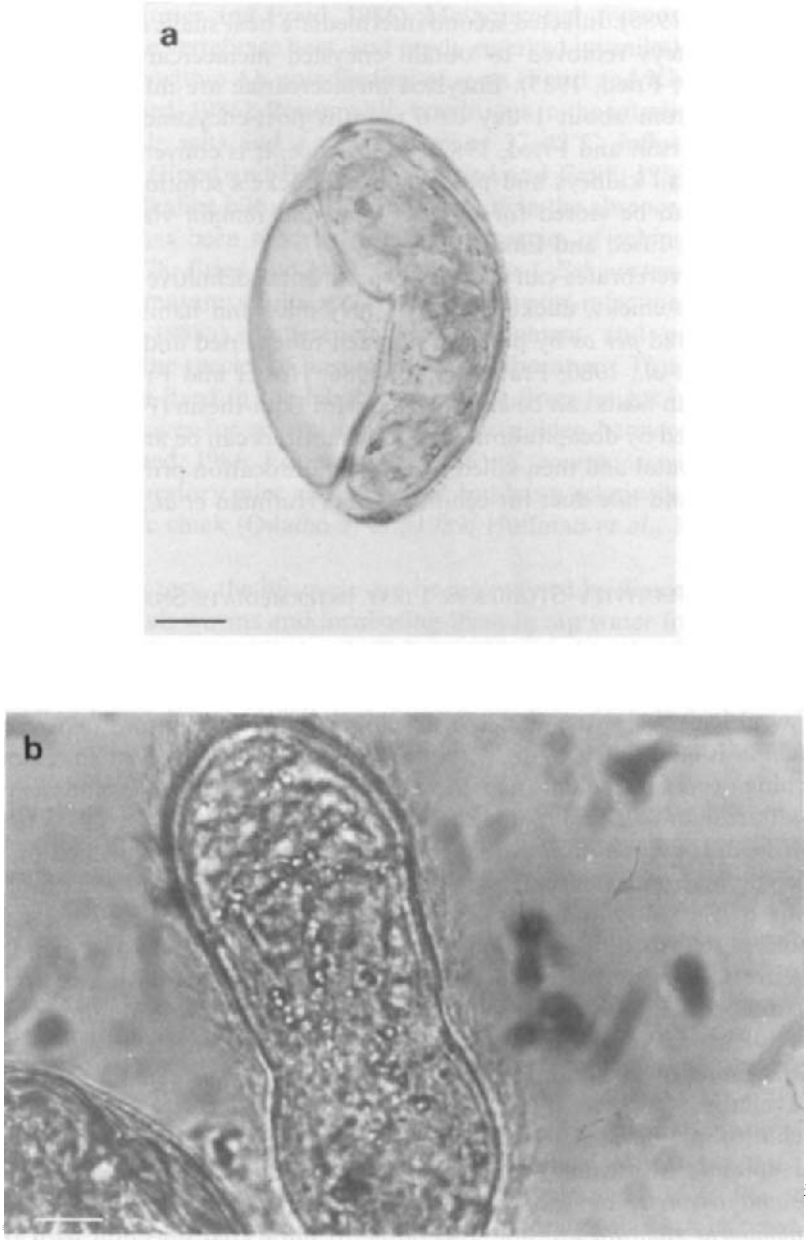

FIG. 10. (a) Fully developed miracidium of *E. caproni* embryonated in tap water for 10 days at 27°C. Scale bar = 25 μm. (b) Live miracidium hatched from an *E. caproni* egg. Scale bar = 15 μm.

susceptible as younger ones (Fried *et al.*, 1987b). Other than incidental observations by Beaver (1937), detailed studies on sporocysts and rediae of *E. trivolvis* are not available.

The Asiatic strain of *E. revolutum* miracidia infects both *Lymnaea ollula* and *L. swinhoei* (Lo and Cross, 1975). *L. ollula* snails exposed to five miracidia were 95% infected, whereas *L. swinhoei* showed a 40% infection rate. Miracidial penetration of *L. ollula* took about 1 h and about 100–200 daughter rediae were present at 8 days post-infection. Cercariae emerged (about 350 cercariae per day per snail) from the mantle collar area and were negatively phototactic (Lo and Cross, 1975).

Young mother sporocysts of *Echinostoma* are in the heart or aorta of the snail host (Jeyarasasingam *et al.*, 1972) and mother rediae released from the mother sporocyst migrate to the digestive gland–gonad complex. The morphology of mother and daughter rediae are not easy to distinguish and most descriptions of *Echinostoma* rediae are based on daughter rediae in the digestive gland–gonad complex. The rediae are typical of digeneans with a large sac-like gut often filled with orange, red or black gut contents (ingesta from the hemolymph and tissues of the snail host). The gut may extend more than one-half the length of the redia and daughter rediae may contain another generation of rediae or cercariae, but rarely both (Jeyarasasingam *et al.*, 1972). A birth pore is usually present along with ambulatory buds. Light and scanning-electron micrographs (SEM) of daughter rediae of *E. trivolvis* are shown in Fig. 11.

Studies are available on the miracidial infectivity of *E. caproni* in *B. glabrata* snails (Jeyarasasingam *et al.*, 1972; Fried *et al.*, 1988b). *B. glabrata* is the preferred intermediate host, although *B. alexandrina* is also susceptible; young *B. glabrata* are more susceptible than are older snails (Jeyarasasingam *et al.*, 1972).

Labeling of *E. caproni* with radioselenium was used to study miracidial infectivity of this echinostome. The labeling did not affect miracidial behavior or infectivity and *B. alexandrina* snails infected with such miracidia retained the label (Christensen, 1980). Infectivity of *E. caproni* miracidia to *B. glabrata* snails declined markedly when eggs were incubated for 30–42 days as compared with snail infectivity with miracidia derived from eggs incubated for 10–24 days (Kuris, 1980). A surprisingly high rate of infectivity of *B. glabrata* snails was achieved (54–66%) when 25 snails were exposed to 125 miracidia in 1 l of water (Kuris, 1980). Exposure of 1–2 mm *B. glabrata* snails to *E. caproni* miracidia affected the survival and longevity of the snails whether they became infected or not; detrimental effects of exposure or infection with miracidia of *E. caproni* in 4–6 mm shell diameter *B. glabrata* were not as apparent (Kuris, 1980).

The effects of miracidial infection by the European/Asiatic strain of *E.*

revolutum in lymnaeid snails have been studied. *Lymnaea luteola* snails infected with the miracidia of *E. revolutum* showed alterations in the type-B neurosecretory cells in the cerebral ganglia 4 weeks post-infection (Krishna and Simha, 1980). *L. luteola* infected with *E. revolutum* miracidia also had a lower rate of oxygen consumption than did uninfected lymnaeids (Krishna, 1981).

E. revolutum alters the biochemical profile of *L. luteola* and causes significant reductions in total proteins and phospholipids but increases the neutral lipids of infected snails (Krishna, 1980a). Acid and alkaline phosphatase activity was greater in *L. luteola* infected with *E. revolutum* than in the controls (Krishna, 1980a). *L. luteola* experimentally infected with *E. revolutum* had heavier shells than the uninfected controls; the concentration of calcium was also greater in the body tissues of the infected snails (Krishna, 1980b).

Echinostomes contribute additional bands of enzyme activity, particularly in glucose phosphate isomerase and malate dehydrogenase systems, in *Bulinus senegalensis* from the Gambia and Senegal (Wright *et al.*, 1979). This African strain of echinostome was described as *E. revolutum*, but in all probability is *E. caproni*.

Stagnicola palustris (Lymnaeidae) from North America infected with a species described as *E. revolutum* (it is not known if this echinostome is a synonym of *E. trivolvis* from the planorbid, *Helisoma trivolvis*) showed disrupted architecture of the hepatopancreas and gonad, a decline in egg laying and in the rate of oxygen consumption and shell formation (Zischke, 1972). Light micrographs of *H. trivolvis* infected with *E. trivolvis* rediae and an uninfected control are shown in Fig. 12.

Parasitism with *E. trivolvis* in *H. trivolvis* alters the ability of the snail to locate lettuce in a chemo-attraction bioassay. Uninfected snails were significantly attracted to hydrophilic factors released from the lettuce (Boland and Fried, 1984).

IV. Infectivity Studies in Second Intermediate Hosts

Carcariae of *Echinostoma* emerge from the first intermediate host and encyst within the same or other snail hosts. Second intermediate hosts include snails, clams, tadpoles, fishes, other invertebrates, and natural products such

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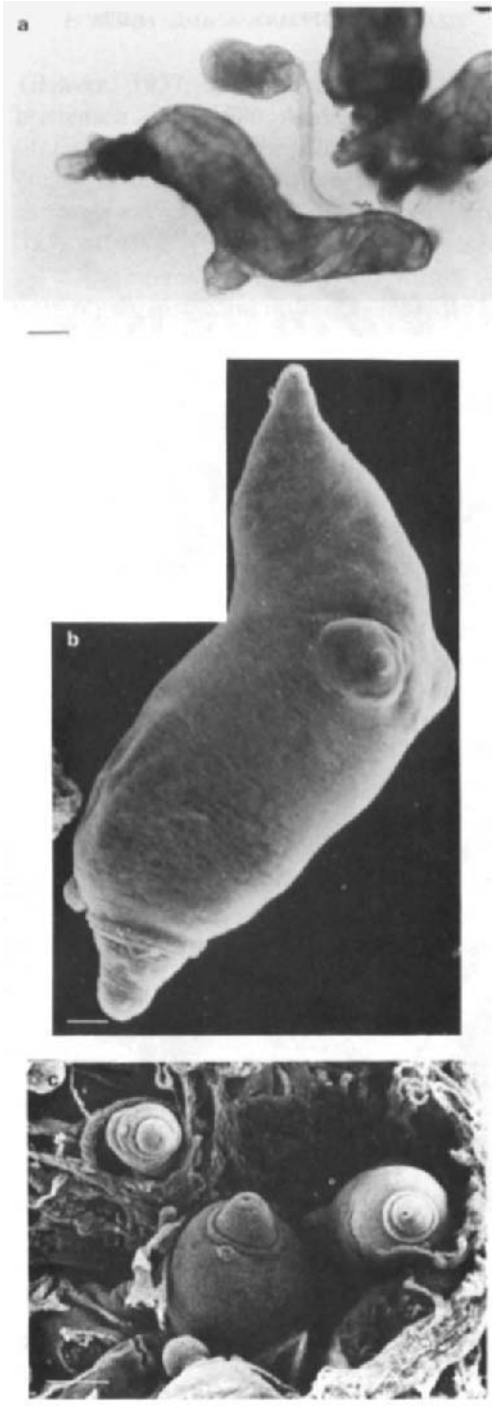
FIG. 11. (a) Light micrograph of a daughter redia of *E. trivolvis*. Scale = 25 μm. (b) SEM of *E. trivolvis* redia showing: A, ambulatory buds; B, birth pore; C, collar; P, pharynx. Scale bar = 5 μm. (Courtesy of Debby Pearsall.) (c) SEM of *E. trivolvis* rediae within the digestive gland–gonad complex of *H. trivolvis*. R, redia. Scale bar = 25 μm. (Courtesy of Debby Pearsall.)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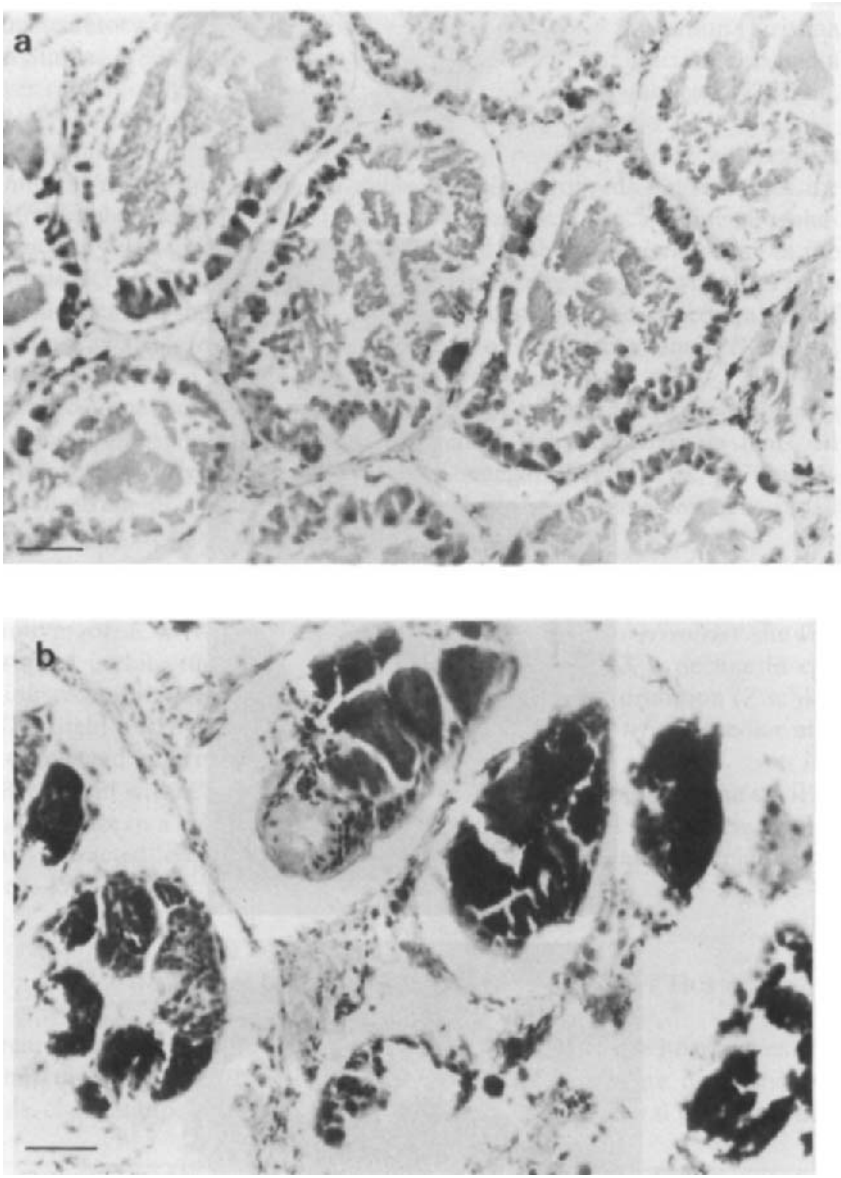

FIG. 12. Light micrographs of gonadal tissue of *H. trivolvis* experimentally infected with *E. trivolvis* rediae, 6 weeks post-infection. (a) Control; (b) experimental. Scale bar = 20 μm.

as snail mucus (Beaver, 1937; Jeyarasasingam *et al.*, 1972; Fried and Bennett, 1979; Christensen *et al.*, 1980; Anderson and Fried, 1987).

The cercariae of *Echinostoma* are short-lived and rarely survive beyond 48 h at ambient temperatures. Temperature is a factor in survival and longevity and as expected survival is shorter at higher temperatures, e.g. 8 h at 40°C versus 75 h at 10°C (Evans, 1985). Maximal penetration and encystment of most *Echinostoma* cercariae occur within 24 h post-emission from snails and probably cercariae are optimally infective within the first 8 h of release from snails.

Cercariae of *Echinostoma* are typically digenean, i.e. distomate, gymnocephalous with a prominent oral collar of spines and a simple tail (Fig. 13(a,b)). They are about 0.6 mm in length and are active swimmers showing typical wobble-like motion with constant flexion and extension of the tail. The tail of *Echinostoma* is often attenuated and bent at the distal tip and most *Echinostoma* have a pair of dorsal, ventral, and ventrolateral finfolds (Fig. 14(a)). Some finfolds are difficult to see by light microscopy, but can be seen by scanning electron microscopy (SEM) (Fried and Fujino, 1987) (Fig. 14(b)). The body of the cercaria has extensive cystogenous glands which often occlude flame cells making it difficult to obtain an accurate flame-cell count. However, flame-cell formulae for *Echinostoma* are available, e.g. $2(3+3+3+3+3+3) = 36$ for *E. caproni* as described by Jeyarasasingam *et al.* (1972). In addition to openings of penetration and cystogenous glands, some cercariae contain paraesophageal and esophageal glands of unknown function (Jeyarasasingam *et al.*, 1972). Silver staining (argentation) along with SEM reveal the presence of uniciliate and multiciliate papillae in echinostome cercariae which are undoubtedly sensory structures. The papillae patterns of some species of *Echinostoma* have been described (Lie, 1966; Fried and Fujino, 1987). Papillae patterns between species are quite similar, but some species differences do occur (Fried and Fujino, 1987).

Although most cercariae of *Echinostoma* must enter a second intermediate host for the continuance of the life cycle, some ectopic encystment does occur, mainly on snail mucus or *in vitro* in a tissue culture medium (Stein and Basch, 1977; Fried and Bennett, 1979; Christensen *et al.*, 1980). Ectopic cysts are infective to definitive hosts (Fried and Bennett, 1979; Christensen *et al.*, 1980). Inducement of *in vitro* encystment in 1:1 Locke's solution + glucose has been done for *E. trivolvis*; factors related to *in vitro* encystment of echinostomes are not understood (Laurie, 1974; Fried and Bennett, 1979).

Although cercariae of *Echinostoma* encyst in a broad range of second intermediate hosts there are cercarial preferences for second intermediate hosts. For example, *E. trivolvis* encysts more readily in *Biomphalaria glabrata* than in *Helisoma trivolvis*, *Physa heterostropha* or *Corbicula fluminea* (Anderson and Fried, 1987; Fried *et al.*, 1987a). Cercarial encystment

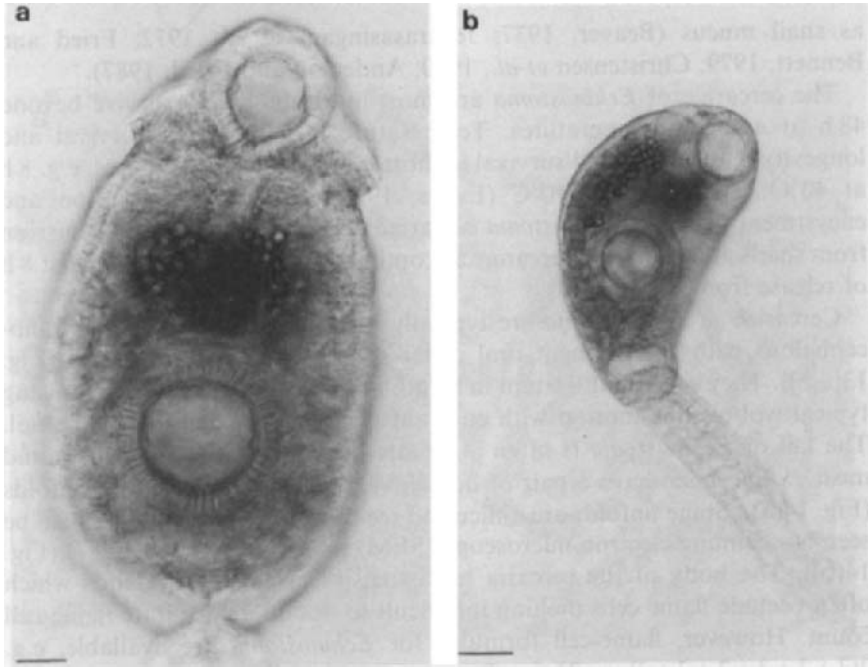

FIG. 13. (a) Cercaria of *E. trivolvis* showing collar spines. Scale bar = 30 μm. (b) Cercaria of *E. trivolvis*. Scale bar = 60 μm.

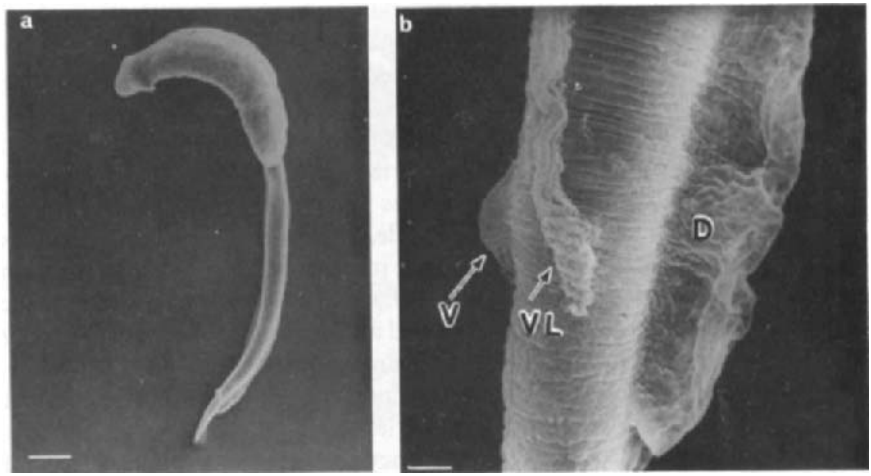

FIG. 14. (a) SEM of the cercaria of *E. trivolvis*. Scale bar = 50 μm. (Reproduced with the permission of the editors of the *Journal of Parasitology* from Fried and Fujino, 1987.) (b) SEM of *E. trivolvis* cercaria. D, dorsal finfold; V, ventral finfold; VL, ventrolateral finfold. Scale bar = 3 μm. (Courtesy of Takahiro Fujino.)

for *E. trivolvis*, at least in snails and in *Rana* tadpoles, occurs mainly in the kidneys; factors associated with attraction toward and migration into the kidneys are poorly understood (Kemp and Devine, 1982; Anderson and Fried, 1987).

Cercariae of *E. caproni* encyst in the heart, pericardium and kidney of their second intermediate host snails (Jeyarasasingam *et al.*, 1972). Five species of the genus *Bulinus*, and *Physa acuta* were very susceptible intermediate hosts, whereas three species of *Biomphalaria*, along with *Planorbarius corneus*, *Lymnea natalensis* and *Helisoma duryi* were less susceptible (Christensen *et al.*, 1980). Laboratory studies on *E. caproni* in France showed that cercariae encyst in various molluscan hosts, i.e. *Planorbis corneus*, *Lymnaea stagnalis*, *Pisidium casertanum* and *Melania tuberculata*, in addition to tadpoles of *Rana temporaria* (Richard and Brygoo, 1978).

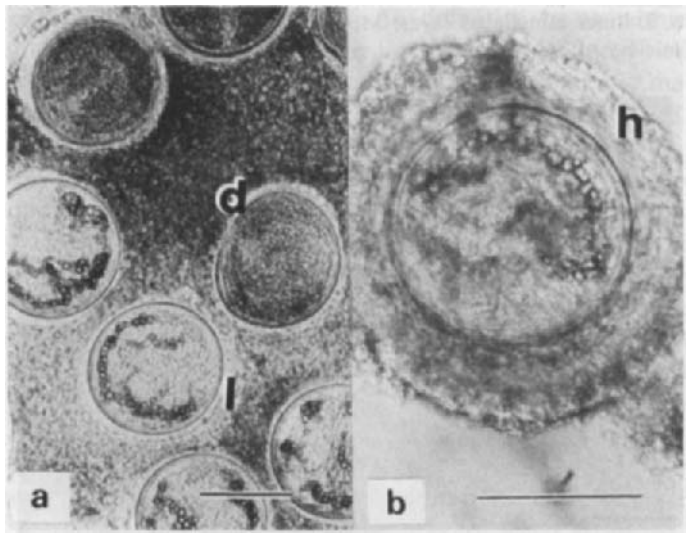

FIG. 15. (a) Live (L) and dead (D) cysts of *E. trivolvis* from *Biomphalaria glabrata*. Scale bar = 100 μm. (b) Live cyst of *E. trivolvis* surrounded by host hemocytes (h). Scale bar = 100 μm. (Reproduced with the permission of the editors of the *Journal of Parasitology* from Anderson and Fried, 1987.)

Metacercarial cysts of *Echinostoma* are about 150 μm in diameter and contain a relatively wide and transparent outer cyst and a narrow but thicker inner cyst (Fig. 15(a); Fried and Bennett, 1979; Fried and Emili, 1988). The larva within the cyst is transparent in viable organisms and excretory concretions and cephalic spines are visible; nonviable cysts are opaque and granular (Figs. 15(a,b)). Cysts invested with amoebocytes (hemocytes) are

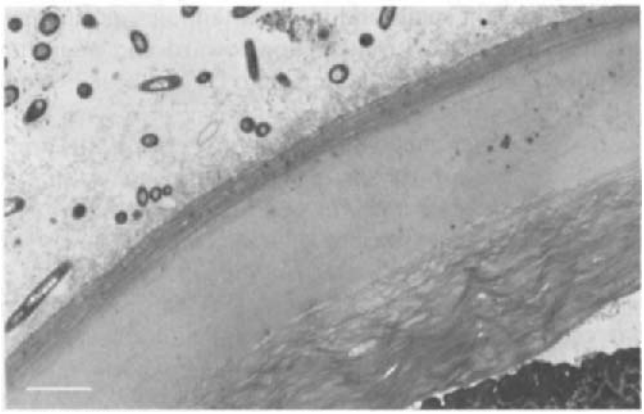

FIG. 16. TEM of an untreated cyst of *E. trivolvis* removed from *B. glabrata* kidney. W, worm, I, inner lamellated layer; M, middle layer; O, outer layer. Scale bar = 3 μm. (Courtesy of Sam Irwin.)

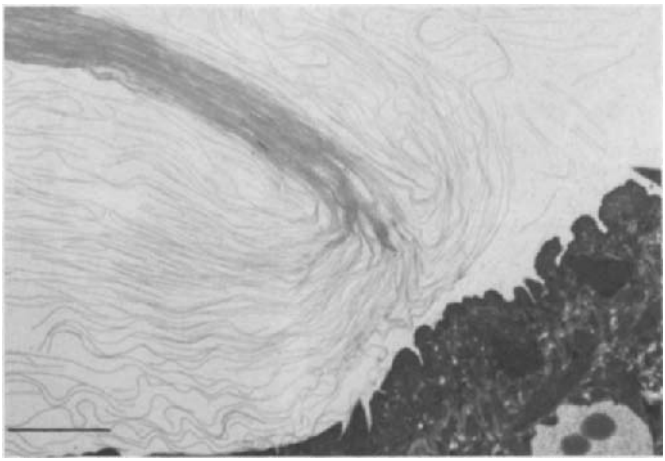

FIG. 17. Breaching of the lamellated layer of an *E. trivolvis* cyst by the larva prior to final disruption of the outer cyst. Scale bar = 1 μm. (Courtesy of Sam Irwin.)

usually viable if the larvae within appear transparent (Fig. 15(b); Anderson and Fried, 1987). By transmission electron microscopy (TEM) the cysts may show two or three layers, with the inner cyst layer being lamellated (Fig. 16; Stein and Basch, 1977; Gulka and Fried, 1979). During excystation, the organism breaches the lamellated layer prior to final disruption of the outer cyst (Fig. 17). Both extrinsic (host) and intrinsic (parasite) enzymes are probably involved in excystation (Sommerville and Rogers, 1987), but details of the process are obscure in *Echinostoma*. *In vitro* excystation for

some species of *Echinostoma* has been accomplished in an alkaline trypsin–bile salt medium maintained at 37–41°C in the absence of an acid pepsin pretreatment (Fig. 18; Fried and Butler, 1978; Fried and Emili, 1988). Although only subtle differences may exist in the morphology of encysted metacercariae of closely related echinostome species, considerable differences in the percentage and rate of excystation have been noted between *E. trivolvis* and *E. caproni* metacercarial cysts (Fried and Emili, 1988).

Cysts may remain viable within second intermediate hosts for months. Survival of *E. caproni* for 4 months and *E. trivolvis* for 6 months within planorbid intermediate host snails has been reported (Christensen *et al.*, 1980; Anderson and Fried, 1987). Furthermore, cysts of *Echinostoma* can be removed from snails and stored for more than 1 year in 1:1 Locke's solution at 4°C and retain their infectivity to definitive hosts (Fried and Perkins, 1982; Fried and Emili, 1988).

Cysts of some species are infective to definitive hosts within 4 h of encystment in the second intermediate host (Anderson and Fried, 1987). However, for other species a longer period (up to 7 days) of cyst maturation is needed before the metacercaria is infective to the definitive host (Dönges, 1969).

Relatively little information is available on the gross or histopathology of *Rana* tadpoles or snails infected with *Echinostoma* metacercariae. The mortality of *Physa* snails and *Rana* tadpoles infected with *E. trivolvis* has been reported, but mainly as incidental observations (Beaver, 1937; Fried and Weaver, 1969). Cercarial penetration and metacercarial encystment of *E. caproni* in 3–8 mm shell diameter *B. glabrata* caused high mortaility in 4–6 days of continuous exposure to about 150 cercariae per day (Kuris and Warren, 1980).

V. Infectivity, Growth and Development in Vertebrate Hosts

The growth and development of *Echinostoma trivolvis* in natural and experimentally infected avian and mammalian hosts was studied by Beaver (1937). Senger (1954) studied growth, development and survival of *E. trivolvis* in domestic chicks and rats. Infectivity of this worm in domestic chicks has been reported (Senger, 1954; Fried and Weaver, 1969; Fried and Butler, 1978; Fried and Alenick, 1981; Fried, 1984).

A. INFECTIVITY

Many factors influence infectivity of *E. trivolvis* in the domestic chicken including age and pretreatment of metacercarial cysts, and the host-gut

emptying time. The use of preselected cysts, as done by Hayes *et al.* (1972) with *Fasciola hepatica*, enhances infectivity of *E. trivolvis* in the domestic chick. Fried (1984) reported 100% infectivity with *E. trivolvis* cysts and a worm recovery of 25%. Worm recovery in the domestic chick has been reported to be up to 44 days post-infection.

Franco *et al.* (1986) reported that 100% of the hamsters became infected with *E. trivolvis* and worm recovery averaged 38%. Hamsters have remained infected for up to 123 days post-infection (Mabus *et al.*, 1988).

Hosier and Fried (1986) reported that ICR and Swiss Webster (SW) mice can be infected with metacercariae of *E. trivolvis*. The worm burden of SW mice was reduced at 3 weeks post-infection and eliminated by 4 weeks. In ICR mice the worm burden was reduced at 2 weeks and eliminated by 3 weeks. Christensen *et al.* (1981a) reported that inbred albino mice exposed to more than 15 metacercariae of *E. caproni* retained consistent numbers for up to 70 days.

Hosier and Fried (1986) reported a recovery rate of 88% of *E. trivolvis* in mice. Christensen *et al.* (1981a) and Sirag *et al.* (1980) reported recoveries as high as 94% and 100%, respectively, of *E. caproni* in mice.

B. WORM DISTRIBUTION

E. trivolvis occupies numerous sites in the intestine of the domestic chicken including the ileum, rectum, cloaca, cecum, and the bursa of Fabricius (Beaver, 1937; Senger, 1954; Fried and Weaver, 1969; Fried and Butler, 1978; Fried and Alenick, 1981). Fried and Freeborne (1984) examined the effects of *E. trivolvis* in domestic chickens for up to 3 weeks on various dimensions of the chicken intestine, and also examined the effects of worm crowding in the intestine. As worms aged they tended to move more posteriad in the gut. This observation is in accord with previous studies on *E. trivolvis* by Senger (1954), Fried and Weaver (1969), and Fried and Alenick (1981).

Franco *et al.* (1986) reported that worms of *E. trivolvis* were found singly, in pairs, or in clusters and were found along the length of the small intestine. Franco *et al.* (1988) found *E. trivolvis* in the cecum when crowding occurred. Huffman *et al.* (1986) reported that in heavy infections the adult parasite could be found in the stomach, liver, gall-bladder and pancreas.

Hosier and Fried (1986) reported that in mice, *E. trivolvis* was recovered mainly in the ileum. Huffman *et al.* (1988b) found that *E. caproni* were

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FIG. 18. (a) SEM showing partial emergence of the larva during excystation of *E. caproni*. Scale bar = 20 μm. (Courtesy of Takahiro Fujino.) (b) SEM of an excysted metacercaria of *E. caproni*. Scale bar = 25 μm. (Courtesy of Takahiro Fujino.)

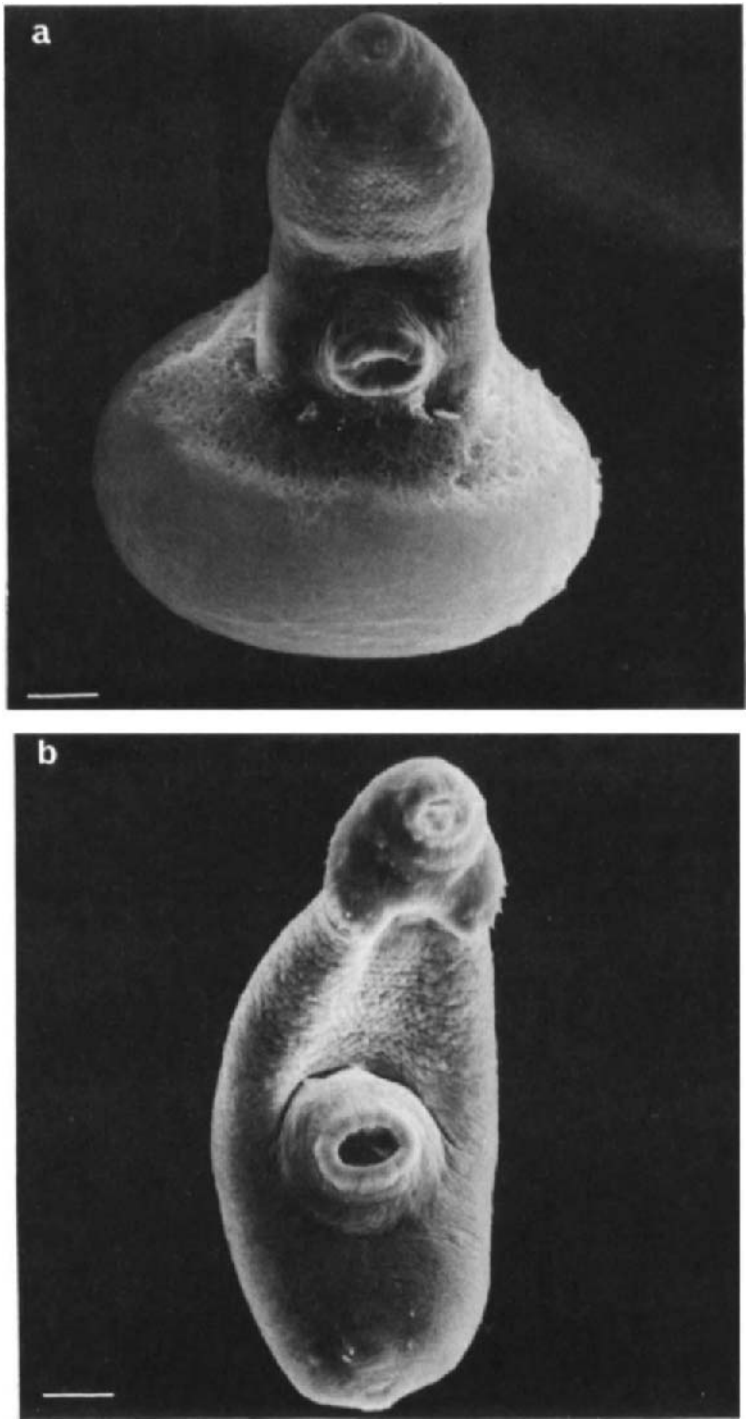
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TABLE 4 *Various parameters illustrating the differences encountered in a particular host and species of parasite*

Parasite	*Experimental host*	*Length of infectivity (days)*	*First appearance of eggs* in utero *(days)*	*Infectivity (%)*	*Worm recovery (%)*	*Small intestine*[c]		
						I (Duodenum)	*II (Jejunum)*	*III (Ileum)*
E. trivolvis	Hamster	123	8	100	38	Y	Y	Y
	Chicken	44	7	50–69	6–21	N	N	Y
	Swiss Webster mice	28	14	—	75	N	Y	Y
	ICR mice	21	14	—	22	N	N	Y
E. caproni	NMRI mice[a]	42	14	—	93.6	N	Y	Y
	NMRI mice[b]	112	14	—	84.7	N	Y	Y
	Hamster	89	7	100	37	N	Y	Y
	Chicken	32	7	100	28	N	N	Y

[a] Exposed to six metacercariae. [b] Exposed to 25 metacercariae. [c] Y, yes; N, no.

TABLE 5 *Worm distribution*[a] *of* E. trivolvis

Rectum	*Ceca*	*Cloaca*	*Bursa of Fabricius*	*Stomach*	*Liver*	*Pancreas*	*Gall-bladder*	*Reference*
N	Y[b]	N	N	Y[b]	Y[b]	Y[b]	Y[b]	Franco *et al.* (1986)
Y	Y	Y	Y	N	N	N	N	Fried (1984)
N	N	N	N	N	N	N	N	Hosier and Fried (1986)
N	N	N	N	N	N	N	N	Hosier and Fried (1986)
N	N	N	N	N	N	N	N	Odaibo *et al.* (1989)
N	N	N	N	N	N	N	N	Odaibo *et al.* (1989)
N	Y[c]	N	N	N	N	N	N	Huffman *et al.* (1989)
N	N	N	N	N	N	N	N	Fried *et al.* (1989)

[a] Y, yes; N, no. [b] Crowded conditions. [c] Exposed to 25 metacercariae.

clustered mainly in the posterior third of the small intestine in golden hamsters.

C. DEVELOPMENT IN VERTEBRATE HOSTS

1. *Echinostoma trivolvis*

Fried and Freeborne (1984) reported considerable differences in the body area of worms of the same age, but from different sites. Body-area measurements of worms in particular sites with 1–10 worms were significantly greater than those from the same site with over 25 worms. In *E. trivolvis* under crowded conditions the number of eggs in the uterus was about one-half that of worms in uncrowded sites.

The length of flukes recovered from golden hamsters is similar to those recovered from domestic chicks. Fried (1984) determined that 3-day-old flukes were 1.0 mm in length whereas in the golden hamster they averaged 0.77 mm. At 14 days, flukes from the domestic chick were 6 mm in length as compared with 6.3 mm in the golden hamster. In the chick the midacetabular width is equal to the greatest width, but in the hamster this is not the case. The region of greatest width in worms from the hamster was that posterior to the acetabulum and anterior to the ovary and contained the uterus. The ratio of length to midacetabular width of preovigerous worms was 6 in both the hamster and the chick. The ratio increased to 10 in ovigerous flukes from the chick, but remained at about 6 in the hamster. The overall result was a greater body area in flukes from the hamster versus the chick.

Wet and dry weights for flukes from the hamster up to day 14 post-infection were similar to those reported by Sleckman and Fried (1984) in the chick. As worms aged in hamsters they showed a greater increase in wet and dry weights than flukes from chicks. Day-21 flukes from hamsters weighed 8.7 mg (wet) and 3.0 mg (dry). The day-21 wet weight in chicks was 5.3 mg and the dry weight was 1.7 mg. By day 28 post-infection, wet and dry weights in the hamsters were 12.3 mg and 3.6 mg in hamsters versus 7.5 mg and 2.3 mg, respectively, in chicks.

Fried (1984) reported vitellaria by day 7 in some flukes from the chick. This occurred by day 8 in flukes from the hamster. Fried (1984) observed eggs infrequently in 8-day-old worms with most becoming ovigerous by day 9. In the hamster there were some ovigerous worms by day 9, but most became ovigerous by day 10 (Franco *et al.*, 1986). Fried and Freeborne (1984) reported a maximum of 500 eggs in the uterus of flukes from chicks. Flukes from hamsters contained approximately five times more eggs than those from chicks. Worms from hamsters produced eggs capable of hatching (Fried *et al.*, 1988b).

TABLE 6 *Avian hosts infected with* E. trivolvis

Common name	*Species or subspecies*	*Reference*
Common pintail	*Anas acuta*	Gower (1937)
American widgeon	*Anas americana*	Gower (1937)
Domestic duck	*Anas borchas*	Gower (1937)
	Anas brasiliensis	Beaver (1937)
Cinnamon teal	*Anas cyanoptera septentrionalium*	Wilkinson, *et al.* (1977)
Blue-winged teal	*Anas discors*	Shaw and Kocan (1980)
Eurasian widgeon	*Anas penelope*	Gower (1937)
Mallard	*Anas platyrhynchos platyrhynchos*	Gower (1937)
Mottled duck	*Anas platyrhynchos fulvigula*	Kinsella and Forrester (1972)
Black duck	*Anas rubripes*	Gower (1937)
Australian black duck	*Anas superciliosa rogersi*	Beaver (1937)
Emden goose (domestic goose)	*Anser* sp.	Griffiths *et al.* (1976)
Greytag goose	*Anser anser*	Beaver (1937)
Magpie goose	*Anseranas semipalmata*	Beaver (1937)
Lesser scaup	*Aythya affinis*	Gower (1937)
Redhead	*Aythya americana*	Gower (1937)
Ring-necked duck	*Aythya collaris*	Gower (1937)
Greater scaup	*Aythya marila*	Gower (1937)
Great-horned owl	*Bubo virginianus*	Ramalingam and Samuel (1978)
Rough-legged hawk	*Buteo lagopus*	Beaver (1937)
Muscovy	*Cairina moschata*	Gower (1937)
Domestic pigeon[a]	*Columba livia*	Beaver (1937)
Domestic chicken[a]	*Gallus gallus*	Beaver (1937)
Common scoter	*Oidemia nigra*	Gower (1937)
American flamingo	*Phoenicopterus*	Threlfall (1980)
Common grackle	*Quiscalus quiscula*	Stanley and Rabalais (1971)
Mourning dove	*Zenaida macroura*	Barrows and Hayes (1977)

[a]Experimental infection.

Hosier and Fried (1986) reported that the dry weights of week-old *E. trivolvis* worms from mice and chickens were comparable, but differed at 2 and 3 weeks. Sleckman and Fried (1984) reported average body weights for worms in chickens of 0.1, 1.4, and 1.7 mg at 1, 2, and 3 weeks, respectively. Worms from mice averaged 0.1 mg at 1 week and 0.7 mg at 2 and 3 weeks.

Worms from mice produced eggs capable of hatching (Hosier and Fried, 1986) as did worms in chickens (Fried and Weaver, 1969). The number of eggs in worms from chickens (Fried and Freeborne, 1984) was greater than

in mice. Although worm recoveries in mice were higher than in chickens, body area, weight and egg data suggest that worm growth and fecundity were suboptimal in ICR and SW mice (Hosier and Fried, 1986).

TABLE 7 *Mammalian hosts of* E. trivolvis

Common name	*Species*	*Reference*
Dog[a]	*Canis familiaris*	Beaver (1937)
Guinea-pig[a]	*Cavia porcellus*	Beaver (1937)
Cat[a]	*Felis catus*	Beaver (1937)
Golden hamster[a]	*Mesocricetus auratus*	Franco *et al.* (1986)
House mouse[a]	*Mus musculus*	Beaver (1937)
Muskrat	*Ondatra zibethica*	Leidy (1888)
Rabbit[a]	*Oryctolagus cuniculus*	MacKinnon and Burt (1978) Beaver (1937)
Norway rat[a]	*Rattus norvegicus*	Beaver (1937)
Pig[a]	*Sus scrofa*	Beaver (1937)
Red fox	*Vulpes vulpes*	Smith (1978)
ICR mouse[a]	—	Hosier and Fried (1986)
Swiss Webster mouse[a]	—	Hosier and Fried (1986)

[a] Experimental infection.

Fried *et al.* (1988a) reported that wet weights for 7-day-old *E. caproni* in chickens averaged about 0.3 mg, increased to about 2.5 mg at 14 days and peaked at 3.0 mg at 21 days; average dry weights for the identical days were 0.1, 0.15 and 0.8 mg, respectively. Worm body area increased slowly to about day 5 (average 0.5 mm^2), rapidly to about day 8 (average 1.6 mm^2), and less rapidly to day 32 (average 6.5 mm^2).

Odaibo *et al.* (1988) reported on the establishment, survival and fecundity of *E. caproni* infections in NMRI mice at different doses. As the infection progressed the parasites moved posteriorly in the small intestine. Mice receiving 25 metacercarial cysts of *E. caproni* were positive for egg production for up to 112 days. Those mice receiving six metacercariae maintained the infection for only 42 days. The worm loss in individual mice was gradual.

Odaibo *et al.* (1989) reported that worm recovery averaged 93.6% (range 83–100%) and 84.7% (range 64–100%) in six and 25 metacercarial infections, respectively. Approximate body areas for NMRI mice infected with six metacercariae of *E. caproni* was 17 mm^2 (35 days post-infection), 18 mm^2 (55 days post-infection) and 23 mm^2 (112 days post-infection). Similar body areas were obtained for NMRI mice infected with 25 metacercariae of *E. caproni*, 11 mm^2 (day 15 post-infection), 16 mm^2 (day 20 post-infection)

and 17 mm^2 (day 35 post-infection). Uterine egg counts from day-14 worms from mice exposed to six metacercariae averaged 500 eggs and from 25 metacercarial exposure averaged 600 eggs.

Other strains of mice have been infected with *E. caproni*. These include SS, SVS (Christensen *et al.*, 1985, 1986) and athymic nude mice (Bindseil and Christensen, 1984).

TABLE 8 *Avian hosts infected with* E. caproni

Common name	*Species*	*Reference*
Domestic chicken[a]	*Gallus gallus*	Fried *et al.* (1988a)
Domestic duckling	*Anas bochas*	Jeyarasasingam *et al.* (1972)
Pigeon[a]	*Columba livia*	Jeyarasasingam *et al.* (1972)
Finch[a]	*Lonchura striata*	Jeyarasasingam *et al.* (1972)
Falcon	*Falco newtoni*	Richard and Brygoo (1978)

[a] Experimental infection.

TABLE 9 *Mammalian hosts infected with* E. caproni

Common name	*Species*	*Reference*
Rabbit[a]	*Oryctolagus cuniculus*	Moravec *et al.* (1974)
Rat	*Rattus rattus*	Jeyarasasingam *et al.* (1972)
Egyptian giant shrew	*Crocidura olivieri*	Jeyarasasingam *et al.* (1972)
Golden hamster[a]	*Mesocricetus auratus*	Huffman *et al.* (1988b)
Mouse[a]	*Mus musculus*	Jeyarasasingam *et al.* (1972)
White rat[a]	*Rattus rattus*	Jeyarasasingam *et al.* (1972)

[a] Experimental infection.

TABLE 10 *Avian hosts infected with* E. revolutum

Common name	*Species*	*Reference*
Pigeon[a]	*Columba livia*	Lie and Umathevy (1965)
Domestic duck[a]	*Anas borchas*	Lie and Umathevy (1965)
Domestic chicken	*Gallus gallus*	Nasincova (1986)
Little cuckoo dove[a]	*Macropygia ruficeps*	Lie and Umathevy (1965)
Blackheaded or Chestnut munia[a]	*Lonchura ferruginosa*	Lie and Umathevy (1965)
Spotted munia	*Lonchura punctulata*	Lie and Umathevy (1965)
Java sparrow	*Padda oryzivora*	Lie and Umathevy (1965)

[a] Experimental infection.

D. WORM CROWDING

Relatively few experimental studies have been done on the effects of intraspecific crowding of digeneans (Willey, 1941; Fried and Nelson, 1978; Mohandas and Nadakal, 1978; Nollen, 1983; Fried and Freeborne, 1984). Franco *et al.* (1988) examined the growth and maturation, fecundity and niche of *E. trivolvis* at various intensities in the golden hamster. Their study revealed that increased dosage levels and thus infrapopulation size influences several aspects of the parasite's development in the host intestine. It extends the maturation time and reduces total weight gain and overall body size, while enlarging the spatial distribution of the parasites in the gut. These effects may be the products of a crowding effect facilitated by some form of intraspecific competition. Mohandas and Nadakal (1978) found that crowding reduced the length of *E. malayanum* in rats. Fried and Freeborne (1984) reported that under crowded conditions the number of eggs in the uterus of *E. trivolvis* was about one-half that of worms in uncrowded sites. Franco *et al.* (1988) reported that the appearance of *E. trivolvis* eggs in the feces of crowded hamsters was delayed. In uncrowded conditions eggs appeared by day 10 post-infection and in crowded conditions it was delayed in some animals until day 14 post-infection. It has been suggested that chemical "factors" released by worms associated with crowding may be important influences on worm growth (Fischthal *et al.*, 1982; Roberts and Insler, 1982).

VI. IMMUNOLOGY OF ECHINOSTOME INFECTIONS IN VERTEBRATE HOSTS

A complex set of interacting and interrelated factors may govern the immune response that occurs in echinostome infections. Sirag *et al.* (1980) developed models for studying homologous and heterologous interactions in *Echinostoma caproni* infections in the mouse. They demonstrated that a primary, 20- or 28-day-old *E. caproni* infection of 11–15 worms induced complete resistance to a homologous challenge, and that a primary, patent 79-day-old *Schistosoma mansoni* infection induced complete resistance to a heterologous challenge with *E. caproni.* Resistance persisted after anthelmintic removal of worms. It was suggested that homologous *E. caproni* resistance is mediated by immunologically specific factors.

Christensen *et al.* (1981a) suggested that threshold levels do not exist in the *E. caproni*–mouse model, because low-level infections were expelled. These low-level infections do not give rise to any pronounced inflammatory changes in the intestinal wall and this indirectly supports the assumption by Sirag *et al.* (1980) that homologous *E. caproni* resistance is mediated by

immunologically specific factors. Christensen *et al.* (1981a) reported that immunotolerance is possible in high-level infections that are not expelled for a period of at least 61 days. Possible mechanisms to be considered include the active depression of immune responses and a depression of the effectiveness of immune reponses owing to pathophysiological alterations induced by the parasite.

Hosier *et al.* (1988) showed that ICR mice can develop resistance against a homologous challenge infection with either *E. trivolvis* or *E. caproni*. After homologous challenge, established worm burdens of *E. trivolvis* and *E. caproni* were significantly reduced by 87.5% and 42%, respectively. There was a 17% reduction in the worm burden of *E. caproni* superimposed upon a primary infection of *E. trivolvis* and a 55% reduction of *E. trivolvis* superimposed upon a primary *E. caproni* infection (Hosier *et al.*, 1988). These were not significant reductions, but the dry-weight data suggest that conditions for growth of *E. trivolvis* and *E. caproni* were suboptimal in heterologous infections.

Barus *et al.* (1974) singly and concurrently infected golden hamsters with metacercariae of *E. caproni* and *Echinoparyphium recurvatum*. In single infections *E. caproni* was found along the length of the small intestine. The highest concentration of flukes occurred 6.5–9.8 cm from the anterior end of the small intestine. A marked preference for the anterior half of the small intestine was seen with *E. recurvatum*. The same authors found a quantitatively balanced double infection with both trematode species on day 10 post-infection. Both fluke species were sexually mature. *E. recurvatum* preferred the anterior half of the small intestine and *E. caproni* shifted to the posterior half. A mixed simultaneous infection with *E. caproni* and *E. recurvatum* was evaluated on day 17 post-infection. In this case, the two flukes colonized separate sections, always leaving a short unoccupied space between the two species. In double infections the final distribution of both fluke species in the intestine was the result of interactions between the two parasite species. In single infections, both trematodes colonized approximately the same sites, although site specificity was stricter in *E. recurvatum* than in *E. caproni*. Both parasites selected the same optimal microhabitat. The mechanism proposed for this phenomenon by Barus *et al.* (1974) is *interactive site segregation*. The two parasites, however, can co-exist in the intestine of the golden hamster.

Christensen *et al.* (1981b) reported that a primary 90-day-old single-sex *S. mansoni* infection did not confer any resistance to challenge with *E. caproni*. This indicated that the heterologous resistance depends on the deposition of schistosome eggs in the host tissues. Indirect evidence supports the importance of the egg in *Schistosoma* sp. induced resistance to a challenge with *E. caproni*. It was discovered that a primary *S. bovis* infection, aged 100 days with high tissue egg counts (6914 $\pm$ 3465 eggs per g liver tissue), induced an

almost complete resistance, Sirag *et al.* (1980) failed to demonstrate resistance to *E. caproni* in mice harboring 56-day-old *S. bovis* infections with only negligible tissue egg counts (10 ± 2 eggs per g liver tissue).

Early loss of *E. caproni* in mice harboring primary patent *S. mansoni* may be due to a reduction in the metacercarial excystation capacity and/or an early killing/elimination of excysted juvenile larvae. Adult echinostomes can co-exist with *S. mansoni* in mice. These mice are completely resistant to a challenge with *E. caproni* metacercariae, which points to the larval stages as the target of the mechanism of resistance.

Heterologous resistance to *E. caproni* in *Schistosoma* sp. infected mice has an immunological background. This resistance may be mediated directly through specific factors such as cross-reacting antigens. An indirect mechanism may involve the non-specific effect of the schistosome egg-induced inflammatory reactions in the intestinal wall. This specific immunological mechanism has yet to be demonstrated.

Mabus *et al.* (1988) reported several conclusions about the course of *E. trivolvis* infection in the golden hamster. No demonstrable humoral response was evident, although certain characteristics of the cellular response were. In the intestinal inflammatory response, a prominent lymphocytic infiltration was observed, although intestinal eosinophilia was minimal. Mabus *et al.* (1988) were able to reinfect hamsters with *E. trivolvis* after the initial infection was eliminated with Zanil (oxyclozanide).

Christensen *et al.* (1986) demonstrated an equal level of resistance following an oral metacercarial infection and an intraduodenal implantation of newly excysted juvenile worms of *E. caproni.* This shows that the newly excysted juvenile worm is the target stage of the host immune response and indicates that the ability of metacercariae to excyst is probably not affected. Simonsen and Andersen (1986) reported that antibodies to the surface of juvenile *E. caproni* appeared in the serum of infected mice at the same time as resistance to reinfection developed.

Tani (1979) reported an evident increase in peripheral eosinophil counts in human volunteers each given 30 metacercariae of *E. hortense.* However, the antibody production was very slight with individual results differing.

Tani and Yoshimura (1988) indicated that spontaneous expulsion of *E. hortense* occurred in mice experimentally infected with *E. hortense.* Their study indicated that worm expulsion began at week 3 post-infection and was closely associated with an increase in intestinal goblet cells, but not with the kinetics of mucosal mast cells.

VII. Clinical and Pathological Effects of Echinostomes

Case reports on the clinical aspects of echinostomiasis have given insights

into the manifestations of this disease. Hilarrio and Wharton (1917) reported occasional headache, dizziness, and a slight anemia caused by toxic effects of echinostomes in man. Ujiie (1936) infected himself with *Echinochasmus japonicus* and reported gastric pain and slight diarrhea. The disturbances caused by echinostomes in man are closely related to the number of worms (Yamashita, 1964). Faust *et al.* (1970) reported that intestinal colic and diarrhea develop due to the inflammatory reaction at the site of attachment to the intestinal wall by the parasite.

Echinostomes are parasites of man in south-east Asia and the Far East and infections are acquired from the consumption of raw snails or tadpoles containing encysted metacercariae (Rim, 1982).

Human echinostomiasis has been reported from Japan (Tani *et al.* 1974), and Korea (Seo *et al.*, 1983; 1985). Tani (1979) reported on the clinical effects of *E. hortense* in five volunteers each given 30 metacercariae orally. Three of the volunteers discharged eggs in feces from days 16 to 106 post-infection. An increase in peripheral eosinophilia was reported in all cases.

Results derived from experimental infection of laboratory rodents have provided insight into the pathogenesis of echinostomes. Pathological changes were reported by Bindseil and Christensen (1984) in the small intestine of conventional mice and congenitally athymic, nude mice infected with *E. caproni*. Mohandas and Nadakal (1978) reported that pathological changes in the rat intestine produced by *E. malayanum* included lysis and destruction of mucosa, increased activity of goblet cells, edema and reticulated appearance of lamina propria and hyperplasia of epithelial cells. Experimental infections of *E. trivolvis* in hamsters produced a severe and fatal disease in this host (Huffman *et al.*, 1986). Clinical signs in hamsters infected with *E. trivolvis* included progressive unthriftiness, watery diarrhea and weight loss. Packed cell volume, hemoglobin and red blood cell counts increased in those animals with severe diarrhea. No increase in eosinophils was noted in peripheral blood smears. The histopathological responses of hamsters to the parasite showed erosion of intestinal villi with lymphocytic infiltration being the primary response (Fig. 19). Periportal lymphocytic infiltration and focal hepatic necrosis were observed in livers from some infected animals.

The pathology of extraintestinal infections with *E. trivolvis* in the golden hamster was reported by Huffman *et al.* (1988b). The occurrence of *E. trivolvis* in extraintestinal sites probably results from worm crowding in heavy infections. The worms migrate up the common bile duct and can be found in the liver, gall-bladder and pancreas (Fig. 20). The migration of parasites through the liver damages blood vessels resulting in hemorrhage. Granulomas composed of multinucleate giant cells developed in the liver (Fig. 21). In extraintestinal infections serum bilirubin concentrations

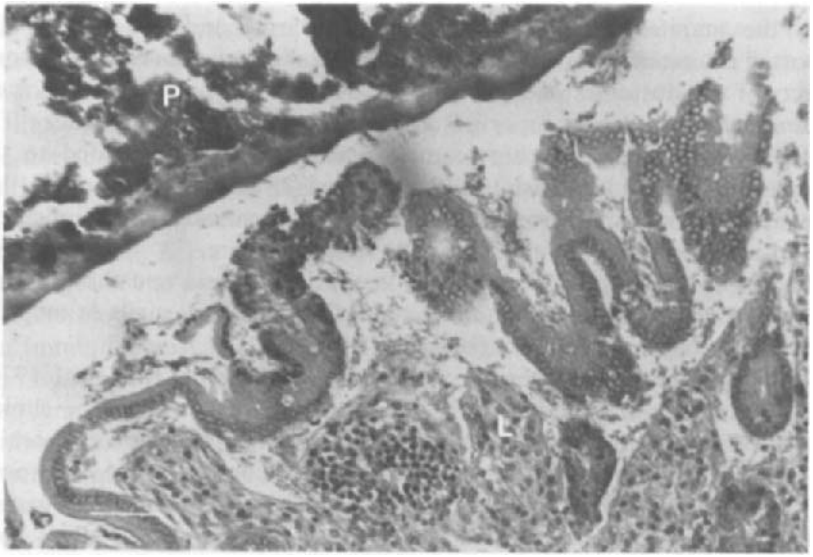

FIG. 19. Histopathological response of hamster intestinal tissue to *E. trivolvis*. Erosion of intestinal villi with lymphocytic infiltration are the primary response. Hematoxylin and eosin. Scale bar = 0.05 mm.

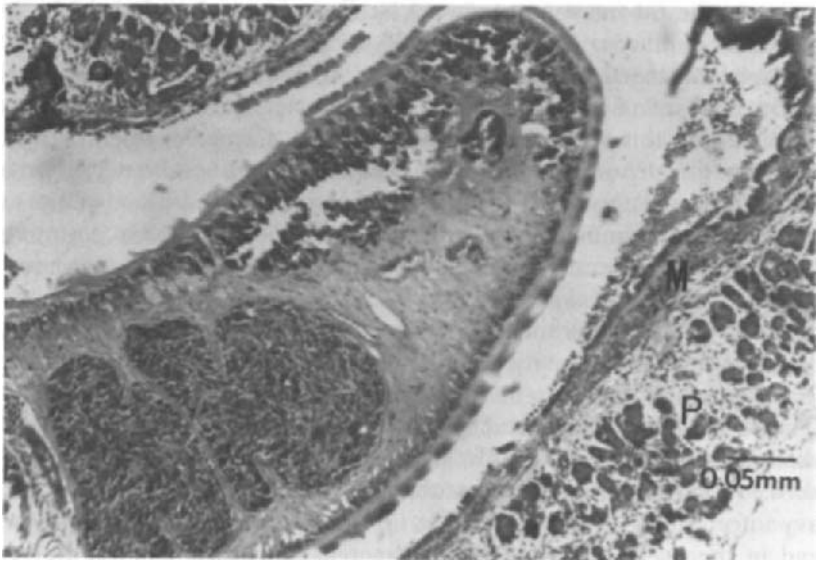

FIG. 20. *E. trivolvis* in the pancreatic duct of the golden hamster. Pancreatic tissue (P), mononuclear cell infiltration (M) of the lamina propria. Hematoxylin and eosin. Scale bar = 0.05 mm.

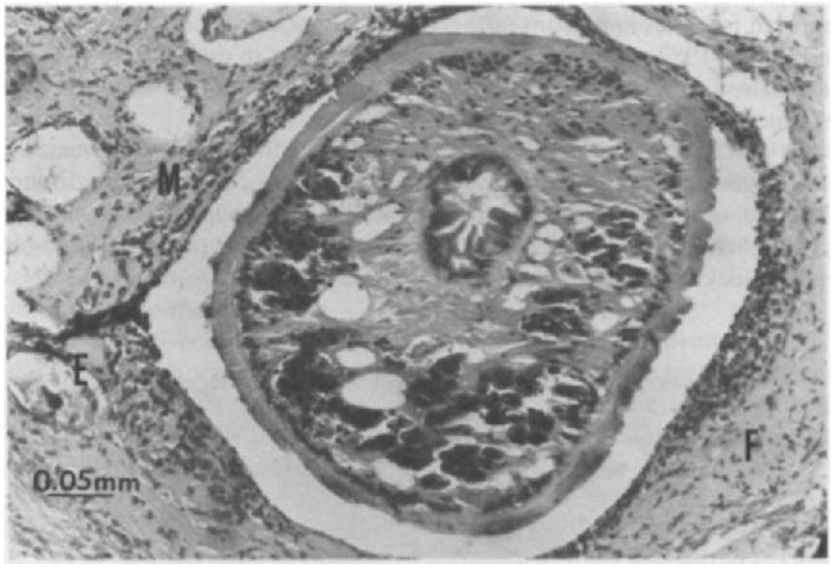

FIG. 21. Cross-section of adult *E. trivolvis* and eggs (E) in the liver of the hamster. Fibrous tissue (F) adjacent to the parasite and mononuclear leucocytic (M) response adjacent to the parasite eggs are shown. Hematoxylin and eosin. Scale bar = 0.05 mm.

increased, splenic relative weights decreased and adrenal relative weights increased.

Huffman *et al.* (1988a) infected golden hamsters with *E. caproni*. In infections with *E. caproni*, hemoglobin and packed cell volumes decreased due to hemorrhage seen in association with the damage to the intestinal villi (Fig. 22). In this study *E. caproni* did not migrate into the common bile duct and did not infect the liver or gall-bladder as does *E. trivolvis*. Thorndyke and Whitfield (1987) suggested that the pathological changes induced by *E. caproni* may be generated directly or indirectly by vasoactive intestinal polypeptide-like materials secreted from the tegument.

Biester and Schwarte (1965) and Muraleedharan and Pande (1967) reported that light infection with echinostomes in avian hosts caused little injury, although in acute infections hemorrhagic inflammation, severe enteritis and the presence of lymphocytes were observed. Gower (1937) reported no pathological consequences of *E. trivolvis* infections in the waterfowl he examined. Gower (1937) reported a 70% incidence of such infection in all ducks examined with as many as three or four age groups of the parasite present. Lie (1964) reported that heavy infections of *E. echinatum* caused death in ducklings, goslings and white mice. Hossain *et al.* (1980)

reported fatalities in pigeons as the result of *E. revolutum* infections. Kishore and Sinha (1982) reported *E. revolutum* infections in domestic ducks. Fried and Wilson (1981b) reported a decrease in body weight of domestic chicks experimentally infected with *E. trivolvis*.

An outbreak of trematodiasis (*E. trivolvis* and *Notocotylus attenuatus*) in Emden geese was reported by Griffiths *et al.* (1976). Lesions were found in the small intestine consisting of patches of mild hyperemia with severe catarrhal enteritis. A renal abscess occurred in one bird. A total of 285 birds from a flock of 9500 geese died of the infection.

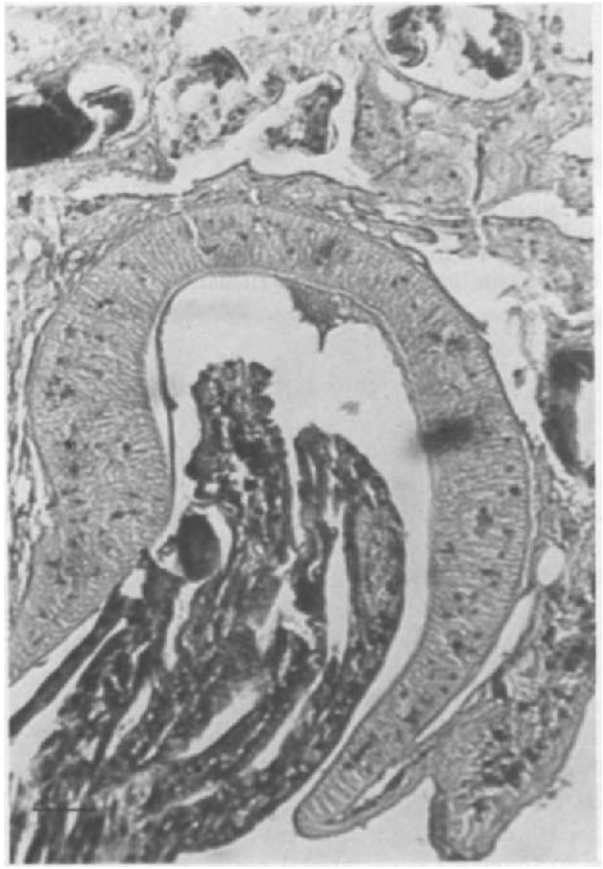

FIG. 22. *E. caproni* in the intestine of a hamster drawing villi into its oral sucker. Hematoxylin and eosin. Scale bar = 0.05 mm.

VIII. Epidemiology and Disease of Echinostomes

A. SOURCE OF INFECTION

The numerous species of flukes belonging to the family Echinostomatidae are cosmopolitan in distribution and have been recorded from many kinds of vertebrates, particularly aquatic birds. The most frequently encountered species among the group of echinostomes are *Echinostoma trivolvis*, *E. caproni*, *E. echinatum*, *E. hortense*, *E. cinetorchis*, *E. malayanum*, *E. revolutum* and *Euparyphium ilocanum*.

Among the 15 echinostomes which have been reported from man, *E. revolutum*, *E. echinatum*, *E. malaynum*, *E. hortense* and *Euparyphium ilocanum* are the most frequently encountered. These parasites are found in the Far East and south-east Asian countries and infections are acquired by consuming raw snails, tadpoles, or freshwater fishes containing encysted metacercariae.

The sources of human infection of *E. hortense* are various species of amphibia and freshwater fishes (Asada, 1926; Ono, 1930; Mori, 1935; Tani, 1976a,b). The ingestion of raw loach, *Misgurnus angiullicaudatus*, has been confirmed as the main source of *E. hortense* infection (Tani, 1976a; Arizono *et al.*, 1976). More than 20 cases have been reported in Japan and Korea (Makino *et al.*, 1982; Seo *et al.*, 1983).

The source of human infection by *E. malayanum* in Malaysia is the freshwater snail, *Bellamya ingallsiana* and *Pila* sp. (Hadidjaja and Oemijati, 1969). *Euparyphium ilocanum* is found mainly in natives of Luzon in the Philippines (Arambulo and Cada, 1970). The freshwater snail which serves as second intermediate host of *Euparyphium ilocanum* is *Pila luzonica*.

Infection with *E. echinatum* is confined to the Lake Lindoe district of Celebes, Indonesia. The metacercariae are found in several pulmonate snails and in the mussel, *Corbicula lindoensis*, which occurs in large numbers in certain parts of Lake Lindoe. Man is a favored host of *E. echinatum* (Sandground and Bonne, 1940). The adult parasite is capable of persisting in the intestine for 10 months. In rats and mice most of the adult worms are eliminated spontaneously from the intestine soon after reaching maturity.

Anazawa (1929) reported *Corbicula fluminea* in Taiwan as a second intermediate host of *E. revolutum* and implicated this bivalve as a vector of echinostomiasis in man. Fried *et al.* (1987a) demonstrated that *C. fluminea*, which has become established in south-eastern Pennsylvania, could serve as a second intermediate host of *E. trivolvis*. An *in vitro* excystation experiment (Fried *et al.*, 1987a) suggested that cysts from *Corbicula* can excyst and presumably infect vertebrate hosts, including man, in Pennsylvania.

B. DIAGNOSIS AND TREATMENT

The diagnosis of echinostomiasis is made by recovering the eggs in the feces. The time of deposition of eggs in the feces will vary between species. The eggs are operculate, ellipsoidal, and yellow to yellowish brown (Fig. 8). The size of the egg differs with the species of echinostome. However, there is considerable overlap of egg sizes between species. Species diagnosis is based mainly on the morphological study of the adult worm which can be obtained after anthelmintic treatment.

For the treatment of *E. caproni*, mebendazole has been found to be very effective (Notteghem *et al.* 1980). Other flukicides which have been shown to be active against *E. caproni* include brotianide, niclofolan, oxyclozanide and rafoxanide (Leger and Notteghem, 1975). Carbon tetrachloride in doses of 1–2 ml administered orally with about 3 ml of liquid paraffin has been reported to be effective as a treatment of *E. trivolvis* (Soulsby, 1968). Zanil (oxyclozanide) at a dose of 1 ml per 100 g body weight has been used effectively in treating hamsters experimentally infected with *E. trivolvis* (Mabus *et al.*, 1988), and for treating mice infected with *E. caproni* (Sirag *et al.*, 1980).

Seo *et al.* (1985) terminated human cases of *E. hortense* by giving a single dose of praziquantel (Distocide) 15 mg kg^{-1}, followed by purgation with 30–40 g magnesium sulfate. Seo *et al.* (1980) treated a human case of *E. cinetorchis* with 35 mg kg^{-1} bithionol after premedication with cathartics.

Other drugs used in treating echinostome infections include hexylresorcinol crystoids, tetrachlorethylene, oleoresin of aspidium and niclosamide (Yomesan) (Rim, 1982).

IX. PHYSIOLOGY AND BIOCHEMISTRY OF *ECHINOSTOMA*

In this section the reported physiological and biochemical studies on *Echinostoma* are examined. Most studies have involved the use of *Echinostoma trivolvis*.

A. PHYSIOLOGY

1. *Transplantation studies*

E. trivolvis adults are adaptable digeneans and can be removed from the lower intestines of avian and mammalian hosts and implanted cloacally into new avian hosts (Beaver, 1935, 1937; Fried and Vonroth, 1968). *E. trivolvis* adults can also be transferred surgically into the intestines or celom of new

avian hosts (Fried and Vonroth, 1968; Fried and Fink, 1968). Adults in the celom feed on the host peritoneal lining (Fried and Fink, 1968). *E. trivolvis* adults have also been transplanted into a surgically altered chicken gut, which is well nourished by an intact blood supply, but does not contain host ingesta (Fried and Vonroth, 1968). That study showed that worms can feed on host mucosal tissue in the absence of ingesta in the host gut.

Transplantation studies have been used to examine the ability of *E. trivolvis* adults to regenerate or heal wounds following amputation of parts. Wounded echinostomes lacking suckers do not survive when implanted into the chick cloaca. Anterior halves transected posterior to the acetabulum and implanted into the chick cloaca heal the tegument and close their protonephridial tubes; *E. trivolvis* is capable of wound healing, but shows a limited capacity to regenerate missing parts (Fried *et al.*, 1971).

2. *Cultivation* in vivo *and* in vitro

In vivo and *in vitro* excysted metacercariae of *E. trivolvis* have been cultivated on chick chorioallantoic membrane to the uterine coiling stage, i.e. to a stage about equivalent to the growth and development achieved in the domestic chick after 7 days (Fried *et al.*, 1968; Fried and Butler, 1978; Fried and Pentz, 1983). Scanning electron microscopy (SEM) showed that the surface topography of worms grown on the chorioallantois was normal and appeared identical to that seen in preovigerous worms grown in domestic chicks (Fried and Fujino, 1984). Worms grown on the chorioallantois feed on blood, whilst those grown in chicks feed on the host intestinal mucosa (Wisnewski *et al.*, 1986). Temperature-tolerance studies of *E. trivolvis* adults on chick chorioallantois show that this digenean is quite adaptable and can survive and develop at temperatures ranging from 30 to 43°C (Fried and Foley, 1969). Single adults of *E. trivolvis* placed in chick embryos locate on the chorioallantois above the embryo. Worm-location changes in the presence of a second worm suggests that site location may be altered by the release of worm chemotractants (Fried and Diaz, 1987).

Only limited success has been achieved in attempts to cultivate *Echinostoma in vitro* in defined media supplemented with natural products (Fried, 1978; Clegg and Smith, 1987). Excysted metacercariae of *E. trivolvis* doubled their body area, but showed no genital development after 14 days of culture at 37°C in the defined medium NCTC 135 supplemented with 20% hen's egg yolk (Butler and Fried, 1977).

3. *Pairing behavior*

E. trivolvis adults show a tendency to pair or aggregate both *in vivo* and *in*

vitro and the behavior is chemically mediated (Fried, 1986; Haseeb and Fried, 1988). Single adults of *E. trivolvis* are attracted to lipophilic but not hydrophilic excretory–secretory products of conspecifics (Fried *et al.*, 1980). The sterol fraction isolated preparatively by thin-layer chromatrography from excretory–secretory products of adult worms is chemoattractive, but an authentic cholesterol standard is not (Fried *et al.*, 1980). In addition to cholesterol, numerous minor sterol fractions are present in *E. trivolvis* adults and excretory–secretory products, but information is not yet available on the chemoattractive nature of any of the minor sterol constituents (Chitwood *et al.*, 1985).

In vitro studies on the pairing tendency of *E. trivolvis* show that this echinostome pairs interspecifically with trematodes from cold-blooded hosts, e.g. *Haematoloechus medioplexus*, and other digeneans from warm-blooded hosts (Fried and Jacobs, 1980; Fried and Leiby, 1982). Interspecific pairing is more transient than intraspecific pairing and often shows more non-contact than contact pairing (Fried and Wilson, 1981a). The significance of *in vitro* pairing is not clear since *E. trivolvis* is capable of self-fertilization in situations that preclude cross-fertilization (Fried and Alenick, 1981; Fried *et al.*, 1988b). Pairing may be important in feeding and social behavior in addition to the reproductive physiology of this echinostome (Fried, 1986; Haseeb and Fried, 1988).

B. BIOCHEMISTRY

1. *Lipids*

Total lipids of *E. trivolvis* adults constitute about 2.5 and 15% of worm wet and dry weights, respectively (Fried and Boddorff, 1978). As determined by thin-layer chromatography (TLC), the major neutral lipids present in adults are free sterols, with lesser amounts of triacylglycerols, sterol esters and free fatty acids (Fried and Boddorff, 1978). Adults excrete mainly free sterols into a non-nutrient medium and densitometric-TLC has been used to quantify sterol release (Fried and Appel, 1977; Bennett and Fried, 1983). Neutral lipid release occurs mainly through the excretory system, but some release of lipids from other systems, i.e. tegumentary, alimentary and reproductive, cannot be excluded (Gallo and Fried, 1984). Release of lipids is involved in the chemical communication of digeneans (Fried, 1986; Haseeb and Fried, 1988).

Phospholipids in *E. trivolvis* have also been identified by TLC, the major fractions being phosphatidylcholine, phosphatidylethanolamine and

phosphatidylserine, with numerous other minor constituents (Fried and Shapiro, 1979). Histochemical lipid studies on metacercariae and adults of *E. trivolvis* show that Oil Red O positive staining material localizes mainly in the excretory system (Fried and Morrone, 1970; Butler and Fried, 1977).

2. *Carbohydrates*

The Periodic acid–Schiff technique was used to show localization of glycogen mainly in the musculature and parenchyma of adult *E. trivolvis*; during starvation in a non-nutrient medium, glycogen was depleted; however, transfer of worms to Tyrode's solution plus glucose or the chick chorioallantois resulted in a subsequent resynthesis of glycogen (Fried and Kramer, 1963). Oxygen consumption of *E. trivolvis* adults was shown *in vitro* both in the presence and absence of an exogenous supply of glucose; when available this organism utilizes oxygen; the Nadi reaction for cytochrome oxidase in the tegument of the worm was positive (Taft and Fried, 1968). The glycogen content was determined in preovigerous worms of *E. trivolvis* as 45% of the dry weight; the glycogen content in older ovigerous worms ranged from 17 to 37% (Sleckman and Fried, 1984). Aerobic and anaerobic fermentation of glucose by *E. caproni* has been studied; incubation of adults with labeled glucose showed that glucose was incompletely oxidized and that qualitatively the same end products were produced under air or nitrogen (Schaefer *et al.*, 1977).

3. *Proteins and amino acids*

The protein content of *E. trivolvis* adults has been examined and was found to be relatively constant (26 to 32%) in both preovigerous (7-day-old worm) and ovigerous (14- to 28-day-old) worms (Sleckman and Fried, 1984). Electrophoretic studies were done on the water-soluble protein fractions of adults of *E. trivolvis* from the USA and *E. revolutum* from Europe. The number of water-soluble protein fractions reported for *E. trivolvis* was 20 as compared with 21 for *E. revolutum*; differences in the staining characteristics and motility of these fractions were also noted between the two species (Vasilev *et al.*, 1978). Isoenzyme analyses by isoelectric focusing of protein extracts of echinostome adults have been used by Voltz *et al.* (1988) to examine the differences between various strains of *E. caproni.*

Various free-pool amino acids were identified by TLC from worm tissues and incubates of adult *E. trivolvis*; the amino acids identified from worm egesta or from the protonephridial system were qualitatively similar to those identified in the incubation fluid (Bailey and Fried, 1977).

X. Concluding Remarks and Future Prospects

Kanev's (1985) revision has been useful in clarifying some of the species in the 37-collar-spined *Echinostoma revolutum* group. A clear-cut distinction now exists between *E. trivolvis* from *Helisoma trivolvis* snails in North America, *E. caproni* from *Biomphalaria* and *Bulinus* snails in Africa and *E. revolutum* from lymnaeid snails in Europe and Asia. Further revision of other species of *Echinostoma* is needed using morphological, life-history and biochemical information as described by Kanev (1985).

Routine maintenance of *E. caproni* and *E. trivolvis* is available (Christensen *et al.*, 1980; Fried, 1985). These echinostomes can be cycled in inexpensive and easily maintained hosts. As adult parasites, they are of reasonable size, 0.5–2 cm in length and 5–10 mg in wet weight, in certain hosts. They provide ideal models for numerous experiments in parasite physiology and biochemistry. Of the two models, *E. caproni* is easier to maintain, because it uses *B. glabrata* snails and laboratory mice as hosts. Therefore, this cycle can be conveniently maintained in the same hosts used for *Schistosoma mansoni* research.

Miracidial infectivity studies of echinostomes in first intermediate planorbid snail hosts should be pursued based on the plethora of previous studies on site finding of *S. mansoni* miracidia in *B. glabrata* (for reviews see, Saladin, 1979; Sukhdeo and Mettrick, 1987). It would be interesting to compare the site finding of *E. caproni* miracidia in *B. glabrata* with the numerous studies available on the site-finding of *S. mansoni* miracidia in the same snail host.

Little information is available on how echinostome cercariae find their second intermediate hosts. Anderson and Fried (1987) suggested that renal secretions of snails are involved in cercarial location, penetration and encystment. *B. glabrata* is an excellent second intermediate host for both *E. trivolvis* and *E. caproni* (Anderson and Fried, 1987; Jeyarasasingam *et al.*, 1972). Cercarial site-finding studies modeled on schistosome miracidial–*Biomphalaria* work might be helpful in elucidating mechanisms of attraction, attachment, penetration and encystment of echinostome cercariae in second intermediate hosts. The studies by Motzel and Haas (1985) on the cercarial attachment of *Isthmiophora melis* (Echinostomatidae) to various substances and by Feiler and Haas (1988a,b) on the attachment and host finding of the avian cercaria, *Trichobilharzia ocellata*, should be consulted for further details.

Numerous studies are now available on vertebrate hosts, particularly the domestic chick, the golden hamster, the laboratory mouse (Fried *et al.*, 1988a; Huffman *et al.*, 1988a; Hosier *et al.*, 1988) that serve as experimental definitive hosts of echinostomes. The echinostome–rodent model as a conve-

nient system for examining host–parasite relationships in intestinal trematode infections has been reviewed (Christensen *et al.*, 1988). Interestingly, similar studies using experimental duckling models are not available, but may be of more than just academic interest because ducks are naturally infected with echinostomes and are of considerable economic importance as a food and game animal.

Studies on the immunology of echinostomes in mice and hamsters have recently been started (Christensen *et al.*, 1986; Hosier *et al.*, 1988; Mabus *et al.*, 1988; Tani and Yoshimura, 1988). Homologous and heterologous resistance occurs with *E. trivolvis* and *E. caproni* in mice (Christensen *et al.*, 1986; Hosier *et al.*, 1988). The mouse is resistant to a superimposed infection of *E. caproni* and this host–parasite system shows concomitant immunity because the immunological mechanism responsible for the expulsion of the superimposed infection has no effect on the number of primary worms present (Christensen *et al.*, 1986). The mechanism of concomitant immunity in echinostomes is poorly understood. The phenomenon of worm expulsion in echinostomes will be interesting to explore because *E. trivolvis* is expelled rapidly from mice, whereas *E. caproni* is retained (Hosier and Fried, 1986; Odaibo *et al.*, 1988, 1989). A study has been made recently on the mechanism of *E. hortense* worm expulsion in the mouse (Tani and Yoshimura, 1988). In the *E. hortense*–mouse system, rapid expulsion of worms from the small intestine at week 3 is associated with an increase in the number of goblet cells, but not mast cells.

The epidemiology of *E. trivolvis* has been well explored, but less information is available on *E. caproni* and *E. revolutum*. Since echinostomiasis is a zoonotic disease and can cause serious human infection, more attention to the epidemiology of echinostomes is warranted.

The use of echinostomes in physiological, biochemical and behavioral studies is an untapped resource. Many of the studies done on *E. trivolvis* (see Section IX) need to be performed on *E. caproni*, *E. revolutum* and other echinostomes for comparative purposes. The reviews of Sukhdeo and Mettrick (1987), Sommerville and Rogers (1987) and Haseeb and Fried (1988) should be helpful to those contemplating physiological and behavioral work with echinostomes. The books of Smyth and Halton (1983) and Barrett (1981) should be consulted by those interested in undertaking physiological or biochemical work on these parasites.

The gross and histopathological effects of echinostome infections have been well documented in experimental infections in mice and hamsters (Bindseil and Christensen, 1984; Huffman *et al.*, 1986; Huffman *et al.*, 1988b). Details of the cellular phenomena involved in the host intestinal response are lacking and further study is required at the ultrastructural and molecular level.

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Physiological and Behavioural Interactions Between Parasites and Invertebrate Hosts

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I. Introduction 272
II. Parasitic Castration 273
A. Trematode infections of molluscs 274
B. Insects as hosts 280
III. Host Growth 286
A. Gigantism 287
IV. Host Sex 290
A. The influence of host sex upon the parasite 290
B. The influence of parasites upon host sex 291
V. Parasite Modification of Host Behaviour 291
A. Modification of host reproductive behaviour 291
B. Strategies which improve parasite transmission 292
VI. Host–Parasite Synchrony 299
VII. Endocrinological Interactions 300
A. Protozoa 301
B. Nematodes 301
C. Crustaceans 302
D. Endoparasitic insects 302
VIII. Conclusions 304
Acknowledgements 305
References 306
Appendix 317

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I. Introduction

A study of host–parasite interactions can provide new insights into the biochemistry, physiology, ethology and ecology of the respective partners. This is particularly so in the case of parasitic infections of invertebrates for the following reasons: (i) the short generation time and large population size of many invertebrate hosts make them convenient models for study and the need to understand the role of invertebrate vectors in the transmission cycles of parasitic disease is of particular relevance (Maier *et al.*, 1987); (ii) invertebrates are utilized by a variety of protozoan and helminth parasites as definitive or intermediate hosts in complex life cycles, some of which involve vertebrates. Thus parasites may exploit two or more environments which are markedly different with respect to physicochemical characteristics. A comparison of such factors, e.g. biochemical pathways and surface membrane structure and function at different points in the life cycle, may provide insights into the mechanism by which establishment and survival in different environments can be achieved (Arme, 1988). (iii) Proposals to use parasites as biological control agents for invertebrates (Platzer, 1981; Peterson, 1985; Combes and Cheng, 1986) have generated interest in the host as a biochemical environment, knowledge of which is important in ascertaining the conditions required for large-scale culture of these parasites.

Many authors have attempted to classify symbiotic relationships by grading them in terms of host pathological responses. However, the notion of a beneficial/harmful continuum has largely been superseded by Read's (1970) concept of a parasite–host complex defined in terms of the degree of metabolic integration (see review by Thompson, 1985). Attention is now focused upon the biochemical, physiological, behavioural and immunological integration of the invertebrate–host/parasite complex. To attempt to discuss all these aspects would be to paint too broad a canvas; thus, it is intended to restrict this examination to a few interlocking themes. Recent and very thorough reviews by Freeman (1983) and Sparks (1985) cite numerous examples of the pathology associated with invertebrate infections and this aspect of the host response will not be dealt with here, unless directly pertaining to an understanding of the aspects of host–parasite integration chosen for discussion. Similarly, many parasite-induced changes in host biochemistry have been described by Thompson (1983) and others have described immunological adaptations (e.g. Lackie, 1986). Therefore, these aspects will not be specifically addressed here. Rather, my intention is to survey and discuss the effects of parasite–invertebrate associations upon the life histories of the respective partners, emphasis being placed, wherever possible, upon attempts to elucidate underlying physiological mechanisms. Using examples of parasitic protozoans, helminths, crustaceans and insects in association with a wide range of invertebrate hosts, strategies such as the

synchronization of host–parasite life cycles, alteration of host growth rates, the impairment of host reproductive capacities, parasite establishment in hosts of different sexes and alterations in host behaviour will be examined. An assessment of the evidence pointing to endocrinologically active substances as effectors in the subtle interplay between parasite and host is also made.

Although the parasite–host complex can be regarded as an integrated unit, a potential conflict in terms of resource management clearly exists. A parasitic association will only succeed if the host survives long enough to enable the parasite to complete the particular phase of its life cycle and if the host population is able to persist in time. The resolution of this conflict is paramount to the evolution of this type of association and may be achieved using one or more of the strategies outlined above.

Price (1980), amongst others, has discussed the negative impact of parasites upon their hosts, parasites reducing host-fitness and thereby acting as a factor for selection and evolutionary change. The literature is replete with examples of the potential of the parasite genome for manipulating the invertebrate host, and many of these are cited below. Dawkins (1982) regarded the host as an extension of the parasite phenotype, host modifications being part of the phenotypic expression of particular parasite genes. However, parasitic relationships are generally asymmetric, the host biomass being the greater, and the possibility for the host to influence the parasite also clearly exists (Bouletreau, 1986). Smith-Trail (1980) discussed the application of kin selection theory to parasitic relationships, and coined the term "host suicide" to describe aberrant host behaviour which might lower the risk of infection of host kin. Minchella (1985) discussed the effect of parasite-induced early reproduction, termed fecundity compensation, as an example of a strategy which has no cost to uninfected organsims but which is able to redress, to some extent, later loss in reproductive output of parasitized individuals (see examples below).

Whilst the majority of the physiological and behavioural interactions discussed below would appear to fall within the jurisdiction of parasite-induced modifications, it is clear that, in many cases, both partners contribute to relationships involving a high degree of intimacy at many levels of biological organization. The remainder of this chapter will be devoted to an examination of the mode and degree of integration of a variety of invertebrates and their parasites.

II. Parasitic Castration

Well over a century ago, biologists observed that parasitic infections in invertebrates could result in host sterility (McCrady, 1874; Malm, 1881).

Giard (1911–1913) first used the phrase "parasitic castration" to describe this phenomenon and the scientific literature is full of examples from diverse host–parasite systems (see review by Baudoin, 1974). True castration is an irreversible destruction of reproductive tissue. However, the term parasitic castration was defined by Noble and Noble (1971) as a "destruction or *alteration* of gonad tissue by the parasite" and, in a similar vein, by Malek and Cheng (1974) as a total or partial inhibition of gamete formation in hosts by parasites. Thus reproductive tissue, although not functioning, may still be present in the host.

These precise, but restrictive, uses of the term are, however, of limited value in discussing the impact of parasites upon invertebrate host life histories and population dynamics. For the purpose of the present review, the phrase "parasitic castration" will encompass infections resulting in a negative effect on host reproductive success. Thus, in addition to parasite-induced gonad damage producing host sterility or reduced fecundity, associations which result in altered host behaviour, leading to reduced sexual or parental activities, host juvenilization due to a reduction in host secondary sexual characteristics, host sexual reversal, and alteration in the functioning of host accessory glands or endocrine systems are included in this discussion.

Baudoin (1974), considered parasitic castration in the wider sense, outlined above as an evolutionary strategy, and concluded that a parasite-induced manipulation of host resources away from reproduction may produce increased host survival, thus leading to increased parasite fitness as a result of an improved environment. Following this, he predicted that castration will only occur when the timing of infection coincides with a period of host-energy investment in reproduction.

There are numerous examples of parasites affecting invertebrate host reproduction. For the sake of brevity only a few have been selected to illustrate both the widespread occurrence of parasitic castration and also the diverse mechanisms underlying its induction.

A. TREMATODE INFECTIONS OF MOLLUSCS

Parasite-induced curtailment of host reproduction is a phenomenon widely recognized throughout the spectrum of animal symbioses. It has, however, attracted the particular attention of invertebrate pathophysiologists studying trematode/molluscan associations. Parasitic castration of molluscs was first recognized by McCrady (1874), who described the destruction of the gonads of the oyster *Crassostrea virginica* by sporocysts of *Bucephalus*. A plethora of reports have since appeared, describing a wide variety of pathological responses. In some cases, trematodes are reported to cause direct damage to gonads, and in others indirect suppression of gonadal

development occurs. The associated pathophysiology ranges from partial to total cessation of egg laying and permanent castration to eventual recovery. Host responses may vary with initial time of infection and parasitization may affect reproductive organs other than the gonads or cause sex-reversal, and has been associated with gigantism (reviewed by Wright, 1966, 1971).

Various authors have attempted to impose a semblance of order upon these diverse responses. Giard (1911–1913) suggested that parasitic castration may be direct or indirect, depending upon the proximity of the parasite to gonadal tissue. In contrast, according to Wilson and Denison (1980), the effect of digenetic species with daughter rediae will differ from those with sporocysts alone, with the former being capable of ingesting cellular material while usually residing in the gonads. Although neither of these two classifications is comprehensive, it is evident that associations that cause host castration can be broadly divided into two categories. The scheme of classification proposed by Cheng *et al.* (1973) and reproduced in Table 1 would appear to encompass the majority of these pathological responses and thus prove to be a valuable working scheme. Thus, according to Cheng *et al.* (1973) rediae of *Halipegus occidualis*, residing in the ovotestis of *Helisoma anceps*, cause mechanical castration, whereas sporocysts of *Diplostomulum scheuringi*, located in the same site, cause direct chemical castration (Crews and Esch, 1987). Indirect chemical castration takes place in the *Trichobilharzia ocellata* infection of *Lymnaea stagnalis*, where reduction in egg production occurs before sporocysts invade the gonads (McClelland and Bourns, 1969). Cheng *et al.* (1973), however, observed that, should the chemical factors responsible for parasitic castration in different species prove to be identical, then distinction between types of chemical castration is unnecessary.

TABLE 1 *A proposed classification for types of parasitic castration in molluscs due to parasites*[a]

Mechanical castration	Parasite in gonads
Chemical castration	Direct type: parasite in gonads Indirect type: parasite not in gonads

[a] Reproduced with permission from the *Journal of Invertebrate Pathology* (Cheng *et al.*, 1973).

Sparks (1985), in his exhaustive review of invertebrate pathology, has given a thorough account of the pathology associated with mechanical damage inflicted by platyhelminths upon molluscan gonadal tissue, and this aspect will not be dealt with here. In recent years, this largely histological, and therefore descriptive, approach has been augmented by more analytical examinations of the detailed timing of perturbations in host reproductive

functioning and their place in a strategy for long-term survival of the host-parasite complex. A further avenue of research that has attracted increased attention in the past decade is that of the physiological basis underlying a reduction in molluscan–host fecundity, particularly applicable to cases of chemical castration. It is these latter approaches that will be discussed below.

The classical study of Pan (1965) showed that, in both snails infected as juveniles and as adults, suppression of egg laying coincides with cercarial release (at approximately 6 weeks post-infection) in the *Schistosoma mansoni*/*Biomphalaria* (=*Australorbis*) *glabrata* association. However, the timing of infection in this association has been shown to cause variations in host response. Thus, when young *Biomphalaria* are infected with *S. mansoni*, sterility resulted, whereas in snails infected when nearing maturity, oviposition was initially reduced and finally inhibited after patency was achieved (Sturrock and Sturrock, 1970; Meuleman, 1972). These findings were substantiated by Meier and Meier-Brook (1981) who monitored egg laying by *S. mansoni* infected *B. glabrata* for 15 weeks, by which time snails infected at 8 weeks were still producing a limited number of eggs. Both Minchella and Loverde (1981) and Thornhill *et al.* (1986), examining the above association, described an initial significant increase in egg laying in susceptible snails which were mature, or almost mature, at the time of exposure to miracidia. These authors also reported that both parasitized snails, as well as snails exposed but not infected, exhibited initial fecundity compensation (see discussion above). Minchella and Loverde (1981) discussed this strategy in terms of the cost of future reduced reproductive output of snails exposed but not infected, and concluded that the effect varies according to the reproductive capacity at the time of exposure, and overall snail population dynamics. Etges and Gresso (1965) also investigated reduced host fecundity in *S. mansoni* infections of *B. glabrata* and noted that castration was reversible, with snails resuming egg laying before infection was eliminated.

The examples of trematode-induced castration of gastropod molluscs cited above give an indication of what is undoubtedly a widespread phenomenon. The elucidation of a mechanism underlying this pathological response has provided a stimulating challenge to many research groups, and some of their findings will be examined below.

A parasite-induced curtailment in egg production could create a pool of nutrients which, although in normal animals would be utilized for vitellogenesis, would be available for parasite development. Alternatively, if larval trematodes were in direct competition with the host for scarce nutrient resources, deemed "food-robbery" by Von Brand (1979), material would be unavailable for egg production. Many workers have sought to correlate reduced reproductive output with the physiological stress resulting from

parasite-induced nutrient deprivation. Becker (1980) emphasized the similarity between the effects of starvation and *Schistosoma* infection on the snail *B. glabrata*, particularly with regard to decreases in haemolymph glucose levels, and an increase in activity of the urea cycle: 30 days of infection were equivalent to 5 days of starvation. Becker suggested that the flukes were competing for host nutrients, possibly causing starvation with a resultant adverse effect on oogenesis.

Trematode-induced nutrient depletion in the intermediate host is well documented. Thompson (1983) cited examples of decreases in the concentration of host glycogen (*Bithynia tentaculata* infected by sporocysts of *Cercaria helvetica* and *Biomphalaria alexandrina* and *Bulinus truncatus* infected by *S. mansoni* and *S. haematobium*), amino acids (*B. glabrata* parasitized by *S. mansoni* and lipids *Lymnaea truncatula* infected by *Fasciola hepatica*), although the effect of infection upon fatty acids was varied and often resulted in alteration in the composition of free fatty acid pools. However, gastropod-induced changes in the concentrations of metabolites, reported by these and other authors, are highly variable and could reflect differences in experimental conditions, such as altered feeding regimes, or be attributable to interspecies variation.

Other authors are of the opinion that competition for nutrients between parasite and gonad is not the prime or sole cause of castration. Meuleman (1972) noted that reproductive output in *B. pfeifferi* was impaired 7 days post-infection, i.e. before the production of daughter sporocysts of *S. mansoni*. At this stage of infection, the few mother sporocysts present in the foot region were unlikely to produce a significant drain on host nutrient resources and indeed, even at 15–16 days post-infection, no pathology was detected on histological examination of the gonads. Meuleman concluded that the parasite produces an endocrinologically antagonistic substance which, via its action on the host hormonal system, indirectly affects gonad development. This explanation had been propounded previously by McClelland and Bourns (1969) to account for *Trichobilharzia ocellata* induced castration in *Lymnaea stagnalis*. Indeed, the implication that substances secreted by sporocysts influence host gametogenesis was proposed in the 1940s by Neuhaus (1940, 1949) who studied the infection of *Bithynia tentaculata* by *Pleugenes medians*.

The observation that initial reduction in host reproductive output occurred at a time when few mother sporocysts were present, also led Meier and Meier-Brook (1981) to conclude that a substance secreted by sporocysts of *S. mansoni* may directly or indirectly inhibit oogenesis in the gastropod *B. glabrata*. This hypothesis has been extended to encompass the view that trematode products may control host reproduction indirectly via manipulation of the host endocrine system.

Three types of neurosecretory cells, designated A, B and C, have been observed in the cerebral ganglion of *Lymnaea luteola*. A significant increase in cell diameter, nucleus diameter and the intensity of staining of neurosecretory material was detected in B cells of snails infected with metacercarial stages of *Echinostoma revolutum* (Krishna and Simha, 1980). Gonadal invasion by the parasite was thought to account for reduction in host fecundity and indirectly cause changes in the B type neurosecretory cell. These authors also suggested that a toxic substance may be secreted by the parasite.

Sluiters and colleagues (e.g. Sluiters *et al.*, 1980; Sluiters, 1981) made an intensive study of the pathophysiology of the avian schistosome *Trichobilharzia ocellata* infections in the pulmonate snail *Lymnaea stagnalis*. They found that the maturation of the accessory sex organs (for example the albumen and prostate glands) was severely delayed in infected snails (Sluiters, 1981). This finding was confirmed by Joosse and Van Elk (1986) who further demonstrated that, in albumen glands from infected snails the deoxyribose nucleic acid (DNA) content did not increase with snail age and galactogen did not accumulate, as it did in controls. The latter observation was explained by the adverse effect of infection upon UDP-galactose 4-epimerase (EC 5.1.3.2), an enzyme essential for galactogen synthesis. No mechanical damage was inflicted upon the host and no tissue response was observed. However, mean gonad volume relative to snail wet weight declined after 7 days in infected snails, and gonadal growth was blocked by day 19 post-infection, being only 1% of the mean actual volume of controls by day 83. Gametogenesis was not inhibited; both oocyte maturation and sperm production occurred in infected snails, although no eggs were laid. The effect of infection in this association was thus found to be one of degree, gonad growth and hence gametogenesis being reduced rather than completely inhibited. In view of this, Sluiters and co-workers hypothesized that two hormones regulating pulmonate snail reproduction, namely the dorsal body hormone which stimulates vitellogenesis and growth and differentiation of the female accessory gland and the caudodorsal cell hormone, or ovulation hormone, must still be present in infected snails, although the parasite may cause a reduction in secretion or a reduction in the target tissue sensitivity.

Morphometric analyses of the dorsal bodies of control and infected *L. stagnalis* have been performed (Sluiters *et al.*, 1984) using phase contrast and electron microscopy. They observed a 45% increase in dorsal body cell cytoplasmic volume and a 40% increase in Golgi apparatus volume, demonstrating that parasitism resulted in increased dorsal body cell synthetic activity, although secretory granules were released. The conclusion that the parasite-induced inhibitory effects upon host gonads in this system were not

the result of decreased dorsal body hormone secretion was verified by experiments in which addition of hormone supplement in the form of dorsal body implant was performed on control and infected snails. Elevated dorsal body hormone levels advanced ovipository activity and increased fecundity in control snails, but had no effect on their infected counterparts. Cercarial output was also unaffected and the development of *T. ocellata* in *L. stagnalis* was not found to be dependent upon the presence of dorsal body hormone (Sluiters and Geraerts, 1984a,b). Cerebral commisure extract containing the second gonadotropic hormone (caudodorsal cell hormone) similarly had no effect when injected into infected snails. Extracts taken from infected snails produced a limited response (measured as packaging of egg cells by albumen gland secretions and egg membranes) in recipient non-infected snails. However, the response was much lower than that elicited by extract from non-infected animals (Sluiters and Dogterom, 1984). The authors concluded that the caudodorsal cell/cerebral commisure system of infected snails was not fully mature and that, as maturation is controlled by the dorsal body hormone, it appears likely, based upon the above data, that sporocysts inhibit the action of dorsal body hormone on its target organs.

The investigations outlined above imply the existence of parasite-derived endocrinologically antagonistic substance(s), which are produced early in the infection. One such substance, named schistosomin, has recently been identified in the haemolymph of *T. ocellata* infected *L. stagnalis*.

Calfluxin is a neuropeptide released by the cerebral commisures of the snail. It causes an influx of Ca^{2+} into the mitochondria of the secretory cells of the albumen gland. Glucose and galactose incorporation by albumen glands was reduced 4 weeks post-infection as measured by using quantitative cytochemistry (Dictus *et al.*, 1987) and with the antimonate precipitation technique (Slocum and Roux, 1982). A series of elegant experiments have shown that schistosomin, a heat stable, pronase-labile peptidergic agent, has an inhibitory effect upon albumen gland response to this gonadotropin. From 3 weeks post-infection onwards, albumen glands from host snails exhibit a decreased response to calfluxin *in vitro*. Furthermore, the inhibitory action of this agent upon albumen glands from non-infected snails was detected in the haemolymph of snails 6 weeks or more post-infection, its effect being dose dependent (De Jong-Brink *et al.*, 1988a). Preincubation of normal glands in serum from infected snails, followed by varying times of rinsing prior to incubation with calfluxin, demonstrated that schistosomin binds quite firmly to receptor sites. The results also indicated that a loss of receptor sites occurred in infected organisms. Data from a determination of the percentage of Ca^{2+} mitochondria in the light cells of albumen glands from infected snails also indicated that some loss of receptor sites may have taken place *in vivo* (De Jong-Brink *et al.*, 1988b; Joosse *et al.*, 1988).

Schistosomin appears to be a gonadotropin antagonist acting at the level of the receptor site, and its action is clearly detectable during the prepatent period of infection. As such, it is a strong contender for the role of a causative agent in a trematode-induced reduction in host fecundity.

Preliminary evidence has been produced (De Jong-Brink and Elsaadany, 1987) indicating that dorsal body hormone activation of adenylate cyclase activity in the follicle cells of *L. stagnalis* is also inhibited by incubation of the tissue in haemolymph from infected snails. Whether this is an additional effect of schistosomin or is indicative of the existence of additional endocrinologically antagonistic substances remains to be determined.

Investigation of the *T. ocellata–L. stagnalis* model has demonstrated unequivocally that some degree of endocrinological interaction plays a role in the orchestration of parasitic castration, as opposed to being mediated by nutritional stress alone. A similar rigorous approach is now required to determine when and how host reproduction is curtailed in those snails which act as vectors for trematodes of medical and economic importance.

Pearson and Cheng (1985) have also focused their investigations on the causative agent of parasitic castration. Working with the *Ilyanassa obsolata–Zoogonus lasius* model they have investigated the incorporation of [^{3}H]-thymidine into testes incubated with or without an extract of *Z. lasius* sporocysts. The incorporation of thymidine by testicular cells is indicative of the presence of spermatogonia undergoing mitosis or differentiation into spermatocytes. The [^{3}H]-thymidine label was associated with a significantly greater number of cells in testes incubated without parasite extract; these data led the authors to conclude that *Z. lasius* sporocysts produce a substance capable of causing partial inhibition of spermatogenesis.

The studies cited above herald a new and exciting era in the investigation of host–parasite interactions. It is to be hoped that the new tools of the molecular biologist will be applied to the characterization of what may be novel parasite products and to the further elucidation of the role that they play in the manipulation of host reproductive strategy. For example, identification of the gene(s) encoding schistosomin could lead to the design of novel methods to control reproduction of uninfected snails.

B. INSECTS AS HOSTS

1. *Protozoan infections*

Microsporidian infections of insects are common and often associated with a reduction in host fecundity. Milner (1972) examined *Nosema whitei* infection of the fat body of *Tribolium castaneum*, and reported a reduction in host egg production that was not dependent upon parasite burden; egg

viability was unaffected. These findings were confirmed by Armstrong (1982) and Armstrong and Bass (1986) who also demonstrated a reduction in mating frequency in infected beetles. *Microsporidium itiiti*, a midgut tissue parasite of the Argentine stem weevil, was shown to delay the period of host egg laying by 6 weeks although, here too, eggs from infected hosts were viable. Impaired host nutrient assimilation was thought to be responsible for the reduction in reproductive output (Malone, 1987). Microsporidian species that invade the ovariole have, however, been associated with reduced egg viability (Malone and Wigley, 1981; Mercer and Wigley, 1987a,b). Reduction in fecundity was also associated with vertical transmission of *Nosema algerae* via eggs of the hosts *Culex fatigans* (*C. quinquefasciatus*) and *Aedes aegypti*; however, no F1 progeny became infected by transovariole transmission (Nnakumusana, 1986). The possible use of *N. algerae* as part of an integrated programme of biological control of vectors thus appears feasible. The reproduction of *A. aegypti* was reduced when infected with *Plasmodium gallinaceum* (Wülker, 1964). However, Hacker (1971) noted strain differences in host response and Zharova *et al.* (1983) were unable to demonstrate any effect upon host reproduction in this association. These latter studies highlighted the need for careful investigation of local strain response as a necessary part of any assessment of the feasibility of incorporation of protozoan pathogens into pest- or vector-control programmes.

2. *Nematode infections*

Evidence that certain nematode species can reduce the fecundity of their insect hosts is well documented (for example, Massey, 1962; Ashraf and Berryman, 1970; Gaaboub, 1976). Mermithid-induced suppression of normal ovarian development leading to parasitic castration has been described by several authors (for example, Wülker, 1971). Gordon and Webster (1971) and Gordon *et al.* (1973), investigating the mechanism underlying parasitic castration of *Schistocerca gregaria* by *Mermis nigrescens*, reported that vitellogenesis was entirely suppressed 11 days post-infection, the contents of terminal and penultimate oocytes being resorbed 3 weeks post-infection and that no egg pods were laid by infected locusts. Electrophoretic separation and quantitative analysis of proteins in the fat body and haemolymph revealed that they were significantly depleted in the former tissue 1 week prior to depletion in the blood. Similarly, a reconstruction of haemolymph proteins later in the infection reflected earlier changes in fat body metabolism. The authors suggested that the nematode stimulated catabolism of host fat body proteins at a time of maximum amino acid requirement by the parasite. The cessation of vitellogenesis 11–18 days post-infection was ascribed to an impairment of the ability of the follicular epithelium to

sequester vitellogenic proteins rather than to a decrease in available haemolymph vitellogenins, as these proteins were not significantly depleted until 3 weeks post-infection. However, an accumulation of vitellogenins in the haemolymph was not associated with the cessation of ovarian sequestration (see Hurd and Arme, 1986a and below). Experimental infection of *Locusta migratoria* with metacestodes of *Hymenolepis diminuta* (Hurd and Arme, 1987a) also produced a significant decrease in size of terminal oocytes without a concomitant increase in haemolymph vitellogenins. Gordon *et al.* (1973), speculating upon possible interaction between mermithids and the host endocrine system, suggested that parasite-induced changes in host neurosecretory activity could affect fat body protein synthesis and that a reduction in circulating juvenile hormone may inhibit protein sequestration by the ovaries.

The mermithid–locust symbiosis is an ideal model for the investigation of possible interference with host hormonal systems for the following reasons. First, the pathophysiology is clearly defined as the parasite causes total castration of its host if infection occurs 3 days after the imaginal moult. Secondly, the locust is a large insect, amenable to physiological investigation; and thirdly, much information is already available concerning orthopteran endocrinology (see Hurd and Arme, 1987a).

Lieutier (1982a,b, 1984a) described a delay in oocyte maturation and a reduction in ovary weight, size of terminal oocytes and oviposition in the coleopteran, *Ips sexdentatus* infected by the nematodes, *Parasitaphelenchus* spp., *P. sexdentati* and *Contortylenchus diplogaster*. Both the concentration of total fat body protein and the rate of protein incorporation into ovaries were found to be reduced in parasitized insects, although variations in the degree of host response occurred depending on nematode species. Another species of *Contortylenchus*, the bark beetle nematode, *C. reversus*, has also been reported to reduce the fecundity of a coleopteran host (Thong and Webster, 1975a,b). A 20% reduction in oocyte size did not, however, affect egg viability. Polyacrylamide gel electrophoresis (PAGE) demonstrated that nematode-induced depletion of haemolymph proteins was proportional for all proteins, thus suggesting that the reduction in sequestration of proteins seen in ovaries from infected beetles could be due to an overall depletion of haemolymph proteins.

Several nematode-infected Diptera also exhibit reduced fecundity (Javadian and Macdonald, 1974; Cheke *et al.*, 1982). Christensen (1981) found decreased egg production to be correlated with increased parasite burden in *Dirofilaria immitis* infections of *Aedes trivittatus*, and Courtney *et al.* (1985) suggested that reduced fecundity in this system could be attributed to the decrease in uptake of infected blood as compared with uninfected blood. Reduced feeding rates were observed in *Simulium ornatum* exposed to hosts infected with *Onchocerca lienalis*, a negative correlation between parasite

burden and host egg production being demonstrated (Ham and Gale, 1984).

Some of the investigations into the pathophysiology of parasitized Coleoptera and Diptera outlined above have attempted to link reduced host reproductive output to parasite-induced depletion of available nutrients. In contrast, a similar phenomenon observed in nematode-infected locusts is thought to result from an intimate interaction between parasite and host endocrine systems (Gordon and Webster, 1971). Some degree of parasitic castration is clearly a common occurrence during parasitism of adult insects. The examples cited here highlight the need for further studies, particularly those designed to clarify the physiological status of infected insects, many of which are important vectors of parasitic diseases. The model system described below, although not of medical importance, has been used to elucidate the relationship between a blood parasite and the reproductive system of its female insect host.

3. *Cestode infections*

Metacestodes of *Hymenolepis diminuta* have been shown to reduce the fecundity of the intermediate host *Tribolium confusum* (Keymer, 1980), in a manner related to the total parasite biomass present rather than the absolute number of invading parasites. A long-term study (60 weeks) demonstrated a 60% depression in host population levels (Keymer, 1981). This reduction in host reproductive output was found to be age related (Maema, 1986), being greatly reduced on, or by, 14 days post-infection. Hurd and Arme (1986b), investigating aspects of the pathophysiology of parasitism by *H. diminuta* in *Tenebrio molitor*, also detected a reduction in egg laying 13–16 days post-infection in beetles infected 3 days after eclosion. However, these data represented a temporary retardation in oviposition during this period of the infection, and only infected beetles maintained in very crowded conditions (12–14 females plus 8–10 males per Petri dish) had laid fewer eggs than their non-infected counterparts 30 days post-emergence.

Egg viability in this system was decreased by 9.4% in parasitized beetles maintained in single pairs and 12% in those kept in very crowded conditions (see above). Thus, in this larger host, parasite-induced depression of host population is not so marked as in *T. confusum*, but longer term studies are needed fully to assess parasitic effects on the population dynamics of this model. Hurd and Arme (1986b) also showed that the volume of yolk within eggs retained in the lateral oviducts of 15- and 30-day-old virgins was significantly reduced by infection, as was the total TCA-insoluble protein content of eggs from 15-day-old beetles. Specifically, 15.26 μg per egg was found in control insects, as compared with 13.6 μg per egg in eggs from parasitized beetles.

Vitellins represent 90% of the total soluble yolk proteins present in the

eggs of *Tenebrio molitor* (Harnish and White, 1982). Thus, the reduction in the yolk volume and protein content of eggs, associated with the cestode infection of *T. molitor*, is likely to be due to a decrease in vitellogenin sequestration by the ovaries. Vitellogenins pass from the haemolymph through the spaces between the epithelial cells which develop at patency (Telfer, 1954) and are selectively absorbed at the oolemma by micropinocytosis (Bradley, 1983). Examination of the telotrophic ovaries of infected *T. molitor* revealed a reduction in the degree of patency exhibited by terminal follicles from infected 9-, 15- and 30-day-old mated and 9-day-old virgin females (Hurd and Arme, 1987b), which may have resulted in a retardation in oocyte development. A highly significant increase in resorption of terminal oocytes was also associated with infection in 15-day-old virgins and 30-day-old mated insects. Hurd and Arme (1984b) demonstrated that in a 15-day-old female *T. molitor*, harbouring 12-day-old metacestodes of *H. diminuta*, haemolymph protein concentration was elevated by 46.7%. Although not occurring earlier in the infection, protein elevation could also be detected at 17 and 27 days post-infection; this pathophysiology was confined to female hosts. Electrophoretic separation of female haemolymph proteins revealed the presence of two sets of protein subfractions (designated bands 2/3 and 7/8) with relative mobilities similar to polypeptides extracted from eggs (see Fig. 1). Histochemical evidence and calculation of the apparent molecular weights of these proteins supported the view that these fractions were vitellogenins. Furthermore, densitometric analysis of gels revealed that the parasite-induced elevation in haemolymph protein was entirely due to an increase in the concentration of vitellogenins and did not reflect an overall enhancement of protein biosynthesis.

In an attempt to identify the origin of these female-specific proteins, Hurd and Arme (1986a) determined the rate of synthesis and secretion of protein by the fat body as well as the subsequent sequestration of these proteins by the developing oocytes of infected and control insects. Using a combination of *in vivo* and *in vitro* incubations with radiolabelled leucine, it was possible to demonstrate that storage and secretion of labelled protein was reduced by parasitization. In addition, ovaries from infected females contained 51.5% less protein than non-infected females. Electrophoretic separation of ovarian protein revealed that the majority of the [^{14}C]-label was incorporated in vitellin subunits during *in vivo* incubation. The decrease in ovarian vitellogenin sequestration associated with infection, appeared to be correlated with an accumulation of these proteins in the haemolymph, despite a concomitant reduction in fat body protein synthesis and secretion. Furthermore, this decrease in protein uptake may be associated with the reduction in patency discussed above.

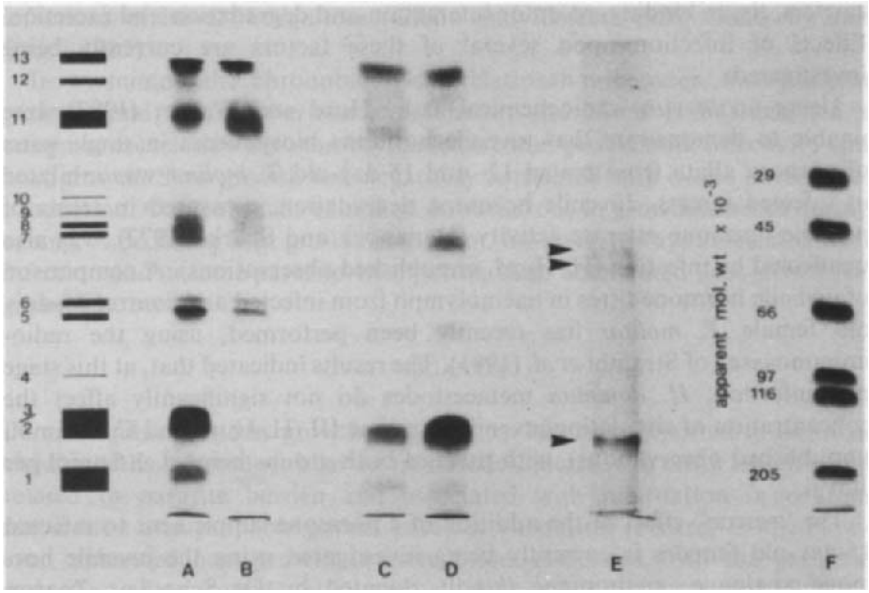

FIG. 1. (A, B) Haemolymph from non-infected 15-day-old beetles, run on the same gel: A, female; B, male. (C, D) Haemolymph from 15-day-old beetles, run on the same gel: C, non-infected; D, infected. (E) Egg proteins prepared by the method of Harnish and White (1982). (F) Molecular weight markers. Reproduced with permission from *Parasitology* (Hurd and Arme (1984b).)

The mechanism by which metacestodes of *H. diminuta* interfere with host vitellogenesis is unknown. However, it has been proposed that the parasite may interfere in some way with the endocrinological control of host reproduction (Hurd and Arme, 1986a). A study by Mercer *et al.* (1987) showed the presence of ecdysteroids in both the egg and adult *H. diminuta*, but no free ecdysteroid activity was found in the metacestode stage, moreover Hurd *et al.* (1987) were unable to demonstrate detectable effects of metacestodes upon uniformly low titres of circulating free ecdysteroids. It is well established that juvenile hormone, produced by the corpora allata, controls both the development of patency in the ovaries and the initiation and regulation of vitellogenin synthesis in the fat body of the majority of insects (see, for example, Engelmann, 1983). In the case of *T. molitor*, it has been demonstrated that only juvenile hormone III is produced by the corpora allata (Weaver *et al.*, 1980). Regulation of vitellogenesis via fluctuations in juvenile hormone titre is the result of a dynamic equilibrium between biosynthesis and release into the haemolymph, uptake by protein

carriers, tissue binding, receptor interaction and degradation and excretion. Effects of infection upon several of these factors are currently being investigated.

Using an *in vitro* radiochemical assay Hurd and Weaver (1987) were unable to demonstrate that juvenile hormone biosynthesis in single pairs of corpora allata from mated 12- and 15-day-old *T. molitor* was inhibited in infected insects. Juvenile hormone degradation, measured in terms of juvenile hormone esterase activity (Hammock and Sparks, 1977), was also unaffected by infection (H. Hurd, unpublished observations). A comparison of juvenile hormone titres in haemolymph from infected and control 15-day-old female *T. molitor* has recently been performed, using the radioimmunoassay of Strambi *et al.* (1981). The results indicated that, at this stage post-infection, *H. diminuta* metacestodes do not significantly affect the concentration of circulating juvenile hormone III (H. Hurd and C. Strambi, unpublished observations), with titres of both groups being *c.* 1.5 pmol per 100 µl.

The "rescue" effect of the addition of a hormone supplement to infected 15-day-old females is currently being investigated using the juvenile hormone analogue, methoprene (kindly donated by Dr Schooley, Zoecon Corporation). Preliminary data show that an elevation in haemolymph protein concentration does not occur in hormone-treated infected insects (H. Hurd, unpublished observations), thus indicating that a parasite-produced or induced competitive inhibitor of possible juvenile hormone receptor sites may be involved, with the addition of excess hormone overriding its effect.

The investigations outlined above did not substantiate the original hypothesis that parasite-induced alterations in female host reproductive functioning are a result of depressed titres of juvenile hormone. It is, however, possible that the mechanism by which these cestodes affect their intermediate host mirrors that occurring in the *T. ocellata*/*L. stagnalis* association. Attention is now being directed to the possibility that *H. diminuta*-induced perturbations of host juvenile hormone mediated events are perpetrated via interference with hormone binding sites.

Further discussion of the interaction between parasite and host endocrine systems is given below.

III. Host Growth

In many instances, the growth rate of parasitized invertebrates declines as infection proceeds. This may be accompanied by impaired food consumption and a concomitant alteration in gross conversion efficiency (see discussion in Thompson, 1983), as has recently been described in mermithid

infections of larval mosquitoes (Giblin and Platzer, 1985; Galloway and Brust, 1985).

In examining the chronobiological relationship between *Biomphalaria glabrata* and *Schistosoma mansoni*, Théron and Moné (1984) detected a temporal correlation between rhythmic cercarial production, infectivity and host growth. Slow growth was negatively correlated with cycles in cercarial production. Control snails exhibited no variations in growth kinetics during ageing. A declining host growth rate is not, however, always associated with infection and in some parasite–host partnerships accelerated host growth, or gigantism, occurs.

A. GIGANTISM

Parasite-induced excess growth or gigantism has been reported to occur in chaetognaths infected with larval trematodes, increase in mean length being related to parasite burden and associated with retardation in ovarian maturation and probable partial parasitic castration (Pearre, 1976). However, enhanced host growth is more often associated with the parasitic castration of gastropods, although opinion concerning the causal relationship of these phenomena is by no means unanimous (Wright, 1971; Meuleman, 1972; Sluiters *et al.*, 1980). Data from a field study of snail growth rates (Sousa, 1983) failed to demonstrate any enhanced growth in *Cerithidea* infected with seven common trematodes. However, Sousa suggested that findings from several snail–trematode infections revealed a general pattern in that snails infected as juveniles exhibited retarded or unaffected growth, whereas growth was enhanced in snails first parasitized as sexually mature adults. It is evident that, although the trend is not ubiquitous, gigantism is frequently associated with reduced host fecundity and, as such, merits some consideration here.

Strategies concerning the commitment of the resources of an organism to gamete production have been discussed by Calow (1979), and this notion of a parasite-induced redistribution of finite host nutrient resources has already been outlined with respect to host reproductive needs versus parasite growth. The possible reapportionment of large amounts of nutrient reserve to somatic growth in castrated hosts was investigated by Wilson and Denison (1980) using the *Lymnaea truncatula–Fasciola hepatica* model. Growth was assessed in terms of shell length and dry mass (tissue, shell and parasite). Growth curves expressed in terms of dry mass (see Fig. 2) diverged at the time when infected snails ceased to lay eggs, and the latter grew at a significantly faster rate thereafter. By day 56 post-infection, the dry mass of parasitized snails was twice that of controls and the shell was 1.34 times larger. However, in control snails, considerable biomass production had

been diverted into egg production and, when the cumulative dry mass of eggs produced by these snails was considered, infected snails had not produced a significantly greater biomass. These data thus support the view that gigantism arises from a rerouting of resources from reproductive effort to growth. The life span of host snails was not affected by infection and the authors suggested that, in field populations, infected *L. truncatula* may be better fitted to survive than non-infected snails carrying the burden of reproductive effort.

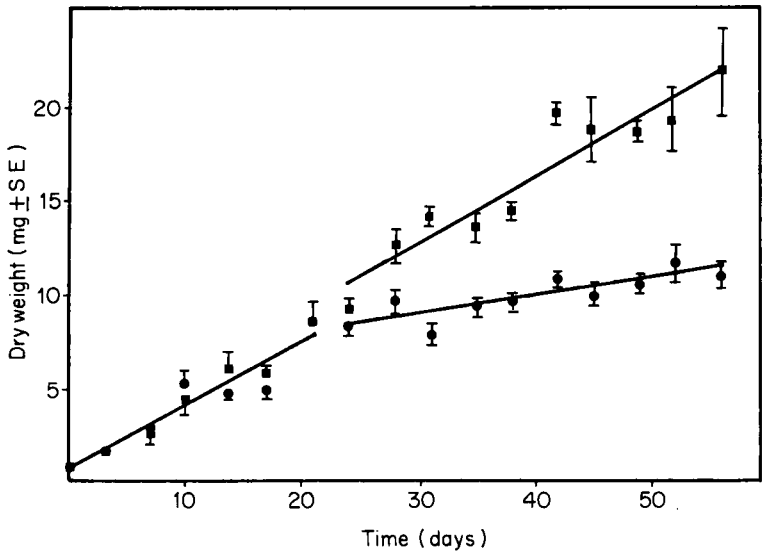

FIG. 2. Increase in total dry weight of infected (■) and control (●) snails with time after exposure. Each value is the mean of five individuals; the error bars are standard errors (SE). (Reproduced with permission from *Zeitschrift für Parasitenkunde* (Wilson and Denison, 1980).

Although Wilson and Denison (1980) found a very precise relationship between castration, gigantism and host mortality in their model system, reports vary as to the effect of *S. mansoni* upon *Biomphalaria* spp. Pan (1965), and Sturrock and Sturrock (1970) identified an initial acceleration in shell growth that was not maintained throughout the infection, and growth was eventually retarded when compared with controls. This adverse affect upon host growth has also been described by Théron and Moné (1984). Meier and Meier-Brook (1981), using different strains of this parasite–host system, detected no growth acceleration and, indeed, those snails infected early in life exhibited an initial retardation of growth relative to controls. In

contrast, gigantism was effected and maintained in another species, *B. pfeifferi* also infected with *S. mansoni* (Meuleman, 1972).

In the majority of these studies, growth was assessed in terms of increase in shell size. However, the value of this criterion as a true measure of growth is contentious. Cheng (1971) examined shell deposition in *Nitocris dilatatus* infected with sporocysts of *Acanthatrium anaplocani* and *Physa sayii* infected with rediae of *Echinostoma revolutum* to determine whether the weight increase associated with infection in these species could be attributed to shell growth and not increase in soft tissue. The concentration of Ca^{2+} was greater in both soft tissue and aqueous extracts from infected snails. Moreover, ultrastructural studies demonstrated an increase in Ca^{2+} spherites in the calcium cells of digestive gland acini and an increase in secretory granules in the secretory cells, as well as ruptured cells devoid of calcium. Cheng suggested that calcium, released into the haemolymph from ruptured cells, is deposited in the shell by the nacre-secretory mantle epithelium, thus accounting for increased shell weight.

An assessment of normal or proportional growth in non-infected *Ilyanassa obsoleta* and snails naturally infected by one of seven digenetic trematodes was made by examining wet and dry tissue weights and shell length and weight (Cheng *et al.*, 1983). Using these criteria, no enhancement of soft tissue growth or increase in shell length was detected. Increased shell weight in snails infected with *Zoogonus lasius* (a species causing parasitic castration) was tentatively attributed to increased calcification of the shell. The existence of a lytic enzyme or hormone-like parasite secretion which resulted in enhanced host growth was suggested.

In normal pulmonate snails there is an inverse relationship between female reproductive activity and growth. Geraerts (1976) discussed the role of dorsal body hormone, and hormones from the light green cells and lateral lobes in controlling reproductive activity and growth, with the lateral lobe hormone governing calcium deposition and, therefore, shell thickness. Sluiters *et al.* (1980) also demonstrated an increase in body wet weight of *L. stagnalis* infected with *T. ocellata*, Joosse and Van Elk (1986), however, attributed this increase to larger haemolymph volume rather than increased somatic growth in infected snails. The latter authors linked body characteristics of parasitized snails to those of juveniles, which maintain a large haemolymph volume. This condition can also be induced by removal of the lateral lobes of the cerebral ganglion. No increase in shell calcium deposition was observed in this study.

It is clear from the above discussion that the link between parasitic castration and gigantism in gastropods is by no means universal, nor indeed, is the occurrence of enhanced growth *per se*. A standardized method of growth assessment must be adopted before the extent of this phenomenon

can be determined, as must timing of infections, host maintenance and feeding regimes.

IV. Host Sex

A. The influence of host sex upon the parasite

In some parasitic associations, host sex is an important factor in a consideration of the overall relationship and the question of whether parasites are more successful in terms of survival, rate of development, incidence or burden of infection. The most detailed investigations have concerned hymenolepid infections, but these have yielded conflicting conclusions. A higher incidence of *Hymenolepis diminuta* infections in female insects was described for *Tribolium confusum* and *T. castaneum* infections by Mankau (1977) and Mankau *et al.* (1971), but no such differences were detected in *T. brevicornis* infections (Mankau, 1977). Parasite burden was also found to be higher in female *Tribolium* spp. (Mankau, 1977), although no such difference was detected by Mankau *et al.* (1971) and Kelly *et al.* (1967) reported an age-related increase in resistance to *H. diminuta* metacestode infections in female *Tribolium*. Parasite development was found to be more rapid in female hosts by Soltice *et al.* (1971), using timing of scolex invagination as a measure of *H. diminuta* development in *T. confusum* and *T. castaneum*. However, Schom *et al.* (1981), examining *H. citelli* infections of *T. confusum*, found development time to be greater in female hosts, with infected females having a shorter survival time than males.

In many of the above reports, important details such as host age at time of infection, initial host starvation period and host maintenance temperature were not reported. The rigorous standardization of such factors would facilitate any comparison made between different investigations of this type. With this in view, sex differences affecting the incidence of infection, parasite burden, development time and size attained by the metacestode of *H. diminuta* in infections of *T. molitor* were monitored using defined experimental conditions (Hurd and Arme, 1987c). Data obtained from 15-day-old infections of 18-day-old insects, maintained at 26°C demonstrated that 98% of male beetles were infected after exposure to cestode eggs for 24 h, a significantly higher percentage than found for female beetles (82%). In contrast, parasite burden did not differ between the sexes. No evidence of a crowding effect existed within the range of parasite burden encountered in this study (3–304 metacestodes per insect), but mean parasite midbody length and width were significantly larger in metacestodes developing in male hosts. In contrast, the width of presumptive adult scolex and scolex

retraction cavity were unaffected. Approximately 50% of all metacestodes in both sexes had undergone scolex retraction 9 days post-infection; however, a delay in early parasite development occurred in female beetles, 5.9% still remaining at stage I (Voge and Heyneman, 1957) at this age (*cf.* 0.8% in male beetles). Thus, an influence of sex-related variations in hormonal milieu and haemolymph metabolites (such as amino acids, described by Hurd and Arme, 1984a) upon parasite development appears plausible.

Wilson and Hubschman (1985) investigated the infection of copepods by cystacanths of *Tanaorhamphus longirostris*, and reported no significant difference in susceptibility to infection between the sexes in adult copepods. Parasites in female hosts attained a larger size, probably because female hosts were larger than male hosts.

Host sex has been shown to affect factors other than parasite burden and growth. For example, the sex of developing mermithid nematodes was shown to be influenced by the sex of their mosquito host, the ratio of males to females increasing as parasite burden increased (Petersen, 1977).

B. THE INFLUENCE OF PARASITES UPON HOST SEX

The influence of endoparasites upon the structure of the sex organs of their invertebrate hosts has been described by many authors and, in most cases, would appear to be the result of a general parasite-induced juvenilization of the host (Baudoin, 1974). Tomalak *et al.* (1984) reported that nematode infections resulted in underdeveloped male genitalia in coleopteran hosts, and the relationship between the presence of intersexes and dinoflagellate infections in populations of *Paracalanus parvus* (Copepoda) was analysed by Ianora *et al.* (1987). Infection of the rhizocephalid *Sacculina* in crabs induced such modifications as feminization of male appendages and degeneration of pleopods in females, with sexual reversal occurring in males (see Reinhard, 1956).

The occurrence of intersexes in stylopized bees parasitized as larvae and harbouring mature triungulinids as adults has long been recognized (Wülker, 1975). Although the underlying mechanism is not understood, Beckage (1985) speculated that parasite disruption of host endocrine control of sexual differentiation occurred in this association.

V. PARASITE MODIFICATION OF HOST BEHAVIOUR

A. MODIFICATION OF HOST REPRODUCTIVE BEHAVIOUR

In addition to the well-documented parasite curtailment of host fecundity,

some metazoan parasites have been shown to affect aspects of host behaviour associated with reproduction. For example, Lieutier (1981) demonstrated that, in the field, nematode infection delays the swarming of bark beetles by 3–4 days; however, this parasite-induced delay was reduced in cold weather.

Alteration in egg gallery construction has also been noted in nematode-infected beetles. Data obtained by Thong and Webster (1975b) demonstrated that *Contortylenchus reversus* infected *Dendroctonus pseudotsugae* females excavated shorter galleries, fewer eggs being deposited in the egg niches along gallery sides, although gallery shape was not affected. This had previously been described for other bark beetle/nematode associations (Massey, 1960). Gallery shape was also shown to be affected in nematode-infected *Scolytus ventralis* and *S. rugulosus* by Ashraf and Berryman (1970), who suggested that parasite secretory or excretory products may affect nervous coordination of the beetle host. MacGuidwin *et al.* (1980) found a similar reduction in the length of egg galleries constructed by *C. brevicornis* infected Southern Pine beetles. Shortened galleries were associated with the production of fewer eggs, although egg viability and progeny survival was not affected. Alteration of gallery construction was not, however, found to be a feature of parasitism of *Ips sexdentatus* by *Parasitorhabditis ipsophila* (Lieutier, 1984b).

B. STRATEGIES WHICH IMPROVE PARASITE TRANSMISSION

For many parasites, the utilization of an invertebrate host represents but one phase in a complex life cycle which could include free-living stages, other vertebrate or invertebrate intermediate or final hosts. Such life-styles have their attendant problems, one of which is transmission from one host stage to the next. Various strategies have been adopted to facilitate this transfer. For those parasites lacking a free-living stage, intimate contact between intermediate and definitive host is essential and several authors have discussed examples of parasite-induced host modifications which increase the likelihood of this contact occurring (for example, Holmes and Bethel, 1972; Pearre, 1979; Moore, 1984a,b; Molyneux and Jeffries, 1986). The tactics which have evolved to fulfil this end appear to be related to the exact mode of transmission, be it ingestion of the intermediate host/parasite (e.g. many cestodes and acanthocephalans), surface contact between successive hosts, or inoculation of parasites by intermediate hosts (e.g. Protozoa). These strategies will be divided into parasite-induced changes in host behaviour or host appearance, although some parasites appear to employ both methods.

1. *Modification of host behaviour*

Holmes and Bethel (1972), in an excellent review of parasite modification of intermediate host behaviour, distinguished between changes which increase the probability of accidental ingestion and those which increase the likelihood of intentional ingestion by the definitive host. In the former case, an increase in the time spent in the vicinity of feeding definitive hosts may suffice to increase transmission, whereas, in the latter case, more sophisticated changes occur, some of which may render non-infected and infected individuals visually distinguishable. Few parasitic life cycles investigated to date maintain the former mode of transmission, a notable exception being that of the liver fluke, *Dicrocoelium dentriticum*, the brain-worm of Hohorst and Graefe (1961). The effect of these metacercaria on the behaviour of their second intermediate hosts, formicine ants, is described in many texts (see review by Carney, 1969) and, briefly, results in a temperature-dependent migration of infected insects to the top of vegetation (see Fig. 3), the plant choice being dependent upon relative abundance (Spindler *et al.*, 1986). A paralysis of the mandibular muscles causes the host to remain attached to vegetation throughout the grazing period of the ungulate definitive host, and infected ants only return to the nest during the hot, midday period. The majority of these trematodes encyst within the host haemocoel and it is the one or two which migrate to the suboesophageal ganglion that are responsible for the alteration in host behaviour which greatly aids accidental ingestion and thus transmission.

As part of a theoretical appraisal of parasites in predator–prey relationships, Holmes and Bethel (1972) identified prey-selection procedures and suggested four means by which parasites increased the likelihood of host predation. Two of these strategies are particularly prevalent in infections of vertebrate hosts. Thus, reduction in stamina, and decreased locomotory efficiency, have been noted in many piscine hosts (seen, for example, as part of the pathology associated with the plerocercoid *Schistocephalus solidus* infection of sticklebacks (Lester, 1971). Another effect is host disorientation, produced by parasitic infection of the central nervous system (for example, gid in ungulates infected with coenuri of *Taenia multiceps*). Examples of invertebrate hosts rendered more conspicuous, and thus more susceptible to predation, are discussed below. The fourth strategy distinguished by Holmes and Bethel (1972), that of altered host response to external stimuli, appears to be the basis of the *Dicrocoelium*-infected ant pathology described above and, in various other guises, is documented throughout the literature.

Alterations in host phototaxis and geotaxis are commonplace and may

result in increased exposure of infected individuals to predation by causing more overlap between the territory of predator and prey. For example, the molluscan hosts *Macoma balthica* infected with metacercaria of gymnophallid trematodes are rendered more conspicuous to predators by leaving crawling tracks on the sand surface rather than remaining buried without trace (Swennen, 1969) and *Venerupis staminea*, heavily infected with plerocercoids of the cestode *Echeneibothrium* sp., also remain exposed (Sparks and Chew, 1966). Pearre (1979) reported that larval trematode infections of chaetognathans rendered intermediate hosts more conspicuous, due to the larger size of the host and presence of opaque parasites in this near-transparent animal. In addition, a shallower than normal habitat made these intermediate hosts more accessible to the zooplanktonic-feeding definitive host.

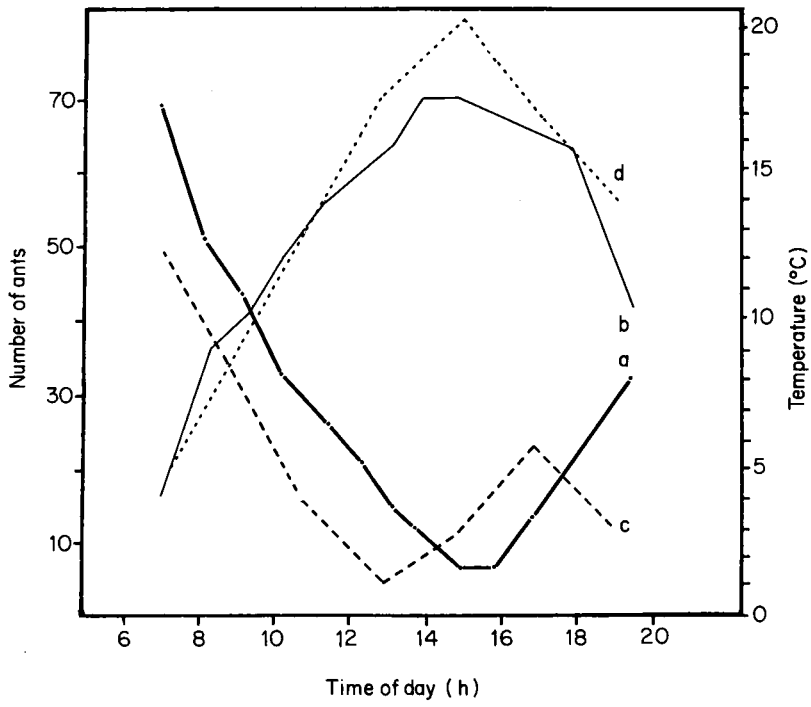

FIG. 3. (a) Numbers of *Dicrocoelium dendriticum*-infected *Formica* ants attached to plants during day. (b) Temperature. (c) Number of ants attached in the morning and newcomers during day 2. (d) Temperature. Redrawn with permission from *Zeitschrift für Parasitenkunde* (Spindler *et al.* (1986).)

Loss of photophobic behaviour has been reported for infected insects. Carpenter ants infected with metacercariae of *Brachylecithum* are, thereby, made more vulnerable to predation by insectivorous birds including robins, the definitive hosts for these trematodes (Carney, 1969) and *Tribolium confusum* infected with *Raillietina cesticillus* incurs greater risk of predation by the chicken final host (Graham, 1966). Webber *et al.* (1980, 1987) reported behavioural modifications in *Plagiorchis noblei* (Trematoda) infected third-instar *Aedes aegypti* larvae, with the degree of modification dependent upon parasite burden. Larvae infected with more than three metacercariae spent greater amounts of time at the water surface and thus were more vulnerable to predation by the definitive host, but less susceptible to further infection by bottom-inhabiting cercariae. Conversely, lightly infected individuals exhibited an extended aquatic phase of the life cycle and were more active in lower waters, thereby increasing exposure to further infection.

The examples of manipulative exploitation of intermediate host behaviour to facilitate transmission cited above are only a selection of those that abound in the literature. However, this phenomenon is nowhere better illustrated than by the classic studies of Bethel and Holmes (1973, 1974, 1977) and Moore (1983a,b; 1984a,b) of acanthocephalan infections. The former work is well-cited and will be touched upon only briefly here.

Using several model systems, Bethel and Holmes demonstrated that acanthocephalan-infected gammarids exhibited an altered response to environmental stimuli, intermediate hosts moving into the zone of the respective definitive host, rather than other predator species, thus maximizing the chance of predation. For example, *Gammarus lacustris* infected with *Polymorphus paradoxus* become strongly photopositive, alter their evasive behaviour, rising to the surface when disturbed, and exhibit an abnormal clinging response to floating vegetation or skim over the surface. These changes occur only after the parasite has developed to the stage at which it is infective to the surface-feeding definitive hosts, dabbling ducks such as mallards, and muskrats. Results obtained from feeding experiments showed that four times as many infected as non-infected gammarids were taken.

Data obtained from field and laboratory investigations demonstrated that *Plagiorhynchus cylindraceus* infection resulted in an increase in encounters between intermediate host isopods, *Armadillidium vulgare*, and the definitive host *Sturnus vulgaris*, the starling (Moore, 1983a). In contrast with normal isopods, infected females were more active, found more frequently on light-coloured backgrounds in less-humid areas and behaved randomly with respect to shelter availability. However, photoresponse was unaffected. In the field, nestling starlings exhibited a higher incidence of infection than

expected from prevalence levels of isopod infection, and in laboratory trials adult starlings consumed more infected than non-infected conspecifics when presented with the same number of each. Moore concluded that parasite-induced changes in isopod behaviour predisposed them to predation. Using a different acanthocephalan model system Moore showed that *Periplaneta americana* infected with *Moniliformis moniliformis* exhibited positive phototaxis, spent more time actively moving and, after exposure to light, paused significantly longer than non-infected cockroaches (Moore, 1983b). Wilson and Edwards (1986), also working with this model, confirmed the increase in locomotory activity with infection and also demonstrated an altered response to aggregation pheromone. They suggested that altered behaviour may serve to isolate infected individuals from dense groups and thus render them more susceptible to predation.

Moore and Lasswell (1986), investigating the hypothesis that altered host behaviour may be a generalized response to infection, chose the nematode *Dispharynx nasuta* infection of *Armadillium vulgare* as a model for comparison with *P. cylindraceus* infections. Their conclusions were tentative because of differences exhibited by controls in both studies. However, nematode-infected crustaceans differed significantly in photoresponse, spending more time in lighter areas. Here too, behavioural response to infection increased vulnerability to predation.

Altered behaviour resulting in selective predation of infected individuals could clearly be conceived of as host suicide, according to the hypothesis put forward by Smith-Trail (1980) and outlined earlier. This model would thus predict that the complex life cycles of acanthocephalans and other parasites, discussed above, had evolved as a result of the parasitized host sacrificing itself to a predator, thus eliminating the parasite(s) contained within them at minimum expense to the host population, particularly if the infected individual had reduced reproductive potential. Establishment of the parasite in the predator would extend the dimensions of the life cycle and host suicide would then be of ultimate benefit to the parasite.

Reviewing acanthocephalan strategies which increase transmission, Moore (1984a) stated that every acanthocephalan so far investigated (11) altered host behaviour and that all infected arthropods tested to date showed greater vulnerability to predation. She used data obtained from these studies to evaluate the Smith-Trail model and observed that, in modern acanthocephalan associations, host modifications were of benefit to the parasite and not the host. Three arguments were presented to support this view: (i) that the trait is not necessarily associated with female-host castration as behaviour changes are observed in both sexes; (ii) in the one system that has been investigated, the timing of host behavioural changes coincide with parasite infectivity to the definitive host; and (iii) aquatic crustacea have

planktonic larvae which are widely dispersed and thus kin are unlikely to benefit from host suicide. Nevertheless, whatever the evolutionary history, modifications of host behaviour such as those outlined above are of high selective value and, as survival strategies, have obvious value to the parasite–host complex, ensuring parasite transmission only at the expense of the more expendable members of the host population.

In none of the studies outlined above was any attempt made to ascertain the functional basis for the observed changes in host behaviour. Helluy (1983) compared the behavioural modifications induced in gammarids by acanthocephalans with those caused by a trematode *Microphallus papillorbustus*. Changes in host phototactic and geotactic response and an altered response to mechanical disturbance resulting in hyperactivity at the air–water interface, facilitated transmission of the parasite to the definitive host, *Larus cachinnans*. Unlike the models investigated by Bethel and Holmes, parasites were not confined to the haemocoel. Metacercariae were located in the thorax and abdomen of *Gammarus insensibilis*, but migrated to the cerebral ganglion in *G. aequicauda*. Helluy (1983) suggested that larvae are attracted to the amphipod brain by neurosecretory products. Reviewing the phenomenon of altered host behaviour in both the gammarid infections outlined above and *D. dendriticum* infection of ants, Helluy (1984) concluded that, in all cases, the underlying mechanism is chemical rather than mechanical, and that studies need to be directed towards biochemical ecology if light is to be shed upon the molecular basis of this fascinating area of study. I strongly support this view.

2. *Modification of vector feeding patterns*

Gathering examples from many haematophagous arthropods, Molyneux and Jeffries (1986) reviewed evidence suggesting that changes in vector feeding behaviour enhanced parasite transmission. This work drew on empirical evidence from numerous sources and will be summarized very briefly here, attention being directed to this excellent review for detail and sources. *Leishmania* infection of phlebotomine sandflies has been demonstrated to result in repeated host probing, reduced likelihood of engorging and impaired fluid flow. The hypothesis that this pathology may result in impaired sensilla functioning was examined. Trypanosome infections of *Glossina* have also been associated with increased probing frequency, impairment of receptor functioning being implicated here too. Adverse effects upon engorgement have also been noted, but conflicting evidence exists concerning this altered behaviour trait.

The effect of *Trypanosoma rangeli* on engorgement and feeding behaviour of *Rhodnius prolixus* was clearly demonstrated and it has been suggested that

this may result from damaged pharyngeal muscles. Although this aspect of the pathophysiology of *Plasmodium*-infected mosquitoes has largely been neglected, it was elegantly demonstrated that apyrase activity in salivary glands infected with sporocysts was reduced, thus possibly increasing the chance of blood clotting in the bite wound and, thereby, affecting probing behaviour. Investigations of fleas infected with plague bacilli and virus-infected mosquitoes also furnish evidence of altered host feeding behaviour that could enhance parasite transmission. However, the data obtained from studies of filarial infections in mosquitoes and mites are inconclusive.

As part of a quest for a greater understanding of vector-borne disease, attempts to construct quantitative transmission models are being undertaken (Ribeiro *et al.*, 1985). Molyneux and Jeffries (1986) have stressed the importance of the accurate assessment of pathological changes such as vector feeding behaviour, the information provided being a necessary component of such models.

3. *Hosts made more conspicuous*

As discussed above, if prey is actively hunted by visually oriented predators, it may be rendered more conspicuous by changed behaviour. This effect may be enhanced by altered appearance, particularly if camouflage is disrupted. Holmes and Bethel (1972) discussed examples of vertebrate and invertebrate hosts that are visually recognizable when infected, and cited the classic example of the pulsating tentacles of snails infected with sporocysts or brood capsules of *Leucochloridium* or *Neoleucochloridium* (see Ulmer, 1971). However, it has not been determined whether this very strong visual clue enhances parasite transmission.

More recently, changes in pigmentation associated with crustacean infections have been investigated. Brattey (1983) noted a significant increase in susceptibility to predation by perch in *Acanthocephalus lucii*-infected *Asellus aquaticus*. The parasite caused a change in pigmentation, infected individuals having darkened respiratory opercula. It was concluded that this alteration, combined with possible changes in behaviour, may have resulted in increased conspicuousness of infected isopods. Pilecka-Rapacz (1986) noted that changes in pigmentation corresponded to the transformation of acanthellae to cystacanths and also observed an increase in vulnerability to predation. The author cited several reports of altered pigmentation associated with this host–parasite complex (see Oetinger and Nickol, 1981, 1982) and discussed the work of Needham (1974) correlating darkening with increasing amounts of xanthommatine and pigment dystrophy with competition between parasite and host for amino acid resources of the ommochrome pathway. Finally, Brown and Thompson (1986) applied orange

paint to mimic *Pomphorhynchus laevis* infections in *G. pulex*. Their findings led them to speculate that host intraspecific interactions have played a role in the evolution of modified host behaviour. They suggested that uninfected shrimps attacked infected ones and, in time, the escape response of infected gammarids was used by the parasite to induce altered behaviour. This work provides a novel addition to current ideas on the subject. The possibility that visual and other cues render infected individuals recognizable to conspecifics is largely undetermined, although Hamilton and Zuk (1982) suggested that this may be the case for North American passerines infected with blood parasites.

VI. Host–Parasite Synchrony

Invertebrate hosts which undergo metamorphosis and, in particular, holometabolous insects, provide an environment which may be subject to marked fluctuations in physiology, endocrinological milieu and behavioural patterns. Such hosts are utilized by a vast number of parasitic species, many of which adopt a strategy of developmental synchrony to counter the difficulties imposed by a habitat liable to changing regimes. Of these, the endoparasitic insects of the orders Diptera, Hymenoptera and Strepsiptera provide a focus for investigations and numerous developmental interactions, many via endocrine systems, have been described. Comprehensive coverage of this work in recent reviews by Thompson (1983), Beckage (1985) and Lawrence (1986a) and entire volumes of two journals, *Journal of Insect Physiology* (1986, **32**) and *Archives of Insect Biochemistry and Physiology* (1989, **10**) obviates the need for all but a selective examination of this field.

Parasitoid infections are regarded by some as a special case of parasitism (Kuris, 1974; Bouletreau, 1986). These infections usually result in the eventual death of the host, but highly integrative relationships of a varied nature may be formed early in the infections. Strand (1986) pointed out that the existence of closely interwoven life histories often results in host specificity and that characteristics of the host (particularly size range and age) can affect parasite survival, growth and sex ratio.

Many of these insect–insect associations display a high degree of temporal synchronization (Riddiford, 1975); parasite emergence, for instance, may be timed to coincide with a particular stage of host metamorphosis, as in *Hyposeter exiguae* parasitizing *Manduca sexta* (Beckage and Templeton, 1985). Emergence occurred 24 h post-host-apolysis preparatory to moulting, and was regarded by the authors as highly adaptive, since consumption of host tissue was facilitated. Examination of the infection of a smaller host, *Trichoplusia ni*, led to the further conclusion that host size was related to the

timing of emergence and that a specific nutritional requirement may exist. In addition, in the *Cotesia congregata* (formerly *Apanteles*)–*M. sexta* association, parasite moulting occurred 48–72 h after that of the host, with the initial development of the parasite being delayed in the first instar until host ecdysis to the terminal stadium (Beckage, 1985, 1986). Recently, Ramadhane *et al.* (1987) reported four phases of parasitoid development synchronized with host physiological events in *Ostrinia nubilalis* larvae parasitized by the tachinid fly *Pseudoperichaeta nigrolineata*. In diapausing hosts, parasitoid development was arrested at the second instar.

It is clearly disadvantageous for the emergence of endoparasitic insects to coincide with host ecdysis, when two cuticles would need to be penetrated. Furthermore, once emerged, movement of the host could endanger metamorphosing parasitoids. Thus, temporal synchrony of parasite development, suppression of host moult and developmental arrest are strategies commonly observed in parasitoid relationships (Beckage, 1985). These developmental interactions may result in manipulation of host metamorphosis, often in conjunction with some degree of synchronization of host–parasite development. In *Chelonus* spp. infection of *T. ni*, it has been reported that host and parasite moult in synchrony until the penultimate stadium, when precocious host metamorphosis is initiated due to a significant decrease in the critical size parameter which normally signals the onset of metamorphosis (Jones, 1985a,b). Further examples of parasite-induced precocious metamorphosis and of the induction of supernumery moults, delayed host pupation and arrested development were cited in recent reviews (see above).

The elucidation of the functional basis of these complex and highly integrative associations is a matter of some difficulty. However, progress is being made by several workers and their findings are discussed in the following section.

VII. Endocrinological Interactions

Solomon's review (1969) of host hormones and parasitic infections was almost exclusively devoted to parasites of vertebrate hosts. Recently, although there has been some increase in our knowledge, this is still a largely neglected field with little data available to substantiate considerable speculation. The hypothesis that complex pathophysiological interactions between invertebrate hosts and parasites may be mediated via endocrinologically active substances is not new. In 1948, Palm suggested that the nematode *Sphaerularia bombi* secreted a toxic substance that suppressed host corpora allata activity. Endocrinological interaction between parasite and host has now been shown to be a two-way process with active

substances of parasite origin affecting host metabolism, development and reproduction, and host hormones regulating parasite development. Examples of these phenomena have already been discussed with respect to both cestode infections of insects and trematode–molluscan associations. Other examples, reflecting the theme of this article, are presented below.

A. PROTOZOA

The supernumery moults observed in *Tribolium* infected with the sporozoan *Nosema* were associated with juvenile hormone activity in a parasite extract, although isomers of farnesol were not detected in the extract (Fisher and Sanborn, 1964). Listov (1977), however, attributed the disturbance in metamorphosis of *Tribolium* spp. infected by *Nosema whitei* and *Adelina tribolii* to fat body destruction. The neogregarine, *Farinocystis tribolii* also inhabits the fat bodies of this host, destroying the organ. Moulting was delayed or severely affected with the production of larval–pupal or pupal–adult intermediates. Rabindra *et al.* (1981) suggested that this parasite induced a hormonal imbalance resulting in juvenile hormone accumulation, possibly due to lack of the juvenile-hormone degrading enzyme, epoxy hydrolase, normally present in fat body tissue. Clearly a rigorous experimental approach is required to determine the hormonal status of infected insects. The synchronization of sporulation in *Amblyospora* infection of *Culex salinarius* with host ovarian development is an essential prerequisite of transovarian transmission. Lord and Hall (1983) demonstrated that 20-hydroxyecdysteroid stimulated sporulation *in vivo* but juvenile hormone and methoprene were ineffective.

B. NEMATODES

In reviewing relationships between nematodes and their insect hosts, both Davey and Hominick (1973) and Gordon (1981) concluded that there was very little evidence to support the view that endocrine signals passed between parasite and host. Gordon *et al.* (1973) suggested that mermithid-induced changes in locust endocrine functioning may be responsible for impairment of vitellogenesis (see above) and mermithid infection of larval blackflies was found to result in an increase in DNA/RNA activity in host corpora allata and in an increase in the neurosecretory material stored by the corpus cardiacum (Condon and Gordon, 1977). However, Craig and Webster (1974) failed to find changes in ecdysteroid titres in desert locusts infected with *Mermis nigrescens* that could be associated with parasite-induced inhibition of moulting. Despite this lack of evidence of an endocrinological interaction between parasite and host, the presence in nematodes of both

ecdysteroids (Mendis *et al.*, 1983; Rees and Mercer, 1986) and biologically active juvenile hormone and analogues (Rogers, 1973) has been demonstrated. The latter hormones had inhibitory effects upon hatching of non-infective eggs of several species of nematode and it was suggested that juvenile-hormone-like molecules may act by controlling gene activity (see references in Jones *et al.*, 1983). An analogue of juvenile hormone was shown rapidly to penetrate the nematode cuticle (Jones *et al.*, 1983). In view of these findings, it is perhaps time that the endocrinological relationships between mermithids and their hosts were reassessed, using the sensitive techniques now available for hormone detection.

C. CRUSTACEANS

Inhibition of moulting in crustaceans infected by *Sacculina* spp. has been attributed to suppression of host endocrine activity (Hartnoll, 1967) and depression of ecdysone titres has been demonstrated in *Sacculina carcini*-infected *Carcinus moenas* (Andrieux *et al.*, 1976). More recently, rhizocephalan extract was found to alter crab testicular germinative zones (Rubiliani, 1985).

D. ENDOPARASITIC INSECTS

Endoparasitic insects have been shown to interact with the endocrine system of both adult and larval hosts; thus, cessation of egg production in the wasp *Polistis* infected with a strepsipteran parasitoid was reversed by corpora allata implantation (Strambi and Girardie, 1973). Furthermore, Strambi and Strambi (1973) demonstrated that the reduced corpora allata volume observed in infected wasps was associated with hypoactivity of the neurosecretory cells of the pars intercerebralis of the brain. This evidence suggests that reduced juvenile hormone titre in parasitized insects was responsible for effective parasitic castration. However, determination of juvenile hormone titres *in vivo* would need to be performed to confirm this. By contrast, Girardie (1977) showed that inhibition of oocyte development in the grasshopper *Metacemyia calloti* infected with *Anacridium aegyptium*, was due to impairment of vitellogenin synthesis by the fat body. Corpora allata activity was found to be unaffected by infection, although electrical stimulation of, and [^{35}S]-cystine incorporation into, the pars intercerebralis demonstrated hypoactivity of the neurosecretory cells in parasitized females. Furthermore, these workers showed that the inability of eggs to mature was related to burden of infection and thus argued for the existence of an inhibitory parasite product acting upon median neurosecretory cells.

The disruptive effects of protelean endoparasitic insects upon host devel-

opment has already been discussed (see also Vinson and Iwantsch, 1980). Many of these studies have provided substantial evidence that endocrinologically active substances of both host and parasite origin play an essential role in temporal and developmental synchronization (Riddiford, 1975). Changes in host hormone titres may act as triggers for developmental synchrony (Baronio and Sehnal, 1980) or parasite-induced perturbations in host endocrinology may disrupt metamorphosis (Beckage, 1985).

Lawrence (1986a) divided endoparasitic insects into regulators and conformers, based upon their strategies of endocrinological interaction. Regulator parasitoids have a variety of effects upon their hosts, but all entail alterations in the titre of one or both of the hormones governing insect development, namely juvenile hormone and the ecdysteroids. Elevation of host juvenile hormone titres and depression of the moulting hormone, 20-hydroxyecdysone, gives rise to arrested or decelerated development and depression of juvenile hormone titres to precocious metamorphosis (Beckage, 1985; Lawrence, 1986a). For example, *Manduca sexta* parasitized by *Cotesia congregata* moulted to the fifth instar. Here juvenile hormone titres sufficient to inhibit metamorphosis occur, possibly as a result of increased corpora allata biosynthesis, absence of juvenile hormone esterase activity and a possible alteration in juvenile-hormone-binding proteins (Beckage, 1986). Ecdysteroid titre is also affected by infection, conversion of ecdysone to the active 20-hydroxyecdysone being depressed due to low 20-monoxygenase activity (Beckage and Templeton, 1986), and see also the *T. ni–Hyposoter exiguae* relationship (Thompson, 1983). In contrast, precocious metamorphosis of *T. ni* parasitized or pseudoparasitized by *Chelonus* spp., occurs during the penultimate stadium. The parasitic wasps emerge from host prepupae after cocoon formation, killing the host. In pseudoparasitized hosts, development is arrested at the prepupal stage. These events were thought to be the result of an alteration or reprogramming of tissues involved in establishment of critical growth thresholds (Jones, 1985a). Comparsion with non-parasitized *M. sexta* revealed a decline in corpora allata activity and an increase in juvenile hormone-esterase activity, probably resulting in depressed ecdysteroid titres (Jones, 1985b; Jones *et al.*, 1986). Further examples of host regulation as a result of endocrinological disturbance have been discussed in recent reviews cited above.

The means by which these perturbations are brought about is unknown, although several authors have addressed the problem. The relationship between hormones and metabolic function in infected insects was discussed at length by Thompson (1983), and Beckage (1985) discussed a possible relationship between high corpora allata activity and low haemolymph trehalose titres (associated with corpora allata stimulation in starved larvae; Jones *et al.*, 1981). The contribution played by hormones of parasite origin is

largely unexplored. However, some evidence exists to support the view that both juvenile hormone and ecdysteroids are secreted by endoparasitic insects. The homologue juvenile hormone III, normally absent from *M. sexta*, has been detected in larvae infected with *C. congregata* (Beckage and Riddiford, 1982) and juvenile hormone of parasite origin has been detected in the *Pieris brassicae–A. glomeratus* complex (Schopf, 1984). Beckage and Templeton (1985) discussed the possibility that ecdysteroids secreted by *H. exiguae* initiate host apolysis as did Schneider (1950) with respect to the wasp *Diplazon*. Lawrence (1986a,b) suggested that endocrinological alterations could be mediated via pathological changes in insect fat body.

The nature and functional basis of host regulation in insect–insect relationships is undoubtedly complex and requires a varied analytical approach. Beckage (1985) discussed the use made of juvenoids, antijuvenoid hormone compound and ecdysteroids as probes with which to examine these interactions; Jones *et al.* (1981) proposed pseudoparasitized insects in the *T. ni–Chelonus* complex as an excellent model for biochemical analysis.

It has recently been recognized that virus particles and abiotic factors injected at oviposition, may have a role to play in the regulation of host metamorphosis and alteration of host physiology and immune response (see, for example, the review by Stoltz, 1986).

Many of the associations discussed above contain elements of both regulation and conformity, initial parasite development occurring in response to exogenous endocrine triggers, disturbance of host metamorphosis taking place later. In her review, Beckage (1985) cited examples of relationships in which host ecdysteroid titre regulates parasite moulting and metamorphosis, the emergence of the brachonid wasp, *C. congregata* in synchrony with increases in circulating host ecdysteroid being an example. More recently, in studies of *Galleria mellonella* infected with the tachinid parasitoid *Pseudoperichaeta nigrolineata*, a relationship between ecdysteroid titres in the respective partners was obsereved, low host titres being associated with developmental arrest in the parasite (Plantevin *et al.*, 1986). Lawrence (1986b) and Lawrence and Hagedorn (1986) assessed the role of ecdysteroids in the development of the endoparasitic wasp *Biosteres longicaudatus* and demonstrated *in vitro* uptake of ecdysone and conversion to 20-OH-ecdysone.

VIII. Conclusions

This review of some of the integrative aspects of invertebrate-host–parasite

associations has examined a patchwork of information from a conceptual rather than descriptive view point. However, whilst trying to avoid a species-by-species approach, it has become evident that particular parasite–host complexes have been used as research models with which to investigate each concept. The multifarious nature of invertebrates and their parasites has enabled this broad survey to encompass insights from many fields of research. Amongst this diversity, however, generalizations do emerge. Strategies designed to minimize the deleterious effects of parasitism at the level of the individual and of the host population are commonplace, as are those which help to perpetuate the relationship. The examples outlined in this article serve to illustrate the complex manner by which these strategies are executed and are indicative of highly co-evolved associations. We are thus beginning to appreciate the effects of parasitic infections, but not, as yet, the cause of these effects. For example, the means by which host behaviour is altered to favour parasite transmission and the triggers responsible for initiation of different phases of parasite life cycles are, so far, unknown. Physiological data are, however, beginning to provide insights into the mechanisms underlying strategies such as host resource management, parasitic castration and developmental synchrony.

Recent technological advances should provide methods applicable to the analysis of the minute samples usually obtained from invertebrates and thus accelerate the progress of our understanding of the functional basis of these interactions. In particular, the nutritional and endocrinological status of hosts, and the mediating role that these factors play in many stable associations needs to be fully assessed.

The study of invertebrate-host–parasite interactions has been regarded as a fallow field by some researchers (e.g. Jones *et al.*, 1986). It is hoped that this review has highlighted some of the many areas under investigation and indicated a number of fruitful avenues for further research.

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Appendix

Since submission of this review there have been a number of developments. In particular, investigations into the endocrine interaction in the *Trichobilharzia ocellata–Lymnaea stagnalis* association have made considerable advances. In brief, de Jong-Brink and co-workers have concluded that the parasite induces the snail to produce schistosomin. A bioactive peptide, extracted from the snail CNS has been identified as schistosomin. It has a molecular weight of 12.8 kD and 50% of the molecular structure has now been elucidated. This substance has not been detected in

cercariae. Bioassays revealed that schistosomin reacts with three different hormones at the receptor complex and initial work indicates that this peptide may be "genus-specific" (findings reviewed by M. de Jong-Brink *et al.*, *Journal of Invertebrate Biology*, in press). Crews and Yoshino (1989) monitored the onset of castration, which occurred between 14 and 21 days post-exposure in *S. mansoni* infected *B. glabrata*. They were, however, unable to detect a reproductive burst prior to this period.

Developmental synchrony between *Spodoptera littoralis* and a braconid endoparasite has been reported, elevated juvenile hormone titres being associated with parasitism (Hegazi *et al.*, 1988). Some evidence now exists that acanthocephalan-induced modification of the escape behaviour of *Gammarus* is modulated by monoamines (Helluy, 1988).

A computer model has been developed by Rossignol and Rossignol (1988) which explores the impact of vector and host pathology upon malaria transmission. Dobson (1988) has reviewed the population biology of parasite-induced changes in host behaviour and the regulatory role that parasites play in determining host behaviour has also been surveyed recently (Scott and Dobson, 1989).

Index

Acanthatrium anaplocami, 289
Acanthocephalus lucii, 298
Acidic-basic repeat antigen, 82–3
Adelina tribollii, 301
Adenosine deaminase, (ADA) 113, 127
Aedes aegypti, 281, 295
Aedes trivittatus, 282
Alanine aminotransferase, 4
Amacridium aegyptium, 302
Ambylospora, 301
Amodiaquine resistance, 122
Ankyrin, 173–4
Antibody–ferritin conjugates, 156–7
Antigens
  acidic-basic repeat, 82–3
  CS protein related, 95–6
  cytoplasmic, 98
  erythrocyte associated, 89–97
  falciparum interspersed repeat (FIRA), 92
  mature parasite-infected erythrocyte surface (MESA), 91–2
  merozoite surface, 85–9, 99
  octapeptide repeat (ORA), 97
  ring-infected erythrocyte surface (RESA), 89–91
  S-antigen, 78–81, 99, 100
  schizont-infected cell agglutinin, 96–7
  secreted, 78–83
  serine-rich, 82
Aotus, 90, 94, 160, 166
Apyrase, 298
Armidillidium vulgare, 295, 296
Ascellus aquaticus, 298
Asparagine-rich proteins (ARPs), 97
Avidin, 157–8
Avidin–biotin–peroxidase complex (ABC) technique, 156

Bellamya ingallsiana, 253
Biomphalaria, 236, 258, 288
Biomphalaria alexandrina, 226, 230, 277
Biomphalaria glabrata
  as host to *E. caproni*, 226–8, 230, 232, 234, 238, 258
  relationship with *S. mansoni*, 276, 287
Biomphalaria pfeifferi, 277, 289
Biosteres longicaudatus, 304
Bithionol, 254
Bithynia tentaculata, 277
Brachylecithum, 295
Brotianide, 254
Bucephalus, 274
Bulinus, 236, 258
Bulinus senegalensis, 232
Bulinus truncatus, 277

Calfluxin, 279
Calmodulin, 163
Carcinus maenas, 302
Cercaria helvetica, 277
Cerithidea, 287
Chelonus, 300, 303
Chloroquine, 77
  resistance, 117, 122–5
Circumsporozoite precipitation reaction (CSP), 178
Circumsporozoite (CS) protein, 77, 83–5, 99, 100, 179–86
Circumsporozoite related antigen (CRA), 95–6
Colloidal gold probes, 156–7
Contortylenchus brevicornis, 292
Contortylenchus diplogaster, 282
Contortylenchus reversus, 282, 292
Contour-clamped homogeneous electric field (CHEF) apparatus, 101
Corbicula fluminea, 234, 253
Corbicula lindoensis, 253
Cotesia congregata, 303, 304

Cotesia congregata—M. sexta association, 300
Crassostrea virginica, 274
Crocidura olivieri, 224
Cryofixation, 195
Cryo-ultramicrotomy, 154
Culex quinquefasciatus, 281
Culex salinarius, 301
Cyclohexylamine, 153
Cyclophosphamide, 3
Cytoadherence molecule, 77

Dendroctonus pseudotsugae, 292
3,3'-Diaminobenzidene, 156
Dicrocoelium dentriticum, 293, 294, 297
Diethylmalonimidate as fixative, 153
Dihydrofolate reductase (DHFR), 117–21, 127
Dimethylsuberimidate as fixative, 153
Dinitrophenyl (DNP) hapten sandwich staining (DHSS) procedure, 156
Diplazon, 304
Diplostomulum scheuringi, 275
Dirofilaria immitis, 282
Dispharynx nasuta, 296
Distocide, 254
DNA probes, 15, 38

Echeneibothrium sp., 294
Echinochasmus japonicus, 249
Echinoparyphium recurvatum, 247
Echinostoma
  biology of 37-collar-spined, 224–8
  clinical and pathological effects, 248–52
  collar spine counts, 216–18
  confusion over species identification, 216
  epidemiology and disease, 253–4
    diagnosis and treatment, 254
    source of infection, 253
  hosts, 224–6, 228–46
  immunology of infections in vertebrate hosts, 246–8
  infectivity studies
    first intermediate host, 228–32
Echinostoma (cont.)
    infectivity, growth and development in vertebrate hosts, 238–46
    second intermediate hosts, 232–8
  life cycle, 224–8
  morphology, 217–23
  physiology and biochemistry, 254–7
Echinostoma caproni, 216
  biology, 224–8
  carbohydrates, 257
  clinical and pathological effects, 249, 251, 252
  development in vertebrate hosts, 244–5
  encystment, 236
  epidemiology, 259
  excystation, 238, 239
  flame-cell formula, 234
  hosts, 224–6
  immunology of infections in vertebrate hosts, 246–8
  infection, 253
  infectivity, 240
  life cycle, 226–8
  maintenance, 258
  miracidia development, 228–9
  miracidial infectivity, 230, 232
  morphology, 217, 219, 220, 222, 223
  proteins and amino acids, 257
  resistance to, 259
  species definition, 258
  treatment, 254
  worm distribution, 240–2
Echinostoma cinetorchis, 253, 254
Echinostoma echinatum, 251, 253
Echinostoma hortense, 248, 249, 253
  treatment, 254
  worm expulsion, 259
Echinostoma malayanum, 246, 249, 253
Echinostoma revolutum
  avian hosts, 245
  clinical and pathological effects, 252
  epidemiology, 259
  hosts, 226, 230, 278, 289
  infection, 253
  miracidial infection, 232
  proteins and amino acids, 257
  species definition, 258
  systematics, 216

Echinostoma trivolvis, 216
biology, 224–8
carbohydrates, 257
cercaria, 234, 235
clinical and pathological effects, 249–52
cultivation, 255
development in vertebrate hosts, 242–4
encystment, 234, 236
epidemiology, 259
excystation, 238
growth and development, 238
hosts, 224–6, 228–9
immunology of infections in vertebrate hosts, 247, 248
infection, 253
infectivity, 240
life cycle, 226–8
lipids, 256–7
maintenance, 258
morphology, 217–19, 221, 222
pairing behaviour, 255–6
parasitism in *H. trivolvis*, 232, 233
proteins and amino acids, 257
rediae, 230, 231
resistance to, 259
species definition, 258
transplantation studies, 254–5
treatment, 254
worm crowding, 246
worm distribution, 240–1
Eimeria, immunoelectron microscopy of, 187–8
Erythrocyte associated antigens, 89–97
antigens of sexual stages, 98
application of immunoelectron microscopy to, 160–86
Escherichia coli, 83, 92, 107
Euparyphium ilocanum, 253

Falciparum interspersed repeat antigen (FIRA), 92
Fansidar, 127
Farinocystis tribolii, 301
Farnesol, 301
Fasciola
immunoelectron microscopy of antigens, 192–4
Fasciola hepatica, 277
immunoelectron microscopy of antigens, 194
Ferriprotoporphyrin IX (FP), 124
Food-robbery, 276
Formaldehyde fixation, 152
Freeze-fracture techniques, 195
Freeze-substitution techniques, 195

Galleria mellonella, 304
Gambian trypanosomiasis, 20, 33, 39
Gamma-irradiation, 3
Gammarus aequicauda, 297
Gammarus insensibilis, 297
Gammarus lacustris, 295
Gammarus pulex, 299
Genetic recombination of *Plasmodium falciparum*, 108
Giardia, immunoelectron microscopy of antigens, 190
Gigantismn, 287–90
Glossina spp. 15, 190, 297
Glossina pallidipes, 37
Glutamic acid-rich protein (GARP), 95
Glutaraldehyde fixation, 152
Glycophorin, 81
Glycophorin A, 173
Glycophorin-binding protein, 81–2

Haematoloechus medioplexus, 256
Halipegus occidualis, 275
Helisoma anceps, 275
Helisoma duryi, 236
Helisoma trivolvis, 226–8, 232, 234, 258
Heterostropha, 228, 234
Hexyl resorcinol crystoids, 254
Histidine-rich protein (HRP), 78, 92–3, 94
Host behaviour
hosts made more conspicuous, 298–9
modification, 293–7
modification of vector feeding patterns, 297–8
parasite modification of host reproductive behaviour, 291–2
strategies which improve parasite transmission, 292–9

Host growth, 286–90
Host–parasite endocrinological interactions, 300–4
  crustaceans, 302
  endoparasitic insects, 302–4
  nematodes, 301–2
  protozoa, 301
Host–parasite synchrony, 299–300
Host sex
  influence of, on parasite, 290–1
  influence of parasite on, 291
Host suicide, 273
Hymenolepis citelli, 290
Hymenolepis diminuta, 282–6, 290
Hyposoter exiguae, 299, 303, 304
Hypothetical taxonomic unit, 12
20-Hydroxyecdysone, 301, 303
Hypergammaglobulinaemia, 101

Ilyanassa obsolata, 280, 289
Ilyanassa obsolata–Zoogonus lasius model, 280
Immunocytochemistry
  controls, 158–9
  post-embedding, 154–5
  pre-embedding, 154
Immunoelectron microscopy
  accessibility of antigenic sites, 153–5
  fixation, 152–3
  of *Plasmodium*, 160–86
  of *Schistosoma* and *Fasciola*, 192–4
  of *Theileria*, 188–90
  of *Toxoplasma*, *Sarcocystis* and *Eimeria* 187–8
  of *Trichinella*, 190–2
  of *Trypanosoma*, 190
  visualization of antigenic sites, 155–8
    enzymatic markers, 155–6
    particulate markers, 156–8
Isthmiophora melis, 258

Kinetoplast DNA (kDNA) maxicircles, 15, 35, 38
Knob-associated histidine rich protein, 93

Larus cachinnans, 297
Leishmania, 297
Leishmania major, 118, 122
Leucochoridium, 298
Locusta migratoria, 282
Lymnaea, 226
Lymnaea luteola, 232, 278
Lymnaea natalensis, 236
Lymnaea ollula, 230
Lymnaea stagnalis
  E. caproni encystment in, 236
  Trichobilharzia ocellata infection of, 275, 277–80, 289
Lymnaea swinhoei, 230
Lymnaea truncatula, 277, 287, 288
Lymnaea truncatula–Fasciola hepatica association, 287

Macoma balthica, 294
Manduca sexta, 299, 303, 304
Mature parasite-infected erythrocyte surface antigen (MESA), 91–2
Mefloquine, 122
Melania tuberculata, 236
Mermis nigrescens, 281, 301
Merozoite surface antigens, 85–9, 99
Mesocricetus auratus, 224
Metacemyia calloti, 302
Microphallus papillorobustus, 297
Microsporidium itiiti, 281
Misgurnus angiullicaudatus, 253
Moniliformis moniliformis, 296

Nannomonas, 20
Neoleucochoridium, 298
Niclofolan, 254
Niclosamide, 254
Nitocris dilatatus, 289
Nosema algerae, 281
Nosema whitei, 280, 301
Notocotylus attenuatus, 252

Octapeptide repeat antigen (ORA), 97
Oleoresin of aspidium, 254

Onchocerca lienalis, 282
Ondatra zibethica, 224
Osmium tetroxide as fixative, 153, 156
Ostrinia nubilalis, 300
Oxyclozanide, 248, 254

Paracalanus parvus, 291
Parasitaphelenchus spp., 282
Parasitaphelenchus sexdentati, 282
Parasitic castration, 273–86
  cestode infections, 283–6
  definition, 273–4
  insects as hosts, 280–6
  nematode infections, 281–3
  protozoan infections, 280–1
  trematode infections of molluscs, 274–80
Parasitorhabditis ipsophila, 292
Patristic distance, 6, 11
Peptococcus magnus, 157
Periplaneta americana, 296
Phosphatidylcholine, 256
Phosphatidylethanolamine, 256
Phosphatidylserine, 257
6-Phosphogluconate dehydrogenase, 113
Physa, 238
Physa acuta, 236
Physa heterostropha, 228, 234
Physa sayii, 289
Pieris brassicae–Apanteles glomeratus association, 304
Pila, 253
Pila luzonica, 253
Pisidium casertanum, 236
Plagiorchis noblei, 295
Plagiorhynchus cylindraceus, 295, 296
Planorbis corneus, 236
Plasmodium
  antigens, 78–101
    acidic-basic repeat antigen, 82–3
    antigens of sexual stages, 98
    circumsporozoite (CS) protein, 83–5, 99, 100
    CS protein related antigen, 95–6
    erythrocyte associated antigens, 89–97
    falciparum interspersed repeat antigen (FIRA), 92
Plasmodium (cont.)
    glutamic acid-rich protein (GARP), 95
    glycophorin-binding protein, 81–2
    histidine and alanine-rich proteins, 92–3, 94
    knob-associated histidine rich protein, 93
    mature parasite-infected erythrocyte surface antigen (MESA), 91–2
    merozoite surface antigens, 85–9, 99
    nomenclature, 78
    ring-infected (RESA) erythrocyte surface antigens, 89–91
    S-antigen, 78–81, 99, 100
    schizont-infected cell agglutinin antigen, 96–7
    secreted antigens, 78–83
    serine-rich antigen, 82
  immunoelectron microscopy, 160–86
    asparagine-rich proteins (ARPs), 97
    chromosome size polymorphisms, 101–7
    conservation of housekeeping genes, 99
    cytoplasmic antigens, 98
    erythrocytic stages, 160–78
    exoantigens, 169–71
    exoerythrocytic stages, 182–6
    genetic crosses between malaria parasites, 108–16
    host–cell alterations, 172–8
    meiosis and genetic recombination, 108–16
    merozoite surface coat, 160–2
    octapeptide repeat antigen (ORA), 97
    recombination and parasite life cycle, 108
    repetitive antigens and immune evasion, 97, 100–1
    rhoptry–microneme complex, 162–8
    rhoptry proteins, 97
    sporogonic stages, 178–82
Plasmodium berghei
  chloroquine resistance, 123, 124

Plasmodium berghei (cont.)
chromosomes in, 102
CS proteins in, 180, 182–4
dihydrofolate reductase (DHFR) inhibitor in, 117, 122
DNA synthesis, 108
hybridization, 104
pyrimethamine resistance, 118
Plasmodium brasilianum
exoantigens, 170, 171
host–cell alterations, 174–7
rhoptry-microneme antigens, 166
Plasmodium chaubaudi
antigenic variation, 77
chloroquine resistance, 123, 125
chromosomes in, 102–3
drug resistance in, 117
erythrocyte associated antigens in, 89
genetic crosses, 109, 110–11
pyrimethamine resistance, 118
Plasmodium cynomolgi
CS protein in, 83, 84, 85, 184
Plasmodium falciparum
actin genes, 99
chloroquine resistance, 122–5
chromosome size polymorphisms, 103–6
CS protein in, 83, 84, 85, 97, 180, 183, 184
CS protein-related antigen, 95–6
cytoadherence molecule in, 77
diversity of antigens in, 99, 113
drug resistance, 127
exoantigens, 170
FIRA in, 92
gene deletions, 106–7
genetic crosses, 109, 110–13
glycophorin-binding protein, 81
host–cell alterations, 172–4, 176
merozoite surface antigens, 160, 161
molecular karyotype, 101–3
MSA1 gene, 86–7, 113–15
multidrug resistance (MDR) gene, 124, 125
Pf11–1 gene in, 94–5
pyrimethamine resistance, 118–22
recombinant DNA technology in, 76
recombination, 108
repetitive antigens, 100–1
rhoptry–microneme complex of merozoite, 163, 164, 166–8
Plasmodium falciparum (cont.)
S-antigen, 78, 80
schizont-infected cell agglutinin antigen, 96–7
vaccines against, 126
Plasmodium fragile, 77
Plasmodium gallinaceum
and reproduction of *Aedes aegypti*, 281
chloroquine resistance, 124
drug resistance, 117
Plasmodium knowlesi
CS proteins in 84, 85, 182
deletion of gene for antigen, 100
DNA synthesis inhibition, 117
merozoite surface antigens, 160, 161
microspheres, 164, 165
schizont-infected cell agglutinin antigen, 77, 96–7
Plasmodium lophurae
DHFR in, 118
histidine-rich protein of, 92–3
Plasmodium malariae, 174
CS protein in, 180, 182
Plasmodium ovale
CS protein in, 180
Plasmodium vinckei
chloroquine resistance, 123
chromosomes in, 103
Plasmodium vivax, 85
CS protein in, 183, 184
host–cell alterations, 178
Plasmodium yoelii
CS protein in, 83
drug resistance, 117
genetic crosses, 109
merozoite surface antigens, 160
rhoptry–microneme antigens, 166
Pleugenes medians, 277
PLP fixative, 153
Polistis, 302
Polyacrylamide gel electrophoresis (PAGE), 126, 127, 282
Polymorphism
DNA, 35
enzyme, in *Trypanozoon*, 21–2, 38
maxi-circle, 15
Polymorphus paradoxus, 295
Pomphorhynchus laevis, 299
Praziquantel, 254
Proguanil resistance, 117

Protein A, 157, 158
Protein A-peroxidase technique, 156
Protein L, 157
Proteinase K, 101
Pseudoperichaeta nigrolineata, 300, 304
Pulsed field gradient (PFG) electrophoresis, 76
Pyrimethamine, 76
Pyrimethamine resistance, 99, 117–22

Quinine resistance, 122

Rafoxanide, 254
Raillietina cesticillus, 295
Rana, 236, 238
Rana temporaria, 236
Rattus rattus, 224
Rhodesian trypanosomiasis, 39
Rhodnius prolixus, 297
Rhoptry proteins, 97
Ring-infected erythrocyte surface antigens (RESA), 89–91

Sacculina, 291, 302
Sacculina carcini, 302
Saimiri, 160
S-antigen, 78–81, 99, 100
Sarcocystis, 164
Sarcocystis muris, 188
Satellite DNA, 99
Scanning electron microscopy (SEM), 255
Schistocephalus solidus, 293
Schistocerca gregaria, 281
Schistosoma, 192–4, 277
Schistosoma bovis, 247, 248
Schistosoma haematobium, 277
Schistosoma mansoni
  and *Biomphalaria* (= *Australorbis*) *glabrata*, 276, 277, 287, 288
  gigantism in *B. pfeifferi*, 289
  immunoelectron microscopy of antigens, 192–4
  infection, and resistance of *E. caproni* in mouse, 246, 248, 258
Schistosomin, 279, 280
Schizont-infected cell agglutination (SICA) antigen, 77, 78, 96–7
Scolytus rugulosus, 292
Scolytus ventralis, 292
Serine-rich antigen (SERA), 82
Simulium ornatum, 282
Small histidine- and alanine-rich protein (SHARP), 94
Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), 78
Spectrin, 173
Sphaerularia bombi, 300
Stagnicola palustris, 232
Staphylococcus aureus, 157
Streptavidin, 158
Streptavidin-peroxidase technique, 156
Streptomyces avidini, 158
Sturnus vulgaris, 295

Taenia multiceps, 293
Tana, 226
Tanaorhamphus longirostris, 291
Tenebrio molitor, 283
Tetrachlorethylene, 254
Theileria, 188–90
Theileria parva, 188–90
Thin-layer chromatography (TLC), 256
Thin layer starch gel electrophoresis, 2–3
Toxoplasma, immunoelectron microscopy of, 187–8
Toxoplasma gondii, 187
Transmission electron microscopy (TEM), 237
Tribolium, 301
Tribolium brevicornis, 290
Tribolium castaneum, 280, 290
Tribolium confusum, 283, 290, 295
Tribolium molitor, 284, 285, 286, 290
Trichinella spp., 190–2
Trichinella spiralis, 192
Trichobilharzia ocellata, 258
  castration in, 275, 277, 278, 279, 289
Trichobilharzia ocellata–L. stagnalis model, 280, 286
Trichoplusia ni, 299, 300, 303
Trichoplusia ni–Hyposter exiguae association, 303, 304

Trypanosoma spp.
immunoelectron microscopy of antigens, 190
Trypanosoma brucei
dendrogram for, 6
experimental hybridization, 15
immunoelectron microscopy of antigens, 190
surface glycoprotein, 88
Trypanosoma brucei brucei, 23, 36–40
Boufle strain, 27–8
Kakumbi strain, 30
Kiboko strain, 29–30
Sindo strain, 28–9
Trypanosoma brucei gambiense
dendrogram, 14, 15
strains, 23, 28, 32–3, 36–9
zymodemes, 11, 34
Trypanosoma brucei rhodesiense
Busoga strain, 24–5
strain groups, 23–7, 39, 40
Zambezi strain, 25–7
Trypanosoma congolense
cladogram, 38
dendrogram, 6
immunoelectron microscopy of antigens, 190
strains, 3
Trypanosoma cruzi, 190
Trypanosoma equiperdum, 35
Trypanosoma evansi
cladogram, 14
dendrogram, 11
distribution, 15
enzyme patterns, 19
strains, 23, 27, 30–2, 36–9
Trypanosoma rangeli, 297
Trypanosoma rhodesiense, 40
Trypanosomes
distribution
pan-African, 14–16
in time, 16
enzyme profiles
enzyme electrophoresis of, 4–6
isolation and preparation, 3
numerical analyses, 6–7
Trypanosomes (cont.)
cladogram, 6–7, 11–14
dendrogram, 6, 9–11
infrequent enzyme patterns, 18–19
limitations, 20–2
isoenzymes, 21–2
sampling, 20–1
trypanosome populations, 21
mixed populations, 16–18
double-population mixtures, 17–18
frequency, 16–17
triple-population mixtures, 17
strain groups, 22–35
Trypanosomiasis
Gambian, 20, 33, 39
human, 35
Rhodesian, 39
Trypanozoon, dispersal of, 35–8
in East Africa, 36–7
in West Africa, 37–8
Two-dimensional polyacrylamide gel electrophoresis, 113

UDP-galactose 4-epimerase, 278

Venerupis staminea, 294
Verapamil, 124

Xanthomatine, 298

Yomesan, 254

Zanil, 248, 254
Zoogonus lasius, 280, 289
Zymodeme, 6
unidentified, 33–5