STUDIES ON TRYPANOSOMATID FLAGELLATES WITH SPECIAL REFERENCE TO ANTIGENIC VARIATION AND KINETOPLAST DNA

by

Stephen Louis Hajduk B.S. (Georgia, USA)

Department of Zoology

University of Glasgow

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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Finally to my wife Ann, new son Patrick and parents I dedicate this thesis.

CONTENTS

Abbreviations.

AMB	Amsterdam molecular biology
AnTAR	Antwerp *Trypanozoon* Antigenic Repertoire
AnTat	Antwerp *Trypanozoon* antigenic type
ATCC	American Type Culture Collection
c	Crop
cDNA	Complementary DNA
DEAE	Diethylaminoethyl
DHAP	Dihydroxyacetonephosphate
DPG	1,3-diphosphoglycerate
EATRO	East African Trypanosomiasis Organization
FCS	Foetal calf sera
FDH	Fructose-1,6-phosphate
FITC	Fluorescein isothiocyanate
GAP	Glyceraldehyde-3-phosphate
αGP	αGlycerophosphate
GPO	Glycerophosphate oxidase
GUP	Glasgow University Protozoology
GUTAR	Glasgow University *Trypanozoon* Antigenic Repertoire
h	Haemocoel
hg	Hindgut
hx	Hypopharynx
I-VAT	Ingested variable antigen type
ITMAP	Institute of Tropical Medicine Antwerp Protozoology
KCN	Potassium cyanide
kDNA	Kinetoplast DNA
LUMP	London University Medical Protozoology
mtDNA	Mitochondrial DNA
PBS	Phosphate buffered saline

mRNA	Messenger RNA
M-VAT	Metacyclic variable antigen type
nDNA	Nuclear DNA
pm	Peritrophic membrane
poly A^+	Poly adenylated
ps	Peritrophic space
r	Rectum
rRNA	Ribosomal RNA
SHAM	Salicylhydroxamic acid
TRITC	Tetramethyl-rhodamine isothiocyanate
tRNA	Transfer RNA
VAT	Variable antigen type
VSG	Variable surface glycoprotein

Summary.

The results presented in this thesis concern two aspects of the biology of trypanosomatids. First, the process of antigenic variation in the African trypanosomes and second the structure and function of the kinetoplast DNA of trypanosomatids.

Part I.

Trypanosomes were cyclically transmitted by the insect vector, Glossina morsitans, and the expression of variable antigen types (VATs) in the metacyclic populations from the salivary glands and the first bloodstream populations in metacyclic initiated infections in mice were analysed. Tsetse flies were fed on the blood of mice containing any one of 5 VATs of Trypanosoma brucei of the AnTAR 1 serodeme. The VATS of the metacyclic trypanosomes subsequently detected in the flies' saliva probes vere investigated using monospecific antisera to AnTAR 1 VATs in indirect immunofluorescence and trypanolysis reactions; these sera included 3 raised against AnTats 1.6, 1.30, and 1.45, previously idnetified as components of the metacyclic population (M-VATs), and against the 5 VATs originally ingested by the flies. The percentage of metacyclics reacting with a particular M-VAT antiserum remained more or less constant (AnTat 1.6, 6.0-8.3%; AnTat 1.30, 13.7-18.2%; AnTat 1.45, 2.0-8.0%), regardless of the age of the fly or the ingested VAT. As these 3 VATs account for no more than one-third of the metacyclic population, the existence of at least one more VAT is envisaged. The ingested VAT could not be detected among the AnTAR 1 metacyclic trypanosomes.

Metacyclic trypanosomes from the salivary glands of infected tsetse flies were also used to initiate infections in mice. Immunofluorescence and trypanolysis reactions employing 24 monospecific antisera were used to analyse the VATs present in the mice following cyclical transmission. Regardless of the VAT used to infect tsetse flies, the first VATs detectable in the bloodstream were those previously identified as M-VATs.

These were present until at least 5 days after infection, at which time lytic antibodies against at least 2 of the M-VATs were detectable in the blood of infected mice. In mice immunosuppressed by X-irradiation the M-VATs were detectable in the bloodstream for longer periods, but the percentage of the population labelled with anti-metacyclic sera showed a decrease on day 5 as in non-irradiated animals. The VAT ingested by the tsetse was always detectable early during the first parasitaemia following cyclical transmission and was usually the first VAT detectable after the M-VATs. Neutralization of selected M-VATs before infecting mice resulted in elimination of the neutralized M-VAT from the first parasitaemia but had no effect on the expression of other VATs in the early infection.

Part II.

In studies on the structure and function of the kinetoplast DNA (kDNA) of trypanosomatids I have examined the kDNA structure and mitochondrial activity of two species of Herpetomonas and also a stock of T. brucei which has lost the ability to activate its mitochondrion during syringe passaging in laboratory rodents.

The structure of the kDNA of Herpetomonas muscarum and Herpetomonas ingenoplastis was compared by electron microscopy, restriction endonuclease digestion and hydridization with cloned portions of the maxi-circle from T. brucei 427. The kDNA of both H. muscarum and H. ingenoplastis has a buoyant density of 1.698 g/cm^3; however, the kDNA of H. ingenoplastis represents 31% of the total cellular DNA as compared with 8% for H. muscarum kDNA. The kDNA network of H. muscarum consists of thousands of mini-circles of 0.6 to 0.7 x 10^6 daltons and a few large circular molecules, maxi-circles, of 21 x 10^6 daltons. The mini-circles of H. muscarum show sequence heterogeneity while maxi-circles of H. muscarum have a unique nucleotide sequence. The kDNA of H. ingenoplastis completely lacks mini-circle size molecules and the network is composed

entirely of large circular molecules of 11 x 10^6, 15.5 x 10^6 and 24 x 10^6 daltons. The 11 x 10^6 and 15.5 x 10^6 dalton molecules show sequence heterogeneity and are the major component of the kDNA. Hybridization studies with cloned fragments of T. brucei maxi-circle suggest that the 24 x 10^6 dalton component of H. ingenoplastis kDNA is functionally equivalent to the maxi-circle of other trypanosomatids. It was concluded that the 11 x 10^6 and 15.5 x 10^6 dalton circles of H. ingenoplastis are functionally similar to mini-circles of other trypanosomatids and that the maxi-circles of H. ingenoplastis differ from those of T. brucei and H. muscarum in major nucleotide sequences.

The structure and activity of the mitochondrion from H.ingenoplastis and H. muscarum have been studied by electron microscopy, respiration studies with different substrates and inhibitors, analysis of oligomycin-sensitive ATPase activity and low-temperature difference spectra of respiratory chain cytochromes. Certain differences in the two species can be correlated with alterations in the maxi-circle of H. ingenoplastis described in the preceding paper. 1) The mitochondrion of H. ingenoplastis is poorly developed and devoid of the plate-like cristae present in the mitochondrion of H. muscarum. 2) The total cellular ATPase activity in homogenates of H. muscarum is sensitive to oligomycin (24% inhibition) while only 5% of the ATPase activity in homogenates of H. ingenoplastis is oligomycin-sensitive. 3) Inhibition studies on the respiration of intact cells and homogenates with cyanide, azide, antimycin A and salicylhydroxamic acid show that respiration is not mediated by a conventional cytochrome chain with cytochrome aa_3 acting as the terminal oxidase in H. ingenoplastis as it is in H. muscarum. 4) Low-temperature difference spectra suggest that cytochromes b, c and aa_3 are present in the mitochondrion of H. muscarum but that H. ingenoplastis completely lacks cytochrome aa_3 and that the absorption maxima peaks for cytochrome b in preparations of H. ingenoplastis differ from those obtained from H. muscarum. It appears that mutations have occured in

the maxi-circle of H. ingenoplastis which result in the typical mitochondrial gene products, cytochrome b, and portions of cytochrome aa_3 and the mitochondrial ATPase complex being either absent or present in an altered form. The loss of cytochrome aa_3 has resulted in H. ingenoplastis using an alternative pathway for energy metabolism possibly using an o-type cytochrome as the terminal oxidase.

Several characteristics of a recently derived population of T. brucei EATRO 1244 which is incapable of infecting the tsetse fly were compared with the parental population which retains infectivity for the insect vector. Oligomycin-sensitive ATPase activity, growth characteristics in the mammal, degree of pleomorphism and the ability to grow in culture at 26°C differ for these two populations. No detectable alterations in the maxi-circle component of the kDNA were correlated with loss of infectivity for the tsetse fly either by electron microscopy or by restriction endonuclease analysis. There are two possible interpretations of our results: 1) minor alterations, such as point mutations in critical mitochondrial genes, have occurred which are undetectable with the methodology used; 2) mutations have occurred in nuclear genes coding for peptides which are imported into the mitochondrion and are essential for mitochondrial protein synthesis.

Part I.

STUDIES ON ANTIGENIC VARIATION IN CYCLICALLY TRANSMITTED *TRYPANOSOMA BRUCEI*

1. INTRODUCTION

1.1 GENERAL

Trypanosomes are parasitic protozoa of the order Kinetoplastida which spend at least a portion of their life-cycle in a vertebrate host. Trypanosomes infecting fishes, amphibians, reptiles and birds are generally not pathogenic to the host. A few of the many species of trypanosome parasitizing mammals are the causative agents of serious diseases, collectively termed trypanosomiasis.

The trypanosomes of mammals have been divided into 2 groups based on the developmental cycle in the insect vector (Hoare, 1972). The group Salivaria, largely confined to sub-Saharan Africa, completes its developmental cycle in the mouthparts or salivary glands of the vector which is usually the tsetse fly (*Glossina* spp.). Infection of the mammalian host is usually initiated by the injection of metacyclic trypanosomes with the saliva when the vector feeds. The other group, the Stercoaria, completes its developmental cycle in the hindgut of the vector and metacyclic trypanosomes are present in the faeces of the vector.

Control of both African and South American trypanosomiasis has relied almost entirely upon eradication of the insect vector or upon drug treatment of the mammalian host. Serious problems exist with both of these approaches and immunological approaches for controlling these diseases are now being considered (Holms, 1980; Murray *et al.*, 1980).

The most extensively studied of the African trypanosomes is *Trypanosoma brucei*. *Trypanosoma brucei brucei*, causative agent of Nagana in cattle, is not infective to man but is morphologically and

biochemically indistinguishable from Trypanosoma brucei rhodesiense and Trypanosoma brucei gambiense the causative agents of acute and chronic human sleeping sickness respectively.

1.2 THE LIFE-CYCLE OF T. brucei

The developmental stages in the life-cycle of T. brucei, in both the mammalian host and the insect vector are shown in figure 1. T. brucei infects both the blood and tissue fluids of the mammal (Losos & Ikede, 1974) and trypomastigotes (Hoare & Wallace, 1966) are probably the only forms present. After inoculation into the vertebrate host the first trypanosomes observed are long, slender multiplicative forms which normally differentiate, through an intermediate stage, to a short, stumpy non-multiplicative trypomastigote form (Robertson, 1912a, b). Intracellular stages have not been demonstrated in the life-cycles of of any of the salivarian trypanosomes. The morphological variation in the bloodstream trypanosomes is termed pleomorphism and production of stumpy forms is associated with the ability of the trypanosome population to infect the insect vector (Robertson, 1912; Vickerman, 1965). The short stumpy forms appear to be preadapted to life in the insect vector by virtue of changes which take place in the structure and function of the flagellates single mitochondrion during differentiation from the slender form (Vickerman, 1965; Brown et al., 1973); these changes are discussed in more detail in Part II.

The bloodstream trypanosomes are ingested by the tsetse fly when it feeds on an infected mammal. The blood meal plus trypanosomes pass through the oesophagus, crop and proventriculus to reach the midgut where the trypanosomes quickly transform to procyclic trypomastigotes (Hoare, 1940). From the midgut the trypanosomes migrate to the

Figure 1

Diagram to show changes in the surface of T. brucei during the course of the life cycle. In the bloodstream, lymphatics and connective tissue of the mammalian host trypomastigote developmental stages undergo antigenic variation and at all times possess a surface coat (shaded). Upon ingestion by the insect vector bloodstream trypomastigotes rapidly lose the surface coat, their antigenic idenity and infectivity for mammals. In the salivary glands uncoated, attached epimastigote forms differentiate to uncoated, attached trypomastigotes and finally to coated metacyclic trypomastigotes which are free in the lumen of the gland. The metacyclic forms are infective for mammals and express a characteristic repertoire of variable antigen types. The cycle in the mammal begins with the injection of metacyclics with the saliva when the tsetse fly feeds. (Wickerman, unpublished)

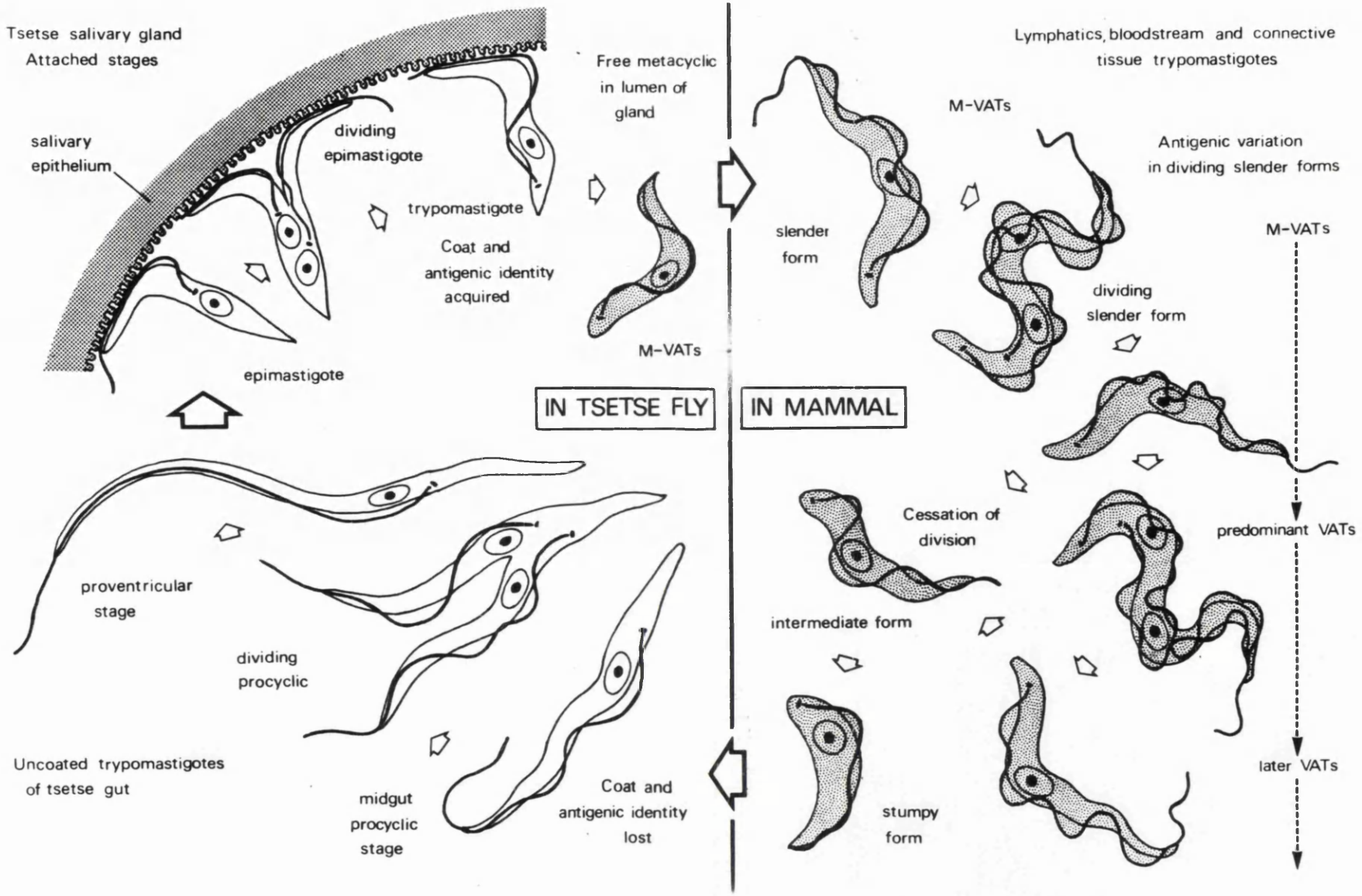
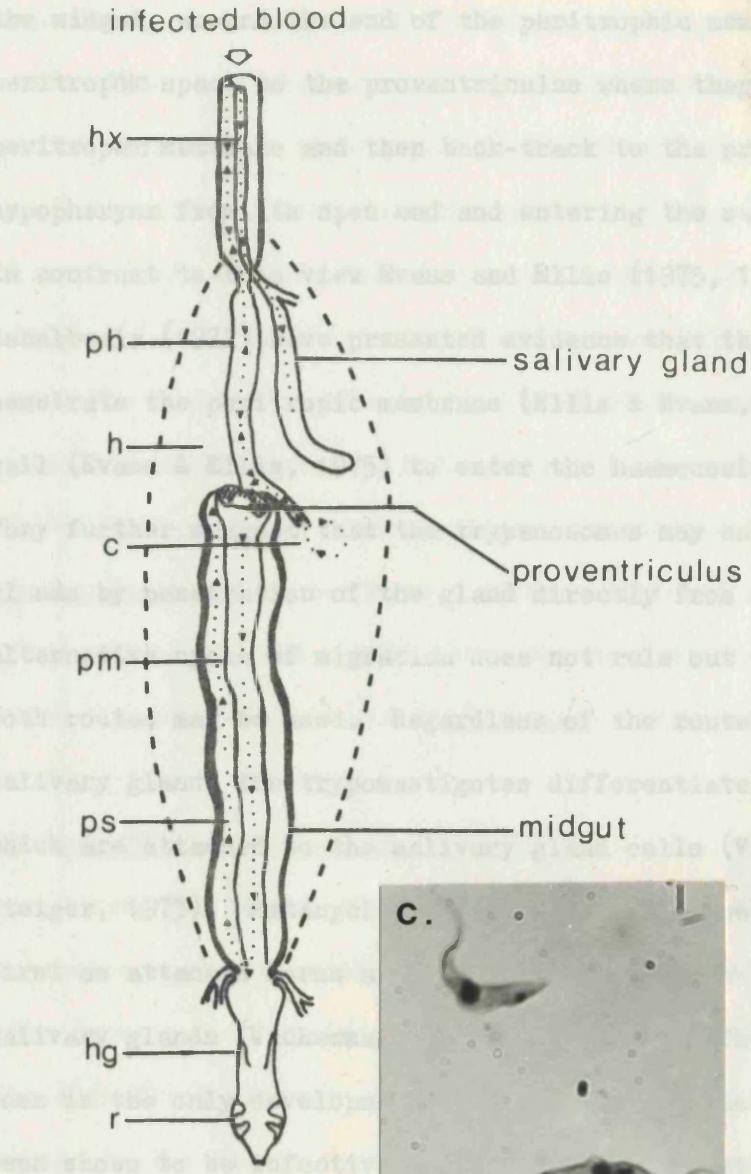
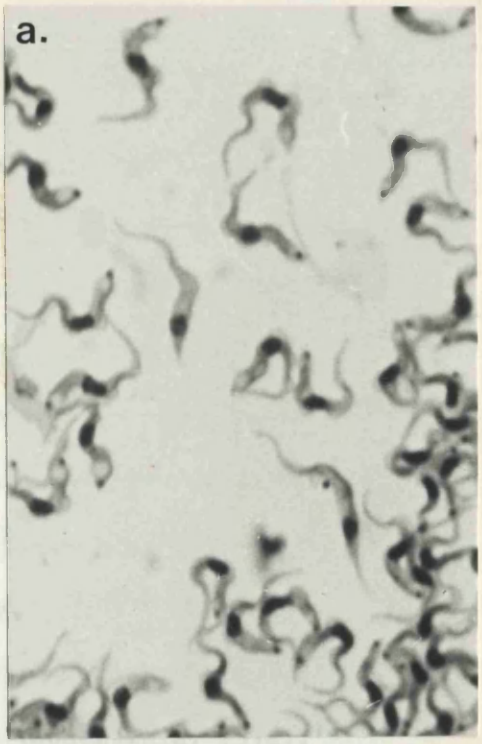

Tsetse salivary gland
Attached stages
salivary epithelium
dividing epimastigote
trypomastigote
Coat and antigenic identity acquired
epimastigote
Free metacyclic in lumen of gland
M-VATs
IN TSETSE FLY
IN MAMMAL
Lymphatics, bloodstream and connective tissue trypomastigotes
M-VATs
Antigenic variation in dividing slender forms
slender form
M-VATs
dividing slender form
Cessation of division
predominant VATs
intermediate form
later VATs
stumpy form
proventricular stage
dividing procyclic
Uncoated trypomastigotes of tsetse gut
midgut procyclic stage
Coat and antigenic identity lost

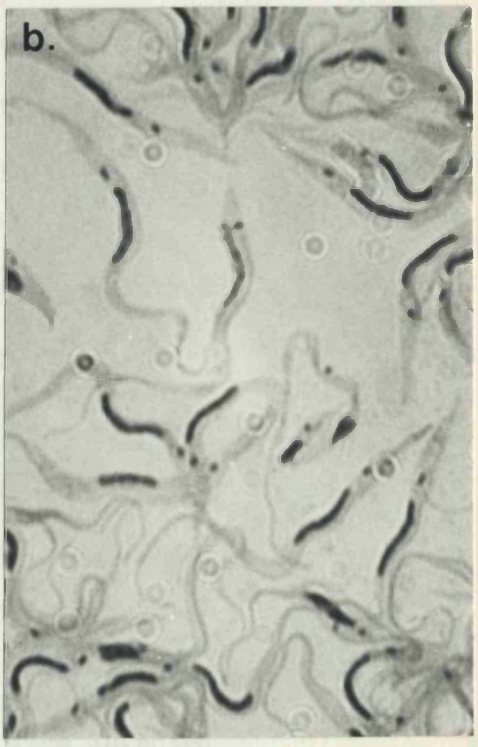
Figure 2

Development of T. brucei in the insect vector, Glossina spp., based upon the cycle described by Robertson (1913). The direction of migration by the trypanosomes is indicated with arrows and dotted lines. The trypanosomes ingested with an infected blood meal pass through the pharynx (ph), crop (c), proventriculus into the midgut. The trypanosomes then migrate around the posterior end of the peritrophic membrane (pm), along the peritrophic space (ps), through the peritrophic membrane into the proventriculus then move back along the pharynx entering the salivary glands via the hypopharynx. Also labelled in this drawing are the rectum (r), hindgut (hg) and haemocoel (h).

Photographs of Giemsa stained T. brucei from the (a) salivary glands, (b) proventriculus and (c) midgut are shown. x1500.


infected blood
hx
ph
salivary gland
h
c
proventriculus
pm
ps
midgut
hg
r


a.


b.

c.

salivary glands of the tsetse. The classical viewpoint (Robertson, 1913) has been that the procyclic trypanosomes migrate to the posterior end of the midgut, around the end of the peritrophic membrane, move up the peritrophic space to the proventriculus where they retraverse the peritrophic membrane and then back-track to the proboscis, invading the hypopharynx from its open end and entering the salivary glands (figure 2). In contrast to this view Evans and Ellis (1975, 1977 & 1979) and Mshelbwala (1972) have presented evidence that the midgut trypanosomes penetrate the peritropic membrane (Ellis & Evans, 1977) and the gut wall (Evans & Ellis, 1975) to enter the haemocoel (Mshelbwala, 1972). They further suggest that the trypanosomes may enter the salivary glands by penetration of the gland directly from the haemocoel. This alternative cycle of migration does not rule out the possibility that both routes may be used. Regardless of the route taken, once in the salivary glands the trypomastigotes differentiate to epimastigotes which are attached to the salivary gland cells (Vickerman, 1969; Steiger, 1973). Metacyclic trypomastigotes arise from the epimastigotes first as attached forms and later are found free in the lumen of the salivary glands (Vickerman et al., in press). The metacyclic trypanosome is the only developmental stage in the insect vector which has been shown to be infective for the mammalian host although other, non-metacyclic forms, are often seen in saliva probes from infected tsetse flies (Otieno, 1978).

During the life-cycle of T. brucei changes in the antigenicity of the organism have been observed (Seed, 1966; LeRay, 1975; Honigberg et al., 1976; Barry & Vickerman, 1979) which can be correlated with loss and reaquisition of the thick surface coat which covers the entire cell (Vickerman, 1969). The coat is the site of the variable antigen

or variant specific glycoprotein (VSG) and is present on all bloodstream stages. By altering the composition of this coat the bloodstream trypanosome evades the host's immune response. This switch in the nature of the variable antigen expressed is termed antigenic variation. Loss of the surface coat occurs in the midgut of the tsetse and is correlated with loss of infectivity for the mammalian host. Only after 17-25 days, when coated metacyclic forms are present in the salivary glands, do the tsetse flies become able to infect the mammalian host. The aquisition of a surface coat at the metacyclic stage is apparently a preadaptation for survival in the mammalian host (Vickerman, 1969). Tetley et al. (in press) have found that the metacyclics of Trypanosoma vivax do not have a surface coat and are insensitive to normal guinea pig and rabbit sera; the same authors found that uncoated, insect developmental stages of T. brucei are lysed by normal sera.

1.3 ANTIGENIC VARIATION IN TRYPANOSOMES

During the course of infection in the mammalian host the number of trypanosomes in the blood and lymphatic fluids fluctuates in a characteristic fashion. As early as 1907 (Massaglia, 1907) it was suspected that this fluctuation in the parasitaemia of African trypanosomes was due to the host's immune response, each remission being the consequence of antibodies destroying trypanosomes bearing a particular antigen and each recrudescencebeing composed of a trypanosome population expressing a different antigen. Antigenic variation - the ability of the trypanosome to change its antigenic characteristics and so circumvent complete destruction of the parasite population by the host's immune response is a feature of all African trypanosomes (Vickerman, 1974; 1978; Gray & Luckins, 1976; Cross, 1978; 1979; Turner, 1980; Doyle, 1977). The change in antigenic composition of the trypanosomes in the relapsing

parasitaemias has been demonstrated by a variety of serolological (McNeillage et al., 1969; Van Meirvenne 1975a, b; LeRay, 1975) and immunochemical methods (Cross, 1975; 1977).

1.4 THE STRUCTURAL AND MOLECULAR BASIS OF ANTIGENIC VARIATION

Vickerman (1969) described a thick (12-15nm) proteinaceous coat covering the entire surface of the trypanosome during its developmental stages in the mammalian host; this surface coat was hypothesized to contain the variable antigen of the trypanosome and to be an adaptation to life in the immunologically reactive mammalian host. Localisation of the variable antigen in the surface coat has been demonstrated by several approaches. 1. Binding of ferritin conjugated antiserum to the surface coat of the homologous trypanosome variant and not to a heterologous variant or to the homologous variant after the surface coat had been removed by trypsinization (Vickerman & Luckins, 1969; Fruit et al., 1977). 2. Trypanosomes from the midgut of the tsetse and from invitro culture at 26°C lack a surface coat, are non-infective for mammals and do not react with antiserum raised against the bloodstream variable antigen type (VAT) used to initiate the infection or culture (Vickerman 1969; Steiger, 1973; Honigberg et al., 1976; Barry & Vickerman, 1979). Hyperimmune antiserum against purified VSG reacts only with the surface of the homologous VAT (Cross, 1975).

The VSG from cloned populations of T. brucei has been purified and studied extensively by Cross and co-workers (Cross, 1975; 1977; Bridgen et al., 1976; Cross & Johnson, 1976). From each antigenically distinct and homogeneous population of T. brucei a single VSG can be isolated. This glycoprotein accounts for roughly 10% of the total cell protein and has a molecular weight of about 65,000 daltons. The

carbohydrate of the VSG differs from one VAT to another both in amount by weight from 7-17%, the proportions of the 3 sugar constituents galactose, mannose, and glucosamine present and the site of attachment to the polypeptide backbone of the VSG (Johnson & Cross, 1977). As discussed by Cross (1978) the carbohydrate component of the VSG does not appear to be a major antigenic determinant and cytochemical evidence (Wright & Hales, 1970) suggests that oligosaccharide groups are attached to the VSG at a site near to the surface membrane and are not exposed in the living trypanosome. Studies on the amino acid sequence of the N-terminal portion of VSG from 4 VATs of a single trypanosome suggest that there is little or no homology in the primary structure of the VSG, at least in this portion of the molecule (Bridgen et al., 1976). This suggests that the immunological uniqueness of each VSG is due to extensive differences in the amino acid composition of the molecule. The C-terminal portion of the VSG, which is probably the region of the molecule involved in attachment to the trypanosome surface membrane, apparently has some regions of homology in different VATs since tryptic digest fragments from this portion of the molecule cross-react serologically (Cross, 1979; Barbet & McBuire, 1978).

Until recently the genetic basis of antigenic variation was completely unknown although the semi-ordered appearence of VATs in trypanosome populations following cyclical transmission through the tsetse fly (Gray, 1965; 1975) and the extensive differences in amino acid sequences of the N-terminal region of the VSG argued against antigenic variation being the result of mutation of a small number of variable antigen genes.

In vitro translation studies with purified mRNA for VSG (Williams *et al.*, 1979; Lheureux *et al.*, 1979; Merrit, 1980) and total polyadenylated (poly A^+) RNA (Hoeijamkers *et al.*, 1980) suggests that the VSG mRNA codes for a pre-VSG which is larger than the "mature" molecule present on the surface of the trypanosome. The portion of the molecule removed might be a "signal peptide" involved in the transfer of the nascent protein to the surface (Hoeijmakers *et al.*, 1980a). When the products of *in vitro* translation of the total poly A^+ RNA from 4 different VATs was analysed by SDS gel electrophoresis and immunoprecipitation with antisera against each of the VATs, the only differences detected were in the variant pre-glycoproteins (Hoeijmakers *et al.*, 1980a). In additional experiments, complementary DNA copies of poly A^+ RNA from each of the 4 VATs were cloned in *Escherichia coli* after linking to a plasmid vector. When the cloned cDNA was reacted with poly A^+ RNA from the 4 VATs, hybridization was detectable only with the homologous RNA and not with that of the heterologous VATs. Hoeijmakers *et al.* (1980a) concluded that this lack of cross hybridization shows that antigenic variation in trypanosomes is not due to the linkage of a large set of genes for a variable N-terminal region of the antigen with a single gene for a constant C-terminal region as is the case for antibodies in mammals (Milstein & Munro, 1973). Thus it seems likely that each VSG is coded for by a single gene and that antigenic variation involves the differential expression of a large number of VSG genes.

Hybridization experiments using the cloned cDNA described above and nuclear DNA (nDNA) digested with restriction endonucleases from the 4 VATs were done to examine the type of genomic rearrangements which may occur when a particular VSG gene is expressed (Hoeijmakers *et al.*, 1980b).

The results of these experiments show that each VAT contains a copy of the other VSG genes in its genome supporting the idea of a gene for each VSG. When cDNA was hybridized with nDNA from the homologous VAT and the 3 heterogous VATs an extra hybridizing fragment was detectable in the nDNA from the homologous VAT. This is interpreted as an "expression-linked copy", each VAT containing a basic copy of the entire repertoire of VSG genes, an extra copy of the VSG gene is present only in the genome of the expressed VAT. Although the nature of the events leading to the presence of this expression-linked copy is unknown it is clear that the nucleotide sequences surrounding this gene are altered, either by movement of the gene to a new (expression) site or insertion of a sequence next to the extra copy.

Early studies on the occurence of new VATs in trypanosome infections in non-immune animals (Lourie & O'Conner, 1937) suggested that the expression of new VATs was induced by antibody against the trypanosome (discussed by Vickerman, 1974). To study in detail the occurrence of antigenic variation in the mammalian host, Van Meirvenne and co-workers (1975a) prepared, from a single cloned trypanosome, a set of 13 VATs identified by immunofluorescence and trypanolysis reactions. The 13 VATs isolated make up only a small fraction of the VATs which can be expressed by this cloned population. The entire repertoire of VATs which can be expressed by a clone is termed a serodeme (WHO, 1978). Van Meirvenne and co-workers (1975a) found that, in non-immune animals, minor VATs (heterotypes) are detectable in addition to the major VAT (homotype) even very early in the course of the infection. In a relapsing parasitaemia resulting from a single infecting trypanosome the first peak is composed of a homotype plus a few heterotypes. Following antibody induced remission, the relapse population consists of one (or more) heterotypes of the previous peak parasitaemia which

Table 2. Summary of trypanolytic activities of single antisera and mixtures against metacyclic trypanosomes before and after purification of metacyclics on DEAE cellulose columns

	Antisera against					
	AnTat 6	AnTat 30	AnTat 45	AnTats 6 & 30	AnTats 30 & 45	AnTats 6, 30 & 45
(A) Before DEAE purification						
No. lysed / No. counted	20/318	44/358	31/433	12/50	60/350	52/183
Percent lysis	6.3	12.3	7.2	24.0	17.1	28.6
(B) After DEAE purification						
No. lysed / No. counted	10/150	38/350	23/350	14/90	49/300	46/150
Percent lysis	6.7	10.9	6.6	15.5	27.4	30.7

Figure 4

FITC immunofluorescence reactions on acetone-fixed metacyclics from a single fly labelled with antiserum against AnTat 1.30 (a), AnTat 1.6 (b) and AnTat 1.45 (c) to show specificity of reactions obtained, (X1500).

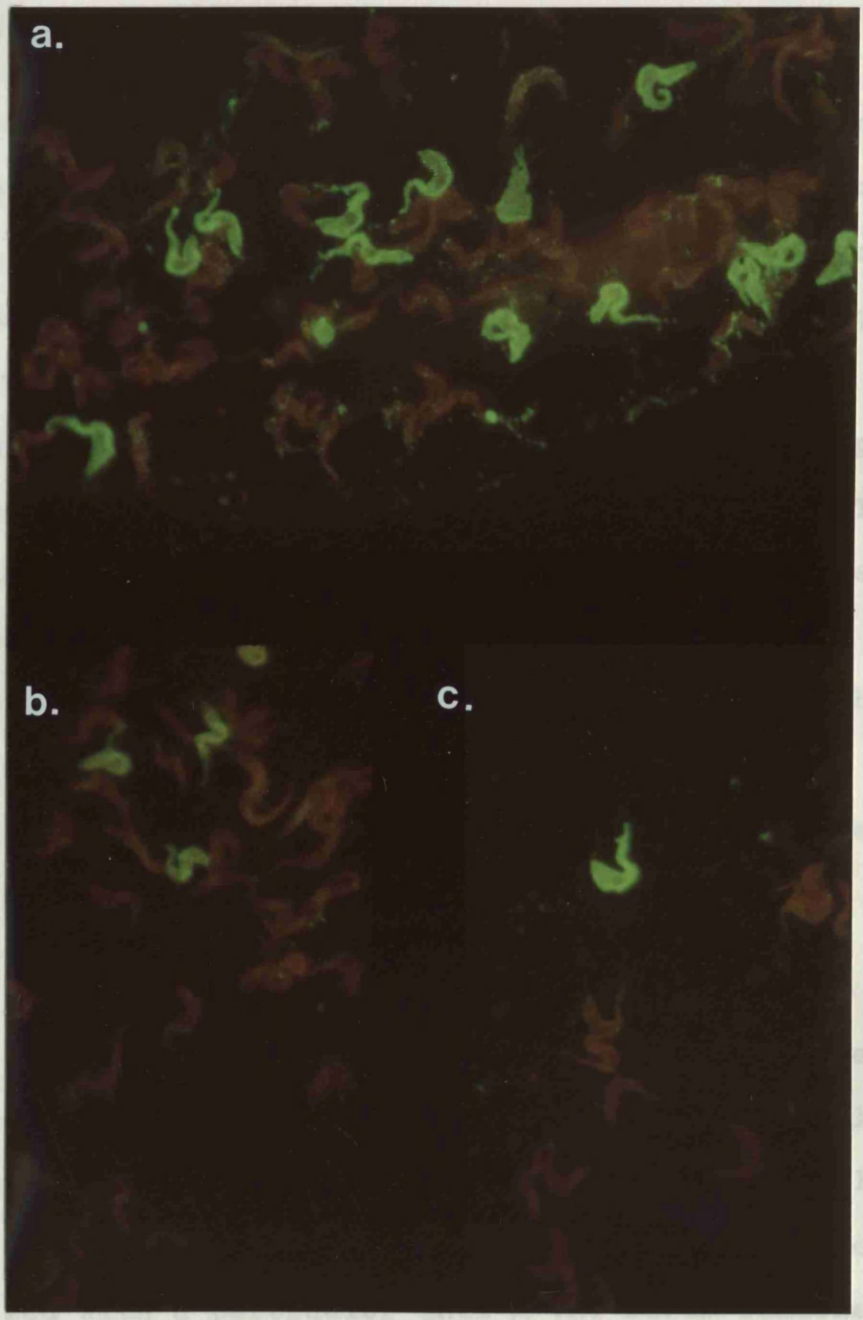

a.
b.
c.

figure for lysis by combined anti-M-VAT sera quoted above. Metacyclic trypanosomes from another serodeme, GUTAR 1, derived from EATRO 1244 (Barry & Vickerman, 1979) were not labelled by antisera against any of the AnTAR 1 M-VATs.

The presence of two different M-VATs within one salivary probe was demonstrated by using a double primary antibody-labelling immunofluorescence test. Monospecific rabbit antiserum against AnTat 1.30 and mouse antiserum against AnTat 1.45 were pooled to give active specific dilutions and applied to saliva probes. The second antibody layer was pooled FITC-conjugated goat anti-rabbit immunoglobulin and TRITC-conjugated rabbit anti-mouse immunoglobulin. By the use of appropriate filters it was possible to visualise both the FITC and TRITC reactions on the same preparation (fig. 5). In this experiment 7.3% metacyclics were labelled with the anti-AnTat 1.45 TRITC reaction and 15.3% with the anti-AnTat 1.30 FITC reaction; the remaining cells were unlabelled indicating the presence of other VATs in the metacyclic population.

The stability of the VAT composition of the metacyclics during the course of the infection in the tsetse fly was examined using the immunofluorescence reaction on sequential saliva probes from the same fly (Table 3). The percentage of metacyclics labelled with a particular anti-M-VAT serum remained relatively constant in all 3 cases, viz. AnTat 1.30, 13.7-18.2%; AnTat 1.45, 2.0-8.0% and AnTat 1.6, 6.0-8.3%.

Figure 5. Metacyclic trypanosomes in a saliva probe labelled with both (a) rabbit anti-AnTat 1.30 antiserum and (b) mouse anti-AnTat 1.45 antiserum, then reacted with goat anti-rabbit FITC and rabbit anti-mouse TRITC. Arrows indicate unlabelled AnTat 1.45 in the FITC anti-AnTat 1.30 photograph (a) and the unlabelled AnTat 1.30 in the TRITC anti-AnTat 1.45 photograph (b). Other metacyclics show only slight background fluorescence and indicate the presence of other metacyclic VATs. x1200.

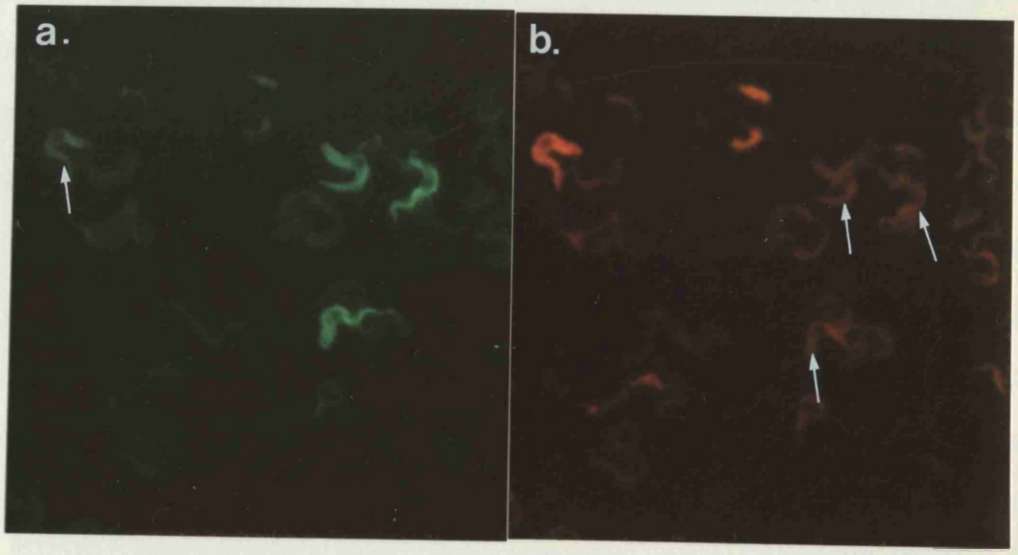

a.
b.

Table 3. Percentage of Metacyclic Variable Antigen Types in Sequential Probes from Individual Tsetse Flies

Age of tsetse infection (Day after infective feed)	Immunofluorescence
Fly A	% AnTat 1.30
22	15.2
24	15.2
27	13.7
29	14.0
36	12.9
38	14.3
42	17.0
44	17.0
46	18.2
Fly B	% AnTat 1.45
43	8.0
50	2.0
55	7.0
Fly C	% AnTat 1.6
25	8.3
41	6.0
44	8.3

Effect of ingested VAT on M-VAT composition of metacyclic population

The possibility that the VAT of the trypanosome population ingested by the fly might influence the VAT expressed by the metacyclic trypanosome population developing from it was investigated. Flies were infected with 5 VAT-defined bloodstream populations and an undefined mixture of VATs, all belonging to the AnTAR 1 serodeme. Table 4 shows the degree of homogeneity of the ingested VAT-defined populations in terms of the percentage of heterotypes present as assessed by immunofluorescence analysis. The metacyclic populations arising in the infected flies were analysed by immunofluorescence, testing for presence of the ingested VAT and the 3 identified M-VATs. The VAT ingested by the fly was never detected in the ensuing trypanosome metacyclic population, whereas the 3 M-VATs were regularly found (Table 5). The only saliva probes in which all 3 M-VATs were not detected were those of the flies that had ingested AnTat 1.18: these probes lacked AnTat 1.6 though this M-VAT has since been identified in other experiments utilising similarly infected flies.

DISCUSSION

The observation that the variable antigen-containing surface coat of T. brucei is acquired during transformation of the epimastigote to the metacyclic trypomastigote stage in the salivary gland of Glossina (Vickerman, 1969, Steiger, 1973) along with Cunningham's (1966) report that the infectivity of metacyclic trypanosomes is neutralised by 14d serum from infected sheep, led to the hypothesis that reversion to a basic antigen occurs at the metacyclic stage (Vickerman, 1969); the metacyclics

Table 4. VAT composition of bloodstream trypanosome populations ingested by Glossina morsitans : immunofluorescence analysis of 1000 trypanosomes in acetone-fixed blood smears.

Ingested VAT homotype	% heterotypes
AnTat 1.8	0.5
AnTat 1.14	20.6
AnTat 1.18	3.9
AnTat 1.19	0.3
AnTat 1.21	0.2

Table 5. Effect of ingested VAT on M-VAT composition of metacyclic populations developing in Glossina morsitans.

Ingested VAT	Tsetse	Percentage of metacyclics labelled by immunofluorescence			
		Antiserum against ingested VAT	Antisera against metacyclic VATs		
			AnTat 1.6	AnTat 1.30	AnTat 1.45
AnTat 1.8	I	0.0	4.0	20.5	10.0
	II	0.0	6.6	14.0	4.0
AnTat 1.14	III	0.0	6.0	22.5	4.7
	IV	0.0	12.5	20.8	0.0
AnTat 1.18	V	0.0	0.0	22.5	2.7
	VI	0.0	0.0	24.8	2.0
AnTat 1.19	VII	0.0	9.0	18.2	3.0
	VIII	0.0	6.5	19.3	0.0
AnTat 1.21	IX	0.0	5.5	19.1	4.9
	X	0.0	6.0	23.7	4.0
Mixture of AnTAR VATs	XI	ND	8.6	22.0	4.25
	XII	ND	6.0	16.6	5.0
	XIII	ND	10.9	12.0	3.5
	XIV	ND	4.2	24.0	7.5

ND = Not done

of a clone line differentiating in the same environment might be expected to put on the same type of coat. The hypothesis has recently received support from Jenni (1977, 1979) and from Hudson et al. (1980) though the latter presented no direct evidence. Our own recent transmission experiments on the AnTAR 1 serodeme (Le Ray et al., 1978; Barry et al., 1979; Barry and Hajduk, 1979) make this view hard to sustain and lead us to a different interpretation of the early events of antigenic variation following fly transmission.

Using the VAT-specific immunofluorescence and trypanolysis tests of Van Meirvenne et al. (1975), we found marked VAT heterogeneity in the first patent trypanosome population following fly transmission (Le Ray et al., 1977), suggesting that either VAT diversity developed quickly after fly-induced infection of the mammal, or that the inoculated metacyclic population was already heterogeneous, or that both these situations occurred. We also found that clones isolated from this early infection were extremely unstable with respect to VAT. It is this property of metacyclic-derived clones that has made it difficult to produce antisera to metacyclic VATs and therefore to identify defined VATs in tsetse salivary probes; Hudson et al. (1980) also recognised the VAT lability of metacyclic trypanosomes in the mammal. The discrepancy between the findings of other investigators and our own can be explained by the different methods used to produce anti-metacyclic antisera and to identify VATs with these sera.

Jenni (1977; 1979) prepared antisera to metacyclic trypanosomes by allowing infected flies to feed on mice or rabbits on single or multiple occasions before collecting serum on d6, d9, or d10; he used these sera in immunofluorescence reactions and obtained 100% serodeme-specific labelling of metacyclics. In our laboratory, rabbit serum, collected 1 week after a bite from an AnTAR 1-infected tsetse, labels or lyses over 90% of AnTAR 1 metacyclics, but use of VAT-specific antisera reveals marked VAT heterogeneity of metacyclics within the same salivary probe (Le Ray et al. 1978). If the immunising metacyclics are antigenically heterogeneous, the antiserum will contain antibodies to this variety of VATs, and might be expected to show activity against most or all metacyclics of that serodeme. Our approach to the identification of individual metacyclic VATs is to employ specific antisera raised by infection of rabbits with antigenically stable clones (Barry et al. 1979). As the variable antigen is more immunogenic than the common antigens of the trypanosome (Le Ray, 1975), specificity of 6 day antisera from such infected animals can be achieved by diluting out antibodies to common antigens (Van Meirvenne et al. 1975). The specificity of the 3 antisera used to identify metacyclic VATs 1.6, 1.30 and 1.45 in our experiments was demonstrated in homologous and heterologous reactions with 21 other VATs of the AnTAR 1 serodeme. Lack of cross reactivity between the 3 M-VATs was further demonstrated by the use of combined sera in trypanolysis reactions with metacyclics.

We have here presented direct evidence that there are at least 4 VATs in the metacyclic populations of the AnTAR 1 serodeme of T. brucei. Three of these VATs have been identified with specific antisera, two of them simultaneously, in a single probe, by double labelling experiments; we infer the presence of at least one additional metacyclic VAT, as only a third of the metacyclics reacted with our anti-metacyclic sera. These results complement our previous findings (Le Ray et al. 1978; Barry et al. 1979; Barry and Hajduk, 1979). Using similar techniques the presence of at least 4 VATs in the metacyclics of another serodeme has now been demonstrated (Barry, unpublished). The possibility that unidentified trypanosomes in the probe represent discharged "immature" metacyclics (Jenni, 1979) has been discounted by the demonstration that the reactivity of fly-probe trypanosomes in immune lysis reactions is unaltered by passage through ion exchange columns.

The mechanisms underlying the generation of M-VAT diversity in tsetse salivary glands, and the significance of the relative constancy of different M-VAT percentages in probes, regardless of the VAT ingested by the fly, remain to be investigated. As yet we cannot discount the possibility that nascent metacyclics express the same VAT, but quickly switch to the expression of other M-VATs while still in the fly. Our recent electron microscope studies (Vickerman, et al. 1980) show that for AnTAR 1 trypanosomes metacyclic coating actually occurs before detachment of the flagellate from the salivary epithelium and not after detachment as was previously supposed (Vickerman, 1969; Steiger, 1973). Immunocytochemical studies at the electron microscope level should therefore enable us to see if nascent metacyclics

belong to more than one antigenic type. There is no a priori reason why antigenic change should not take place in the fly's saliva; the belief that host antibody is necessary for the induction of antigenic change can now be discarded (Vickerman, 1978), especially as VAT changes occur in vitro in the apparent absence of an immune response (Doyle et al. 1980). An alternative interpretation of the constant percentages of M-VATs in fly salivary probes would be that different epimastigotes give rise to metacyclics with antigenically-different coats in different proportions or at different rates.

Our conclusions from this work are (1) the metacyclic population of Trypanosoma brucei is heterogeneous with respect to variable antigen type; there is no evidence for a single "basic" VAT in the salivary glands of Glossina; (2) at least 4 metacyclic VATs are present in the AnTAR 1 serodeme, 3 of which have been shown to form an approximately constant percentage in the metacyclic population; (3) the VAT ingested by the fly is not present in the metacyclic population, nor does the ingested VAT appear to influence the M-VAT composition of this population; the effect of the tsetse ingesting a M-VAT has not yet been investigated.

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Antigenic variation in cyclically-transmitted *Trypanosoma brucei*

II. Variable antigen type composition of the first parasitaemia in mice bitten by trypanosome-infected *Glossina morsitans*.

Summary

Tsetse flies were infected with 5 different variable antigen types (VATs) or with a mixture of VATs of the AnTAR 1 serodeme of Trypanosoma brucei. Metacyclic forms from the salivary glands of infected flies were used to initiate infections in mice. Immunofluorescence and trypanolysis reactions employing 24 monospecific antisera were used to analyse the VATs present in the mice following cyclical transmission. Regardless of the VAT used to infect tsetse flies, the first VATs detectable in the bloodstream were those previously identified as metacyclic VATs (M-VATs). These were present until at least 5 days after infection, at which time lytic antibodies against at least 2 of the M-VATs were detectable in the blood of infected mice. In mice immunosuppressed by X-irradiation the M-VATs were detectable in the bloodstream for longer periods, but the percentage of the population labelled with anti-metacyclic sera showed a decrease on day 5 as in non-irradiated animals. The VAT ingested by the tsetse was always detectable early during the first parasitaemia following cyclical transmission and was usually the first VAT detected after the M-VATs. Neutralization of selected M-VATs before infecting mice resulted in elimination of the neutralized M-VAT from the first parasitaemia but had no effect on the expression of other VATs in the early infection.

Introduction

The cyclical development of Trypanosoma brucei in the tsetse fly culminates in the metacyclic trypomastigote form in the vector's salivary glands; this stage is infective for the mammalian host. The metacyclic trypanosomes differ from the other insect developmental stages in having a surface coat similar in appearance to bloodstream forms in the mammal (Vickerman, 1969). This coat is the location of the variable antigen (Vickerman & Luckins, 1969; Cross, 1975; Le Ray, 1975).

Studies by Gray (1965, 1975) using the agglutination reaction suggested that, following cyclical transmission through the tsetse fly, trypanosomes tend to revert to a "basic strain antigen". He found that tsetse flies transmitted either trypanosomes expressing the basic antigen only, or a mixture of trypanosomes expressing the basic antigen and the variable antigen type (VAT) ingested by the fly in its infecting blood meal. Further experimental evidence (Cunningham, 1966; Jenni, 1977, 1979) suggested that the metacyclics acquire the basic antigen during their development in the salivary glands of the tsetse. Recent results from our laboratory (Le Ray et al. 1978; Barry et al., 1979; Barry & Hajduk, 1979; Hajduk, et al. 1981) show, however, that the metacyclics of the AnTAR 1 serodeme of T. brucei express at least 4 different variable antigen types (VATs) and that the ingested VAT is not present in the metacyclic trypanosome population. In this paper we have analysed the composition of the bloodstream population of trypanosomes in mice infected with metacyclics to determine which VATs are expressed in the bloodstream during the early stages of the

infection and whether the VAT ingested by the tsetse influences the VAT composition of the first parasitaemia in tsetse-bitten mice.

Materials and methods

Trypanosomes and tsetse flies

The infection of Glossina morsitans morsitans with cloned VATs of T. brucei AnTAR 1 serodeme, the maintenance of infected flies, the collection of metacyclic trypanosomes and determination of the VAT composition of bloodstream and metacyclic trypanosome populations were all carried out as described in the preceding paper (Hajduk et al., 1981). To initiate trypanosome infections in experimental animals, tsetse flies with metacyclic infections were allowed to feed on female CFLP mice. In some experiments mice were infected by injection with metacyclics obtained by allowing infected tsetse flies to probe into fresh guinea pig serum. The number of trypanosomes in the blood of infected mice was estimated by the method of Herbert & Lumsden (1976). Blood samples for serological analysis of trypanosome VATs by immunofluorescence or trypanolysis were prepared from day 3 after the infecting tsetse bite. Due to the low parasitaemias (usually less than 2.5×10^5/ml of blood) detectable on day 3 it was necessary to concentrate trypanosomes for identification of the VATs present as follows: 0.2-0.4 ml blood was collected, trypanosomes separated from blood constituents on DEAE columns (Lanham, 1968) and concentrated by centrifugation at 1,500g for 10 min at $4^{\circ}C$. From day 4 onward sufficient numbers of trypanosomes were present in the blood to allow VAT determination from blood smears.

have now become the homotype. Thus antigenic variation is a spontaneous process with antibody not acting as an inducer of the phenomenon, but as a selective agent eliminating the homotype of each wave of parasitaemia and permitting the heterotypes to emerge as the homotypes of subsequent relapse populations.

The development of a cultivation system for the bloodstream stages of T. brucei (Hirumi et al. 1977) has made it possible to examine in vitro, the role of antibody in the expression of new variants. Recent findings by Doyle et al. (1980) show that antigenic variation occurs at low frequency in vitro in the absence of antibody.

The expression of new VATs is not a strictly random process. This has been shown for cyclically transmitted T. brucei (Gray, 1965) and T. gambiense (Gray, 1975) and in syringe passaged infections of Trypanosoma equiperdum (Capbern et al., 1977). Gray (1965) tested for the presence of agglutinating antibodies in the blood of animals infected by tsetse bite and found that antibodies to certain VATs were detectable early in the infection. Regardless of the VAT used to infect the tsetse fly, antibodies to a "basic strain antigen" were detected first. if all trypanosomes of a particular serodeme revert to a single VAT following cyclical transmission in the insect vector, then this VAT might be useful as a potential vaccine against the natural challenge in the field.

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2. PURPOSE OF INVESTIGATION

Recent studies by LeRay et al. (1977; 1978) have shown that the metacyclic trypanosomes of the AnTAR 1 serodeme of T. brucei express a mixture of VATs in the salivary glands of G. morsitans and that in the mammalian host the metacyclic VATs (M-VATs) rapidly change to non-metacyclic VATs. The purpose of my investigation was to define the antigenic composition of the metacyclic population and the first parasitaemia in mice infected with metacyclics using monospecific antisera. The influence of the VAT ingested by the tsetse fly on the VAT composition of both the metacyclic and first bloodstream populations was studied. In addition, I have studied the role of antibody in the change from M-VATs to "predominant" VATs in the first parasitaemia of metacyclic infected mice by analysis of the VAT composition in immuno-suppressed, metacyclic infected mice.

Antigenic variation in cyclically-transmitted

Trypanosoma brucei

I. Variable antigen type composition of metacyclic trypanosome populations from the salivary glands of *Glossina morsitans*.

Summary

Tsetse flies (Glossina morsitans) were fed on the blood of mice containing any one of 5 variable antigen types (VATs) of Trypanosoma brucei AnTAR 1 serodeme. The VATs of the metacyclic trypanosomes subsequently detected in the flies' saliva probes were investigated using monospecific antisera to AnTAR 1 VATs in indirect immunofluorescence and trypanolysis reactions; these sera included 3 raised against AnTats 1.6, 1.30 and 1.45, previously identified as components of the metacyclic population (M-VATs), and against the 5 VATs originally ingested by the flies. The percentage of metacyclics reacting with a particular M-VAT antiserum remained more or less constant (AnTat 1.6, 6.0-8.3%; AnTat 1.30, 13.7 - 18.2%; AnTat 1.45, 2.0 - 8.0%), regardless of the age of the fly or the ingested VAT. As these 3 VATs accounted for no more than one-third of the metacyclic population, the existence of at least one more VAT is envisaged. The ingested VAT could not be detected among the AnTAR 1 metacyclic trypanosomes.

INTRODUCTION

The life cycle of Trypanosoma brucei consists of developmental stages in a warm-blooded host, usually a mammal, and in an insect vector, the tsetse fly (Glossina spp.). For survival in the mammal, T. brucei and the other salivarian trypanosomes have evolved a mechanism, antigenic variation, which allows the antigenic determinants on the trypanosome's surface to change, thus nullifying the effects of the host's immune response (for recent reviews see Gray and Luckins, 1976; Doyle, 1977; Cross, 1978; Vickerman, 1978; Turner, 1980).

Several studies suggest a semi-ordered appearance of VATs in both syringe passaged lines and lines cyclically transmitted through the tsetse fly (Gray, 1965, 1975; McNeillage et al., 1969; Van Meirvenne et al., 1975; Capbern et al., 1977). Gray (1965, 1975) used agglutination reactions to follow the appearance of antibodies to specific variable antigen types (VATs) in the blood of animals infected by tsetse bite and found that following cyclical transmission trypanosomes of a particular stock tend to express first the same "basic antigenic type", though occasionally a mixture of this type and that originally ingested by the fly was transmitted.

The metacyclic developmental stage found in the salivary glands of the tsetse fly is the only form in the insect vector which has a surface coat (Vickerman, 1969). This coat is shed by the bloodstream trypanosome, in the tsetse midgut, within 36-48 hours of ingestion (discussed by Barry and Vickerman, 1979). The metacyclics reacquire infectivity for the mammal along with the surface coat (Vickerman, 1969). The coat contains the variable antigen (Vickerman and Luckins, 1969; Cross, 1975).

Considerable interest now attaches to the question of whether the variable antigen in the coat is the same for all the metacyclic trypanosomes of a given serodeme or whether a mixture of variable antigen types (VATs) is present in the population. Recent studies in our laboratory have shown that the VAT composition of the metacyclic population of the AnTAR 1 serodeme of T. brucei is heterogeneous (Le Ray et al., 1978, Barry et al., 1979; Barry and Hajduk, 1979) and that upon inoculation into the mammalian host the metacyclics rapidly undergo antigenic variation (Le Ray et al., 1977). In this and an accompanying paper we describe further investigations on the VAT make-up of the metacyclic population of T. brucei and the antigenic differentiation which occurs in the bloodstream of tsetse-bitten mice. These findings are discussed in relation to Gray's (1965) original observations on the effect of fly transmission on antigenic variation.

MATERIALS AND METHODS

Trypanosomes:

Cloned VATs of the AnTAR 1 serodeme of T. brucei stock EATRO 1125 (see WHO, 1978 for nomenclature) were used in these experiments. Variable antigen types AnTat 1.1 to 1.13 were isolated from a syringe passaged, monomorphic line of this stock as described by Van Meirvenne and co-workers (1975); AnTat 1.14 to 1.22 were isolated from a line of the AnTAR 1 serodeme which had been cyclically transmitted through Glossina morsitans (Le Ray et al., 1977). AnTat 1.30 and 1.45 were isolated from chronically infected rabbits as described by Barry et al., (1979) and in this paper. The pedigree of Antat

1.30 and 1.45 is given in figure 1. All clones were stored at -196°C with DMSO (7.5%) added as a cryopreservant.

Tsetse flies:

Glossina morsitans pupae were obtained from the Tsetse Research Laboratory, Bristol, UK. Pupae were incubated at 30°C and 70-80% relative humidity on sterile sand in gauze-covered Kilner jars. Recently hatched flies were chilled at 4°C for 15-20 minutes before being transferred to numbered individual plastic tubes, length 80 mm, diameter 25 mm, with gauze-covered ends. These flies were then fed, usually within 48 hours, on female CFLP mice, which had received 600 RADs whole body X irradiation, 1 day before infection with 1 of 5 T. brucei VATs or with an uncharacterized mixture of VATs from the AnTAR 1 serodeme. Following this infective feed, tsetse flies were maintained at 26°C, and 75% humidity. Maintenance blood meals were given 3 times per week on the flank or ears of New Zealand half-lop rabbits. From 15 to 30d following the infecting feed, flies were allowed to probe on a microscope slide warmed to 37°C and the extruded saliva was examined for the presence of metacyclic trypanosomes by phase contrast microscopy. All infected flies were henceforth maintained on uninfected CFLP mice.

Antisera:

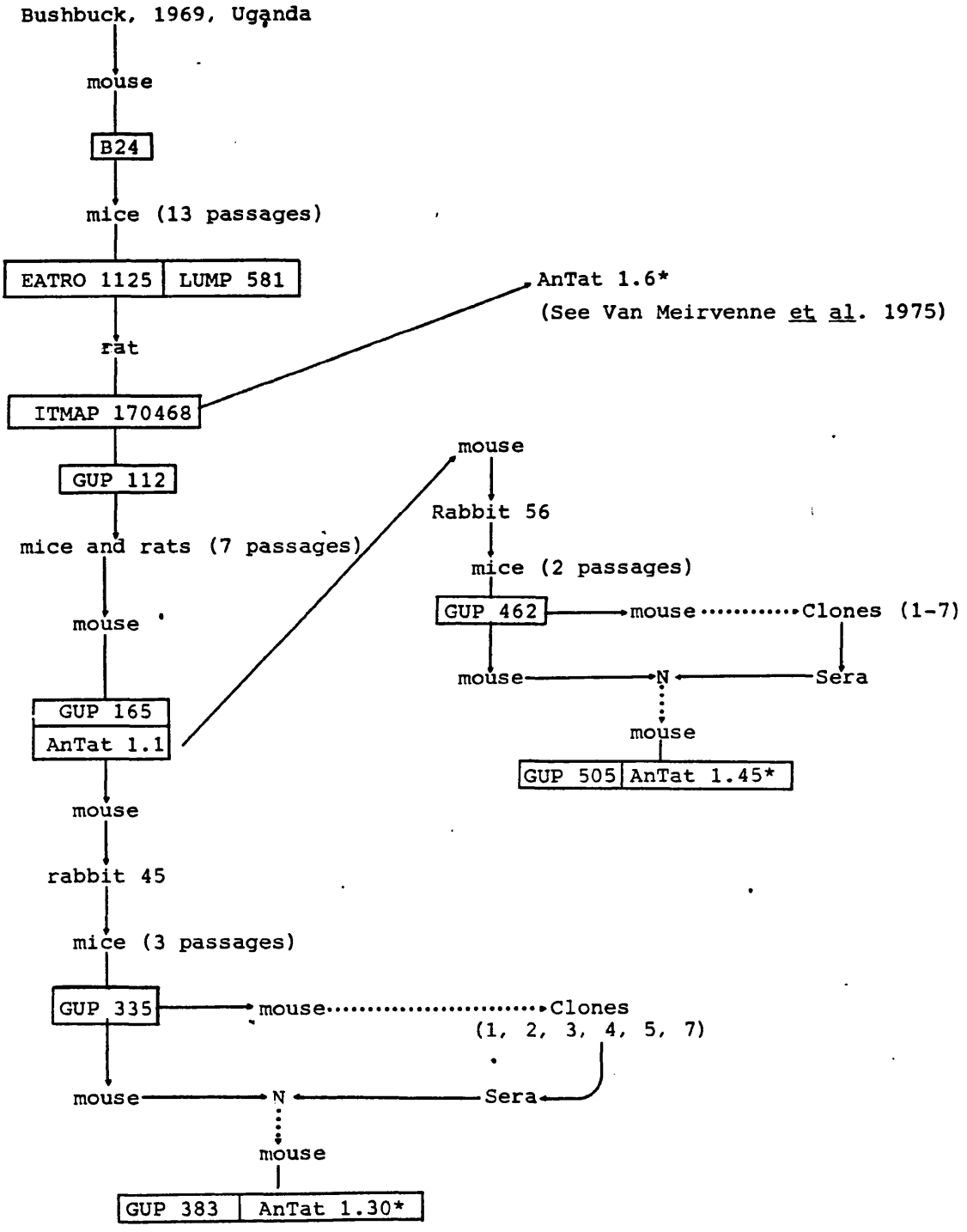
Monospecific antisera against bloodstream VATs AnTat 1.1 to 1.22 were obtained by intravenous injection of 1×10^6 living trypanosomes of a particular cloned VAT into rabbits and collection of serum 6 days later from the marginal ear vein.

Figure 1

Pedigree of the syringe passages AnTAR 1 populations used to obtain antigenically stable clones baring metacyclic VATs. Joined stabilate boxes indicate transfer from one laboratory to another. EATRO, East African Trypanosomiasis Research Organization; LUMP, London University Medical Protozoology; ITMAP, Institute of Tropical Medicine Antwerp Protozoology; GUP, Glasgow University Protozoology.

Figure 1.

Isolation of Stable Metacyclic Variable Antigen Types:



N = neutralization

Broken lines indicate passaging of a single trypanosome

*Indicates a monomorphic bloodstream population expressing metacyclic VAT

Sera were then titrated against the homologous VAT and a heterologous one; end point dilutions for specificity were determined using both immunofluorescence and trypanolysis reactions, and serum samples were stored either whole at -70°C or as freeze dried material at room temperature. Most of the serum samples used in this study were those previously employed by Van Meirvenne et al (1975) or by Le Ray et al (1977) and had been extensively checked for specificity.

Monospecific antisera against 2 metacyclic VATs, AnTats 1.30 and 1.45, were prepared using the method described by Barry et al (1979). Rabbits chronically infected with a syringe passaged, monomorphic line of AnTat 1.1 were bled at 3 day intervals for serum samples and for trypanosomes to infect mice; following 2 passages in mice stabilates were made of each isolate. By trypanolysis reactions using living metacyclics and the serum samples from the chronically infected rabbit, the point in the rabbit's infection when its serum showed incipient anti-metacyclic activity was identified, and clones were isolated from the blood of mice that received trypanosomes at this time. These clones expressing metacyclic VATs were more stable with respect to VAT than clones derived directly from metacyclics and could be used to prepare monospecific antiserum in rabbits as described above.

Immunofluorescence reactions:

Air dried smears of infected mouse tail blood, and tsetse saliva probes of metacyclic trypanosomes, were routinely fixed in acetone at room temperature for 15 minutes. Material fixed in methanol or formalin (Nantulya and Doyle, 1977) and living trypanosomes (Barry, 1979) gave similar results. Fixed probes and smears were rehydrated in phosphate buffered saline (PBS)

pH 7.2 for 15 minutes. Diluted rabbit anti-trypanosome antiserum was added to a marked area of smear which was then incubated in a humid chamber for 15 minutes then washed in PBS. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin (Pasteur Institute) was added at a 1:400 dilution in 1:10,000 Evans blue-PBS solution. After incubation for 15 minutes the preparation was washed again in PBS.

To distinguish between 2 VATs in the same preparation a double labelling, indirect immunofluorescence test was used. Monospecific mouse anti-trypanosome serum raised against one metacyclic VAT was mixed with rabbit anti-trypanosome serum against a second metacyclic VAT, both at their determined specific dilutions. The mixture was applied to acetone fixed, rehydrated saliva probes of metacyclics, incubated 15 minutes, washed with PBS, reacted first with FITC goat anti-rabbit immunoglobulin (1:400), washed again in PBS, then reacted with rabbit anti-mouse immunoglobulin conjugated with tetramethyl-rhodamine isothiocyanate (TRITC), and finally washed with PBS.

Smears were mounted in 50% (w/v) glycerol/PBS and preparations examined with a Leitz Ortholux II microscope using incident light fluorescence with an HBO 50 high-pressure mercury vapour lamp. For the FITC reactions, 2X KP490 (exciting), TK 510 (dichroic mirror) and K515 (suppressing) filters were used. For TRITC, 2mm BG36+S546 (exciting), TK580 (dichroic mirror) and K580 (suppressing) filters were used.

Colour photographs were taken using Kodak Ektochrome 200 ASA film, which was exposed and developed as for 400 ASA. Colour prints were made using Cibachrome Systems filters, chemicals and paper (Ilford).

Trypanolysis reactions:

Antibody-mediated lysis of trypanosomes, *in vitro*, was carried out essentially as described by Van Meirvenne *et al* (1975). Living metacyclics were collected by allowing infected tsetse flies to probe into shallow wells (leucocyte migration plates, Sterilin) containing about 0.2 ml of either fresh guinea-pig serum or Medium 199 (Gibco) supplemented with 15% foetal calf serum. Metacyclic trypanosomes were concentrated by centrifugation at 1,000g for 10 minutes at 15°C in Eppendorf centrifuge tubes. Metacyclics were then resuspended in fresh guinea-pig serum for lysis reactions in disposable microtitre plates. 5 µl of guinea-pig serum containing metacyclics was mixed with 1 µl of monospecific antiserum at the appropriate dilution and incubated at 26°C for 30 minutes. Samples from the plates were examined under the 40x phase contrast objective.

RESULTS

Infection of tsetse with trypanosomes

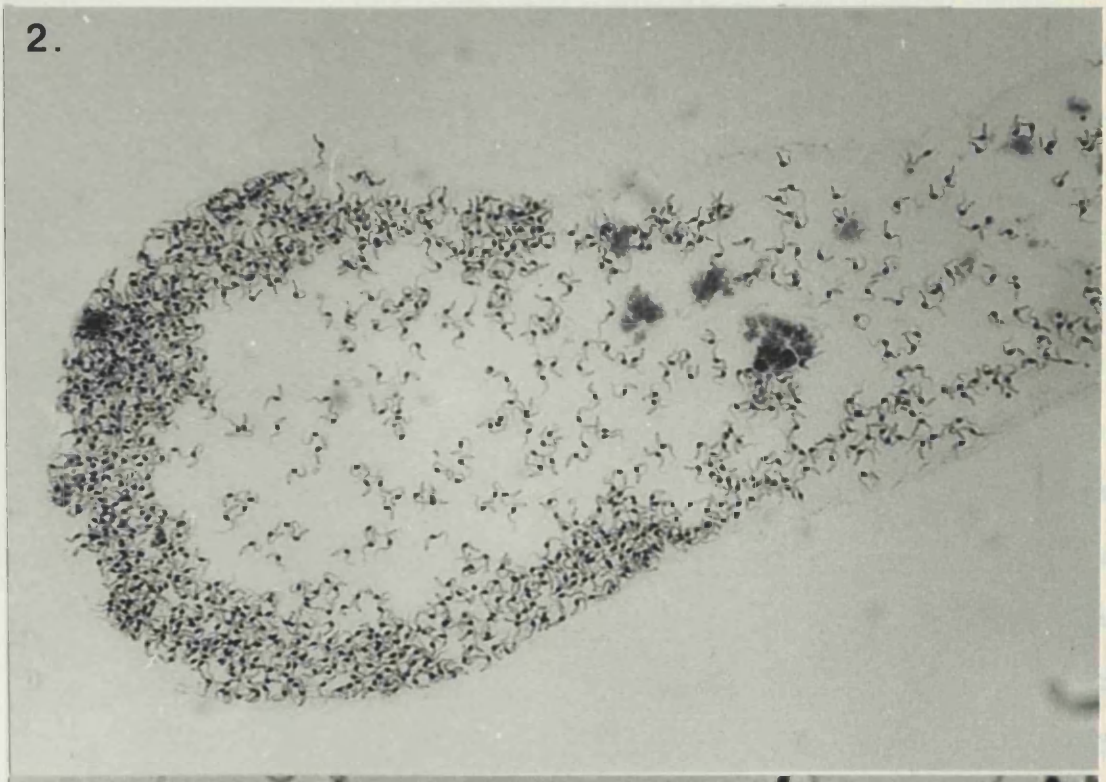
Experimental studies on the effect of cyclical transmission on *Trypanosoma brucei* infections are made difficult by the inefficiency of transmission in the laboratory, as in the field. The percentage of flies which develop metacyclic infections after an infecting meal is usually less than 5 and mortality rates during the long period required for completion of cyclical development may contribute substantially to this inefficiency. Our experiments confirmed the findings of Jenni (1977) that (1) by maintaining the flies in a clean, controlled environment mortality rates can be reduced to less than 1% per day; (2) by utilising flies that have

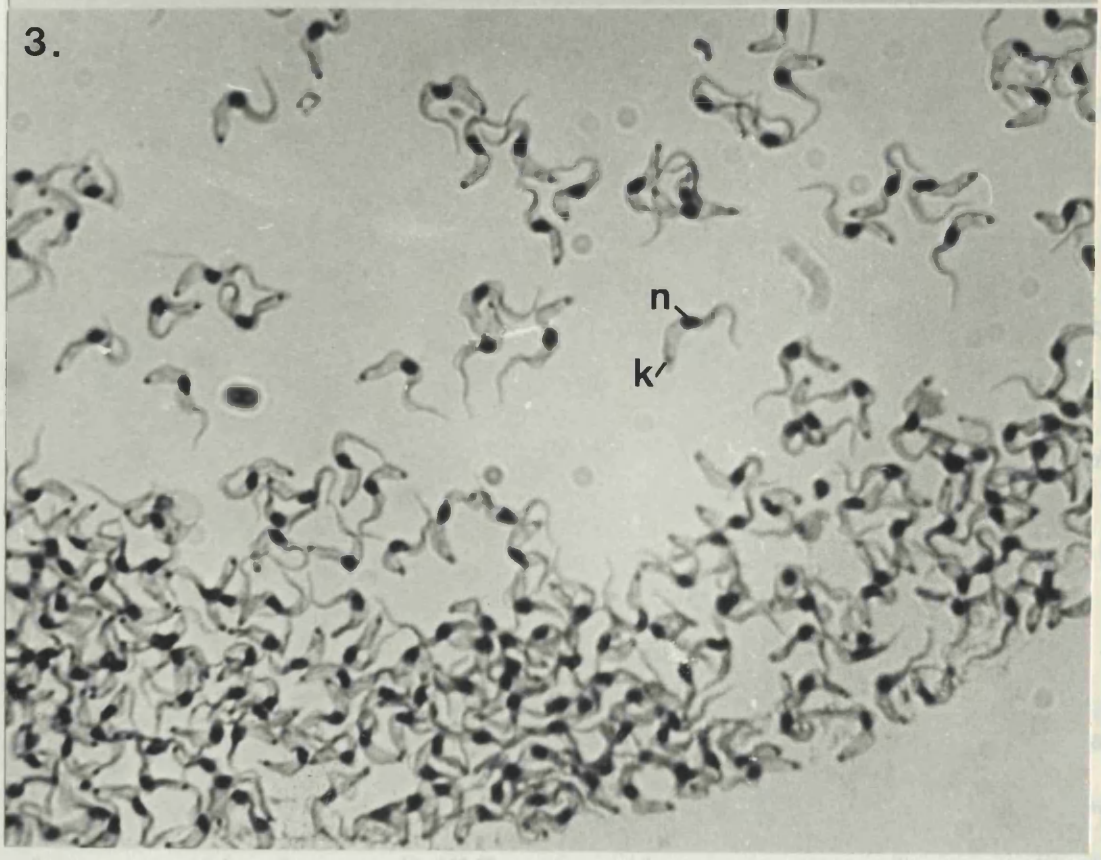
Figure 2

Giemsa stained, methanol fived saliva probe obtained from a tsetse fly with a metacyclic infection to show the large number of trypanosomes extruded. (X400).

Figure 3

Portion of the saliva probe shown in figure 2 at higher magnification. Only trypomastigote (metacyclic) forms are present in this probe; the position of the nucleus (n) and kinetoplast (k) are clearly visible in the trypanosomes, (X1500).


2.


3.
n
k

taken their infecting meal within 24h of hatching, infection rates may be raised to 10%; in our experiments 75% of flies fell into this category. The use of predominantly stumpy bloodstream trypanosomes to infect flies was also found to be important in achieving this elevated transmission rate.

Metacyclics in saliva probes

A representative dried tsetse saliva probe containing metacyclics is shown in fig. 2. The flagellates were usually crowded around the edge of the dried droplet, more dispersed and flattened towards its centre. Fig. 3 shows that at higher magnification the flagellates have readily recognisable metacyclic morphology. Epimastigote trypanosomes, dislodged from their anchorage on the salivary epithelium, and proventriculus or midgut trypomastigotes were occasionally observed in probes from flies with "immature" infections but were not present in significant numbers in the probes utilised for analysis.

Metacyclic trypanosomes obtained in tsetse saliva probes remained viable for several hours in guinea pig serum, while uncoated stages (eg procyclics, epimastigotes) were rapidly lysed in this serum.

Identification of metacyclic VATs

In previous papers the identification of AnTat 1.30 (Barry et al., 1979) and AnTat 1.6 (Barry & Hajduk, 1979) as metacyclic VATs (M-VATs) has been described. AnTat 1.45 was also recognised as a M-VAT using the same procedure as that used to identify AnTat 1.30, and a monospecific antiserum was produced

against the AnTat 1.45 clone. The specificities of the 3 antisera used to identify AnTats 1.6, 1.30 and 1.45 among the metacyclic trypanosomes of tsetse saliva probes were previously tested in homologous and heterologous trypanolysis reactions with the 3 bloodstream clones and also in heterologous reactions with 21 other VATs of the AnTAR 1 serodeme. In trypanolysis reactions the 3 antisera did not cross react with one another (Table 1). This lack of cross reaction was further demonstrated by trypanolysis experiments in which the individual anti-M-VAT sera were pooled in different combinations (Table 2). In reactions where 2 or 3 sera were combined, the percentage lysis obtained was additive; a total of 28.6% of metacyclics were lysed by a mixture of all 3 sera. Unlike AnTat 1.6, AnTats 1.30 and 1.45 were not lysed with the pooled preparation of sera against AnTat 1.1 to 1.22 whereas all 3 M-VATs were lysed by end-infection serum from rabbits (Table 1; cf. Le Ray et al., 1978). The contribution of uncoated epimastigotes and procyclic trypomastigotes and partially coated "immature" metacyclics was minimal in these preparations since the percentage lysis with individual and pooled anti-M-VAT sera was unaltered by passaging of the trypanosomes obtained from tsetse saliva probes through DEAE cellulose columns (Lanham, 1968) prior to the lysis reaction (Table 2).

Figure 4 shows the FITC immunofluorescence reaction obtained when the 3 monospecific sera were applied individually to acetone-fixed salivary probes from a single infected fly. Antiserum against AnTat 1.30 labelled 18.2% of the metacyclics, Anti-Antat 1.6 labelled 9.0% and anti-AnTat 1.45 labelled 3.0%. The total of 30.2% metacyclics labelled compared well with the

Table 1. Specificity of antisera prepared against bloodstream trypanosomes expressing M-VATs.

Bloodstream trypanosome VAT	Percent trypanolysis with sera				
	Anti-AnTat 1.6	Anti-AnTat 1.30	Anti-AnTat 1.45	Pooled anti-AnTats 1.1-1.22	End infection serum
AnTat 1.6	90	0	0	80	98
AnTat 1.30	0	100	0	0	99
AnTat 1.45	1	0	100	2	98

Antisera

Antisera against VATs AnTat 1.1 to 1.22 and AnTat 1.30 and 1.45 were prepared as described previously (Van Meirvenne et al., 1975; Barry et al., 1979; Hajduk et al., 1981) and diluted to concentrations at which they reacted only with the homologous VAT. A preparation of pooled antisera against AnTats 1.1 to 1.22 was prepared to use in trypanolysis reactions by mixing the 22 antisera together so that when added at a 1:6 dilution to an antigen preparation each was at a monospecific dilution. A polyvalent antiserum against metacyclic VATs (M-VATs) and VATs arising from the metacyclics in the mammalian host was prepared by allowing an infected tsetse fly to feed on an uninfected New Zealand half-lop male rabbit and collecting serum 7 days later (Le Ray et al., 1978).

Neutralization of M-VATs

Neutralization of M-VATs AnTat 1.30 and 1.45 was carried out on metacyclics suspended in fresh guinea pig serum with the antisera diluted to 1:80 and 1:50 respectively; incubation was at 26°C for 1 hour. Mice were then infected by intraperitoneal injection.

Effect of Immunosuppression on antigenic variation

Infected tsetse flies which had ingested an undefined mixture of VATs of the AnTAR 1 serodeme were fed on CFLP mice immunosuppressed by subjection to 600 rads total body x-irradiation 1 day previously. Unirradiated mice were similarly infected to provide controls.

Results

Expression of metacyclic VATs in mice following tsetse bite

The number of trypanosomes in the blood of tsetse-bitten mice increased for the first 4 days followed by a slight decline or levelling off in numbers on day 5. The parasitaemia ascended from day 6 until day 10 when a major remission generally occurred (Figure 1a). The decline in parasitaemia on day 5 corresponded with the point at which anti-metacyclic trypanolytic antibodies could first be detected in the bloodstream of tsetse bitten mice (Figure 1b); the titre of antibodies against M-VATs AnTat 1.30 and 1.45 increased from day 5 until at least day 8. The presence of M-VATs on days 3-5 in the bloodstream of mice infected with metacyclics was demonstrated by immunofluorescence (Figure 1b). The percentage of trypanosomes labelled with both anti-AnTat 1.30 and anti-AnTat 1.45 increased to about 39% and 15% respectively on day 4 but had fallen to 0 by day 6.

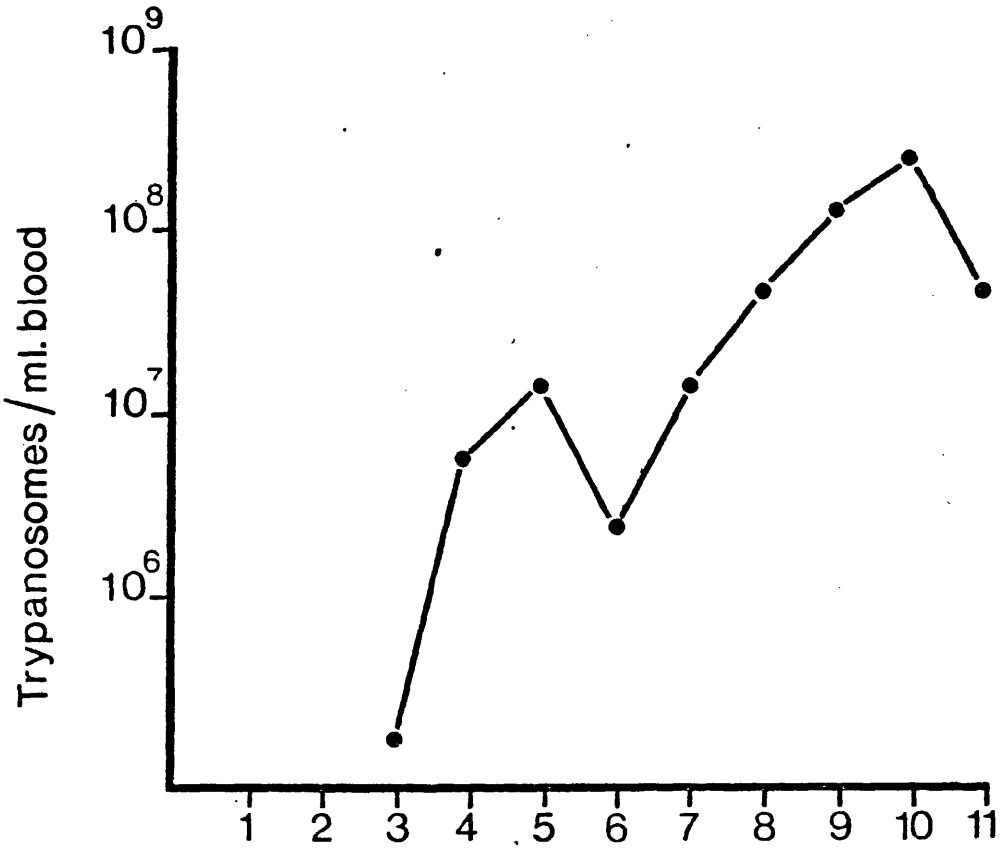
The presence of M-VATs in the bloodstream of tsetse-bitten mice was verified by trypanolysis reactions using monospecific antisera against AnTat 1.6, 1.30 and 1.45 (Table 1). All 3 M-VATs were detectable in the metacyclic initiated infection. The polyvalent 7-day antiserum against M-VATs of this serodeme lysed 83% of the metacyclics in probes and decreasing percentages of the bloodstream forms from day 3 to day 7 after infection with metacyclics. Non-M-VATs arose early in the infections as shown by the increased percentage of lysis with pooled antiserum against AnTats 1.1-1.22 in the bloodstream trypanosome population from day 3 to day 10 (Table 1).

Legends

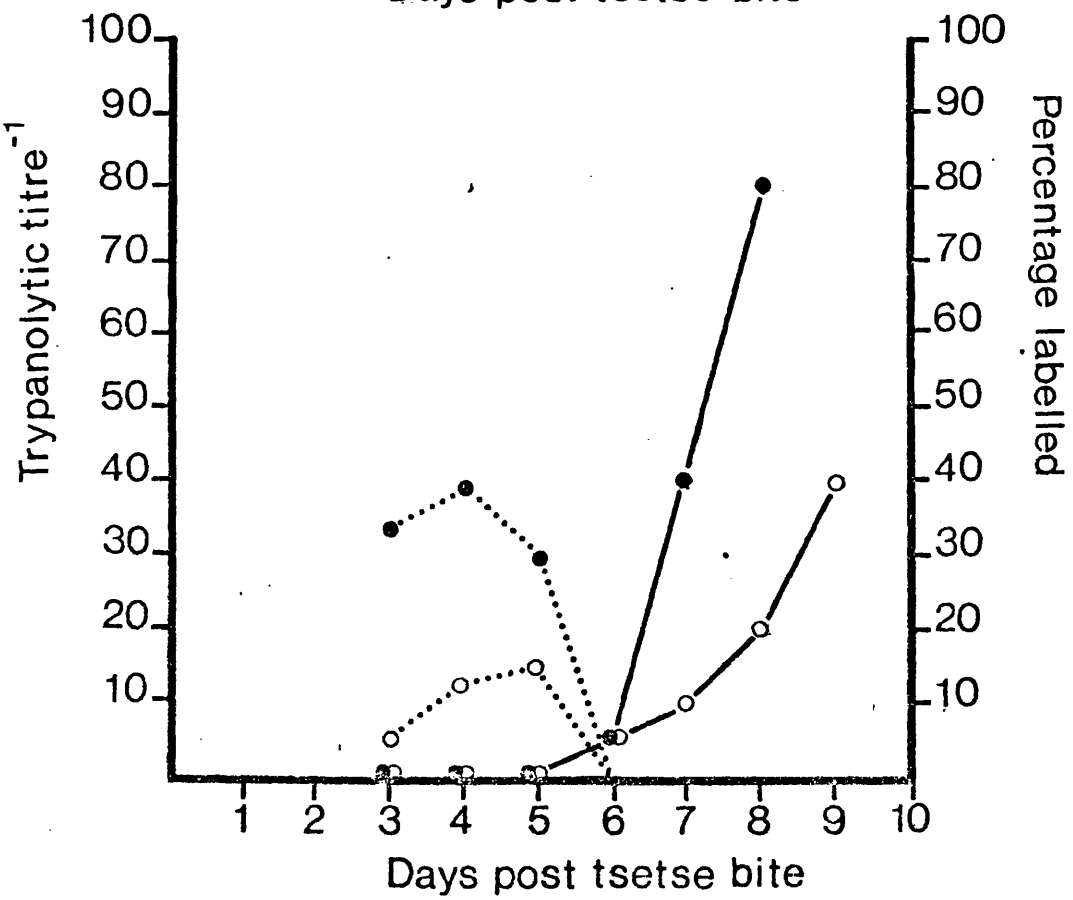
Figure 1. The course of parasitaemia, lytic antibody titres against 2 M-VATs, (AnTat 1.30 and 1.45) and the percentage of trypanosomes labelling by immunofluorescence reactions using anti-AnTat 1.30 and 1.45 sera was determined for 10 days in a mouse infected by the bite of a tsetse fly which had ingested AnTat 1.14. (A) Course of parasitaemia in representative mouse infected by tsetse bite. (B) Trypanolytic titres of serum from the same mouse against AnTats 1.30 (●——●) and 1.45 (o——o); similar results were obtained with sera from 2 other mice. Also shown are percentages of trypanosomes labelled with anti-AnTat 1.30 (●·····●) and 1.45 (o·····o) sera.

Figure 2. The course of parasitaemia and the expression of 5 VATs in the bloodstream of a tsetse-bitten untreated mouse and of a mouse immunosuppressed by 600 rads total body x-irradiation. (A) Course of parasitaemia in irradiated (●·····●) and non-irradiated (O——O) mice. (B & C) Percentages of M-VATs AnTats 1.30, 1.45 and 1.6 (dotted lines) and of non-M-VATs AnTats 1.5 and 1.19 (solid lines) detected by immunofluorescence reactions in blood from fly-bitten mice. AnTat 1.45 persisted in the blood of the irradiated mouse until day seven at a very low level (1% on days 6 and 7).

A.


Trypanosomes/ml.blood
10^9
10^8
10^7
10^6
1 2 3 4 5 6 7 8 9 10 11
Days post tsetse bite

B.


Trypanolytic titre^{-1}
100
90
80
70
60
50
40
30
20
10
Percentage labelled
1 2 3 4 5 6 7 8 9 10
Days post tsetse bite

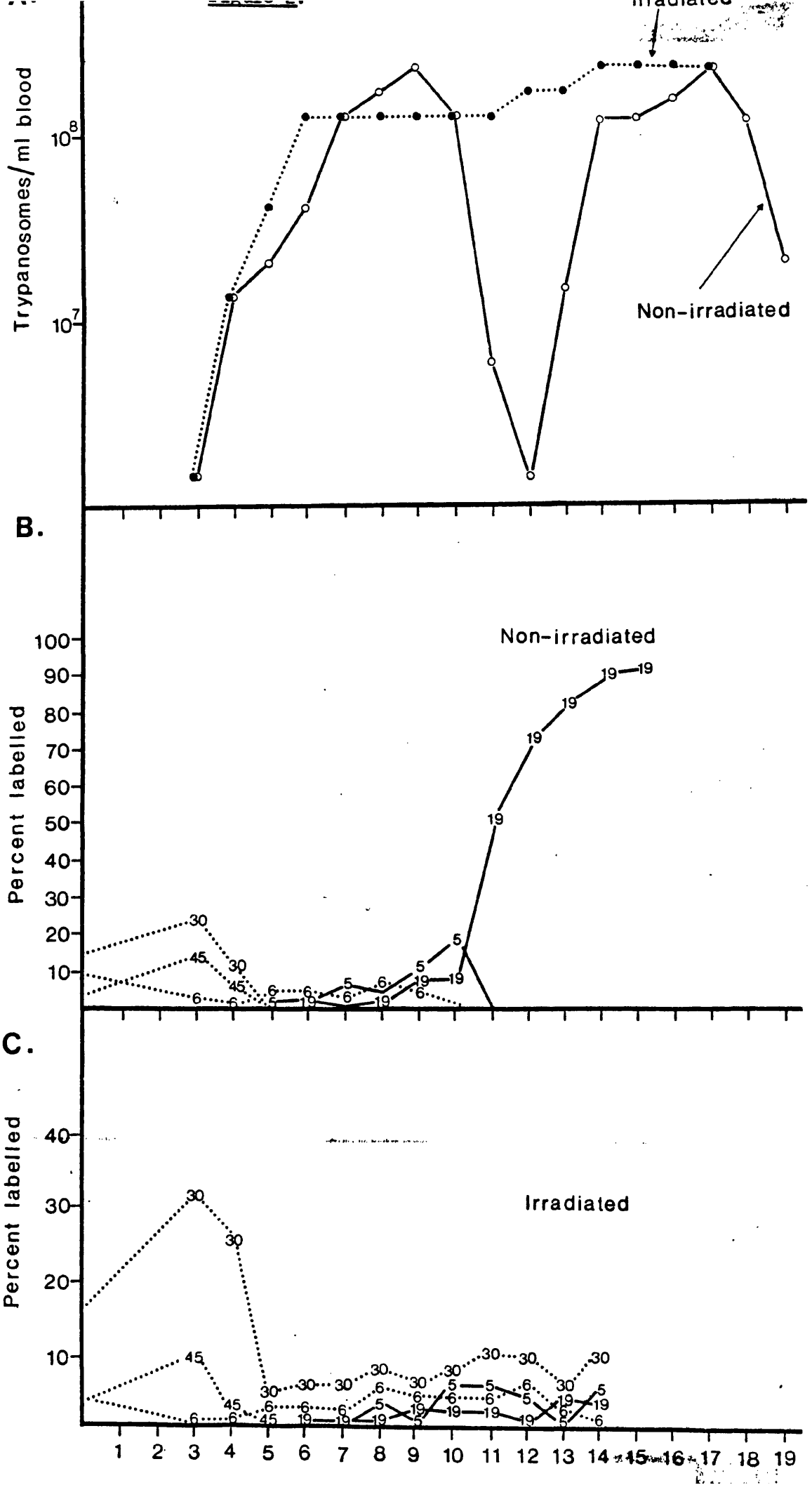

Irradiated
Trypanosomes/ml blood
10^8
10^7
Non-irradiated
B.
Non-irradiated
Percent labelled
100
90
80
70
60
50
40
30
20
10
C.
Irradiated
Percent labelled
40
30
20
10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Table I

Percentage lysis of metacyclic trypanosomes from salivary probes and of bloodstream trypanosomes from first parasitaemia in trypanolysis reactions using monospecific and polyvalent antisera : figures refer to means obtained from 3 different mice and probes from 3 different tsetse flies

Days after infection	Antisera				
	Pooled anti AnTat 1.1-1.22	Polyvalent anti-metacyclic (day 7)	anti-AnTat 1.6	anti-AnTat 1.30	anti-AnTat 1.45
Metacyclics (probe)	4.7	88.3	8.8	19.3	8.0
3	3.3	71.6	1.7	6.3	11.3
4	5.6	65.0	2.3	4.6	6.3
5	21.0	58.0	2.3	1.7	5.0
6	27.0	3.0	1	0	2.7
7	33.7	2.7	0.67	0	0
8	24.7	0.3	0.3	0	0
9	30.0	0	1.3	0	0
10	38.4	0	1.7	0	0

Influence of the VAT ingested by the tsetse on the VATs expressed in the bloodstream

The results of the immunofluorescence analysis, with 24 monospecific antisera, on the trypanosomes in the blood of mice bitten by tsetse flies which had ingested 5 different VATs is presented in Table 2. The M-VATs AnTat 1.6, 1.30 and 1.45 were the first VATs detected in the blood on day 3: the percentage of the population labelled with each anti M-VAT serum was approximately the same regardless of whether the infecting tsetse had ingested AnTat 1.8, 1.14, 1.18, 1.19 or 1.21. AnTats 1.30 and 1.45 were always eliminated by day 6 after tsetse bite, but AnTat 1.6 generally persisted until day 9 or 10; this finding was consistent with the low virulence of AnTat 1.6 reported by Van Meirvenne et al., (1975). Of the 24 different VATs tested for using monospecific sera in these experiments, only AnTats 1.1 and 1.13 were never detected during the first 10 days; VATs rarely expressed were AnTats 1.20 and 1.22. In addition to the M-VATs, AnTat 1.3 and 1.7 were always expressed at high percentages (greater than 10%) sometime during the first 10 days of infection. The expression of several VATs in these infections appeared to be influenced by the VAT used to infect the tsetse (I-VAT). The I-VAT was always a major type in the bloodstream of fly-bitten mice, usually being detectable by day 4 or 5 after tsetse bite, i.e. immediately following or slightly overlapping the M-VATs. Other VATs were also expressed more frequently in the tsetse-bitten mice when a particular VAT had been ingested by the tsetse fly. The increased frequency of AnTat 1.2 when AnTat 1.8 or 1.14 was the I-VAT, and of AnTat 1.15 when AnTat 1.18 or 1.19 was the I-VAT, suggested that the expression of certain VATs was somehow linked with expression of the I-VAT in the early parasitaemia of fly-bitten mice.

Table II

Immunofluorescence reaction : percentage of metacyclic and other VATs of the AnTat serodeme in mice bitten by tsetse flies which had ingested AnTat 1.8, 1.18, 1.14, 1.19, 1.21

Antigenic type ingested	Days after fly bite	Mono-specific antisera against AnTat VATs																								
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	30	45
AnTat 1.8 (3)*	3	0	0	0.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	30	4
	4	0	0	0.2	0	0	0.2	0.5	0.5	0	0	0.2	0	0	0	0	0	0	1	0	0	0	0	28	8
	5	0	2	4	1	0	4	6	5	0	1	1	2	0	5	0	2	1	2	2	0	0	0	10	2
	6	0	1	7	0	0	2	5	18	0	0	1	3	0	13	1	2	1	6	0	1	2	0	0	0
	7	0	3	7	2	0	2	6	21	0	2	1	8	0	11	0	1	0	5	1	0	3	0	0	0
	8	0	11	16	1	0	3	4	35	0	2	3	0	0	0	1	1	0	0	4	0	0	0	0	0
	9	0	15	0	2	0	14	10	37	0	0	0	0	0	0	2	2	0	0	8	0	0	0	0	0
	10	0	14	0	0	0	18	14	44	0	0	0	0	0	0	0	2	0	0	5	0	0	0	0	0
AnTat 1.18 (3)	3	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	28	7
	4	0	0	1	0	0	6	0	0	0	0	0	0	0	0.5	0.5	0	0	0.5	0	0	0	0	30	6
	5	0	1	3	0	1	3	2	0	0	0	0	3	0	4	7	2	1	0	1	0	2	0	0	12
	6	0	1	13	1	4	1	2	1	0	1	0.5	2	0	8	16	4	1	18	0	1	1	0	0	0
	7	0	0	18	0	9	3	5	0	0	1	2	4	0	8	33	4	0	10	1	1	1	0	0	0
	8	0	1	12	0	2	1	9	1	1	0	1	0	0	1	51	3	0	6	1	0	0	0	0	0
	9	0	1	10	1	0	8	10	2	0	0	0	0	0	0	68	3	0	0	1	0	0	0	0	0
	10	0	0	0	0	0	6	5	0	0	0	0	0	0	0	74	0	0	0	6	0	0	0	0	0

Antigenic type ingested	Days after fly bite	Mono-specific antisera against AnTat VATs																							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	30	45
AnTat 1.14 (2)	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	28	10
	4	0	0	0	0	0	1	0	0	0	0	0	0	0	9	1	0	0	0	0	0	0	0	28	6
	5	0	0	0	0	0	2	1	2	0	0	0	1	0	5	0	2	0	0	0	0	0	0	0	0
	6	0	1	1	0	1	3	1	2	1	0	0	1	0	28	1	0	2	0	0	0	1	0	0	0
	7	0	3	2	0	4	4	4	5	4	0	1	6	0	30	2	4	3	0	0	2	0	0	0	0
	8	0	7	7	1	6	9	4	3	2	0	0	6	0	0	3	7	0	0	2	7	0	0	0	0
	9	0	9	8	2	5	10	7	6	0	0	0	0	0	0	3	4	0	0	2	0	0	2	0	0
	10	0	14	13	5	8	10	10	8	0	0	0	0	0	0	4	3	0	0	6	0	0	1	0	0
AnTat 1.19 (2)	3	Not enough cells present for immunofluorescence																							
	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	15	10
	5	0	0	0	0	0	1	0	1	0	0	0	0.5	0	0	1	0	0	1	1	0	0	0	5	1
	6	0	0.5	2	0	2	2	5	1	0	0	0	1	0	0	2	0	0	1	4	0	0	0	1	0
	7	0	0	7	0	0	1	6	0	0	0	0	5	0	1	13	0	0	6	11	0	0	0	0	0
	8	0	1	7	0	3	1	4	1	0	1	0	5	0	4	15	0	5	2	12	0	0	1	0	0
	9	0	3	8	0.5	1	3	4	1	0	1	2	1	0	2	32	0	6	0	22	0	0	0	0	0
	10	0	0	0	4	5	23	24	3	0	2	6	0	0	0	16	0	0	0	10	0	0	1	0	0

Antigenic type ingested	Days after fly bite	Mono-specific antisera against AnTat VATs																							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	30	45
AnTat 1.21 (1)	3	ND	0	0	ND	0	0	0	0	ND	ND	ND	ND	ND	0	ND	ND	ND	0	0	ND	0	0	29	10
	4	ND	0	0	ND	0	2	0	0	ND	ND	ND	ND	ND	0	ND	ND	ND	0	0	ND	0	0	24	9
	5	ND	2	4	ND	7	1	3	0	ND	ND	ND	ND	ND	2	ND	ND	ND	1	0	ND	10	0	0	2
	6	ND	3	7	ND	3	1	5	1	ND	ND	ND	ND	ND	4	ND	ND	ND	2	2	ND	7	0	0	0
	7	ND	4	9	ND	6	1	8	1	ND	ND	ND	ND	ND	6	ND	ND	ND	3	6	ND	0	0	0	0
	8	ND	7	3	ND	4	3	12	3	ND	ND	ND	ND	ND	0	ND	ND	ND	0	12	ND	0	0	0	0
	9	ND	8	6	ND	6	3	14	2	ND	ND	ND	ND	ND	0	ND	ND	ND	0	14	ND	0	0	0	0
	10	ND	3	13	ND	10	6	12	1	ND	ND	ND	ND	ND	0	ND	ND	ND	0	13	ND	0	0	0	0

*Results are from a single mouse but are representative of those obtained from the analysis of parasitaemias from this number of mice.

Effect of neutralization of M-VATs on the VATs present in the first parasitaemia

Live metacyclic trypanosomes from a fly which had ingested AnTat 1.19 were neutralized with Anti-AnTat 1.30 or 1.45 serum and the preparation injected into mice. Regardless of whether neutralized AnTat 1.30 or 1.45 metacyclics, or untreated metacyclics, were injected, the major VATs detectable in the blood of the mice from days 3 to 9 were the same except that the neutralized M-VAT was never detected (Table 3).

Effect of immunosuppression on the course of infection and on trypanosome antigenic variation in tsetse bitten mice

The effect of host X-irradiation on the subsequent parasitaemia of mice infected by tsetse bite is shown in figure 2A. In the non-irradiated host the parasitaemia fluctuated forming 2 major peaks on day 9 and day 17. In the immunosuppressed host the parasitaemia formed a plateau after about 6 days, remaining at about $2x10^8$ trypanosomes per ml of blood until day 17 of the infection.

The percentages of AnTats 1.6, 1.30 and 1.45 (the 3 M-VATs) of AnTat 1.5 (a major non-M-VAT in the first peak parasitaemia), and of AnTat 1.19 (the major VAT in the relapse parasitaemia) in the non-irradiated host are shown in figure 2B. The M-VATs AnTat 1.30 and 1.45 were detectable at maximum percentages on day 3 and were undetectable by day 5. The other known M-VAT, AnTat 1.6, was detectable as less than 10% of the population from day 3 to day 9. AnTat 1.5 was detectable on day 4, reached maximum percentage on day 10 and was eliminated from the population by day 11, i.e. at a time corresponding to the drop in parasitaemia. AnTat 1.19 was undetectable before

Table III

Effect of neutralization of specific M-VATs in metacyclic populations on the VAT composition of subsequent parasitaemias in mice as shown by immunofluorescence reactions on blood samples from single mice

Neutralising anti-serum	Days after infection	Percentage of population labelled with Antisera against AnTats											
		2	3	5	6	7	8	14	15	18	19	30	45
Anti-AnTat 1.30	3	0	0	0	0	0	0	0	0	0	0	0	10
	4	0	1	0	1	1	0	0	0	0	0	0	14
	5	0	0	1	1	1	0	0	2	1	1	0	10
	6	2	8	2	2	8	1	0	8	6	5	0	0
	7	2	4	1	1	8	0	0	9	5	10	0	0
	8	5	6	8	4	9	1	0	28	0	17	0	0
	9	5	6	6	5	13	3	0	31	0	24	0	0
Anti-AnTat 1.45	4	0	0	0	1	0	0	0	0	0	1	20	0
	5	0	0	0	3	4	0	0	1	0	3	21	0
	5	1	7	0	4	4	0	0	5	7	12	9	0
	7	2	5	1	4	3	1	0	6	6	9	0	0
	8	2	5	2	10	11	0	4	18	4	13	0	0
	9	5	7	6	10	13	0	0	24	0	16	0	0
Untreated	4	0	0	0	0	0	0	0	0	0	0	15	10
	5	0	0	0	1	0	0.5	0	1	1	1	5	1
	6	0.5	2	2	2	5	1	0	2	1	4	0.5	0
	7	0	7	0	1	6	0	1	13	6	11	0	0
	8	1	7	3	1	4	1	4	15	2	12	0	0
	9	3	8	1	3	4	1	1	32	0	22	0	0

day 8 and only after the remission of the parasitaemia did the percentage of trypanosomes labelled with anti-AnTat 1.19 serum increase. AnTat 1.19 was the major VAT present in the second peak parasitaemia.

In the irradiated host (Figure 2C) the M-VATs AnTat 1.30 and 1.45 also reached maximum percentages on day 3 and, as in non-irradiated mice, the percentage of trypanosomes labelled decreased on day 4 and 5. However, neither VAT was eliminated completely; AnTat 1.45 was detectable until day 7 and AnTat 1.30 was present in the parasitaemia until the end of the experiment on day 14, though as a reduced percentage of the population. The non-M-VATs AnTat 1.5 and 1.19 were detectable on the same day as in the non-irradiated mouse but never accounted for more than 10% of the population. Both were detectable until day 14 when the experiment ended.

Discussion

Work by Broom and Brown (1940) and Gray (1965, 1975) suggested that following cyclical transmission through the tsetse fly, stocks of T. brucei (including T.b. gambiense) revert to a stock specific "basic" antigen. In his 1965 paper Gray reported that flies which had ingested different VATs "transmitted trypanosomes with either the basic strain antigen only or a mixture of the ingested variant and the basic strain antigen". It is important to recall that the transmitted VATs were identified indirectly by looking for agglutinating antibody production to these VATs in the infected mammalian host.

The metacyclics from the AnTAR 1 serodeme of T. brucei are heterogeneous with respect to VAT (Le Ray et al., 1978; Barry et al., 1979) and preliminary results have shown that following infection of mice with AnTAR 1 metacyclics the trypanosomes rapidly undergo antigenic variation; non-M-VATs are detectable after day 4 or 5 of the infection (Barry et al., 1979). Other workers have reported the existence of heterogeneous populations in the blood of mice infected with metacyclics from other serodemes (Jenni, 1977a, b; 1979; Hudson, 1980; Stanley et al., 1979).

Results presented in this paper confirm the presence of M-VATs in the bloodstream of tsetse-bitten mice up to 5 days after fly bite. The elimination of the M-VATs on day 5 corresponds with the detection of lytic antibodies against M-VATs. The detection of anti-metacyclic antibodies in the blood at this time might be taken as evidence that antibody acts as a stimulus for the trypanosome to switch from expressing M-VATs to expressing the other early bloodstream VATs. This seems unlikely, however, since in immunosuppressed mice infected with metacyclics, non-M-VATs were detected as early as day 4 and the percentage of cells labelled with anti-metacyclic serum decreased on day 5. In the immunosuppressed animals the M-VATs persisted for longer in the infection. It appears therefore that the M-VATs, in the bloodstream of the metacyclic infected mice, are replaced by non-M-VATs, in the absence of antibody and that antibody eliminates the bloodstream trypanosomes expressing M-VATs only after a portion of the population has changed its VAT. This pattern of antigenic variation was proposed by Van Meirvenne et al (1975) to account for the presence of minor VATs in cloned bloodstream populations of T. brucei.

Our detailed analysis of the influence of the ingested VAT on the bloodstream VATs arising following cyclical transmission, supports Gray's (1965) finding that the ingested VAT is often present in the first parasitaemia of tsetse bitten animals. In our experiments, regardless of which of the 5 VATs was ingested by the infecting tsetse, the 3 M-VATs were identified on days 3 and 4 in the cyclically-infected mouse. From day 4 to day 10, however, up to 18 different VATs were identified in the blood of mice, illustrating how rapidly the cyclically-transmitted trypanosomes undergo antigenic variation. After proliferation of M-VAT trypanosomes in the bloodstream, the VATs detected from day 4 included the ingested VAT. The ingested VAT did not necessarily become the major VAT in the first peak parasitaemia, perhaps owing to differences in virulence of the different VATs (Seed, 1978). The accompanying VATs in the first parasitaemia were also influenced by the ingested VAT, although certain VATs (AnTat 1.3 and 1.7) were always present as major types in the first population.

The expression of the same repertoire of M-VATs in both the salivary glands and the bloodstream of tsetse-bitten mice, regardless of the VAT ingested by the tsetse, and the detection of the ingested VAT in the bloodstream of mice following cyclical transmission suggests that the genes specifying the M-VATs and the genes for the bloodstream VATs might have separate control mechanisms.

Further support for this hypothesis comes from the observation that, in the first parasitaemia, a given I-VAT is accompanied by a characteristic spectrum of associated VATs, suggesting that expression of the latter is linked to the expression of the I-VAT. The neutralization of M-VATs experiment described here suggests, however, that suppression of one of the M-VATs has no effect on the non-M-VAT composition of the subsequent first bloodstream population. The tendency of the ingested VAT to be expressed in the first parasitaemia of tsetse bitten mice would suggest that the modifications in the DNA sequence leading to expression of a particular variable surface glycoprotein (VSG)(Cross, 1975; Hoeijmakers, et al., 1980, a, b) might persist even in the insect stages where the VSG is not detectable. In the salivary glands a different promoter sequence might activate the metacyclic VSG genes. The ingested VAT might have a greater chance of being expressed in the bloodstream following cyclical transmission if the gene for the VSG of this VAT was present as an expression-linked copy but in an inactive form. A similar situation might be the presence of an inactive expression linked gene for VAT 121 in nuclear DNA of VAT 221 from T. brucei 427 described by Hoeijmakers et al. (1980 b).

Gray's results, suggesting the presence of a basic antigenic type in cyclically transmitted T. brucei populations must now be re-evaluated on the basis of our findings. We find that, regardless of the VAT used to infect the tsetse, a characteristic set of M-VATs are present in the salivary glands of the fly and in the bloodstream of fly-bitten mice. The techniques of immunofluorescence and trypanolysis identify the VAT of individual

trypanosomes, not that of the major component of the trypanosome population as do agglutination reactions. Thus the mixtures of M-VATs detected both in the salivary glands, and in the blood-stream might be undetectable using agglutination reactions. The existence of a single basic VAT for each serodeme now seems unlikely. A basic repertoire of M-VATs expressed by trypanosomes in both the salivary glands and the bloodstream of tsetse bitten mice seems, however, a reasonable possibility.

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Part II.

STUDIES ON THE RELATIONSHIP OF KINETOPLAST DNA STRUCTURE TO MITOCHONDRIAL FUNCTION IN TRYPANOSOMATIDAE

1. INTRODUCTION

1.1 GENERAL

The kinetoplast DNA (kDNA) of the Kinetoplastida is a unique mitochondrial DNA (mtDNA) composed of thousands of covalently closed circular molecules interlocked into a complicated network structure (Simpson, 1972; Borst & Hoeijmakers, 1979a; Englund, 1980). Despite the large amount present per cell, by analogy with the mtDNA of other eukaryotes, kDNA seems likely to contain the genetic information for only a very limited number of essential peptides and mitochondrial RNAs required by the mitochondrial protein synthesizing system and mitochondrial metabolic pathways. The structure, replication, and transcription of mtDNA has been extensively reviewed (Borst, 1972, 1977, 1980; Borst & Grivell, 1978; Grivell *etal.* 1979; Linnane & Nagley, 1978; O'Brian, 1977; Schatz & Mason, 1974; Wolstenholme, *et al.*, 1974) and I present here only a few important features of mtDNA, in particular that of yeast, as an aid to considerations of kDNA in the papers which follow.

1.2 MITOCHONDRIAL DNA STRUCTURE

All eukaryotes capable of synthesizing functional mitochondria have mtDNA. With the exceptions of *Tetrahymena* and *Paramecium*, which contain linear duplex mtDNA, the mtDNA of all organisms studied exists as covalently closed circular duplex molecules and varies in molecular weight from about 10×10^6 daltons in higher animals to about 70×10^6 daltons in higher plants. The mitochondrial genome only specifies a small fraction of the components necessary for mitochondrial biogenesis. Nuclear genes specify the remaining components which are imported into the mitochondrion after translation on cell sap ribosomes (Chua & Schmidt, 1979); duplicate copies of the mitochondrial genes are not present in the nuclear genome. The mitochondrial gene products of yeast are shown in Table 1. An interesting feature of the peptides coded for by the mtDNA

GENE PRODUCTS OF YEAST MITOCHONDRIAL DNA

TABLE 1.

Mitochondrial complexes	Number of subunits			Mitochondrial gene product	Associated genetic loci
	Total	Made on cell sap ribosomes	Made on mitochondrial ribosomes		
Cytochrome C oxidase	7	4	3	Subunit I	oxi-3
				Subunit II	oxi-1
				Subunit III	oxi-2 (?)
Cytochrome b c_1 complex	7	6	1	Cytochrome b	cob-1, cob-2 box$_r$-1 to 7 ana$_r$, fun^r, din^r muc^r
ATPase complex	9	5	4	Subunit (?)	pho-1
				Subunit (?)	oli-2
				Subunit 6	oli-3
				Subunit 9	oli-1, pho-2
Ribosome	52	51	1	Ribosome ass't. protein	var-1, var-2
				21s RNA	rib$_r$-1,2,3 cap^r, spira^r, ery^r
				15s RNA	
				26 tRNAs	syn$^-$ mutants

is that most are enzyme complexes which are functional only if the mitochondrial and nuclear gene products are present.

Mutants in yeast which have lost from 20 to 99.9% of their mitochondrial genome are incapable of mitochondrial protein synthesis (Borst & Grivell, 1978). These cells are viable under anaerobic conditions, however, since mitochondrial activity is completely repressed. Such petite mutants, ρ^-, contain a mtDNA which is equal in size to the wild type, ρ^+, mtDNA. The sequence retained by the ρ^- cells appears to have been amplified until a wild type size mtDNA molecule is achieved. There does not appear to be a tendency for any particular sequence to be retained and amplified, since petites retaining various portions of the genome have been described. Why the amount of mtDNA remains constant is a mystery, however, Borst and co-workers (Borst et al., 1976) have suggested that molecules of ρ^+ size are preferentially replicated with a "sizing step" being involved.

Recent studies have shown that at least 3 genes in the mtDNA of yeast contain intervening sequences (introns) similar to those found in nuclear genes (Breathnach et al., 1977; Jeffreys & Flavell, 1977). The large, 21s ribosomal RNA (rRNA) gene contains an intervening sequence of 1,200 base pairs (bp) while the apo-cytochrome b gene contains 4 intervening sequences of 2,000, 1,400, 1,200 and 650 bp (Bos et al., 1979, 1980; Grivell et al., 1979). In addition, the gene for subunit I of cytochrome oxidase probably contains intervening sequences (Grivell et al., 1979). Another mitochondrial gene of yeast, that coding for subunit 9 of the ATPase complex, has been subjected to complete sequence analysis and the base pair sequence compared with the amino acid sequence of the peptide. No intervening sequences are present in this gene. The processing of the split gene transcripts is discussed later.

The mitochondrial genome of yeast has been extensively studied by techniques using genetic mapping (Schweyn et al., 1976, 1978;), physical mapping with restriction endonucleases (Morimoto et al., 1975, 1977; Sanders et al., 1977) and more recently mapping of transcripts (Grivell et al. 1979; Van Ommen, 1977). Figure 1 shows the physical and genetic map of Saccharomyces cerevisiae mt DNA.

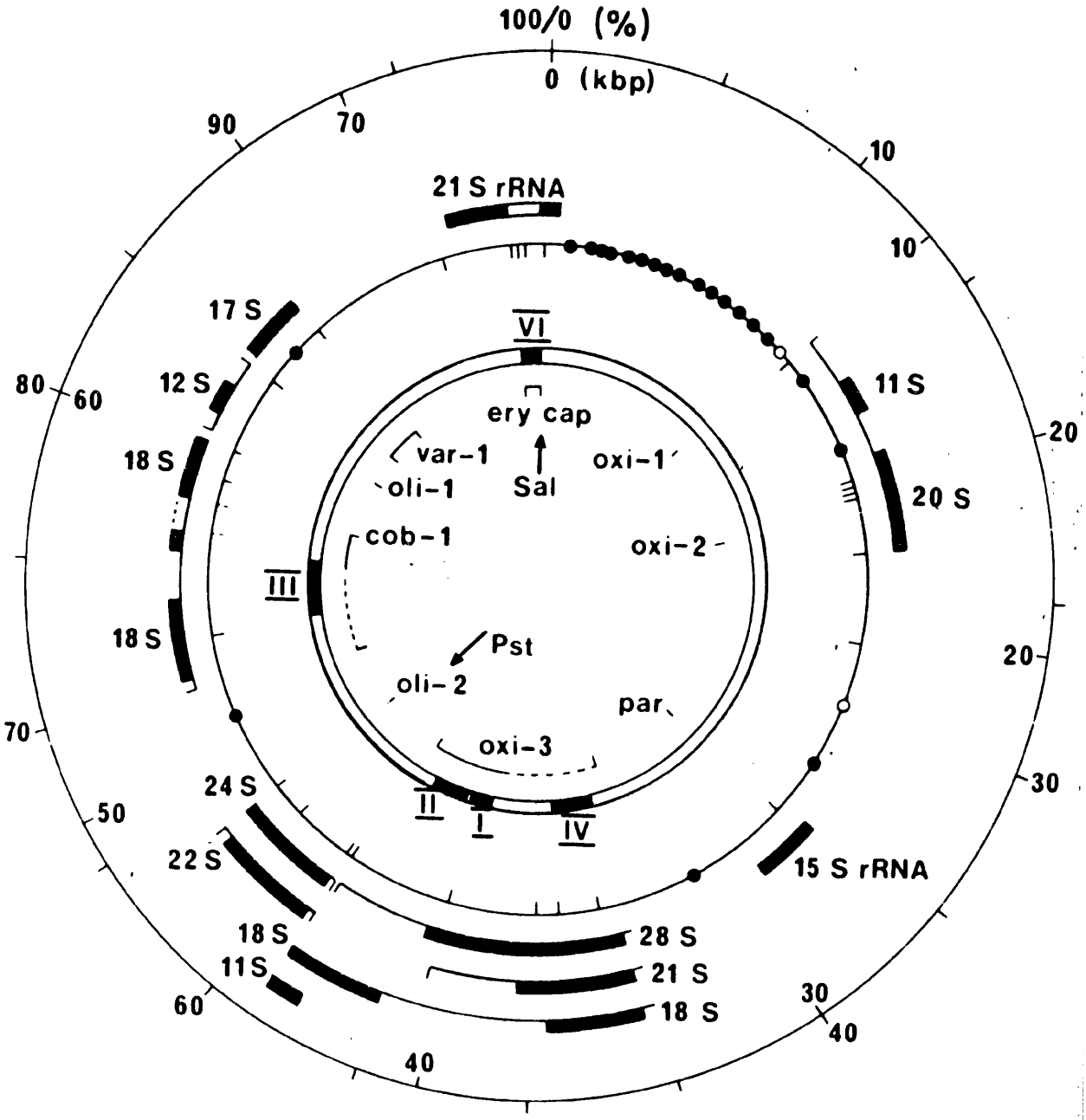
1.3 MITOCHONDRIAL DNA REPLICATION

Replication of mtDNA is semi-conservative and completely independent of nuclear control. Most studies on the replication of mtDNA have been on the process in vivo since, despite many elegant attempts, complete replication of mtDNA molecules in vitro has not been achieved (Eichler et al, 1977). Circular mtDNA appears to replicate according to the Cairns model (Cairns, 1963) of replication for circular viral DNA (Robberson et al., 1972; Kasamatsu et al., 1971). Replicative intermediates have been identified by electron microscopy and replication is initiated by the formation of small displacements loops (D-loops) and DNA synthesis preceeds unidirectionally with the light strand serving as a template. Semi-conservative replication is completed by replication of a new light strand using the old heavy strand as a template.

Replication of the linear mtDNA of Tetrahymena (Arnberg et al. 1974; Cleggs, et al., 1974) and Paramecium (Goddard & Cummings, 1975) differs from that of the closed circular mtDNA of other organisms. The mtDNA of Tetrahymena replicates bidirectionally from a fixed starting point with semiconservative replication taking place on both stands simultaneously. Paramecium mtDNA is replicated unidirectionally and replicated molecules exist as linear dimers which must later be cleaved into 2 equal molecules.

Figure 1.

Genetic and physical map of yeast mtDNA. The genetic map is based on the co-retention of markers in petite mutants (Schweyen et al. 1978). The markers are indicated within the inner ring (see table 1 also). The black bars in the inner ring represent the major insertions present in this mtDNA. The outer ring gives the positions of recognition sites for endonucleases HindII + III and EcoR I and the approximate position of 4s RNA genes (Van Ommen et al., 1977). The open circles are $tRNA_{met}$ genes. The approximate positions of other transcripts are given outside the outer ring (Van Ommen and Groot, 1977), the bars indicating uncertainty in the exact positions. The open part of the 21s rRNA represents the intervening sequence. Sal and Pst indicate the single recognition sites for restriction endonculeases Sal I and Pst I respectively. (Figure taken from Borst and Grivell, 1978).


100/0 (%)
0 (kbp)
10
20
30
40
50
60
70
80
90
21 S rRNA
17 S
12 S
18 S
18 S
24 S
22 S
18 S
11 S
28 S
21 S
18 S
15 S rRNA
11 S
20 S
VI
III
II
I
IV
ery cap
var-1
oli-1
Sal
oxi-1
cob-1
oxi-2
Pst
oli-2
par
oxi-3

1.4 MITOCHONDRIAL DNA TRANSCRIPTS

Transcripts of the mitochondrial genome, are limited to mitochondrial rRNAs, transfer RNAs (tRNAs) and a few messenger RNAs (mRNAs) which are translated within the mitochondrion (Table 1). The proteins synthesized in the mitochondrion represent only about 5% of the total mitochondrial protein and the advantage in retaining these few extra-nuclear genes is still not obvious. Although mitochondrial transcripts have been studied from a variety of organisms the most extensive analysis has been undertaken with yeast (Grivell et al., 1979). A detailed transcription map of S. cerevisiae is given in figure 1. It seems likely, from the transcript mapping, that all genes present on the mtDNA of yeast have been recognized since transcripts to all the genetic markers on the mitochondrial genome have been identified.

The presence of intervening sequences in at least 3 mtDNA genes has been discussed in section 1.2. It seems likely that the 21s rRNA, apo-cytochrome b gene and the gene for subunit I of cytochrome oxidase are transcribed as large precursor molecules which are later processed (Bos et al., 1978). Arnberg et al. (1980) and Hilbriech et al. (1980) have recently found covalently closed circular RNA molecules in the 11s and 18s fraction of yeast mitochondrial RNA. These circular molecules hybridize with fragments of the cytochrome oxidase gene and may represent active mRNA of this gene. The circular RNA molecules may also be nuclease resistant information storage forms or by-products of processing of larger mRNA precursor molecules.

How mitochondrial transcription is regulated, even in the extensively studied yeast system, is still poorly understood. However, the presence of mRNA precursor molecules containing intervening sequences and circular RNA molecules suggests that processing of transcripts may play an important role in regulation.

1.5 KINETOPLAST DNA

Since the discovery by Riou and co-workers (Riou & Paoletti, 1967; Riou & Delain, 1969) that the kinetoplast of Trypanosoma cruzi was composed of thousands of small circular molecules interlocked to form a network structure, kDNA has become the most extensively studied protozoan DNA. This interest in kDNA of trypanosomes is stimulated not only by the intriguingstructural organization of the kDNA network but also because many of the chemotherapeutic compounds used in the treatment of trypanosomiasis bind perferentially to the kDNA (Williamson, 1976, 1970; Hajduk, 1978). The kDNA of trypanosomatids has been reviewed extensively (Borst & Fairlamb, 1976; Borst & Hoeijamkers, 1979a, b; Borst et al., 1980; Cosgrove, 1973; Englund, 1980; Newton, 1979; Vickerman & Preston, 1976). I shall briefly summerize a few of the most important features of kDNA structure, replication and function.

1.6 KINETOPLAST DNA STRUCTURE

The in situ organization of the kDNA has been studied by electron microscopy of sectioned fixed and stained material. As discussed by Simpson (1972) the appearence of the kDNA is dependent upon the fixation procedure used. In most members of the order Kinetoplastida the kDNA appears as an electron dense fibrous band, 0.08 μm to 0.5 μm in width, bounded by the double mitochondrial membrane. The kDNA is situated in a portion of the cell's single mitochondrion (Paulin, 1975) which is adjacent to the basal body of the flagellum. The relationship of the kDNA to the flagellum, if any, is completely unknown.

The in situ appearence of the kDNA is quite variable, in particular among the free-living Bodonidae (Brooker, 1971; Vickerman, 1977; Vickerman & Preston, 1976). In some members of the Bodonidae and also the trypanosomatid Herpetomonas muscarum ingenoplastis (Section 3.)

the kDNA appears in section as a loosely arranged bundle of long fibres extending from the adbasal mitochondrial membrane at least 2.5 μm into the mitochondrion (Vickerman & Preston, 1976; Wallace et al., 1973). Other bodonids apparently lack an organized network of kDNA since small bundles of fibrous material are seen throughout the mitochondrion (Vickerman, 1977). Because of the difficulties in growing the Bodonidae axenically, the molecular organization of their kDNA has not been studied. The kDNA network can be isolated from many species of parasitic trypanosomatid with relative ease due to its high molecular weight, high adenine-thymine content and covalently closed form (Table 2). Isolated kDNA networks vary in size form 4 μm to 20 μm in diameter, and molecular weight form 0.4 to 4 $x10^{10}$ daltons. Preparations of kDNA spread for electron microscopy give the impression of a "fishnet" like structure (figure 2) mainly composed of catenated small circular molecules, mini-circles (Renger & Wolstenholme, 1971, 1972; Riou & Delain, 1969), but also containing a small number of longer molecules usually seen as loops at the edge of the network, as apparently linear molecules, or as occasional free large circular molecules (Kleisen et al., 1976b; Steinert & Van Assel, 1976; Simpson & De Silva, 1971). These larger-than-mini-circle molecules in kDNA preparations could either be contamination with nDNA (Nichols & Cross, 1976), oligomers of mini-circles (Barker, 1980; Newton, 1979), or large circular kDNA molecules, maxi-circles (Kleisen et al., 1976; Steinert & Van Assel, 1976). As discussed later, it is likely that maxi-circles and mini-circles are the only components of the kDNA network.

1.6.1 MINI-CIRCLES

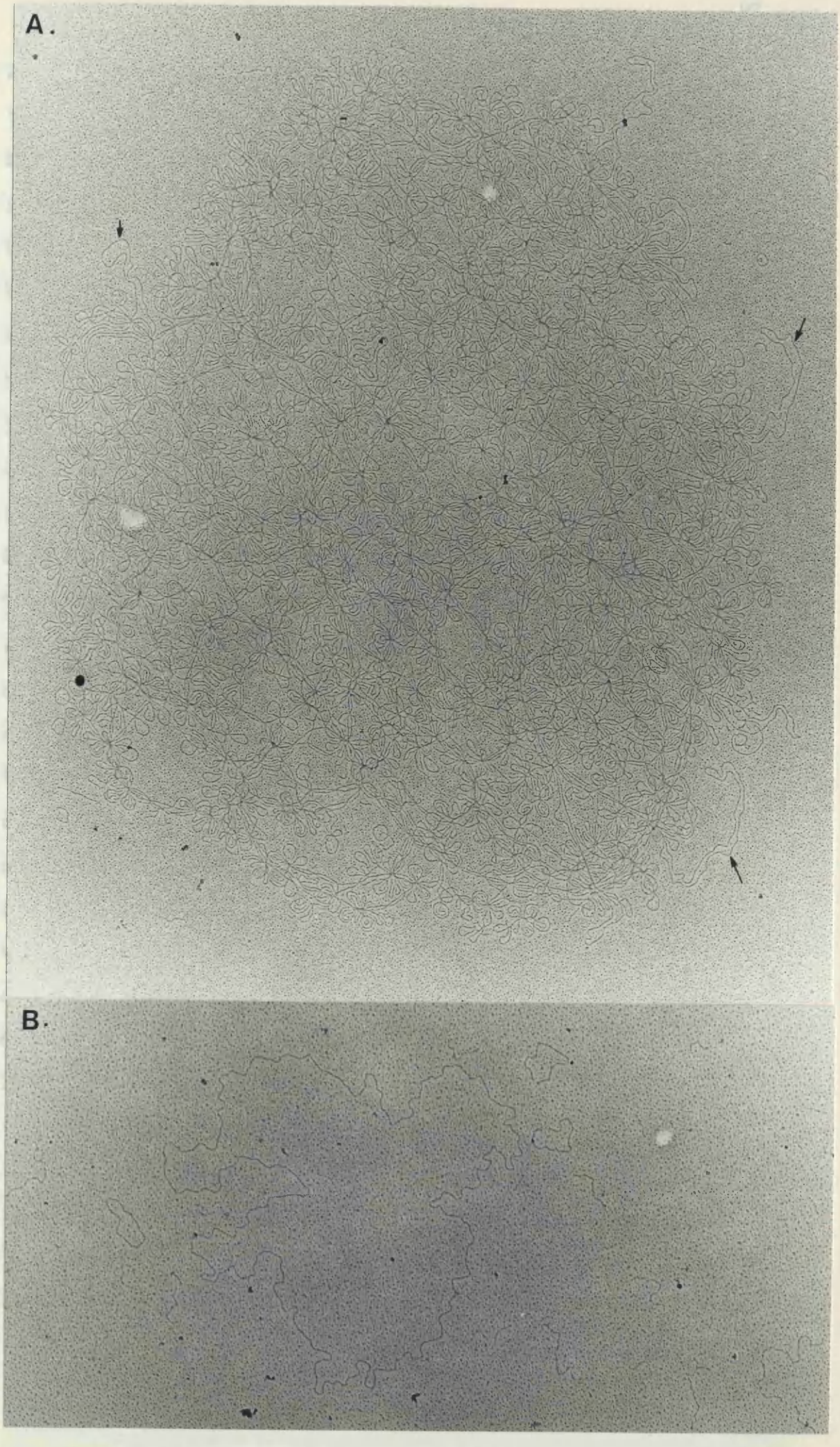
The kDNA network of most species of trypanosomatids is composed primarily of mini-circles which form about 95% of the network by mass. Mini-circles are closed, duplex molecules varying in size from 900 to

PROPERTIES OF kDNA FROM SOME KINETOPLASTIDA

Organism	Mini-circles			Maxi-circles		Mass of network (daltons x10^{-9})	Diameter of spread network (μm)	kDNA width in situ (μm)
	Size (μm)	Mass (x10^{-6})	Sequence heterogeneity	Size (μm)	Mass (x10^{-6})			
Crithidia								
C. acanthocephali	0.80	1.54				41		0.5
C. fasciculata	0.79	1.59	++	12	23		15-20	0.23-0.29
C. luciliae	0.76		++	11.3	23	22		0.43
Herpetomonas								
H. muscarum	0.39	0.7	++		21			
H. ingenoplastis	absent			11.3	24			2.5
Leishmania								
L. tarentolae	0.29	0.55	+		20	10	8-10	0.08
Schyzotrypanum								
T. cruzi	0.49	0.94	+		26	22-60	10-15	0.11-0.43
Phytomonas								
P. davidi	0.37	0.70	++		24			
Trypanozoon								
T. brucei	0.32	0.56	+++	6.3	13	4	4-6	
T. evansi	0.3		-	absent			4-6	
T. equiperdum	0.31		±	absent			4-6	
T. mega	0.74	1.49	+++	9.0	16.1		12-15	0.25

Figure 2.

Electron micrographs of isolated kDNA. A. kDNA network from T. equiperdum spread for electron microscopy following isolation by high speed pelleting and cesium chloride density gradient ultracentrifugation. Networks contain catenated mini-circles and long edge loops, presumably maxi-circles (arrows). X22,000. B. Free maxi-circle and mini-circles in kDNA isolated from Crithidia fasciculata. The mini-circles measure about 0.8 μm; the maxi-circle 11.8 μm. X 32,000.


A.
B.

2500 bp and it is estimated that about 10^4 mini-circles are catenated together to form the kDNA network (Borst & Fairlamb, 1976; Borst & Hoeijmakers, 1979a; Englund, 1980). Restriction endonuclease digestion product analysis of mini-circles from several species of trypanosomatid has shown sequence micro-heterogeneity (Riou & Yot, 1975; Kleisen & Borst, 1975; Borst & Hoeijmakers, 1979a; Englund, 1980). Reassociation studies of T. brucei by Steinert and co-workers are in agreement with a high degree of sequence heterogeneity in T. brucei mini-circle with up to 300 sequence classes being present. Steinert's group also identified a component with low complexity (Steinert & VanAssel, 1976, 1980) which they interpreted as a common sequence of about 200 nucleotides. Studies by Donalson et al. (1979) and Chen & Donalson (1980) using cloned T. brucei mini-circles for hybridization and sequencing studies, also showed extensive heterogeneity in the mini-circles but also regions of homology. The function of these common sequences in the T. brucei mini-circle is unknown although Chen & Donalson(1980) suggest that a transcription initation codon may be present in this region of the mini-circle. This site might also function in the initiation of mini-circle replication or represent a specific site of reattachment of replicated free mini-circles (see Section 1.7). The mini-circles of all other trypanosomatids studied fail to show extensive sequence heterogeneity seen in T. brucei (Borst et al. 1977, Challberg & Englund, 1979; Cheng & Simpson, 1978; Leon et al. 1980; Kleisen et al., 1976a) although limited sequence heterogeneity was detected in all but T. equiperdum (Riou & Saucier, 1979) and T. evansi (Borst & Hoeijmakers 1979b). The mini-circle nucleotide sequence is rapidly evolving since differences in the restriction enzyme analysis of mini-circles from Crithidia luciliae were detected after 3 years maintainance in culture (Borst & Hoeijmakers, 1979a,b).

1.6.2 MAXI-CIRCLES

Because of their small size and sequence heterogeneity and the lack of transcripts in at least 2 species (Section 1.8) the mini-circles seem an unlikely candidate for the genetically functional mtDNA of trypanosomatids. However, until the discovery by Steinert & Van Assel (1975) of free large circular DNA molecules and homogeneous high molecular weight restriction endonuclease fragments by Kleisen et al. (1976) in highly purified kDNA, no other mtDNA had been identified in trypanosomatids. Maxi-circles have now been described for all trypanosomatids studied which can make functional mitochondria.

Maxi-circles vary from 20 to 39 kilo bases (kb) depending on species but there appears to be a high degree of sequence conservation in the maxi-circles from different species (Borst & Hoeijmakers 1979a,b). The number of maxi-circle molecules per cell is low, about 50 copies, and restriction endonuclease analysis suggests a unique nucleotide sequence. Restriction endonuclease maps of maxi-circles from several species are now available (Borst & Fase-Fowler, 1979; Masuda et al., 1979; Stuart, 1979). Although it is difficult to visualize the arrangement of the maxi-circles in the kDNA network because of the accompanying large number of densely-packed mini-circles, two possible arrangements seem likely: either the maxi-circles are simply catenated with the mini-circles in a random fashion, or the maxi-circles are catenated to each other with limited interaction with the mini-circles.

Maxi-circles can be separated from mini-circles on the basis of their higher A-T content (Simpson,1979). In T. brucei the A-T rich nature of the maxi-circle is at least in part due to a segment making up roughly 5 kb of the 20.5 kb molecule which is extremely A-T rich and because of this virtually devoid of restriction endonuclease sites. Borst and co-workers

show that this region of the maxi-circle varies in size in 9 stocks of T. brucei analysed while the rest of the maxi-circle sequences are highly conserved. Based on its size, number of copies per cell and unique, conserved sequence the maxi-circle seems likely to be the trypanosomatid's "true" mitochondrial DNA.

1.7 KINETOPLAST DNA REPLICATION

Early studies on the ultrastructure of several species of trypanosomatid provided information on the structure of replicating kDNA networks (Anderson & Hill, 1969; Burton & Dusanic, 1968; Simpson, 1972). In most species studied by transmission electron microscopy of sections, the kDNA band undergoes lateral elongation until it is about twice its normal length then the kDNA and its surrounding mitochondrial membrane are constricted into 2 daughter kinetoplasts. This duplication of the kDNA band and segregation of the daughter kinetoplasts takes place during a discrete portion of the cell cycle (Cosgrove & Skeen, 1970). The kDNA mass of at least a few trypanosomatids duplicates by vertical rather than horizontal fission (Brack, 1968; Paulin & McGhee, 1971).

Simpson et al. (1974) and Manning & Wolstenholm (1976) have shown that kDNA replication is semiconservative and that each mini-circle is replicated once during a cell cycle. Because of the large number of mini-circles and the catenated structure of the kDNA network, the mechanisms of kDNA replication and segregation are likely to be complex. Recently, Englund (1978, 1979) has presented a model for kDNA replication based on available experimental evidence involving replication of free mini-circles. During the G_1 phase of the cell cycle all of the mini-circles in the kDNA network are covalently closed (Form I). Mini-circles are detached from the network possibly by a topoisomerase-like enzyme (Marini et al. 1980)

during the S phase and replicate by a Cairns-type of mechanism as free mini-circles (Englund, 1979). Following replication the free mini-circles, in a nicked, open, configuration, reattach to the periphery of the network. A topoisomerase-like enzyme might mediate this step as well. Thus for every covalently closed mini-circle which detaches, 2 nicked mini-circles attach following replication resulting in the kDNA network increasing in size. The finished product of kDNA replication is a network twice the size of the starting Form I network and contains only nicked mini-circles; these are called Form II networks. Finally there is a division of the double sized Form II network into 2 equal progeny followed by covalent closure of all the mini-circles.

Replication of the maxi-circle component has not been studied in detail although Hoeijmakers & Weijers (1980) have described the distribution of maxi-circles during replication and segregation in spread preparations of kDNA from T. brucei. Four distinct types of network were identified on the basis of shape, size, number and location of maxi-circle loops and the nicked or covalently closed nature of the mini-circles and maxi-circles.

1.8 KINETOPLAST DNA FUNCTION

Two approaches have been used in studies on the function of kDNA. The first involves the isolation and characterization of kDNA transcripts. The second has taken advantage of mutant bloodstream forms of the African trypanosomes which lack functional mitochondria and cannot multiply in the insect vector (Borst & Fairlamb, 1976). These mutants often have detectable alterations in their kDNA compared with the "wild type".

The transcripts from purified mitochondria have been analysed by Simpson and co-workers for Leishmania tarentolae (Simpson & Simpson, 1978)

and Phytomonas davidi (Cheng & Simpson, 1978). The major RNA's present are 9s and 12s and these selectively hybridize with the maxi-circle fragments following electrophoresis and transfer to nitrocellulose filters (Southern, 1975); no hybridization with mini-circle fragments was detected. The localization of the 9s and 12s RNA genes on a restriction map of the maxi-circle of Leishmania tarentolae has recently been reported (Masuda et al., 1979).

Borst and co-workers (Borst & Hoeijmakers, 1979a, b; Borst et al., 1980; Hoeijmakers & Borst, 1978) have also studied kDNA transcripts. By hybridizing total cellular RNA with endonuclease digests of kDNA blotted onto nitrocellulose filters, Hoeijmakers & Borst (1978) detected hybridization with portions of the maxi-circle of C. luciliae but not the mini-circle fragments. These workers have also hybridized fragments of the T. brucei maxi-circle, which have been cloned in E. coli, with RNA fractionated on agarose gels and transferred to diazobenzyloxymethyl-cellulose paper. Although their cloned fragments cover only about half of the T. brucei maxi-circle they detect hybridization with the 9s and 12s RNAs and at least 6 minor RNAs. All the minor transcripts contain poly A tails and are likely to be mitochondrial mRNAs. As discussed by Borst et al. (1980) the 9s and 12s RNAs are most likely to be the mitochondrial rRNAs of trypanosomatids, though somewhat smaller than other rRNAs. Fouts and Wolstenholme (1979) have obtained results using Crithidia acanthocephali that suggest that transcripts of a portion of the mini-circle may be present. Considering the inability of Borst's and Simpson's groups to detect mini-circle transcripts in T. brucei, C. luciliae, P.davidi or L. tarentolae these results are indeed suprising and certainly additional experiments are required to characterize better the mini-circle transcript of C. acanthocephali.

During the portion of the life cycle of African trypanosomes in the bloodstream of the mammalian host (figure 1, Part I, Section 1.1)

mitochondrial activities are repressed and the trypanosome survives solely on the ATP generated by glycolysis (reviewed by Bowman & Flynn, 1976); upon ingestion by the insect vector cytochrome-mediated mitochondrial respiration begins. This is analagous to the anaerobic to aerobic switch in respiration of yeast (Borst & Hoeijmakers, 1979a, b). As is found in the mtDNA of yeast, the bloodstream African trypanosomes can survive alterations in the kDNA (Borst & Hoeijmakers, 1979a,b; Borst et al., 1980). These mutations in the kDNA are correlated with the inability to infect the insect vector (I^-) (Opperdoes et al., 1976; Borst & Fairlamb, 1976). The I^- mutants studied usually have detectable alterations in the kDNA or have completely lost their kDNA. The most drastic I^- mutants include the so called dyskinetoplastic populations of trypanosomes (Trager & Rudzinska, 1964) which may arise spontaneously or be induced by treatment with DNA-binding compounds (Hajduk, 1978). These forms lack a detectable kDNA in stained preparations but may retain remnants of the kDNA or other DNA dispersed in their mitochondrion (Hajduk, 1976, 1979; Stuart, 1971; Renger & Wolstenholme, 1971; Vickerman, 1977). Some dyskinetoplastic populations have been conclusively shown to lack any kDNA sequences (Borst & Hoeijmakers, 1979a,b). Several stocks of T. evansi have completely lost the maxi-circle component of the kDNA but the mini-circle network structure is retained (Borst & Fairlamb, 1976). Another I^- mutant, T. equiperdum (ATCC 30019) has a mini-circle, maxi-circle kDNA network but the maxi-circle component appears to have suffered a single 1.5 kb deletion in comparison with the maxi-circle of T. brucei 427 which can activate mitochondrial activities and infect the insect vector (Frasch et al. 1980; Hajduk & Cosgrove, 1979). Another class of I^- mutants containing maxi-circles which are normal by all criteria tested have been detected and are discussed later (Section 5.). The alterations, if any, in the maxi-circle must be minimal, ie. point mutations in specific maxi-circle genes. Another explanation might

TABLE 2.

THE STATE OF kDNA IN TRYPANOZOON SUB-SPECIES THAT HAVE LOST THE ABILITY TO MAKE FUNCTIONAL MITOCHONDRIA (I^- TRYPANOSOMES)

Organism	Stock	Oligo-sensitive ATPase	DAPI stained kDNA	Organized networks present	Maxi-circles (μm)	Mini-circles present	Mini-circle sequence heterogeneity	Heterogeneous circular DNA
T. brucei	427[a]	+	+	+	6	+	+	-
	31	-	+	+	6	+	+	-
	GUP 375	-	+	+	?	+	+	-
	LUMP 127	-	+	+	6	+	+	?
	LUMP 1027	-	-	-	-	-	-	-
T. equiperdum	ATCC 30019	?	+	+	5	+	±	-
	ATCC 30023	?	±	-	-	-	-	+
T. evansi	AMB 1	-	-	-	-	-	-	-
	AMB 2	-	-	-	-	-	-	-
	AMB 3	-	+	+	-	+	-	-
	GUP 477	?	?	+	-	+	-	-
	SAK	?	±	-	?	?	?	?
T. equinum	ATCC	?	±	-	?	?	?	+

a) I^+ control stock

be that mutations in nDNA genes coding for components of the mitochondrial protein synthesizing system have occurred.

Even though a wide range of changes in the kDNA and possibly in the nDNA may lead to the I^- phenotype, it is clear that all trypanosomes with detectable alterations in the maxi-circle or lacking the maxi-circle are incapable of mitochondrial activation. Table 3 summarizes some characteristics of the I^- trypanosomes available. The I^- mutants with alterations in the maxi-circle component of the kDNA, but retention of both mini-circles and network structure, support the hypothesis that the maxi-circles are the genetically functional unit in the kDNA. The function of the mini-circles, which make up 95% of the kDNA network is difficult to understand. However, it is clear that the mini-circles are essential for maintenance of the network structure and in every mutant where mini-circles are missing maxi-circles have also been lost. The possibility that mini-circles have a structural role in the kDNA network has been discussed by several workers (Borst & Fairlamb, 1976; Cosgrove, 1973).

1.9 ENERGY METABOLISM IN TRYPANOSOMATIDS

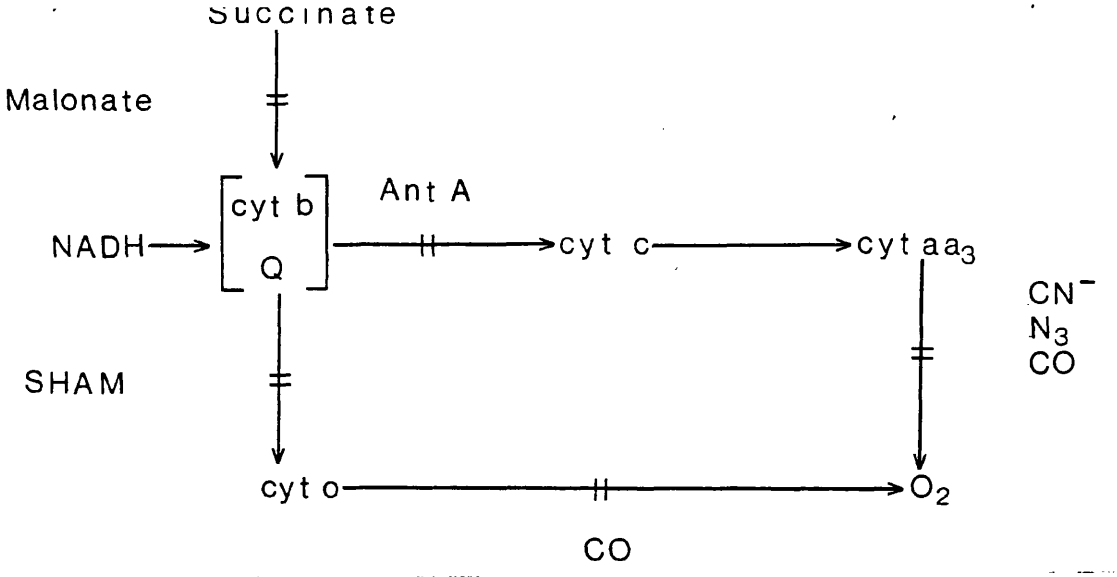
The oxidative metabolism of trypanosomatids has been reviewed by Bowman and Flynn (1976) and it is clear that the metabolic patterns of the members of this family are highly diverse. In most trypanosomatids oxidative metabolism of either carbohydrates or amino acids is complete, with CO_2 being the main product. A functional Krebs cycle is present and NADH is reoxidized by a cytochrome mediated electron transport chain. Morphologically these cells contain a single mitochondrion with numerous plate like cristae (Vickerman, 1965). Respiration in the insect trypanosomatids is inhibited to a large extent by cyanide, azide and antimycin A indicating that electron transport involves cytochromes b, c, and aa_3. The cytochrome chain of the insect trypanosomatids is apparently

branched since a portion of the respiration by these cells is insensitive to cyanide, azide and antimycin A. Figure 3 shows the branched electron transport system proposed by Ray and Cross for Trypanosoma mega (Ray & Cross, 1972) and described for several other species by Hill and co-workers (1976). The presence of cytochrome o as an alternative, cyanide insensitive, terminal oxidase has been controversial (Hill, 1976) but it now seems likely that an o-like cytochrome functions as a terminal oxidase in at least some species (Degn et al., 1977). Njogu et al. (1980) have recently examined the electron transport system of the procyclic trypomastigotes of T. brucei which resemble the developmental stages found in the midgut of the insect vector. Unlike the bloodstream forms of T. brucei the established procyclics have a branched electron transport system with about 60% of the respiration being cyanide sensitive and 30% being sensitive to SHAM an inhibitor of the glycerophosphate oxidase system in the bloodstream trypanosomes. Figure 3 shows this proposed branched pathway.

The bloodstream stages of T. brucei have a repressed mitochondrion, morphologically reduced in size and lacking cristae (Vickerman, 1965). Energy production is entirely by glycolysis, no functional Krebs cycle or cytochromes are detectable and respiration is insensitive to cyanide, azide, and antimycin A. NADH is reoxidized by the glycerophosphate oxidase system (figure 3) localized in the mitochondrion (Opperdoes etal., 1977). This oxidase system is apparently unique to African trypanosomes and is being extensively studied as a potential site for chemotherapy of African trypanosomiasis (Clarkson & Brohn, 1976; Opperdoes et al., 1976b; Van der Meer et al., 1979).

Figure 3.

Respiratory pathways in trypanosomatids. A. Branched cytochrome mediated pathway proposed by Ray & Cross (1972) for Trypanosoma mega and apparently present in most trypanosomatids from insects. B. The proposed arrangement of the respiratory pathways of the insect stages of T. brucei (Njogu et al., 1980). Red. and Ox., are unidentified components that allow transfer of electrons between the glycerophosphate oxidase (GPO) system and the cytochrome system. C. Glycolysis and respiration of the bloodstream stages of T. brucei (Njogu et al., 1980). FDP, fructose-1,6-diphosphate; GAP, glyceraldehyde-3-phosphate; αGP, α-glycerophosphate; DPG, 1,3-diphosphoglycerate; DHAP, dihydroxyacetonephosphate; and salicylhydroxamic acid, SHAM.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Ant A
NADH
cyt c
cyt aa3
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N3
CO
SHAM
cyt o
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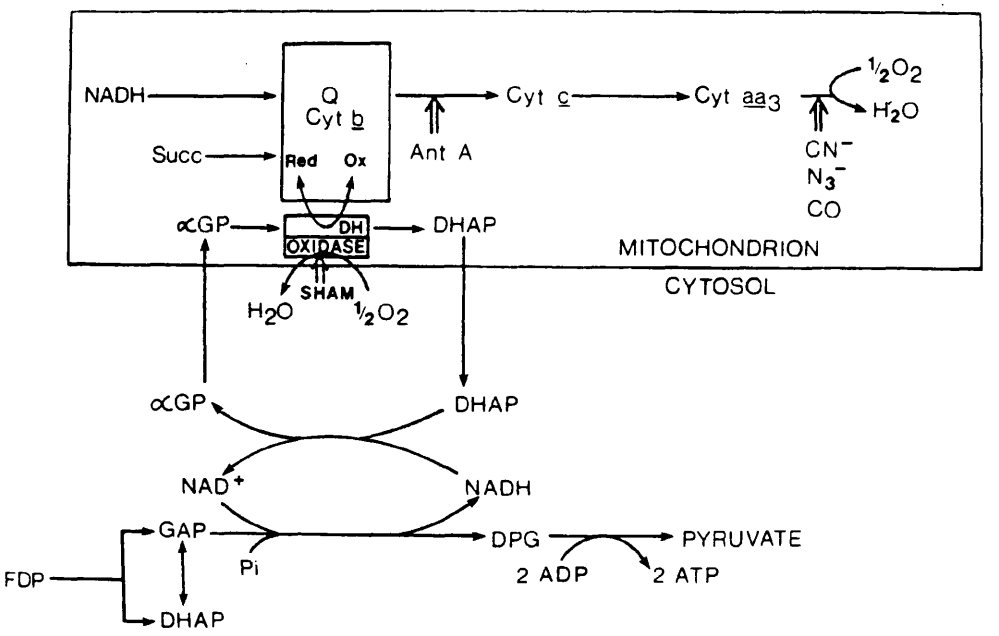
CO

B)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Cyt c
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Succ
Red
Ox
Ant A
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DH
OXIDASE
DHAP
MITOCHONDRION
CYTOSOL
SHAM
H2O
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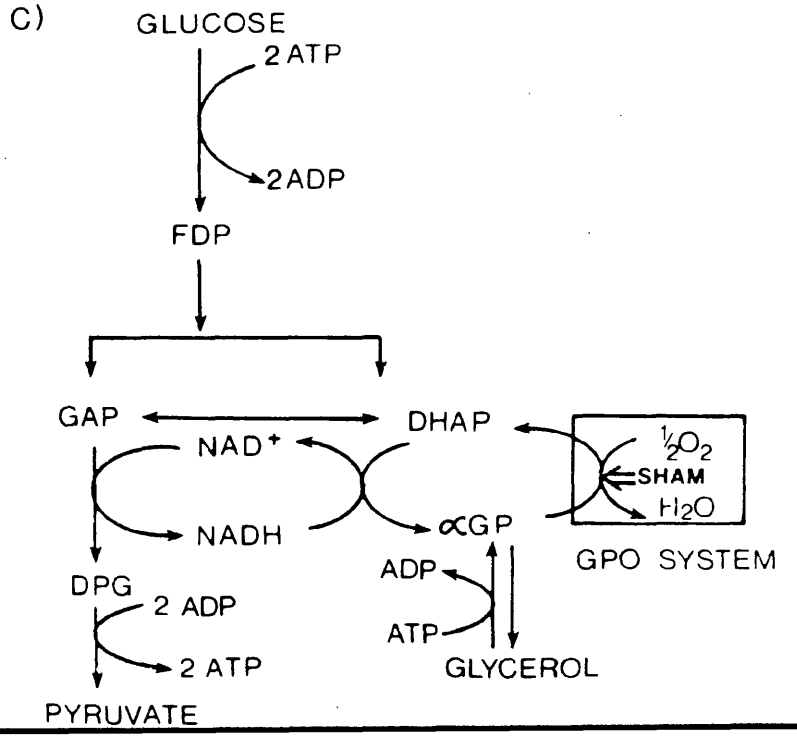
NAD+
NADH
GAP
DPG
PYRUVATE
FDP
Pi
2 ADP
2 ATP
DHAP

C)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2 ADP
FDP
GAP
DHAP
NAD+
NADH
½O2
SHAM
H2O
αGP
GPO SYSTEM
DPG
2 ADP
2 ATP
ADP
ATP
GLYCEROL
PYRUVATE

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2. PURPOSE OF INVESTIGATION

The kinetoplast DNA is the only DNA thus far found in the mitochondrion of trypanosomatids and recent evidence indicates that the maxi-circle is the equivalent of the mtDNA in other eukaryotes. The mini-circles, which make-up the bulk of the kDNA network, apparently are not transcribed in most species and perhaps function as structural elements in maintaining the kDNA network. Such a role would be unique for a DNA molecule.

To study the functional role of DNA making up the kDNA network I have selected cell populations which show morphological and biochemical characteristics which suggest alterations in mitochondrial biogenesis or alterations in kDNA structure. By analysis of these mutant populations some in sight into the function of the kDNA components and network might be obtained. I have attempted to correlate mutations in the kDNA structure, in particular in the maxi-circle, with changes in mitochondrial activities. In all other studies changes in kDNA structure have been restricted to the bloodstream stages of African trypanosomes where mitochondrial activities are repressed. One species I have studied, Herpetomonas ingenoplastis, is unique among the trypanosomatids in being able to survive in culture, at 26^{o}C, with a non-functional mitochondrion.

Relationship of kinetoplast DNA to mitochondrial activity in the flagellate protozoan Herpetomonas muscarum

I. Characterization of the kinetoplast DNA of two subspecies

Summary

We have compared the structure of the kinetoplast (kDNA) of Herpetomonas muscarum muscarum and Herpetomonas muscarum ingenoplastis by electron microscopy, restriction endonuclease digestion and hybridization with cloned portions of the maxi-circle from Trypanosoma brucei 427. The kDNA of both Herpetomonas subspecies has a buoyant density of 1.698 g/cm^3; however the kDNA of H.m. ingenoplastis represents 31% of the total cellular DNA as compared with 8% for H.m. muscarum kDNA. The kDNA network of H.m. muscarum consists of thousands of mini-cricles of 0.6 to 0.7 x 10^6 daltons and a few large circular molecules, maxi-circles, of 21 x 10^6 daltons. The mini-circles of H.m. muscarum show sequence heterogeneity while maxi-circles of H.m. muscarum have a unique sequence. The kDNA of H.m. ingenoplastis completely lacks mini-circle size molecules and the network is composed entirely of large circular molecules of 11 x 10^6, 15.5 x 10^6 and 24 x 10^6 daltons. The 11 x 10^6 and 15.5 x 10^6 dalton molecules show sequence heterogeneity and are the major component of the kDNA. Hybridization studies with cloned fragments of T. brucei maxi-circle suggest that the 24 x 10^6 dalton component of H.m. ingenoplastis kDNA is functionally equivalent to the maxi-circle of other trypanosomatids.

We conclude that the 11 x 10^6 and 15.5 x 10^6 dalton circles of H.m. ingenoplastis are functionally similar to mini-circles of other trypanosomatids and that the maxi-circles of H.m. ingenoplastis differ from those of T. brucei and H.m. muscarum in major nucleotide sequences.

INTRODUCTION:

The mitochondrial DNA of flagellate protozoa of the order Kinetoplastida is organized as a complex network of catenated circular molecules termed the kinetoplast (1,2). The kinetoplast DNA (kDNA) network is situated in a portion of the cell's single mitochondrion adjacent to the basal body of the flagellum. In preparative procedures kDNA is readily separated from the flagellate's nuclear DNA by virtue of its high molecular weight, the covalently closed form of its constituent molecules and its high adenine-thymine content. The kDNA from several species, all from the family Trypanosomatidae, has been studied in some detail (see 3-7 for recent reviews). Each kDNA network is composed of about 10^4 small circular molecules, the "mini-circles" (8,9), and about 10^2 large circular molecules, the "maxi-circles" (10,11), all interlocked to form the high molecular weight structure.

The mini-circles of different species vary in contour length (from about 0.3 μm in Leishmania spp. (12) to 0.8 μm in Crithidia spp. (13)) and in their degree of nucleotide sequence heterogeneity (5,14,15). Two mini-circles from T. brucei have recently been completely sequenced and clearly show a high degree of sequence deviation but also show several regions of homology (16). Studies on the transcription abilities of mini-circles have given conflicting results (17,18,19); in at least 3 species no transcripts have been detected. As discussed by Borst and Hoeijmakers (5) and by Englund (7), the preponderance of evidence suggests that the mini-circles have an as yet undiscovered non-coding function in the species studied.

The minor component of the kDNA network, the maxi-circle, has many of the characteristics of mitochondrial DNA molecules from other cells in

that: 1. maxi-circles are present in similar numbers, i.e. between 50 and 100 copies per cell; 2. maxi-circles are similar in size to mitochondrial DNA molecules, 6-12 μm contour length; 3. maxi-circles have a unique nucleotide sequence whis appears to have been conserved in evolution of the Trypanosomatidae (5); 4. transcripts hybridizing to portions of the maxi-circle of Crithidia, Leishmania tarentolae and T. brucei have been detected (18,19 20); 5. maxi-circles code for the mitochondrial ribosomal RNAs in trypanosomes (20); and 6. mutants lacking all or part of the maxi-circle are incapable of normal mitochondrial activities (6,20).

Although a network composed on mini-circles and maxi-circles is the predominant configuration of the kDNA, at least in the family Trypanosomatidae, different structural arrangements of the kDNA have been found in mutants that have lost the ability to make functional mitochondria, in particular, in the pathogenic African trypanosomes. Mutants lacking all or part of the kDNA network have been described (6,21,22) and in every case these mutants have been found only in the bloodstream form of the trypanosome in which mitochondrial biogenesis is completely repressed, the cells relying entirely upon glycolysis for energy (23). These cells are unable to survive under culture conditions which require mitochondrial activation; this activation occurs naturally in the tsetse fly vector (2).

Our main interest in kDNA stems from its possible role in adaptive activation and repression of mitochondrial activitiy in kinetoplastid flagellates. Further understanding of the function of the kinetoplast, and in particular of the enigmatic mini-circles may come from comparative studies on kinetoplast structure in relation to mitochondrial function. Hitherto such studies have been confined to different stages in the life cycle of Trypanosoma brucei and its evolutionary descendents. We report here, and in the following paper, on the kDNA structure and mitochondrial

activity of two subspecies of the monoxenic trypanosomatid, Herpetomonas muscarum (Leidy); both subspecies occure naturally in the gut of dipterous flies but invitro culture forms were studied here. We find that H.muscarum ingenoplastis (24) has a kDNA network completely devoid of mini-circles and containing an altered maxi-circle component; it is also deficient in mitochondrial activities. Herpetomonas muscarum muscarum (24) on the other hand has a kinetoplast-mitochondrion more closely resembling that of other trypanosomatids parasitising insects.

MATERIALS AND METHODS:

Organisms

Cultures of Herpetomonas muscarum muscarum (ATCC 30260) and Herpetomonas muscarum ingenoplastis (ATCC 30269) were obtained from Dr. W.B. Cosgrove, Department of Zoology, University of Georgia, USA. The 2 subspecies were originally isolated in culture from the gut of Musca domestica and Phormia regina respectively by Rogers and Wallace (24). Both subspecies had been cloned at least once and were stored as frozen stabilates in 10% dimethyl sulphoxide at -196°C.

Cultivation

Both H.m. muscarum and H.m. ingenoplastis were routinely cultivated in brain heart infusion medium with blood agar base as described by Rogers and Wallace (24). Recently time-expired human blood obtained from the Glasgow Western Infirmary Blood Transfusion Service was used in the base. Brain heart infusion agar (Difco) was sterilized as a 5.2% (w/v) solution at 15 PSI for 20 min, cooled to 45°c and blood added to 10% (v/v). After cooling the agar, 1 to 2 volumes of 3.7% (w/v) brain heart infusion (Difco) were added as an overlay. Cultures were maintained at 26°C.

H.m. muscarum (but not H.m. ingenoplastis) was also cultivated in a variety of other media including RPMI 1640 + 25 mM Hepes supplimented with 10% foetal calf serum.

Flagellates of the genus Herpetomonas have two distinct morphological stages in their life cycle, the multiplicative long promastigote form with the kinetoplast and flagellum base in front of the nucleus, and the non-multiplicative shorter opistomastigote form with the kinetoplast and flagellum base behind the nucleus. Only promastigotes were present in our cultures.

Isolation of kDNA

Cells were grown to late log phase in 1 liter flasks with 200 ml of blood agar and 300 ml of overlay. To harvest the flagellates the overlay was filtered through loose, absorbent cotton wool which removed peices of agar, then centrifuged at 2000 g for 20 min at 4°C in an MSE Hi-Speed 18 centrifuge fitted with a 6 x 250 ml angle rotor. Pelleted cells were resuspended in 0.15 M sodium chloride, 0.015 M sodium citrate, 0.1 M disodium ethylenediaminetetra acetate, pH 7.5 (SES) and washed 3 times in the same buffer. Pelleted cells were resuspended in the same buffer, mixed with an equal volume of 6% Sarkosyl (in SES) and incubated, with pronase (Calbiochem) at a final concentration of 1 mg/ml, for 1.5 hours at 37°C with gentle shaking. Pronase was pre-treated for 2 hours at 37°C and 15 min at 80°C before use (22). Lysates were deproteinized by mixing with an equal volume of phenol saturated with SES and leaving on ice for 30 min with gentle shaking at 5 min intervals. Aqueous and phenol phases were separated by centrifugation at 2,000 g for 30 min at 4°C. The aqueous upper layer was removed with a wide-tip pipette and further deproteinized by mixing with an equal volume of a chloroform: isoamyl alcohol (24:1) mixture on ice for 30 min, shaking as before. The 2 phases were again separated by centrifugation and the aqueous phase precipitated with 2 volumes of cold (-20°C) 95% ethanol. The precipitate was collected on a glass rod, dissolved in SES buffer and incubated with

ribonuclease (Sigma, final concentration 200 μg/ml) for 1 hour at 37°C. Ribonuclease was preincubated at 80°C for 15 min to inactivate any deoxyribonuclease activity. Deproteination with chloroform:isoamyl alcohol and centrifugation were repeated and the aqueous phase dialysed against 2 changes of SES buffer for 24 hours at 4°C. The dialysate was then centrifuged at 21,000 RPM in an MSE Prepspin 55 ultracentrifuge with a 3 x 15 ml swing-out rotor for 1.5 hours at 4°C. The supernate containing most of the nuclear DNA was carefully removed and the kDNA pellet resuspended in 10 mM Tris (pH 7.5); pelleting was repeated at least 3 times. The final pellet was resuspended in 10 mM tris and further pruified by centrifugation in sodium iodide gradients (N_D25=1.4335) containing 25 μg ethidium bromide per ml. Centrifugation was for 72 hours at 38,000 RPM in an MSE Prepspin 55, 10 x 10 ml angle rotor, or a Beckman L2-50 ultracentrifuge, type 40 rotor, at 20°C. The upper fluorescent band of kDNA was gently removed and extracted 3 times with water-saturated isoamyl alcohol at 4°C, then dialysed against 10 mM Tris (pH 7.5) for 24 to 48 hours at 4°C. When necessary the kDNA was concentrated by centrifugation in a Sorvall RC-2B centrifuge, HB-4 rotor, at 11,000 RPM for 30 min. Purified kDNA was stored at -20°C.

Light Microscopy

To demonstrate kinetoplast morphology, cells were incubated in 0.1 μg/ml 4,6 diamidino-2-phenylidole (DAPI) for 1 hour at 25°C then examined with a Leitz Ortholux II microscope using incident light fluorescence with a HBO 50 high-pressure mercury vapour lamp, exciting filter UG 1, dichroic mirror TK400 and suppressing filter K430. Photographs were made on Ilford HP5 film.

Electron Microscopy

Cells for thin sectioning were pelleted at 1,500 g for 10 min at 4°C and fixed in 1% glutaraldehyde in 0.1M sodium cocodylate buffer, pH 7.4, for 1 hour at 4°C. Cell were washed 2 times in cacodylate buffer

and post-fixed in 1% osmium tetroxide in the same buffer for 1 hour at room temperature. Following post-fixation cells were washed 3 times in cacodylate buffer then stained for 1 hour at room temberature in 0.5% aqueous uranyl acetate. They were then washed 2 times in cacodylate buffer, dehydrated through graded ethanols and propylene oxide, embedded in an Epon-Araldite mixture and blocks polymerized at 60°C for 48 hours. Sections were stained with uranyl acetate and lead citrate.

Purified kDNA was spread by a modification of Lang and Mitani's microdiffusion technique (25). The spreading solution contained 1.0 μg kDNA, 0.1 mg cytochrome c and 0.15 M ammonium acetate, per ml. In some experiments ethidium bromide was added at 50 μg/ml. After diffusion for 40 min in a formaldehyde-saturated atmosphere, the DNA was picked up on 75-200 mesh grids, with carbon support films, then rotary-shadowed with platinum-palladium at an angle of 9°. In all experiments PM2 phage DNA was co-spread as a length marker. All photographs were taken using either an AEI-EM8 or Phillips EM300 electron microscope.

Contour length measurements were made on tracings of photographed molecules; the contour length of PM2 DNA was taken to be 3.02 μm.

Analytical Ultracentrifugation

Analytical cesium chloride ultracentrifugation of total cellular DNA was carried out in a Beckman Model E at 44,770 RPM for 20 hours at 20°C. Equilibrium bands were photographed at 260 nm and negatives scanned with a Gilford 2400 spectrophotometer. Buoyant densities were calculated as described by Schildkraut et al (26) using Micrococcus luteus DNA as a density marker (ρ=1.731 g/cm^3).

Restriction enzyme anlaysis

Restriction endonucleases XhoI, BglII, XbaI, MboI, MboII and TaqI

were purchased form New England Biolabs. PstI, HapII, HindIII, BspI, KpnI and SalI were prepared by published procedures (see 27 for references). EcoRI was obtained from Boehringer Manheim.

All restriction endonuclease digestions of kDNA were for 2 hours at 37°C except TaqI which was incubated at 60°C. Enzymes XhoI, BglII, XbaI, MboI, MboII, BspI, TaqI, KpnI and SalI were incubated in 10 mM Tris-HCl (ph 7.5), 8 mM $MgCl_2$, 1 mM dithiothreitol and 1% gelatin. Enzymes HindIII, EcoRI and PstI were incubated in 5 mM Tris-Hcl (pH 7.5), 7 mM $MgCl_2$, 50 mM NaCl and 5 mM dithiothreitol. S_1 nuclease was prepared according to Vogt (28) from Aspergellus oryzae but ommiting the Sephadex G-100 filtration and the sulfoethyl-sephadex chromatography. kDNA was incubated for 30 min at 45°C with S_1 nuclease in 0.125 M Na acetate, 0.1 mM $ZnSO_4$, 0.4 M NaCl and 0.04% sodium dodecyl sulphate, pH 4.7.

To facilate layering, all digested kDNA samples were mixed with Ficol and Orange G to final concentrations of 5% and 25 ng/ml respectively. To resolve high-molecular-weight fragments, gel electrophoresis was carried out on 0.5% horizontal agarose slab gels at 20-25 mAmps for 17 hours at room temperature, in a running buffer containing 40 mM Tris-HCl, 20 mM Na acetate, 1mM EDTA,pH 7.7 with 0.5 μg ethidium bromide per ml. kDNA fragments smaller than 1 kilo base bair in size were resolved on 2% agarose gels run at 150 mAmps for 2 hours with a running buffer of 90mM Tris-HCl, 90 mM Na borate, 2.5 mM EDTA (pH 8.3) and 0.5 μg ethidium bromide per ml.

Following electrophoresis gels were destained for 30 min in distilled water, then photographed by ultraviolet light with a Nikon F camera through a Kodak Wratten filter 17 with Agfa Copex Pan Rapid film.

To determine the molecular weight of the kDNA fragments the migration of the fragments was measured relative to marker DNA fragments. Molecular weight markers used were phage lambda DNA, phage lambda DNA digested with EcoRI, phage ØX DNA digested with BspI or MspI.

Hybridization experiments

Following electrophoresis kDNA fragments were denatured *in situ* and the DNA transferred to nitrocellulose filters as described by Southern (29). Filters were hybridized with denatured kDNA probes labelled with 20 µCi of α-^{32}P- TTP and α-^{32}P- CTP by nick translation. DNA probes used were: 1. EC02 (RR2), and 2. EC03 (RR3) fragemnts both from the maxi-circle of *T. brucei* 427 cloned in *Escherichia coli* using lambda-gt-WES. lambda-B as vector (for nomenclature and procedure see 30); 3. mini-circles from *H.m. muscarum* prepared by digesting kDNA networks with XhoI, which cuts the maxi-circle but few of the mini-circles and pelleting the mini-circle network at 11,000 RPM in a Sorvall HB-4 rotor for 30 min at 4°C foll wed by 3 washes. Filters were incubated at 65°C for 3 hours in 3 times concentrated 0.15 M sodium citrate, 0.15 M sodium pH 7.5 (SSC) with 0.2% Ficol, 0.2% bovine serum albumin, 0.2% polyvinyl-pyrrolidone, 0.1% sodium dodecyl sulphate, 50 µg/ml Salmon sperm DNA. The heat denatured radio-labelled DNA probe was added and filters allowed to hybridize for 24 hours. Following hybridization filters were washed exhaustively at 65°C with the hybridization solution (see above) without the ^{32}P labelled DNA probes. Filters were dried and autoradiograms exposed at -70°C for 2 hours to 2 weeks depending on the degree of hybridization.

RESULTS

Fluorescence microscopy of the kinetoplast

DAPI is a highly fluorescent derivative of berenil (31) which

has been useful in detecting small amounts of DNA (32). This compound binds preferentially to A-T rich DNA and when exposed to ultraviolet light fluoresces a brilliant blue. When either H.m. ingenoplastis or H.m. muscarum was incubated with DAPI and examined by fluoreescence microscopy, the kinetoplast appeared as an intensely-stained structure at the base of the flagellum (figure 1a and b). Probably owing to the high A-T content and high concentration of kDNA the kinetoplast fluorescence appeared much more intense than the nuclear fluorescence; an alternative explanation is that the association of nuclear DNA with proteins affects DAPI binding. The kinetoplast of H.m. ingenoplastis was tear-drop shaped both in living and fixed cell preparations. There was a high degree of variation in the size of the kinetoplast of H.m. ingenoplastis and up to 10% of the cells were dyskinetoplastic (33,34); that is, completely lacking a stainable kinetoplast. In contrast the kinetoplast of H.m. muscarum was small and ovoid in shape and the percentage of dyskinetoplastic cell was less than 0.5%.

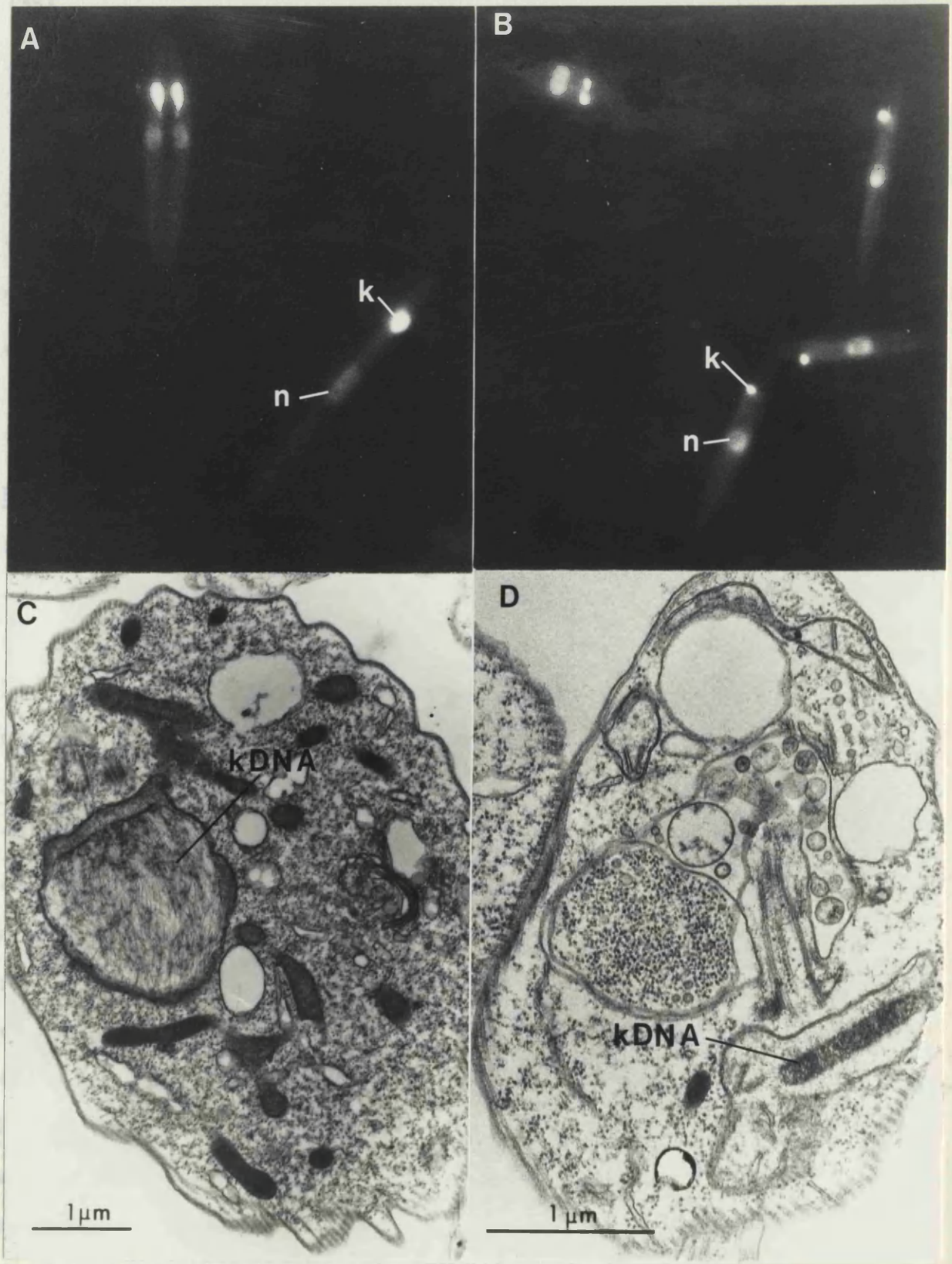
Electron microscopy of the kinetoplast

Electron micrographs of sections of both H.m. ingenoplastis and H.m. muscarum showed the kDNA to be present in that portion of the cell's single mitochondrion adjacent to the basal body of the flagellum (figure 1c and d). The structure of the kinetoplast of H.m. muscarum was similar to that of most other trypanosomatids studied; the kDNA appeared in vertical section as a fibrous band of electron dense material about 0.15 μm in width with the kDNA fibres roughly aligned with the long-itudinal axis of the cell (figure 1d). The kinetoplast of H.m. ingenoplastis was seen in vertical section as a voluminous bundle of fibres, also roughly aligned with the longitudinal axis of the cell, with a distinct electron-dense "edge" at the end nearest the flagellum and loosely packed kDNA fibres extending posteriorly in the mitochondrion for

Figure 1.

Fluorescence photographs of DAPI-labelled (A) H.m. ingenoplastis; and (B) H.m. muscarum. The kinetoplast, k, and the nucleus, n, are visible and dividing cells are present in both preparations. X1500.

Electron micrographs of thin sections of H.m. ingenoplastis (C) X18000 and H.m. muscarum (D) X30000 through the kinetoplast region of the mitochondrion, to show characteristic distribution of kDNA in each subspecies.


A
k
n
B
k
n
C
kDNA
1 µm
D
kDNA
1 µm

distances up to 2.5 μm. These observations on the ultrastructure of both subspecies are in agreement with previous studies by Wallace et al (35).

Extraction and visualization of kDNA molecules

Analytical CsCl equilibrium ultracentrifugation of total cellular DNA from both H.m. muscarum and H.m. ingenoplastis revealed a rapidly banding component which settled at a density of 1.698 g/cm^3 (Table 1). This component represented 8% of the total DNA in H.m. muscarum and 31% of the total cellular DNA in H.m. ingenoplastis.

Following pelleting by high speed centrifugation the kDNA from H.m. ingenoplastis and H.m. muscarum was freed from remaining contaminating nuclear DNA by NaI gradient ultracentrifugation. Figures 2d and 2e show the banding patterns obtained for the two subspecies with the kDNA forming the sharp upper band and the nuclear DNA the faint lower band. The purified kDNA band was removed and spread for electron microscopy. Figure 2a shows a portion of a kDNA network from H.m. ingenoplastis. No mini-circle size molecules were observed in any of the preparations and the kDNA appeared to be composed entirely of large circular molecules (figure 2b). The size distribution of free circular molecules in kDNA preparations from H.m. ingenoplastis is given in figure 3. Spreading in the presence of ethidium bronide induced supercoiling of the kDNA from H.m. ingenoplastis showing that the majority of the circles are covalently closed (not shown). The kDNA networks from H.m.muscarum were more fragile than those from H.m. ingenoplastis and most of the networks observed were extensively fragmented. These networks were composed primairly of catenated mini-circles but a few longer molecules were also present in the networks (figure 2c). The size distribution of free circular molecules from H.m. muscarum kDNA

TABLE 1.

Characteristics of kDNA isolated from H.m. muscarum and H.m. ingenoplastis.

The size measurements are from figure 3; the molecular weights were calculated from the mobility of cleaved molecules on 0.6% agarose gels.

Organism	mini-circle		maxi-circle		HL-circle		buoyant density
	size (μm)	mol. wt. ($\times 10^{-6}$)	size (μm)	mol. wt. ($\times 10^{-6}$)	size (μm)	mol. wt. ($\times 10^{-6}$)	(g/cm^3)
H.m. muscarum	0.39 (54)*	0.6	-	21.0	-	-	1.698
H.m. ingenoplastis	-	-	11.3 (1)	24.0	a. 4.4 (13) b. 6.5 (51)	11.0 15.5	1.698

* Number of molecules measured in spread preparations.

Figure 2.

Electron micrographs of isolated kDNA spread by the protein monolayer technique. (A) Edge of a kDNA network of H.m. ingenoplastis containing only long loops of DNA. (B) A free 6.7 μm HL-circle from H.m. ingenoplastis. (C) A portion of a fragmented kDNA network form H.m. muscarum containing catenated mini-circles and maxi-circles. All micrographs X50000.

Photographs of NaI gradients of partially purified kDNA from (D) H.m. ingenoplastis and (E) H.m. muscarum.

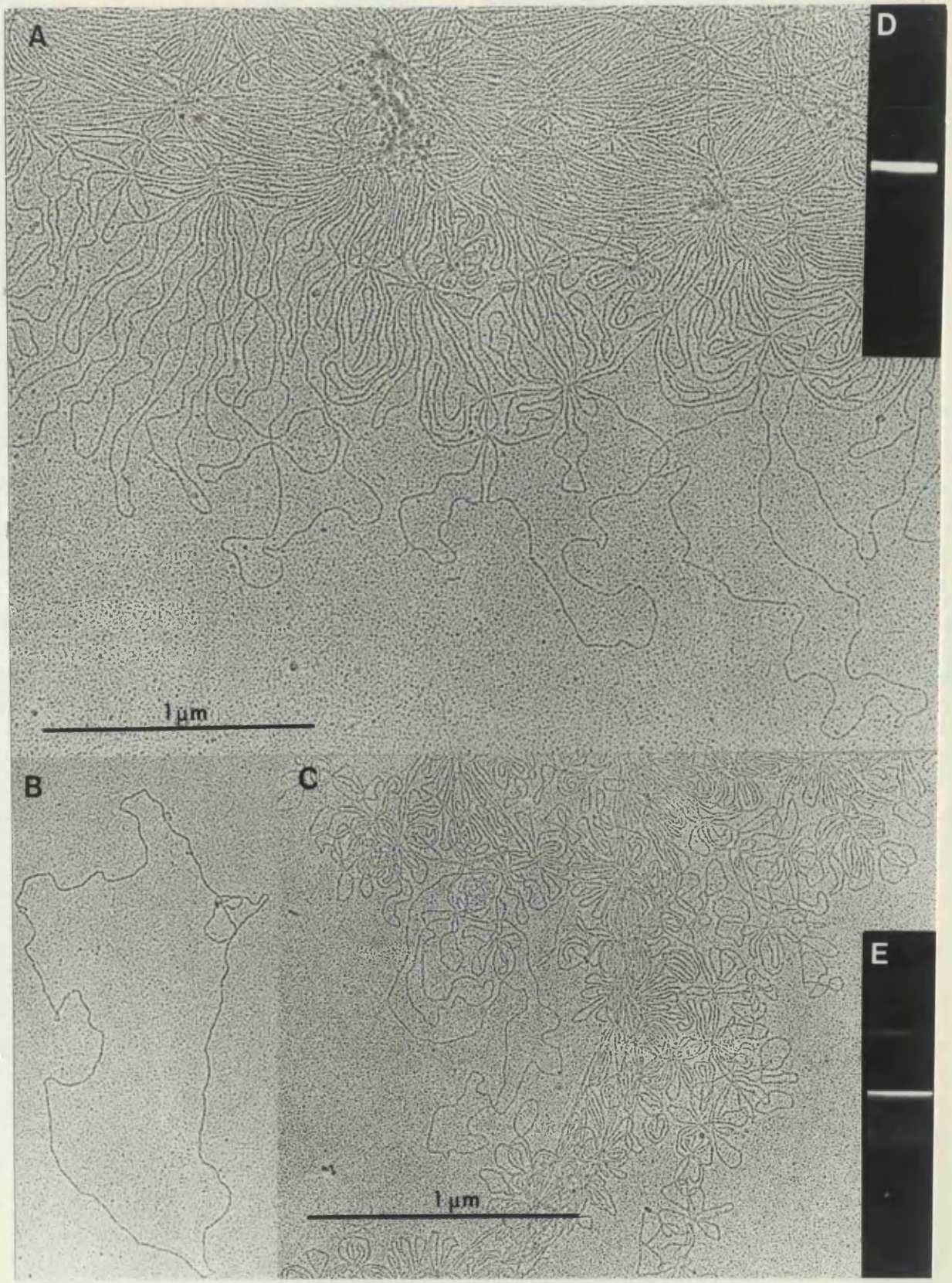
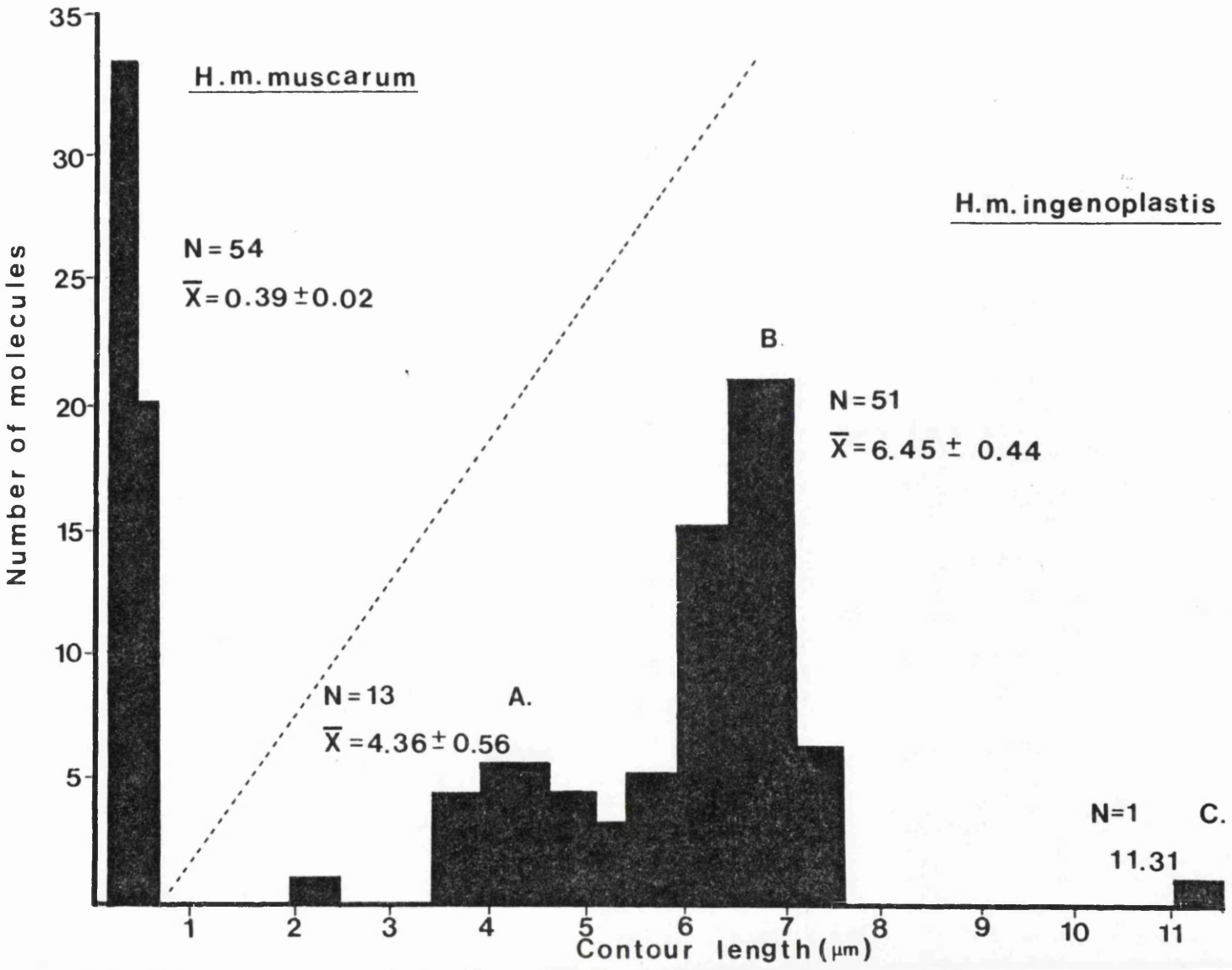

A
D
1 µm
B
C
E
1 µm

Figure 3.

Contour length distribution of free circular molecules in spread preparations of kDNA from H.m. muscarum and H.m. ingenoplastis. The free circles in H.m. ingenoplastis kDNA fall into 3 size classes of (A) 4.4 μm, (B) 6.5 μm, and 11.3 μm.


H.m.muscarum
N = 54
X̄ = 0.39 ± 0.02
H.m.ingenoplastis
B.
N = 51
X̄ = 6.45 ± 0.44
N = 13
A.
X̄ = 4.36 ± 0.56
N=1
C.
11.31
Number of molecules
35
30
25
20
15
10
5
1
2
3
4
5
6
7
8
9
10
11
Contour length (μm)

preparations is shown in figure 3. All free circular molecules in these preparations were of mini-circle size; no circular molecules of maxi-circle size were observed.

Endonuclease digestion experiments

Figure 4 shows the fragments produced when purified kDNA from H.m. ingenoplastis and H.m. muscarum is digested with the restriction endonucleases HindIII, BspI, XhoI and EcoRI and electrophoresed on 0.6% agarose gels. The kDNA of H.m. ingenoplastis yielded a large number of high molecular weight fragments, in non-stoichiometric amounts, which gave an added molecular weight exceeding the molecular weight values obtained for the linearized circular DNA molecules following digestion with S_1 nuclease (Table 2). S_1 nuclease can linearize circular DNA and the kDNA from H.m. ingenoplastis yielded 3 fragments of 11 x 10^6, 15.5 x 10^6 and 24 x 10^6 daltons molecular weight when digested with this enzyme (figure 6). The large number of fragments and lack of stoichiometry were not due to partial digestion or to contamination nuclear DNA since 10 times excess of restriction endonuclease did not alter the number or molecular weight of the fragments obtained and purified nuclear DNA run side-by-side with the kDNA gave an entirely different pattern.

Digests of the kDNA from H.m. muscarum yielded a small number of faint, high-molecular-weight fragments which were present in equimolar amounts and had a combined molecular weight of 20-24 x 10^6 daltons, in agreement with the value of 21 x 10^6 daltons obtained for the maxi-circle cut once with S_1 nuclease (Table 2). The S_1 digest of H.m. muscarum kDNA also yielded small fragments of about 0.6 to 0.7 x 10^6 daltons which corresponded to values expected for mini-circles (figure 6). No low-molecular-weight fragments were present in the S_1 digests of H.m. ingenoplastis kDNA. Table 2 summarizes the results obtained with

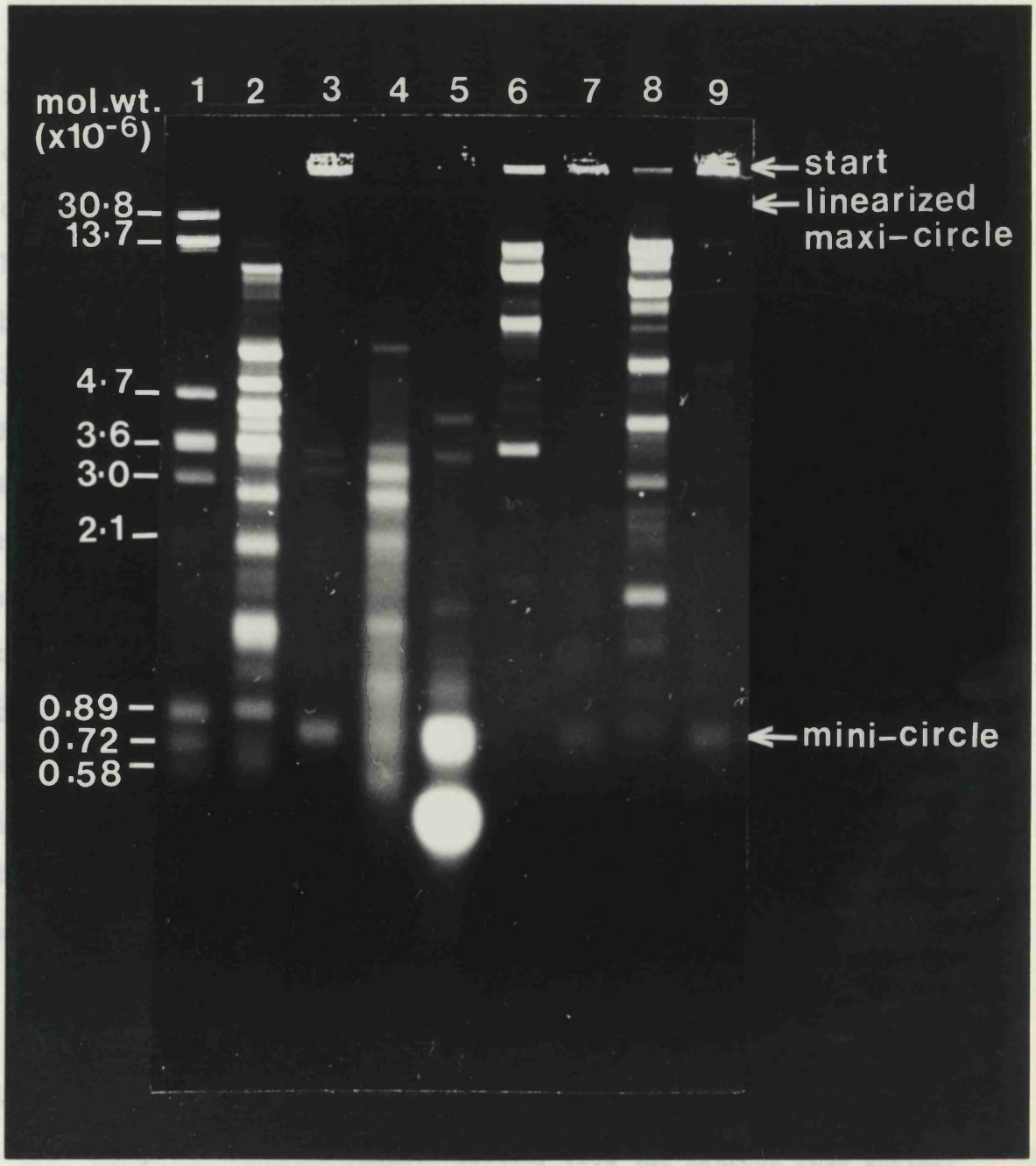
TABLE 2.

Number and sum of molecular weights of high-molecular-weight fragments released from the kDNA of H.m. muscarum and H.m. ingenoplastis by restriction endonucleases and S_1 nuclease.

	H.m. ingenoplastis		H.m. muscarum	
Enzyme	No. of fragments	Sum of mol wts. ($x10^{-6}$)	No. of fragments	Sum of mol. wts. ($x10^{-6}$)
Hap II	23	52	3	20.4
Hind III	24	104	3	20.3
EcoR I	12	66	2	21.3
Xho I	7	63	1	21.0
Bgl II	19	102	3	24.1
Kpn I	7	61	1	21.0
Sal I	6	55	1	21.0
Pst I	26	129	-	-
Xba I	14	103	2	22.5
S_1	3	a. 24.0 b. 15.5 c. 11.0	1	21.0

Figure 4.

Agarose (0.6%) gel electrophoresis of KDNA from H.m. ingenoplastis (lanes 2, 4, 6, and 8) H.m. muscarum (lanes 3, 5, 7, and 9) following digestion with restriction endonucleases HindIII, lanes 2 and 3; BspI, lanes 4 and 5; XhoI, lanes 6 and 7; EcoRI, lanes 8 and 9. Lane 1 contains a mixture of intact phage lambda DNA, phage lambda DNA digested with EcoRI and phage ØX 179 DNA digested with BspI as a molecular weight marker.


mol.wt.
(x10⁻⁶)
1 2 3 4 5 6 7 8 9
30·8
13·7
4·7
3·6
3·0
2·1
0.89
0.72
0.58
start
linearized
maxi-circle
mini-circle

11 restriction endonucleases and S_1 nuclease on the kDNA from H.m. muscarum and H.m. ingenoplastis.

Hybridization experiments

To determine whether any of the mini-circle sequences present in H.m. muscarum kDNA are present in the large, heterogeneous circles of H.m. ingenoplastis kDNA, restriction digests of both kDNAs were run on 2% agarose gels, which resolve the smaller, mini-circle size fragments better than the lower percentage gels. The sequence heterogeneity of the H.m. muscarum mini-circles is clearly demonstrated in the banding patterns in figure 5. The kDNA fragments were transferred to nitrocellulose filters and hybridized with radiolabelled mini-circle probe from an XhoI digest of H.m. muscarum kDNA (figure 5). Although digests of both H.m. ingenoplastis and H.m. muscarum contained low-molecular-weight fragments, smaller than linearized mini-circle, the fragments did not co-migrate and the H.m. ingenoplastis kDNA did not hybridize with the H.m. muscarum mini-circle probe showing that there is little or no sequence homology between the H.m. muscarum mini-cricles and the kDNA of H.m. ingenoplastis. Slot 4 in figure 5 contains kDNA from Crithidia fasciculata, the mini-circle of this species did not hybridize with the H.m. muscarum mini-circle.

Because of the large number of high-molecular-weight fragments in restriction endonuclease digests of the H.m. ingenoplastis kDNA, it was not possible to determine directly from the agarose gels whether maxi-circle sequences were present in the H.m. ingenoplastis kDNA. Two cloned portions of the maxi-circle of T. brucei 427 kDNA were therefore hybridized with kDNA from H.m. ingenoplastis and H.m. muscarum digested with restriction endonucleases, electrophoresed and transferred to nitrocellulose filters and the fragments containing nucleotide

Figure 5.

Hybridization of the mini-circle probe from H.m. muscarum kDNA with restriction endonuclease fragments of kDNA from H.m. ingenoplastis (lanes 2, 5, and 7), H.m. muscarum (lanes 3, 6, and 8), and C. fasciculata (lane 4) following electrophoresis on 2% agarose and blotting of denatured kDNA fragments onto nitrocellose filters. Panel A shows the electrophoresis of the low molecular weight fragments following digestion with restriction endonucleases HapII, lanes 2, 3, and 4; BspI, lanes 5 and 6; and MboI, lanes 7 and 8. Panel B is the autoradiogram of the hybridization of the ^{32}P labelled H.m. muscarum mini-circle probe with the filter blot of the gel shown in panel A. Lane 1, panel A contains a mixture of intact phage lambda DNA, phage lambda DNA digested with EcoRI and phage ØX 179 DNA digested with BspI a molecular weight markers.

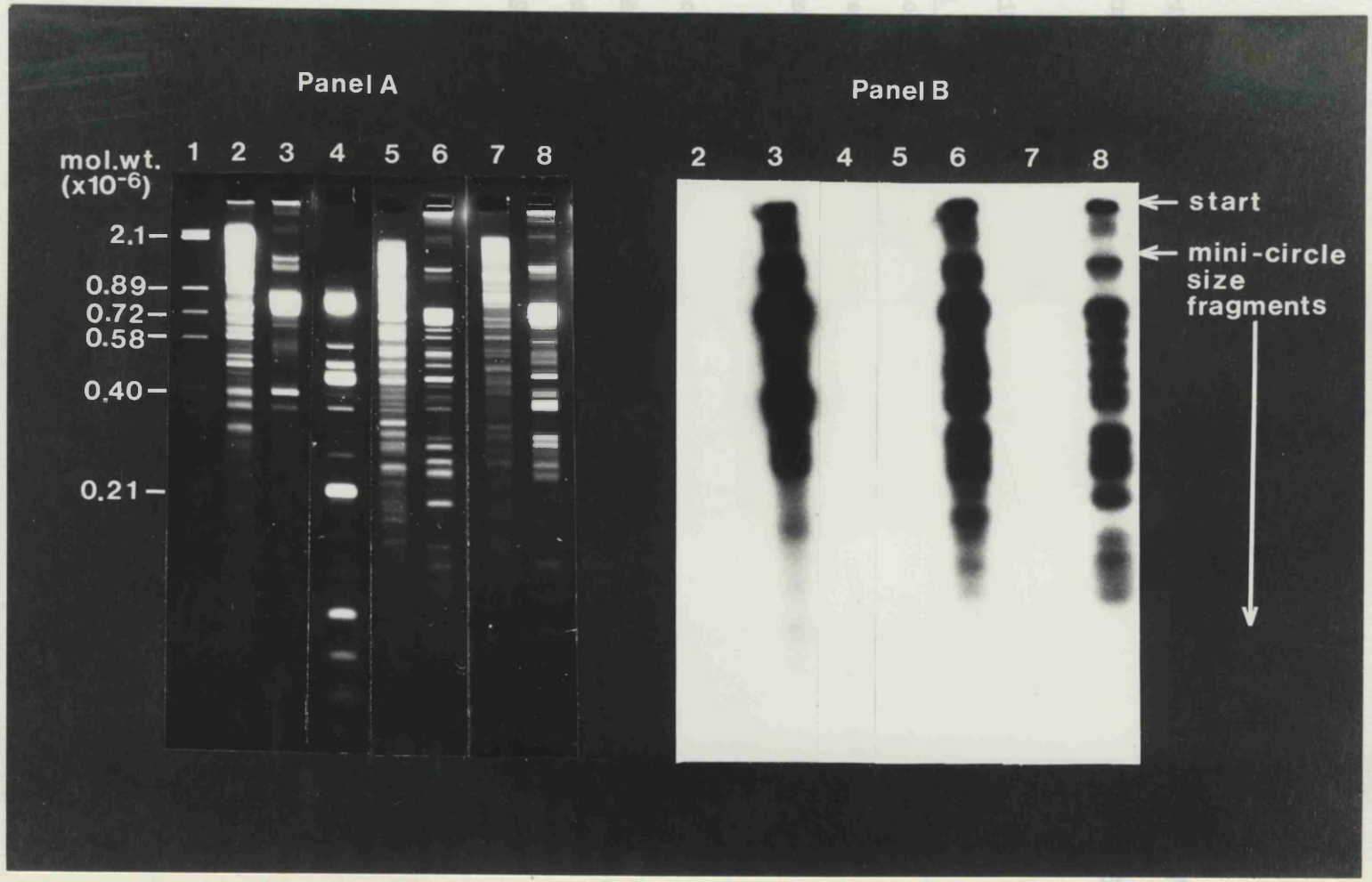

Panel A
mol.wt.
(x10⁻⁶)
1 2 3 4 5 6 7 8
2.1
0.89
0.72
0.58
0.40
0.21
Panel B
2 3 4 5 6 7 8
start
mini-circle
size
fragments

Figure 6.

Hybridization of cloned segments of the maxi-circle of T. brucei 427 with restriction endonuclease and S_1 nuclease fragments of H.m. ingenoplastis (lanes 1, 3, 5, 7, 9, and 11) and H.m. muscarum (lanes 2, 4, 6, 8, 10, and 12) kDNA following electrophoresis on 0.6% agarose and blotting denatured DNA fragments onto nitrocellose filters. Panel A shows the electrophoresis of fragments obtained from HindIII, lanes 1 and 2; BspI, lanes 3 and 4: EcoRI, lanes 5 and 6; MboI, lanes 7 and 8; XhoI lanes 9 and 10; and S_1 nuclease, lanes 11 and 12. Panel B is the autoradiogram of the hybridization of the ^{32}P labelled Eco2 cloned maxi-circle probe form T. burcei and a filter blot of the kDNA from H.m. ingenoplastis and H.m. muscarum shown in Panel A. Panel C shows the hybridization of a duplicate filter with the cloned Eco3 fragment of T. brucei maxi-circle.

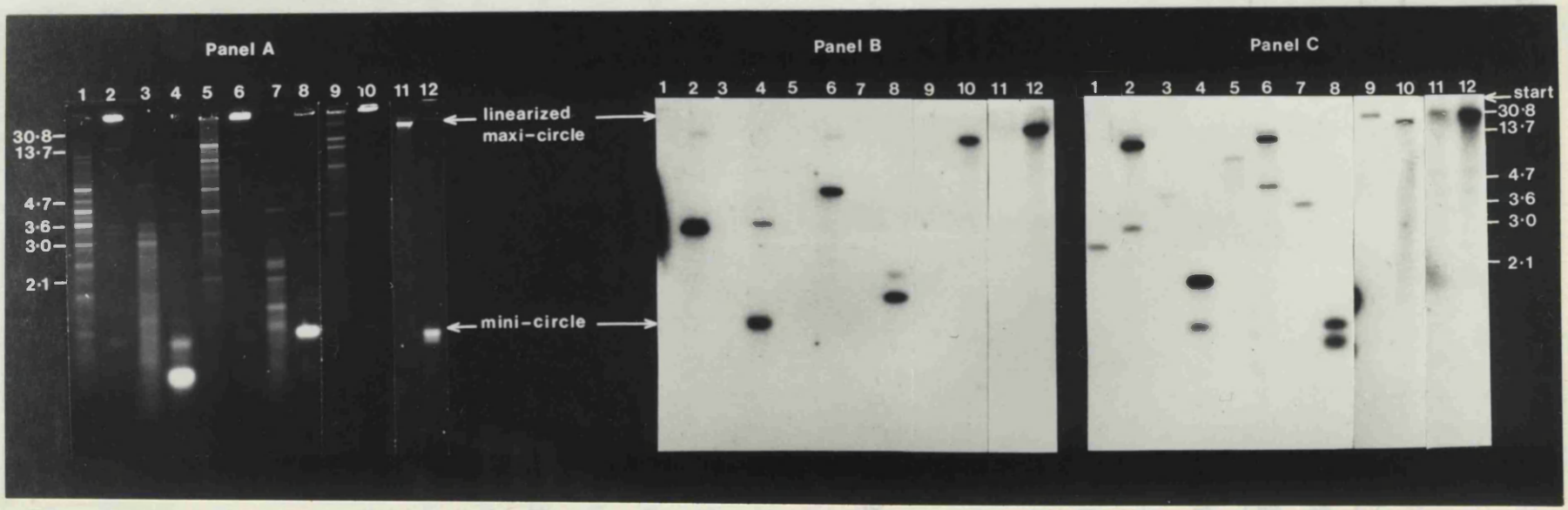

Panel A
1 2 3 4 5 6 7 8 9 10 11 12
30·8
13·7
4·7
3·6
3·0
2·1
linearized maxi-circle
mini-circle
Panel B
1 2 3 4 5 6 7 8 9 10 11 12
Panel C
1 2 3 4 5 6 7 8 9 10 11 12
start
30·8
13·7
4·7
3·6
3·0
2·1

TABLE 3.

Molecular weight ($x10^{-6}$) of kDNA fragments from H.m. muscarum and H.m.ingenoplastis hybridizing with cloned maxi-circle fragments, Eco2 and Eco3, from T. brucei 427.

Restriction endonuclease	Fragment	Eco2 probe		Eco3 probe	
		H.m.m.	H.m.i.	H.m.m.	H.m.i.
EcoR I	1	16.5	-	16.5	11.0
	2	6.0	-	6.0	-
HindIII	1	3.5	-	13.5	2.5
	2	3.3	-	3.5	-
XhoI	1	20.0	-	20.0	24.0
	2	-	-	-	-
BspI	1	3.4	-	1.1	5.6
	2	0.75	-	0.75	-
S_1	1	21.0	-	21.0	24.0

sequences in common with the T. brucei maxi-circle probes identified (figure 6). The high molecular weight fragments of the H.m. muscarum maxi-circle hybridized with both the T. brucei maxi-circle probes (cloned EcoRI fragments Eco 2 and Eco 3). Digests of the H.m. ingenoplastis kDNA gave no detectable hybridization with the Eco 2 cloned fragment of the T. brucei maxi-circle (Panel B, figure 6). However, hybridization occurred with the Eco 3 probe (Panel C, figure 6). In the XhoI and S_1 digests apparent full length fragemnts of the H.m. ingenoplastis maxi-circle hybridized with the Eco 3 probe. The fragments had a molecular weight of 24 x 10^6 daltons and were similar in size to the H.m. muscarum maxi-circle.

DISCUSSION

Our results have shown that the kDNA network of H.m. ingenoplastis differs from that of all other trypanosomatids studied in completely lacking mini-circle size molecules. The kDNA of H.m. ingenoplastis is composed entirely of large circular molecules of 3 size classes: 1. 24 x 10^6, 2. 15.5 x 10^6, and 3. 11 x 10^6 daltons molecular weight. The 24 x 10^6 dalton molecules contain sequences homologous to a portion of the maxi-circles of H.m. muscarum and T. brucei 427 and are likely to represent the maxi-circle of H.m. ingenoplastis. As discussed further below, this putative maxi-circle of H.m. ingenoplastis apparently lacks sequences that are essential for normal mitochondrial biogenesis and this can account for the abnormal mitochondria found in this subspecies (see accompanying paper). The other 2 size classes of circular DNA molecules are heterogeneous in sequence and may fulfil a structural role in the kDNA network similar to the mini-circles of other species. We have termed these molecules heterogeneous large-circles (HL-circles) on the basis of their size and sequence heterogeneity.

The maxi-circle of H.m. muscarum resembles that of other trypanosomatids; it is only slightly smaller than the maxi-circle of Crithidia (see 5) and it hybridizes with both segments of the T. brucei maxi-circle available in cloned form. The maxi-circle of H.m. ingenoplastis is similar in size to that of H.m. muscarum but differences in the nucleotide sequences are evident in restriction endonuclease digests and from hybridization experiments using the cloned T. brucei maxi-circle fragments. Digests of both H.m. ingenoplastis and H.m. muscarum kDNA contain fragments which hybridize with the Eco 3 T. brucei cloned maxi-circle fragment, but the size of the hybridizing fragments differs in digests with 7 different restriction endonucleases. The maxi-circle of H.m. muscarum hybridizes strongly with the other cloned T. brucei maxi-circle fragment, Eco 2, while the maxi-circle of H.m. ingenoplastis completely lacks sequence homology with this DNA probe. The Eco 2 fragment is known to code for at least two prominent transcripts of T. brucei, present in both culture and bloodstream forms (20) and sequences hybridizing to this fragment are also present in the maxi-circle of Trypanosoma cruzi (36) and Leishmania tarentolae (A. Simpson, L. Simpson and P. Borst, unpublished). It seems likely, therefore, that this sequence contains information essential for mitochondrial biogenesis and that the maxi-circle of H.m. ingenoplastis is defective, like the deleted maxi-circle previously found in a T. equiperdum stock (21). Since the H.m. ingenoplastis maxi-circle is slightly larger than its counterpart in H.m. muscarum, the deletion of the sequences hybridizing to the Eco 2 fragment must have been accompanied by a compensatory amplification of some of the remaining sequences; analogous amplification appears to have occurred in the cytoplasmic petite mutants of yeast.

The relationship of the HL-circles of H.m. ingenoplastis to other

kDNA components cannot be determined with certainty, since the HL-circles fail to hybridize with either the mini-circles or maxi-circles of H.m. muscarum (data not shown) or the cloned maxi-circle fragments of T. brucei. However, several properties of these HL-circles suggest that, despite their dissimilarity in size, they are more closely related to mini-circles than to maxi-circles. 1. The HL-circles are present in thousands of copies per cell, as are mini-circles, and apparently play a major structural role in maintaining the kDNA network. 2. The HL-circles show heterogeneity in nucleotide sequence; this suggests a rapidly evolving sequence and is characteristic of most mini-circles. The lack of hybridization with mini-circles form H.m. muscarum is consistent with this rapid sequence evolution. A comparable situation occurs in T. brucei mini-circles where different stocks of the same species show only partial sequence homology (37). 3. The buoyant density in CsCl of H.m. ingenoplastis and H.m. muscarum kDNA networks is identical viz. 1.698 g/cm^3. In all trypanosomatid genera studied thus far the maxi-circle density is low and around 1.682 g/cm^3. We presume that the same situation will hold in Herpetomonas in view of the hybridization of H.m. muscarum maxi-circles with both cloned segments of the T. brucei maxi-circle. The density results therefore suggest that the HL-circles of H.m. ingenoplastis contain sequences more similar to mini-circles than maxi-circles. 4. The HL-circles fail to hybridize with the cloned maxi-circle fragments of T. brucei while the presumptive maxi-circle of H.m. ingenoplastis retains partial homology with the T. brucei maxi-circle. If the HL-circles of H.m. ingenoplastis were derived from a parental maxi-circle than they should retain sequence homology to the T. brucei cloned maxi-circle fragments similar to that of the H.m. ingenoplastis maxi-circle since the rate of mutation of both the non-functional maxi-circles and the HL-circles should be equivalent. Our cloned maxi-circle probes cover only half of the T. brucei maxi-circle,

however, so it is possible that there is sequence homology of the HL-circles with the uncloned half of the T. brucei maxi-circle.

Preliminary experiments in which total-cellular RNA from H.m. ingenoplastis was hybridized with kDNA from H.m. ingenoplastis digested with restriction endonucleases and blotted on to nitrocellulose filters suggest that transcripts are not made on the HL-circles (Hoeijmakers and Hajduk, unpublished results). These results are similar to those reported for the mini-circles of C. lucilae and T. burcei in which transcripts of the maxi-circle but not mini-circles, were found (6,18).

Discussion of the evolutionary relationship of the HL-circles of H.m. ingenoplastis to mini-circles of other trypanosomatids requires establishment of the relatedness of H.m. muscarum and H.m. ingenoplastis. Recent results (Hajduk, Tait and Hoeijmakers, unpublished results) show that these 2 subspecies differ in the electrophoratic mobilities of 12 of 13 enzymes studied, in the sequences of the rRNA genes in the nucleus, and in the buoyant density of the nDNA in CsCl. These findings lead us to consider the subspecies designation invalid and suggest that the two flagellates should henceforth be designated as different species within the genus Herpetomonas, viz. H. muscarum and H. ingenoplastis.

Similarities between the in situ appearence of the kDNA of H. ingenoplastis and that of the kDNA from several free-living species belonging to the family Bodonidae of the order Kinetoplastida (2,38,39,40) raises the intriguing possibility that HL-circle-like molecules are present in the kDNA of members of this family. This would suggest that the HL-circle component of the H. ingenoplastis kDNA appeared early in the evolution of the order, assuming that the parasitic trypanosomatids evolved from free-living bodonid ancestors. Thus it is possible that

during evolution the mini-circles replaced the HL-circle-like molecules as the major structural element of the kDNA network. One possible selective pressure for smaller kDNA size could be the temporal relationship which exists between kDNA and nuclear DNA synthesis, and cell division. To conserve the kDNA structure and the essential maxi-circle genome and at the same time reduce the time and energy required for duplication of the network it is simplest to imagine that the size of the major structural component of the kDNA might be reduced to a minimum. Certainly there is a tendency for the size of the mini-circles in the trypanosomatids to decrease form the evolutionarily primative trypanosomatids having a single host (eg. Crithidia spp., with 0.8 μm mini-circles) to the more recent genera having 2 hosts in their like cycle (Leishmania and Trypanosoma spp., with 0.3 μm mini-circles). Why the HL-circles are conserved in H. ingenoplastis is as yet unclear although this might be related to the unusual metabolic characteristics of this species. Since the kDNA of the bodonids has not been studied either by electron microscopy of spread preparations or restriction endonuclease analysis, the in situ morphological similarities may prove superficial and neither mini-circles or HL-circles may be present.

Alternatively, HL-circles may have been derived from mini-circles by defective mini-circle replication leading to tandem repeats of mini-circle sequences. The lack of hybridization with mini-circles and the lack of detectable repetitive sequences could be due to sequence divergence. Englund (41) has shown that mini-circles from C. fasciculata replicate free of the kDNA network and re-attach following replication. If the free mini-circles replicate many times prior to reattachment or if the free mini-circles were re-attached to the network by insertion into another mini-circle the HL-circles might be produced. We consider it unlikely that the HL-circles evolved in this fashion.

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Relationship of kinetoplast DNA to mitochondrial activity in the flagellate protozoan Herpetomonas muscarum

II. Characterization of mitochondrial activity

Summary

We have compared the structure and activity of the mitochondria of H. ingenoplastis and H. muscarum by electron microscopy, respiration studies with different substrates and inhibitors, analysis of oligomycin sensitive ATPase activity and low-temperature difference spectra of respiratory chain cytochromes. Certain differences in the two organisms can be correlated with alterations in the maxi-circle of H. ingenoplastis described in the preceding paper. 1) The mitochondrion of H. ingenoplastis is poorly developed and devoid of the plate-like cristae present in the mitochondrion of H. muscarum. 2) The total cellular ATPase activity in homogenates of H. muscarum is sensitive to oligomycin (24% inhibition) while only 5% of the ATPase activity in homogenates of H. ingenoplastis is oligomycin-sensitive. 3) Inhibition studies on the respiration of intact cells and homogenates with cyanide, azide, antimycin and saliclhydroxamic acid show that respiration is not mediated by a conventional cytochrome chain with cytochrome aa_3 acting as the terminal oxidase in H. ingenoplastis as in H. muscarum. 4) Low-temperature differnece spectra suggest that cytochromes b, c and aa_3 are present in the mitochondrion of H. muscarum but that H. ingenoplastis completely lacks cytochrome aa_3 and that absorption maxima peaks for cytochrome b in preparations of H. ingenoplastis differ from those obtained from H. muscarum.

It appears that mutations have occured in the maxi-circle of H. ingenoplastis which result in the typical mitochondrial gene products, cytochrome b and portions of cytochrome aa_3 and the mitochondrial ATPase complex being either absent or present in altered form. The loss of cytochrome aa_3 has resulted in H. ingenoplastis using an alternative pathway for energy metabolism possibly using cytochrome o as a terminal oxidase.

INTRODUCTION:

The mitochondrial genome of most eukaryotes codes for a small number of RNAs and peptides essential for mitochondrial protein synthesis and energy production (1,2). The products include mitochondrial ribosomal RNAs, transfer RNAs, cytochrome b and subunits of the mitochondrial ATPase and cytochrome c oxidase complexes. Although most mitochondrial proteins are specified by nuclear genes, translated on cytoplasmic ribosomes and imported into the mitochondrion (3) there are no duplicates of mitochondrial genes in the nucleus and alterations in the mitochondrial genome are generally lethal. Two exceptions are anaerobically grown yeast (4) and the developmental stages of the African trypanosomes in the bloodstream of the mammalian host (5). Both show repressed mitochondrial biogenesis and the pro-mitochondria present lack cristae and a functional Krebs cycle.

In the mammal-parasitising stages of the life cycle of Trypanosoma brucei energy production is strictly a consequence of glycolysis; neither a functional Krebs cycle nor cytochromes are detectable; NADH is reoxidized by an α-glycerolphosphate oxidase system (6,7). This metabolic system is apparently unique to the African trypanosomes since other members of the family Trypanosomatidae have cyanide-sensitive, cytochrome-mediated respiration and a functional Krebs cycle (8,9). Difference spectra and inhibition studies on several species of trypanosomatid (10,11,12,13,14) suggest that in addition to cytochrome aa_3 another terminal oxidase might be functioning in these species. Hill and co-workers (7) have postulated that cytochrome o, generally considered a prokaryotic oxidase (15), serves as an alternative terminal oxidase in a branched cytochrome chain.

The DNA of the kinetoplast (kDNA) is the only mitochondrial DNA found in trypanosomatids and the maxi-circle component of the kDNA network probably carries the genetic information equivalent to that of mitochondrial

DNA of other cells (16). Opperdoes et al. (17) found that oligomycin-sensitive ATPase activity could be used as a marker for an intact mitochondrial genome even in the bloodstream forms of the African trypanosomes where conventional mitochondrial activities are repressed. Borst and co-workers (16,18,19) have described alterations in the maxi-circle of Trypanosoma brucei, Trypanosoma equiperdum and Trypanosoma evansi all of which are unable to activate the mitochondrion or infect the insect vector, as predicted by Opperdoes' findings.

Restriction endonuclease digestion and hybridization studies of the kDNA from 2 subspecies of Herpetomonas muscarum suggest that there are differences between Herpetomonas m. muscarum and H.m. ingenoplastis in the nucleotide sequence of the maxi-circle component (see preceding paper). We have looked for possible correlates of these differences in the kDNA with the metabolism, electron transport system and ultrastructure of the two subspecies.

MATERIALS AND METHODS:

Organisms, cultivation and electron microscopy

H.ingenoplastis and H. muscarum were grown and processed for electron microscopy exactly as described in the preceding paper except that cells used in low temperature difference spectra were grown in RPMI 1640 with 25 mM MES and 2% fetal calf serum at pH 6.7 and 25°C.

Respiration measurements

The oxygen consumption of both intact cells and cell-free homogenates was measured with a Rank Brothers (Cambridge, U.K.) Clark type electrode, in the absence of substrate or with 10 mM substrate added.

For measurement of intact cell respiration mid-log phase cultures were harvested by centrifugation at 1,500 g for 10 min at 4°C, washed 3 times with ice cold 0.017 M phosphate buffered saline, pH 7.2. Cells remained viable in thes buffer without substrate for at least 3 hours on ice. The O_2 electrode was allowed to equilibrate for 5 min with 2 ml of buffer at 26°C and after a stable base line had been obtained cells were added to a concentration of about $5x10^7$ per ml. The rate of O_2 consumption was determined in the absence of substrate then in glucose (British Drug House, BDH), proline (Sigma) or succinate (BDH). The effect of saliclhydroxamic acid (SHAM; Koch-Light), potassium cynaide (KCN; BDH) and sodium azide (BDH) was determined at 0.5mM, 1.0mM and 4.0mM respectively.

Cell homogenates were prepared after about 150 strokes in an all-glass dounce-type homogenized in 20 mM Tris, 1 mM EDTA, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 0.25 M sucrose, pH 7.2 on ice. The homogenate was centrifuged at 1,500 g for 10 min to remove intact cells then washed 3 times in the same buffer by centrifugation at 8,000 g for 15 min. Oxygen uptake was measured in the presence of 0.5% (w/v) bovine serum albumin and 0.1 mM ADP. Substrates and inhibitors were added as described above with sodium α-glycerophosphate (Sigma) used as an additional substrate and sodium malonate (5 mM; Sigma) and antimycin A (10 μg/ml; Sigma) used as additional respiratory inhibitors.

ATPase assay

The ATPase activity in sonicated homogenates of H. muscarum and H. ingenoplastis was determined as described by Opperdoes et al. (20). Oligomycin (Sigma) was added to a maximum concentration or 50 μg/ml. Protein was determined by the method of Lowry et al. (21).

RESULTS:

Electron microscopy

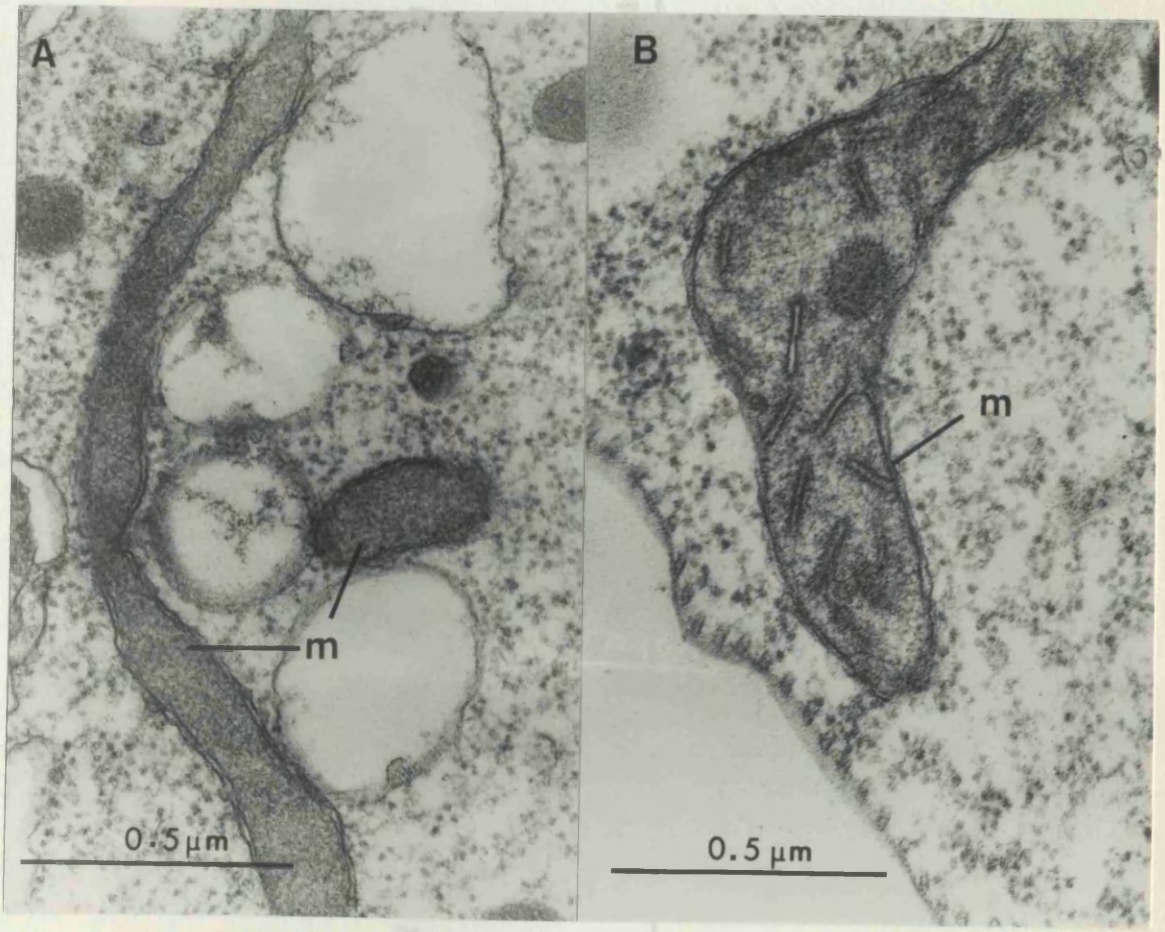
In thin section the mitochondrion of both H. ingenoplastis (figure 1a) and H.muscarum (figure 1b) appeared to be a unitary structure as has been shown for other trypanosomatids (22). The mitochondrion of H. muscarum contained numerous plate-like cristae and was extensively branched; this is the typical appearance of the mitochondrion of other insect trypanosomatids (23) and the insect developmental stages of African trypanosomes. The mitochondrion of H. ingenoplastis was found to be less extensively branched and few tubular cristae were observed. The structure of the pro-mitochondrion of the bloodstream African trypanosomes is similar to that of H. ingenoplastis. Other organelles typical of the trypanosomatids were found in both H. ingenoplastis and H. muscarum including microbody-like structures which have been shown by Opperdoes and co-workers (24,25) to be the location of glycolytic enzymes in the bloodstream forms of T. brucei. Opperdoes et al. (26) have termed these structures glycosomes.

ATPase assays

Table 1 shows the effect of oligomycin of the total cellular ATPase activity in H. ingenoplastis, H. muscarum and purified rat liver mitochondria. An oligomycin concentration of 50 µg/ml gave virtually complete inhibition of the ATPase activity from isolated rat liver mitochondria. This concentration inhibited 24% of the ATP specific ATPase activity in H. muscarum homogenates but had little effect (5% inhibition) on the ATPase activity in H. ingenoplastis homogenates. Figure 2 shows the ATPase activity in homogenates of H. muscarum at various oligomycin concentrations As reported for Crithidia luciliae (20) the half-maximal inhibition of the oligomycin-sensitive ATPase activity was 1.5 µg/mg protein.

Figure 1.

Electron micrographs of thin sections through a portion of the mitochondrion of (A) H. ingenoplastis and (B) H. muscarum showing the morphological differences in the mitochondrial development of the two flagellates. X75,000

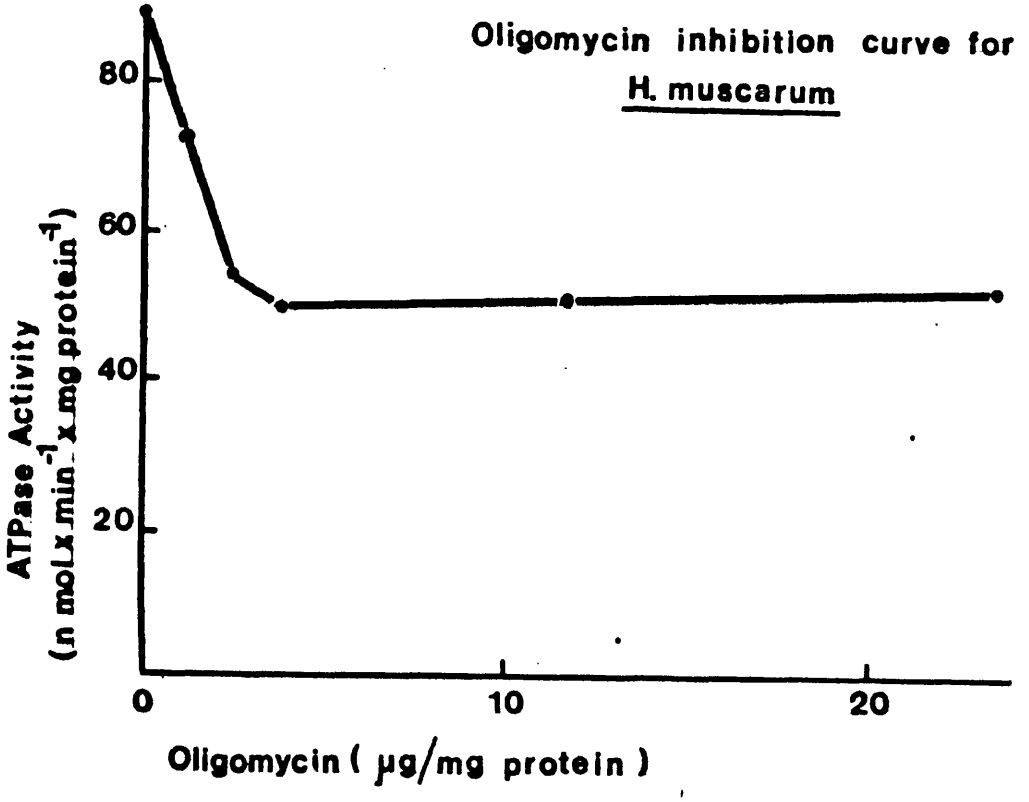

A
m
0.5 µm
B
m
0.5 µm

Oligomycin sensitivity of the ATPase from H. m. muscarum and H. m. ingenoplastis

Organism	ATPase activity (n mol P_i/min/mg) + oligomycin (50 µg/ml)	- oligomycin	% inhibition
H. m. muscarum	32	42	24
H. m. ingenoplastis	21	22	5
Rat liver mitochondria	96	1200	96

Figure 2.

Oligomycin-sensitivity of the ATPase activity in homogenates of H.muscarum.


Oligomycin inhibition curve for
H. muscarum
ATPase Activity
(n mol x min^{-1} x mg protein^{-1})
80
60
40
20
0
10
20
Oligomycin (µg/mg protein)

Respiration and inhibitor studies

Intact cell preparations of H. muscarum showed high levels of O_2 consumption even after extensive washing in substrate-free buffer (Table 2). This is in agreement with reports of high endogeneous respiration in other insect trypanosomatids suggesting intracellular stores of lipids or amino acids (23). H. ingenoplastis had a much lower endogeneous respiratory rate possible suggesting greater dependence on exogenous energy supplies. H. muscarum retained high endogeneous respiration even after 3 hours in substrate-free buffer at 26°C.

H. muscarum was stimulated slightly by both proline and glucose (Table 2); H. ingenoplastis showed a greater preference for glucose with O_2 consumption stimulated to a level more than 4 times that of the endogeneous rate. H. muscarum O_2 consumption was 95% inhibited by 1.0mM cyanide, the remaining 5% being insensitive to either azide or SHAM. Azide alone inhibited respiration by about 70% suggesting that 1.0 mM cynanide may be acting at another site in addition to the terminal oxidase cytochrome aa_3. SHAM had little effect on O_2 consumption in H. muscarum inhibiting respiration by less than 5%. The effect of these 3 inhibitors on the respiration of H. ingenoplastis is also shown in Table 2; inhibition by cyanide was less than 5%, with glucose as a substrate, and the sensitivity to azide was also much lower (13% than for H. muscarum. Respiration was, however, almost completely blocked (98%) by SHAM, suggesting the presence of an alternative terminal oxidase to cytochrome aa_3.

Measurement of O_2 consumption by cell-free homogenates of H. ingenoplastis and H. muscarum (Table 3) gave similar results with azide, cyanide and SHAM inhibition, to those obtained using intact cells. Malonate, a competitive inhibitor of succinic dehydrogenase, suppressed

Intact cellular respiration

	n moles O_2/min/10^8 cells	% inhibition		
		Sham (0.5 mM)	KCN^- (1.0 mM)	Azide (4.0 mM)
H. m. muscarum				
endogenous	84.48	—	—	—
10 mM glucose	91.27	4.27	96.91	65.98
10 mM proline	103.50	4.33	95.35	72.41
10 mM succinate	83.75	1.00	92.50	66.65
H. m. ingenoplastis				
endogenous	25.52	—	—	—
10 mM glucose	112.89	98.63	4.67	13.10
10 mM proline	55.81	86.60	9.09	13.90
10 mM succinate	37.85	97.27	9.08	17.82

Inhibition of respiration in cell homogenates

	% inhibition				
	Sham (0.5 mM)	KCN^- (1.0 mM)	Azide (4.0 mM)	Malonate (5 mM)	Antimycin A (10 µg/ml)
H. m. myscarum					
10 mM succinate	2.48	95.56	67.55	84.85	71.35
10 mM proline	1.91	95.69	86.41	—	93.55
10 mM ∝ glycerolphosphate	2.65	95.80	63.34	—	—
H. m. ingenoplastis					
10 mM succinate	95.5	0	5.0	100	0
10 mM proline	68.7	0	2.0	—	0
10 mM ∝ glycerolphosphate	91.7	0	2.5	—	—

succinate stimulate respiration in homogenates of both H. muscarum and H. ingenoplastis. Antimycin A which specifically blocks electron transport between cytochrome b and cytochrome c_1, inhibited H. muscarum respiration by as much as 93% while having no effect of H. ingenoplastis respiration.

Low temperature difference spectra

Lwo temperature difference spectra were obtained for whole cell suspensions at -196°C (77°K). Figure 3 shows the spectra obtained for H. muscarum and H. ingenoplastis reduced (with sodium dithionite)-minus oxidized control. Absorbance maxima at 604 and 445 mm indicate the presence of cytochrome aa_3 in H. muscarum. These peaks are absent in H. ingenoplastis preparations. The presence of b-type cytochromes was indicated by peaks at 428, 530 and 560 in H. muscarum while H. ingenoplastis spectra showed absorbance maxima at 425 and 528nm. If these do represent cytochrome b then they differ from those found in H. muscarum. Spectra from both organisms indicate the presence of c-type cytochromes typical of most trypanosomatids with absorbance maxima of 515, 523, 550 and 556 nm. The spectral data are summarized in Table 4.

CO-difference spectra

In order to obtain CO-difference spectra, cell suspensions of H. ingenoplastis and H. muscarum were reduced with dithionite and samples gassed with CO. Reference samples were dithionite reduced. Evidence for an o-type cytochrome was obtained in spectra of both H. muscarum and H. ingenoplastis (figure 4). Peaks at 418, 540 and 570 nm are considered characteristic of cytochrome o (7).

DISCUSSION:

Our results suggest that H. muscarum has a respiratory chain which is similar to that discribe for the other trypanosomatids which have functional mitochondria. The cytochrome-mediated electron transport chain is apparently branched at or before cytochrome b, and CO-binding

Figure 3.

Low temperature difference spectra of H. muscarum and H. ingenoplastis cell homogenates. Samples were reduced with dithionite and were cooled to -196°C and spectra determined minus oxidized control.

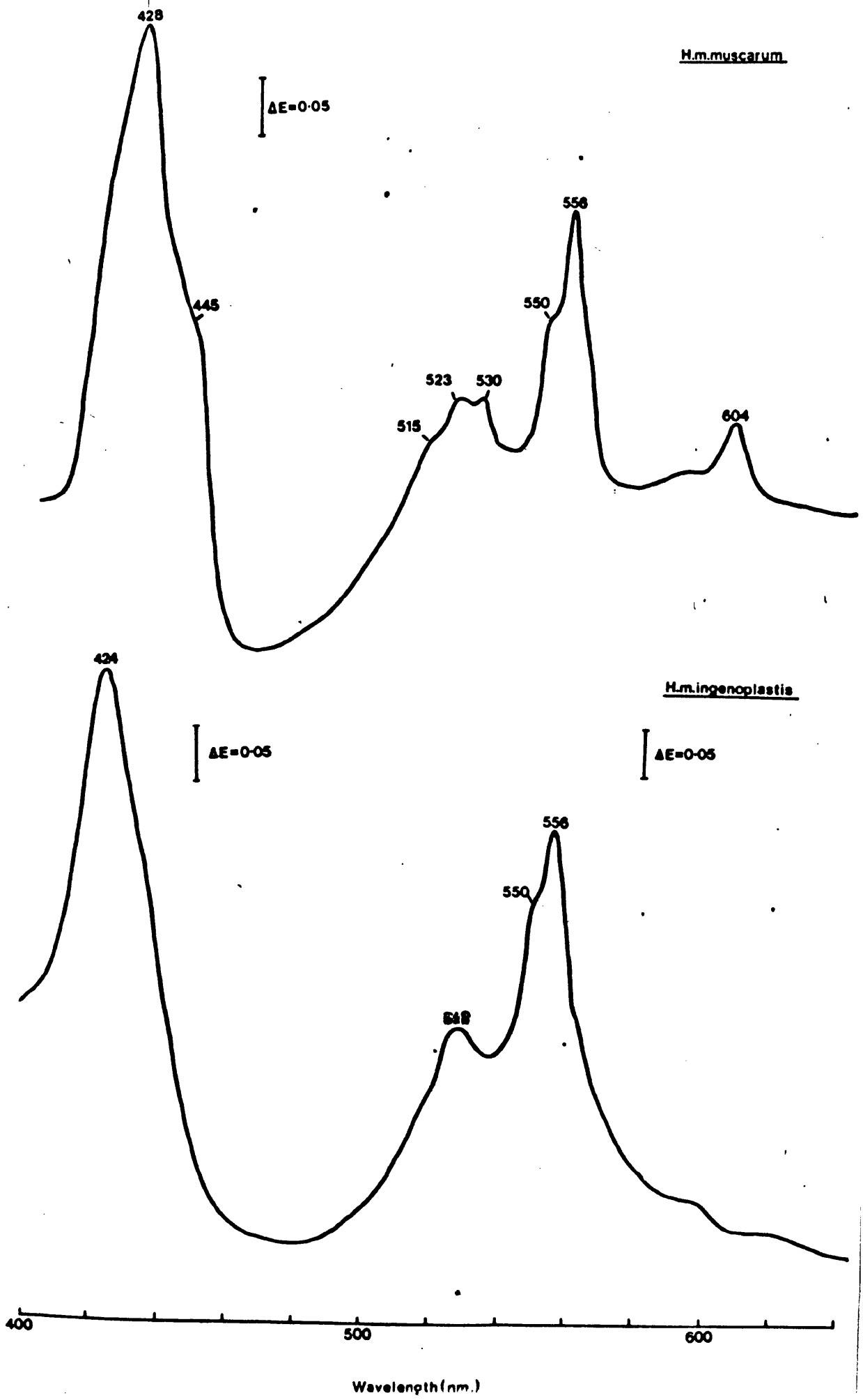
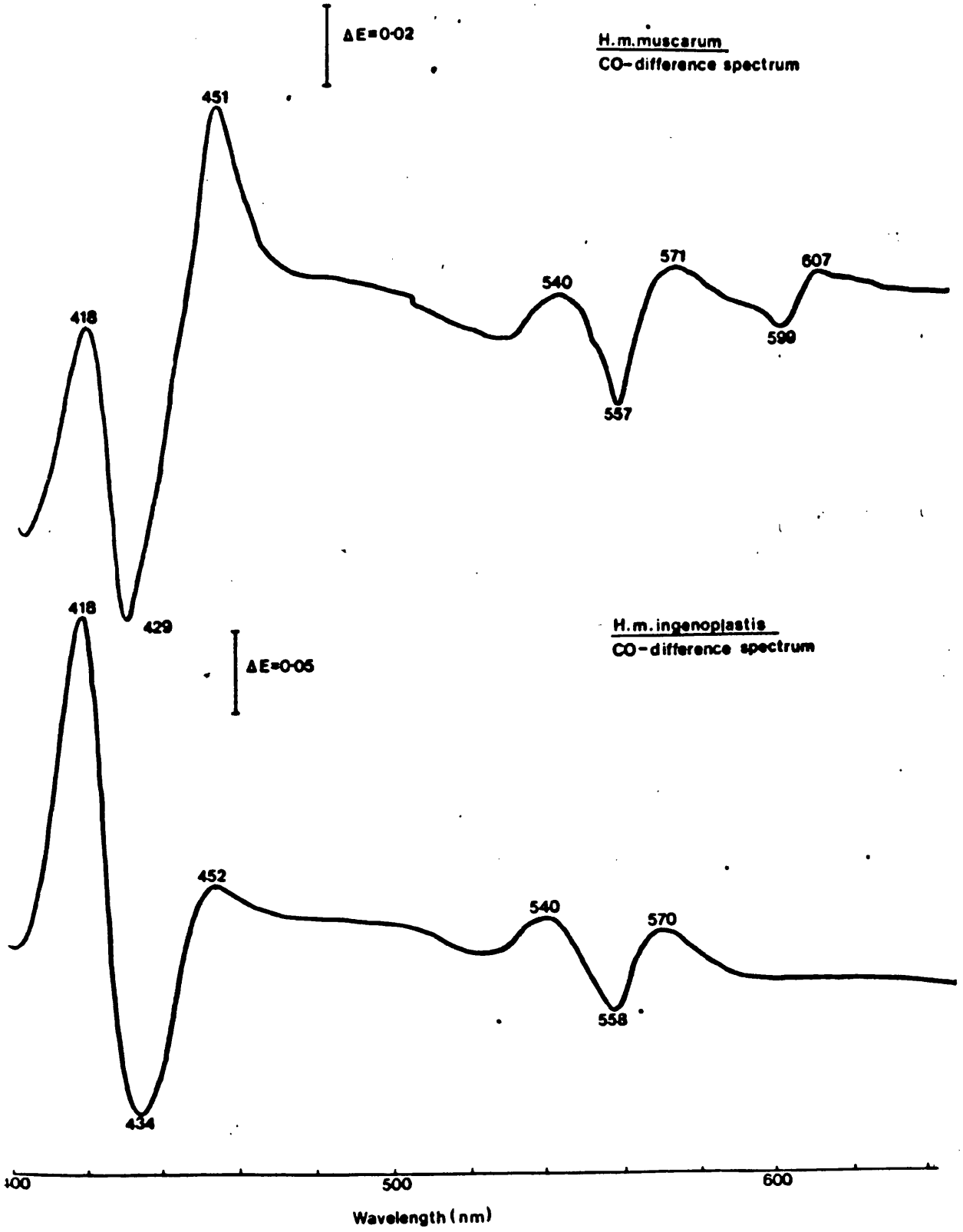

H.m.muscarum
428
ΔE=0·05
445
515
523
530
550
556
604
H.m.ingenoplastis
424
ΔE=0·05
ΔE=0·05
548
550
556
400
500
600
Wavelength (nm.)

Figure 4.

Low temperature CO- difference spectra of H. muscarum and H. ingenoplastis cell homogenates. Samples were reduced with dithionite, bubbled with CO and spectra determined minus dithionite reduced control.

Figure 4.

Low temperature CO- difference spectra of H. muscarum and H. ingenoplastis cell homogenates. Samples were reduced with dithionite, bubbled with CO and spectra determined minus dithionite reduced control.


ΔE=0·02
H.m.muscarum
CO-difference spectrum
451
418
429
540
571
607
557
599
ΔE=0·05
H.m.ingenoplastis
CO-difference spectrum
418
452
434
540
570
558
400
500
600
Wavelength (nm)

LOW TEMPERATURE DIFFERENCE-SPECTRA (REDUCED VS. OXIDIZED)

	Absorption maxima (nm)
H.m. muscarum	
Cytochrome b	428, 530, 560
Cytochrome c	515, 523, 550, 556
Cytochrome aa_3	445, 604
H.m. ingenoplastis	
Cytochrome b	425, 528
Cytochrome c	515, 528, 550, 556
Cytochrome aa_3	

studies suggest the presence of cytochrome o in this branched chain. The nature of alternative terminal oxidases in trypanosomatids and other eukaryotes is far from clear (7,27,28,29,30). Although our results suggest that cytochrome o acts as a terminal oxidase, additional information (in particular photochemical action spectra) is required to substantiate this conclusion.

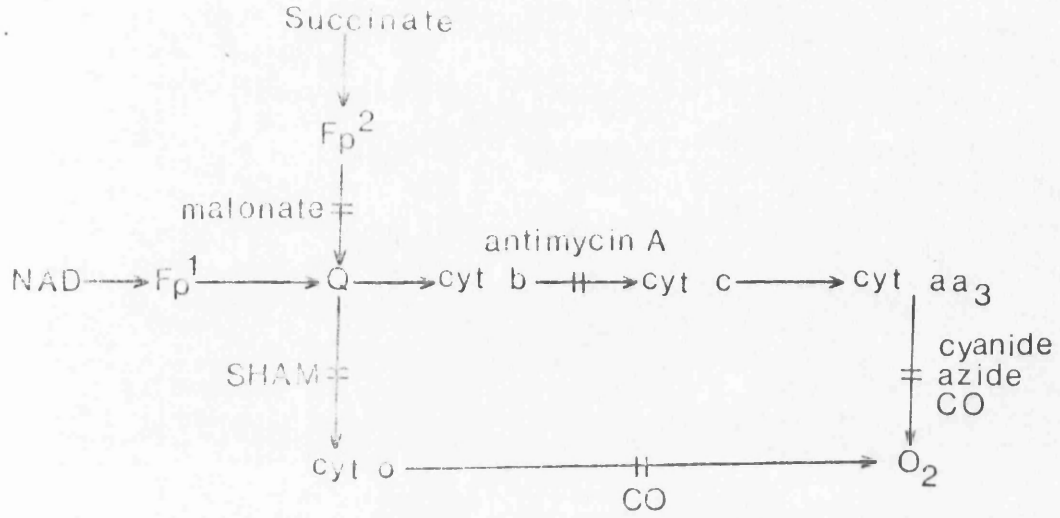
During exponential growth, cytochrome aa_3 is the preferred terminal oxidase in H. muscarum with greater than 90% of the cellular respiration being cyanide-sensitive. Inhibition studies on the respiration of intact cells and homogenates of H. ingenoplastis showed that electron transport did not involve cytochrome aa_3 and that respiration was sensitive to SHAM, an inhibitor of the alternative terminal oxidase in higher plants (31,32) and the α-glycerophosphate oxidase system in bloodstream African trypanosomes (33,34). We conclude that cytochrome aa_3 does not serve as a terminal oxidase in H. ingenoplastis and that an alternative CO-binding oxidase, perhaps cytochrome o, is present.

In figure 5 we present a diagram of possible pathways for respiration in H. ingenoplastis and H. muscarum. The branched electron transport system proposed for H. muscarum is modeled on Ray and Cross' (10) proposed pathway for Trypanosoma mega which is consistent with our data. In H. ingenoplastis only the cytochrome o branch of the pathway appears to be functional, since cytochrome aa_3 is absent and cytochrome b appears to be modified. These findings are of considerable interest in relation to our studies on the kDNA of H. ingenoplastis. We found that the maxi-circle of this flagellate contains major alterations in its nucleotide sequence in comparisons with the maxi-circles of H. muscarum and T. brucei (see preceding paper). Our difference spectra show that cytochrome c is present

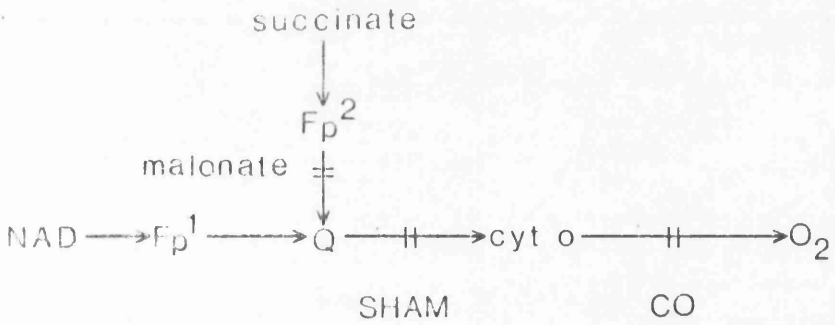
Figure 5.

The proposed arrangement of the electron transport chains of H.muscarum and H. ingenoplastis.

A.


Succinate
$F_p{}^2$
malonate
antimycin A
NAD
$F_p{}^1$
Q
cyt b
cyt c
cyt aa_3
cyanide
azide
CO
SHAM
cyt o
O_2
CO

B.


succinate
$F_p{}^2$
malonate
NAD
$F_p{}^1$
Q
cyt o
O_2
SHAM
CO

in H. ingenoplastis, but it is unlikely to function in electron transport. In yeast, and presumably in other eukaryotes, cytochrome c is coded for by the nuclear genome and synthesized on cytoplasmic ribosomes, so it is unlikely to be affected by changes in the maxi-circle of H. ingenoplastis. The insensitivity of H. ingenoplastis respiration to antimycin A indicates that there is no flow of electrons through cytochrome c in this organism.

In the preceding paper the results of hybridization and restriction endonuclease digestion experiments were presented suggesting that the putative kDNA maxi-circles of H. ingenoplastis and H. muscarum show differences which now can be correlated with differences in mitochondrial activities. As discussed in recent reviews (16,35) the maxi-circle of the kDNA is likely to be equivalent to the mitochondrial DNA of other eukaryotes. The nucleotide sequences differences might be due to mutations in the maxi-circles of H.ingenoplastis which in turn affected only a few specific mitochondrial gene products such as the cytochrome aa$_3$ subunits, cytochrome b and a portion of the oligomycin-sensitive ATPase. Alternatively, changes in the trypanosomatid mitochondrial protein synthesizing system, such as mitochondrial rRNA or ribosomal proteins may have occurred preventing the translation of mitochondrial transcripts. Alterations in the maxi-circle of one trypanosome species, Trypanosoma equiperdum (ATCC 30019), resulting in a 7.5 kilobase deletion are correlated with the inability of this organism to activate the repressed mitochondrion (36). The defective maxi-circle has resulted in the parasite being able to live only in the mammalian host where it obtains its energy wholly by glycolysis.

Mutations in the maxi-circle of H. ingenoplastis may have caused irreversible repression of mitochondrial activity so that respiration is maintained through an alternative pathway; in this case cytochrome o appears to act as the terminal oxidase.

Although alternative terminal oxidases to cytochrome aa_3 have been described for several trypanosomatids, H. ingenoplastis is unique among the insect trypanosomatids in completely lacking cytochrome aa_3.

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Mol.Biochem.Parasitol.

ABSENCE OF DETECTABLE ALTERATION IN THE KINETOPLAST DNA OF A *Trypanosoma brucei* STOCK THAT HAS LOST THE ABILITY TO INFECT THE INSECT VECTOR (*Glossina morsitans*)

S.L.HAJDUK AND K.VICKERMAN

Department of Zoology, University of Glasgow, Glasgow G12 8QQ, Great Britain

(Received ; accepted)

Key words: *Trypanosoma brucei*, kinetoplast DNA, tsetse fly infectivity, restriction endonucleases.

Abbreviations: kDNA, kinetoplast DNA; EATRO, East African Trypanosomiasis Research Organization; GUP, Glasgow University Protozoology.

We have compared several characteristics of a recently derived population of Trypanosoma brucei EATRO 1244 which is incapable of infecting the tsetse fly with the parental population which retains infectivity for the insect vector. Oligomycin-sensitive ATPase activity, growth characteristics in the mammal, degree of pleomorphisms and the ability to grow in culture at 26°C differ for these two populations. No detectable alterations in the maxi-circle component of the kinetoplast DNA were correlated with loss of infectivity for the tsetse fly either by electron microscopy or by restriction endonuclease analysis. There are two possible interpretations of our results: (1) minor alterations, such as point mutations in critical mitochondrial genes, have occurred which are undetectable with the methodology used; (2) mutations have occurred in nuclear genes coding for peptides which are imported into the mitochondrion and are essential for mitochondrial protein synthesis.

INTRODUCTION

In the bloodstream of the mammalian host the long, slender developmental stage of Trypanosoma brucei relies entirely upon glycolysis for energy production [1], the NADH produced in glycolysis being reoxidized to NAD^+ by the cyanide-insensitive glycerophosphate oxidase system; the components of the conventional mitochondrial respiratory chain appear to be completely absent [2,3]. In pleomorphic populations these long, slender forms are capable of differentiation into short, stumpy forms which are apparently pre-adapted for life in the insect vector (Glossina spp.) in that they have a partial Krebs cycle but no functional cytochrome system [4]. The procyclic trypomastigote developmental stage of T. brucei, found in the insect midgut or in

culture at 26°C, has a fully-developed mitochondrion and energy production is coupled to the mitochondrial electron transport chain [5,6]. The potential to develop an active mitochondrion has been lost by certain of the African trypanosomes. Such strains are incapable of cyclical development through the insect vector and are transmissable only by mechanical passage by biting insects (Trypanosoma evansi), as a veneral disease in horses (Trypanosoma equiperdum) and by syringe passage in the laboratory (T. brucei) [7].

Opperdoes et al. [8] have designated trypanosomes which are capable of initiating infections in the insect vector as I^+ and trypanosomes which cannot infect the insect vector or survive in culture at 26°C as I^-. They found a good correlation between known I^+ and I^- stocks and the presence or absence of oligomycin-sensitive ATPase activity. In yeast the peptide conferring oligomycin sensitivity is coded for by the mitochondrial genome and is synthesized in the mitochondrion. Alterations in the mitochondrial genes specifying this peptide or components of the mitochondrial protein--synthesizing system result in the loss of oligomycin sensitivity (a comparable situation may occur in trypanosomes). Trypanosomes lacking the oligomycin-sensitive ATPase activity are apparently unable to develop normal mitochondria. The correlation of oligomycin-sensitive ATPase activity with the ability to grow in certain environments suggests an analogy between the I^- bloodstream trypanosome and the petite mutants of yeast [8-10]. However, the mtDNA ~~of~~ trypanosomes, unlike that of yeast or other eukaryotes, is organized as a uniquely complex structure - the kinetoplast - composed of a large number of catenated, small circular molecules - the mini-circles - and a much smaller number of large circular molecules - the maxi-circles [11-13]. Recent evidence strongly

suggests that the mini-circle component is not transcribed but that major segments of the maxi-circle are [14,15]. The maxi-circle, which is similar in size to other mtDNAs, seems likely to code for the typical mitochondrial gene products while the mini-circles may have some non-coding function [10]. Opperdoes et al. [8] hypothesize that the I^- condition in trypanosomes will usually be the consequence of deletions in or complete loss of the maxi-circle. Such alterations in the mtDNA of the petite mutants of yeast are well documented [16] and preliminary studies of I^- strains of T. brucei, T. evansi and T. equiperdum [17,18] support this hypothesis.

In this study we have used a defined, cloned, parental I^+ stock of T. brucei which has recently been transmitted through the insect vector and an I^- population derived from this clone by 79 syringe passages in laboratory rodents. In the I^- population we were unable to detect any alteration in the maxi-circle component of the kDNA though these trypanosomes have apparently lost the ability to develop active mitochondria.

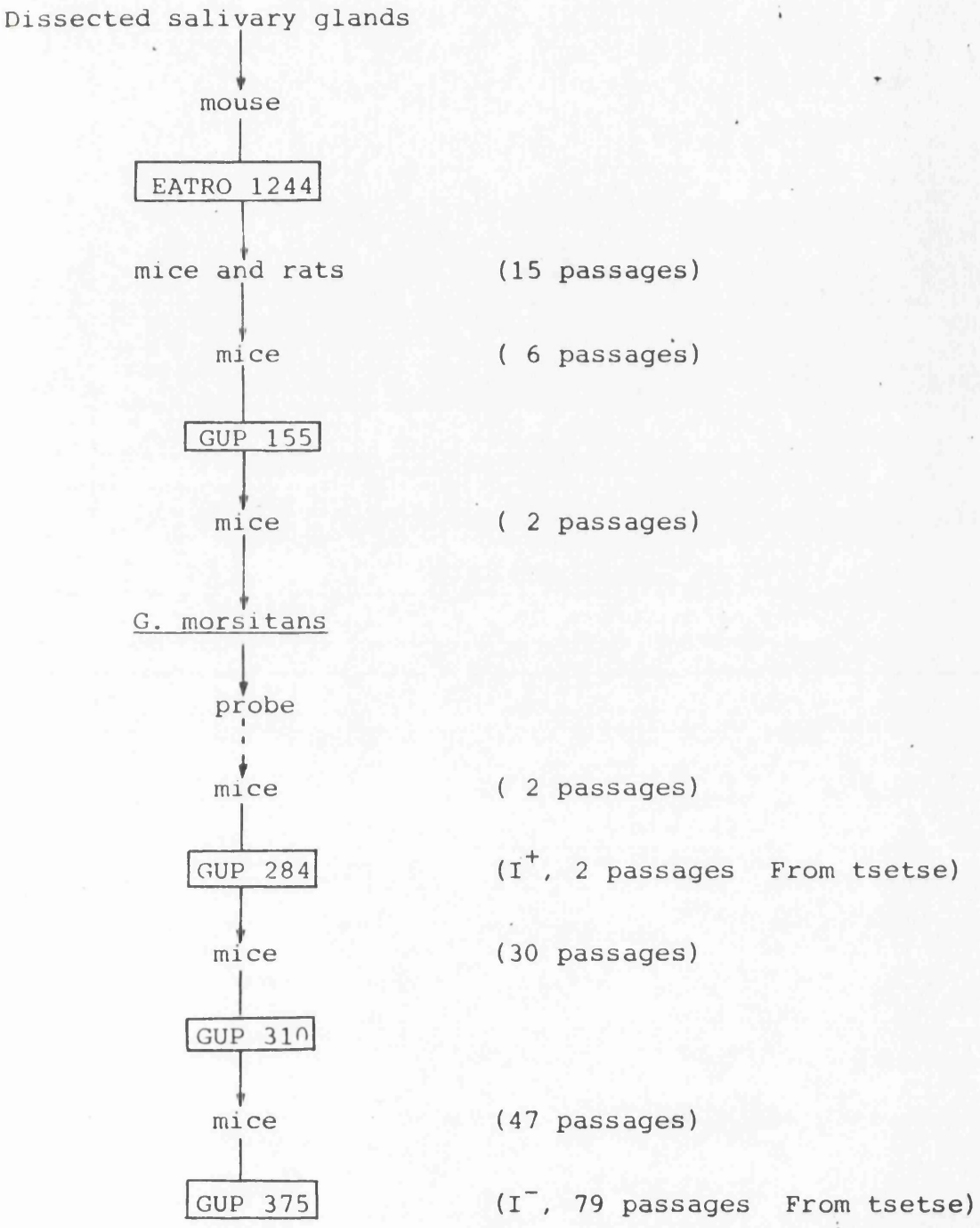
MATERIALS AND METHODS

Organisms

Trypanosoma (Trypanozoon) brucei, EATRO 1244 was isolated from Glossina pallidipes and maintained as a stabilate at -196°C or in laboratory rodents as shown in Fig. 1. The parental I^+ population was cryopreserved following two mouse passages of a single, cloned metacyclic trypanosome from Glossina morsitans. The I^- population was derived following 79 passages in mice. The Amsterdam line of the MITat 1.1 (clone 60) of T. brucei 427 was used as a control in some experiments.

Fig. 1. Pedigree of T. brucei line showing the derivation of the I^+ and I^- populations. Broken lines indicate passage of a single trypanosomes.

Glossina pallidipes Lugala, Uganda (10.4.69)



Growth and isolation of trypanosomes

Female CFLP mice or Wistar rats were routinely irradiated with 600 Rads whole body X-irradiation. About 18 h after irradiation animals were infected by intraperitoneal injection of thawed, stabilated trypanosomes. The doubling times of the trypanosome populations were determined by making cell counts on diluted tail blood samples, taken from non-irradiated, infected mice using a Neubauer haemocytometer. Trypanosomes were separated from the host's blood cells on a diethylamine ethyl cellulose column [19] and concentrated by centrifugation at 1500 x g for 10 min at 4°C. Cells were washed once in 50 mM sodium phosphate, 45 mM sodium chloride, 55 mM glucose (pH 8.0) and held on ice until used.

Tsetse fly infectivity and growth in vitro at 26°C

G. morsitans pupae were obtained from the Tsetse Research Laboratory (Bristol, UK). Pupae were incubated until hatching at 30°C and 60-70% relative humidity. Recently-emerged tsetse flies were chilled at 4°C for 20 min, transferred to individual plastic tubes with gauze-covered ends and then allowed to feed on irradiated mice with 4 or 5 day infections of either the I^+ or I^- populations of EATRO 1244. All infective feeds were taken within 48 h of the fly's emergence. Following the infective feed, flies were maintained at 26°C, relative humidity 60-70% and were fed 3 times a week on the shaven flank of New Zealand male rabbits or on clean CFLP mice. Mature metacyclic infections were detected 17-25 days after the infective feeds by microscopic examination of saliva probes from the flies and the examination of tail blood from mice bitten by the flies.

The in vitro transformation of the bloodstream stages of T. brucei to procyclic forms, similar to the stages found

in the insect midgut, was achieved by incubation of infected mouse blood in a semi-defined medium [20] at 26°C. Irradiated mice were infected with either the I^+ or I^- trypanosome population and blood collected, aseptically, on day 4 of infection. Trypanosomes (5×10^7) were added to 5 ml of media with 50 µg/ml gentamycin (Sigma) and incubated at 26°C, then passaged every 5 days for at least four passages. Cultures started with the I^- trypanosomes showed no live cells past the first passage.

ATPase sensitivity to oligomycin

ATPase activity and oligomycin inhibition were determined using the procedure described by Opperdoes et al. [8] on sonicated homogenates of T. brucei. Protein was estimated using Schecterle and Pollack's [21] modification of the Lowry method.

Isolation of kinetoplast DNA (kDNA)

Trypanosomes were washed twice in 0.15 M sodium chloride, 0.01 M sodium citrate, 0.1 M disodium ethylenediaminetetraacetate (pH 7.5) by centrifugation at 1500 x g for 10 min at 4°C, resuspended in the same buffer and lysed by the addition of an equal volume of 6% sarkosyl in the same buffer. Pronase was added to a concentration of 1 mg/ml and incubation was at 37°C for 90 min with gentle shaking. The lysate was extensively deproteinized with phenol and chloroform:isoamyl alcohol (24:1) extractions and finally precipitated with 2 vols of cold 95% ethanol. Following overnight incubation at -20°C, the precipitate was resuspended in 10 mM Tris-HCl (pH 7.5) and kDNA networks were pelleted by centrifugation in an MSE 55 ultracentrifuge at 21.000 rpm for 90 min at 15°C. The pelleted kDNA was resuspended in 10 mM Tris-HCl and

incubated at 37°C for 1 h with pancreatic ribonuclease (100 µg/ml), extracted with chloroform:isoamyl alcohol and dialysed against 10 mM Tris-HCl for 24 h at 4°C. The kDNA was further purified by NaI gradient ultracentrifugation exactly as described by Fairlamb et al. [22].

Restriction enzyme analysis of kDNA

Purified kDNA was digested with the following restriction endonucleases: EcoRI, HapII, HindIII, MboI, TaqI and HinfI. The digestion fragments were analysed on 0.6% agarose gels using the procedures described elsewhere [23]. The molecular weights of the kDNA restriction fragments were determined by their motility relative to known marker DNA fragments.

Electron microscopy

Purified kDNA networks were spread using a modified version of the micro-diffusion method of Lang and Mitani [24] as described by Fairlamb et al. [22].

RESULTS

The history of the two T. brucei populations used in this study is given in Fig. 1. The trypanosome isolate EATRO 1244 was transmitted through the insect vector G. morsitans and the infected tsetse fly induced to probe into a drop of fresh guinea-pig serum. Metacyclic forms were observed by phase contrast microscopy and single trypanosomes cloned into X-irradiated mice. The I^+ parental population was then stabilated following two passages in mice. The I^- population was derived from this I^+ population by repeated passaging of infected blood from mouse to mouse, usually at 2 or 3-day intervals. 79 passages after the tsetse transmission the

bloodstream population was stabilated and this was used as the I^- population in all further experiments.

Following long-term maintenance in laboratory animals trypanosomes become morphologically monomorphic in appearance and highly virulent for the rodent host [7]. Giemsa-stained blood smears from mice, 2 days after infection with either I^+ or I^- population of T. brucei, showed only the long, slender, multiplicative, developmental stage (Fig. 2a,c). Numerous dividing forms with two kinetoplasts or two nuclei and two kinetoplasts were observed; a short free flagellum was clearly seen on most cells. Following 5 days of infection, the morphology of most I^+ trypanosomes had changed to the short, stumpy developmental stage. These cells were broader, had a prominent undulating membrane, and no free flagellum (Fig. 2b). The frequency of dividing forms decreased in this pleomorphic population. Giemsa-stained smears of the I^- trypanosomes, 5 days post inoculation, were indistinguishable from those seen on day 2. Dividing forms are numerous and only slender forms were observed (Fig. 2d).

The course of parasitaemia in mice infected with the two populations was also different (Fig. 3). When non-irradiated mice were infected with about 10^6 I^+ or I^- trypanosomes, the I^+ population produced a relapsing parasitaemia with mice surviving for about 25 days. The I^- population produces an acute parasitaemia which killed the mice in 4-5 days. Interestingly, the doubling times of both populations was roughly the same - 4.5 h - for the first 72 h of infection, then the growth rate of I^+ populations decreases. This increase in doubling time corresponded with an increased proportion of short, stumpy forms in the population. Growth of the I^- population was exponential until death of the host.

The ability of the I^+ and I^- populations to transform

Fig. 2. Giemsa-stained blood smears from mice infected with I^+ (a,b) and I^- (c,d) populations of T. brucei EATRO 1244. Two days after infection all trypanosomes in both the I^+ and I^- populations are long, slender forms (a,c) kinetoplast (K) and nucleus (N) and a free flagellum (ff) are prominent features I^+ trypanosomes on day 5 of infection are primarily short, stumpy forms (b); I^- populations are entirely long slender (d).

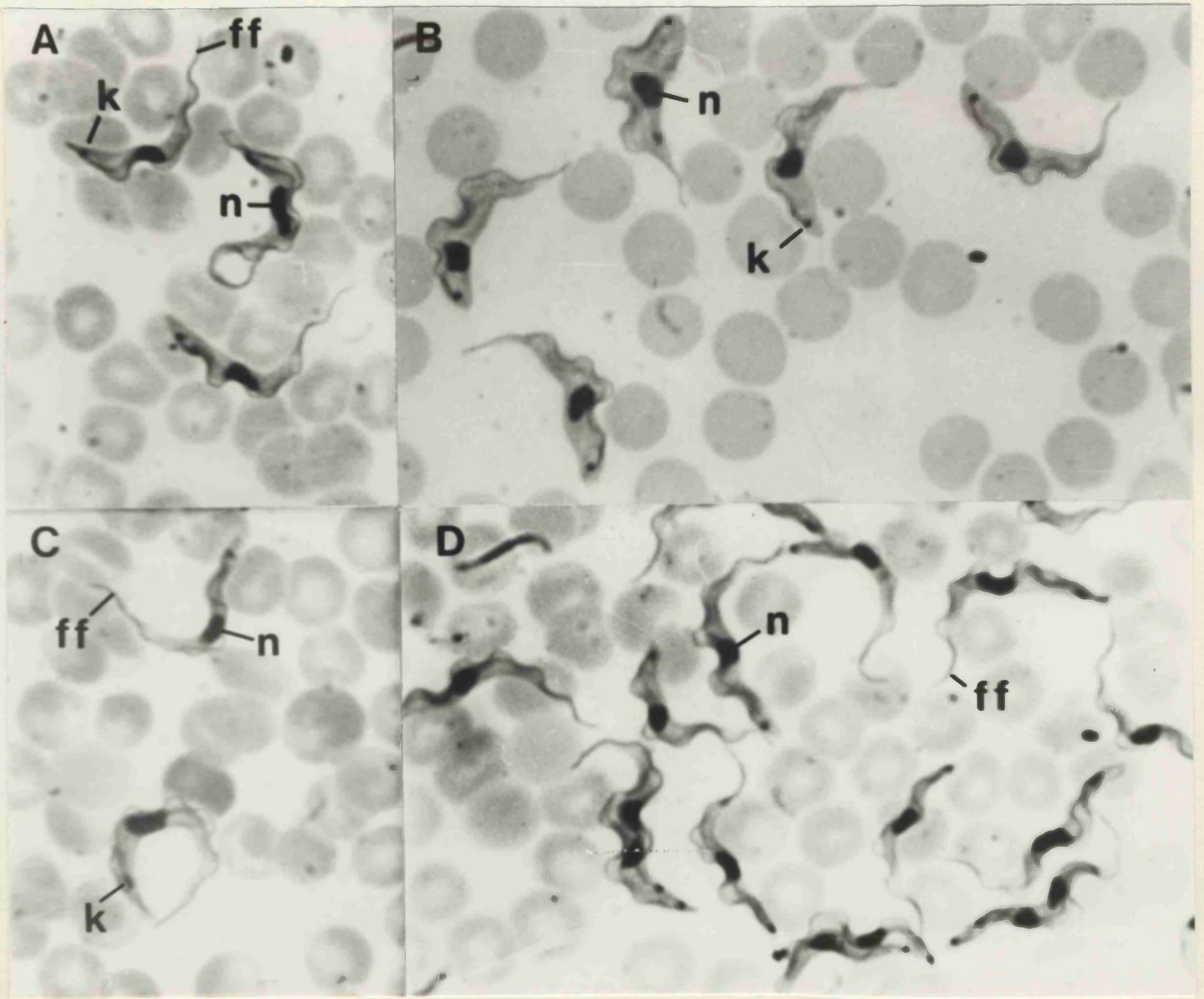
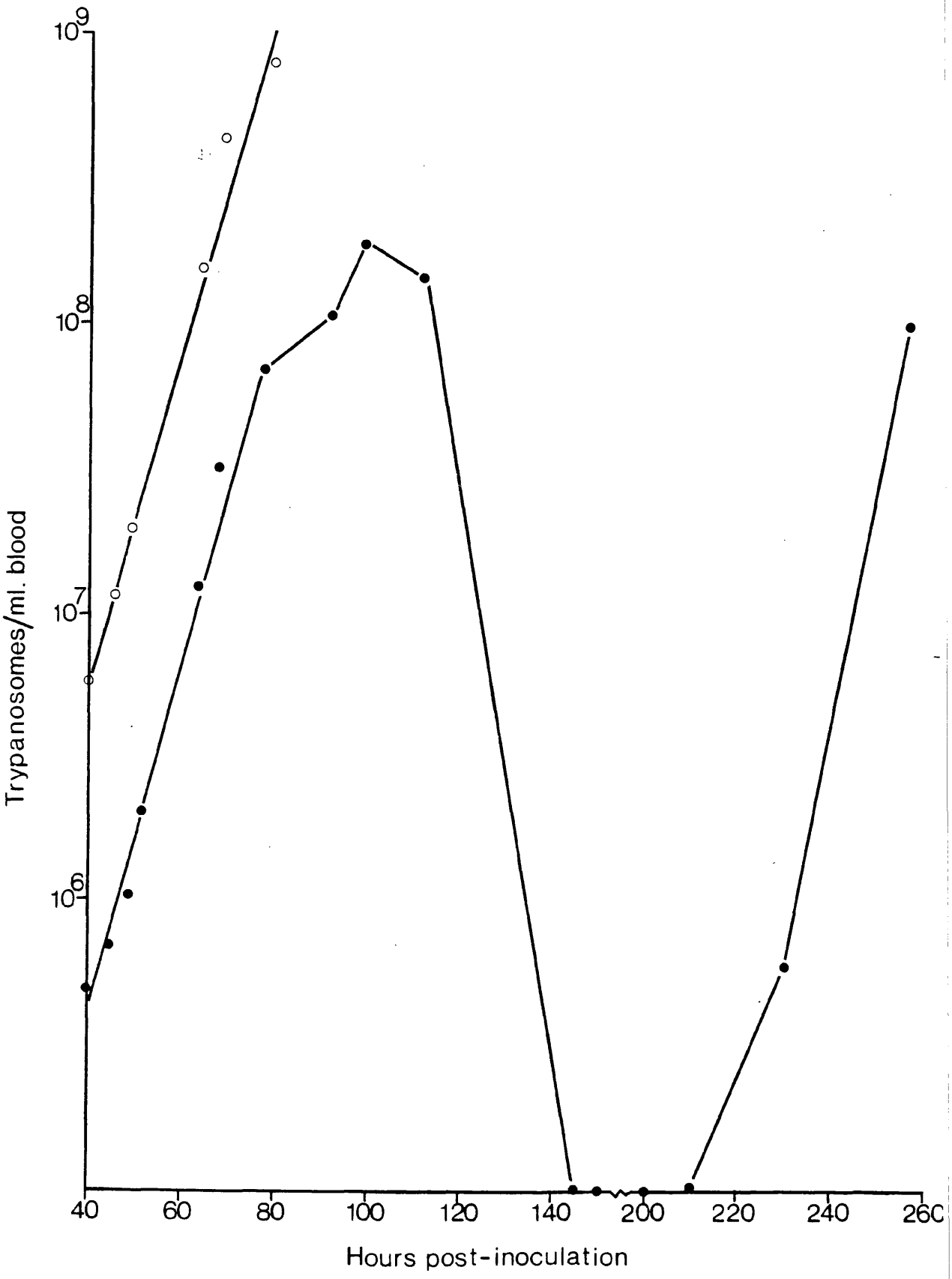

A
ff
k
n
B
n
k
C
ff
n
k
D
n
ff

Fig. 3. Growth characteristics of the I^+ (.—.) and I^- (o—o) populations of T. brucei in the blood of non-irradiated mice.


10^9
10^8
10^7
10^6
Trypanosomes/ml. blood
40
60
80
100
120
140
200
220
240
260
Hours post-inoculation

to the procyclic fly midgut stage was tested directly by infecting tsetse flies and also by cultivation *in vitro* at 26°C (Table I). Recently-hatched flies were fed on irradiated mice which had been infected either the I^+ or I^- *T. brucei* population 4 or 5 days earlier. After a period of about 25 days, flies were induced to probe on warmed microscope slides and batches of 6 flies each fed on clean mice. No infected tsetse flies were detected by either phase contrast examination of probes or by examination of blood samples taken from fly-bitten mice in the 100 tsetse flies which initially fed on the I^- population. Of the flies which fed on the I^+-infected mice, 8.3% developed mature metacyclic infections 17-25 days after the infective feed. We did not dissect the tsetse flies used in these experiments and it is possible that non-infective midgut infections did develop in some of the flies fed on the I^- trypanosomes; however, no trypanosomes were ever observed in the probes from these flies and it is common to observe procyclic forms in probes from flies with midgut infections. Moreover, only I^+ trypanosomes were able to transform to the procyclic stage *in vitro* (Table I).

We compared the oligomycin sensitivity of the mitochondrial ATPase of the I^+ and I^- populations of *T. brucei* (Table II). In sonicated homogenates of the I^+ population of *T. brucei* 28% of the ATPase activity was inhibited by the addition of 50 µg oligomycin per ml. The ATPase activity of the I^- population was completely insensitive to oligomycin.

Electron microscopy of kDNA networks purified by density gradient ultracentrifugation revealed no detectable differences in kDNA from the I^+ and I^- populations. Networks of catenated mini-circles and long-edge loops are seen in the kDNA from both. Contour length measurements of the long-edge

TABLE I

CHARACTERISTICS OF I^+ AND I^- POPULATIONS OF T. brucei EATRO 1244

	I^+	I^-
Infectivity for Glossina		
No. infected/no. fed	3/36	0/100
% Infected	8.3	0
In vitro cultivation		
No. established/no. started	5/5	0/5
Pleomorphism: % short, stumpy forms:		
Day 2	0	0
Day 5	0	75
Doubling time (h) in bloodstream*	4.5	4.5

* Doubling times determined during day 2 and 3 of infection (see Fig. 3).

TABLE II

OLIGOMYCIN SENSITIVITY OF THE ATPase FROM I^+ AND I^- POPULATIONS OF *T. brucei* EATRO 1244

Organism		ATPase activity (nmol P_i/min/mg)		
		- Oligo	+ Oligo (50 µg/ml)	% Inhibition*
T. brucei	I^+	25	18	28
	I^-	28	28	0

* Data given are the means of two experiments.

loops, maxi-circles, were not made.

In Fig. 4 the high-molecular-weight fragments produced by digestion of purified kDNA with the restriction endonucleases HapII, HindIII, TaqI and HinfI are compared for the I^+ and I^- populations of T. brucei EATRO 1244 and T. brucei 427. With these four enzymes and also with EcoRI and MboI no differences in the mobility of the I^+ and I^- kDNA fragments were detectable (Table III). One enzyme, HinfI, revealed a difference in mobility of 1.8×10^6 D for the largest fragment of T. brucei 427 kDNA and that of the I^+ T. brucei EATRO 1244. The difference in the size of the largest fragment of the 427 and the EATRO 1244 was accounted for by the presence of an extra fragment of 1.7×10^6 D present in both the I^+ and I^- EATRO 1244 kDNAs. This was visible as a double band in these gels (Fig. 4). Table III summarizes the results obtained with the six endonucleases tested.

DISCUSSION

Our results show that the inability of certain trypanosomes to develop functional mitochondria and to undergo cyclical development in the tsetse fly vector is not necessarily correlated with detectable alterations in the kDNA. There are two explanations for these observations:

1. Only minor alterations (e.g. point mutations) in critical mitochondrial genes have occurred which are undetectable with the methodology used.

2. Mutations have occurred in nuclear genes coding for peptides which are imported from the cytoplasm into the mitochondrion and are essential for mitochondrial protein synthesis.

The first type of mutation would be analogous to the

Fig. 4. Electrophoretic analysis of kDNA digested with restriction endonucleases HapII (lanes 1-3), HindIII (4-6), TaqI (7-9) and HinfI (11-13) run on 0.6% agarose gels and stained with EthBr. Lanes 1,4,7,11: digests of T. brucei EATRO 1244 I^+ kDNA; 2,5,8,12: digests of T. brucei EATRO I^- kDNA; 3,6,9,13: digests of T. brucei 427 kDNA. Intact phage lambda DNA and phage lambda DNA digested with EcoRI were run as molecular weight markers in lanes 10 and 14 along with phage ØX DNA digested with BspI in lane 10 and phage ØX DNA digested with MspI in lane 14. The positions of linearized maxi-circles and mini-circles are indicated.

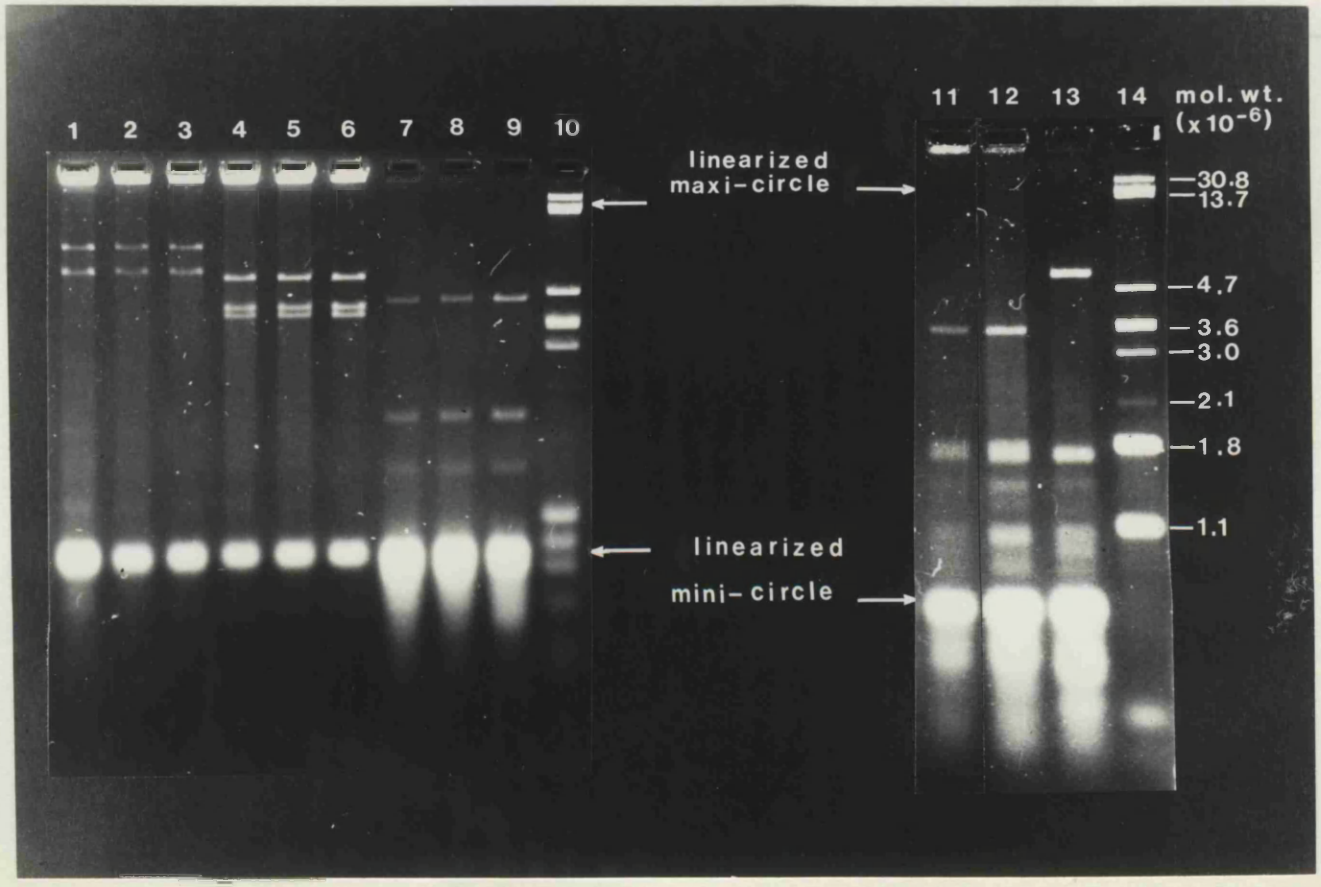

1
2
3
4
5
6
7
8
9
10
11
12
13
14
mol. wt.
(x10⁻⁶)
linearized
maxi-circle
linearized
mini-circle
30.8
13.7
4.7
3.6
3.0
2.1
1.8
1.1

TABLE III

MOLECULAR WEIGHT OF MAXI-CIRCLE FRAGMENTS FROM *T. brucei* EATRO 1244 I^+ AND I^- AND 427 kDNAs DIGESTED WITH RESTRICTION ENDONUCLEASES AND S_1 NUCLEASE

Source	Fragment No.	Molecular weight of maxi-circle fragments (x 10^{-6})						
		HapII	HindIII	EcoRI	HinfI	TaqI	MboI	S_1
EATRO 1244: I^+	1	7.6	5.4	8.0	3.3	4.2	3.9	13.5
	2	5.9	4.0	4.0	1.7	1.7*	1.8	
	3		3.8	2.4	1.5	1.2	1.4	
	4				1.0	0.8	1.2	
	5				0.84		1.1	
Total		13.5	13.2	14.4	8.34	7.9	9.8	13.5
EATRO 1244: I^-	1	7.6	5.4	8.0	3.3	4.2	3.9	13.5
	2	5.9	4.0	4.0	1.7	1.7*	1.8	
	3		3.8	2.4	1.5	1.2	1.4	
	4				1.0	0.8	1.2	
	5				0.84		1.1	
Total		13.5	13.2	14.4	8.34	7.9	9.8	13.5

(Table III - continued)

427	1	7.6	5.4	8.0	5.1	4.2	3.9	13.5
	2	5.9	4.0	4.0	1.5	1.7•	1.8	
	3		3.8	2.4	1.0	1.2	1.4	
	4					0.8	1.2	
	5				0.84			
Total		13.5	13.2	14.4	8.44	7.9	9.8	13.5

• A double band; two different co-migrating fragments.

In HinfI, TaqI and MboI digests, many small maxi-circle fragments cannot be checked on the 0.6% agarose gels since they run with the mini-circle size fragments.

cytoplasmic petite mutants (ρ^-) of yeast; the second to the nuclear petite mutants (p). As pointed out by Opperdoes et al. [8], nuclear petite mutants in yeast are unstable and readily accumulate mutations in mtDNA to become ρ^-p double mutants, if the secondary mutations in mtDNA are not selected against. It is doubtful, however, if the same situation applies in trypanosomes. The unusual sequence characteristics of yeast mtDNA may promote frequent internal recombinations and the deleted molecules generated may readily segregate out, resulting in the high level of spontaneous ρ^- mutants (about 1% per generation) observed in most wild-type strains. Without selection such mutants will accumulate and since usually more than 50% of the wild-type mtDNA sequence is deleted, the alteration in mtDNA is easily detected. The maxi-circles of T. brucei also have a high mole percent AT, but there is no evidence for an alternation of very AT-rich and GC-rich segments like in yeast, from the 2000 base pairs sequenced thus far (Eperon, I.C., personal communication). Moreover, these maxi-circles are catenated into a network and altered maxi-circles will, therefore, not segregate out to yield clones with altered kDNA. The kDNA in nuclear trypanosome mutants, affected in mitochondrial biogenesis, may therefore be stable. Hence, we cannot decide on a priori grounds whether the I^- phenotype of our altered T. brucei population is due to alterations in nuclear or in kDNA.

As discussed by Opperdoes et al. [8] and Borst and Fairlamb [10], the I^- phenotype can apparently result from the following alterations in kDNA structure:

1. Complete loss of the kDNA network; dyskinetoplastic stocks of T. evansi and perhaps T. equiperdum [17] are trypanosomes which show no kDNA in Giemsa-stained preparations [25,26].

2. Retention of the mini-circle network structure, but loss of the maxi-circle component; a stock of T. evansi with kDNA networks lacking long-edge loops in spread preparations and high-molecular-weight fragments in restriction endonuclease digestions, which are considered characteristic of maxi-circles have been described [22].

3. Deletions in the maxi-circle resulting in loss or alteration of vital mitochondrial genes; a kinetoplastic stock of T. equiperdum (ATCC 30019) [18,27].

4. Mutations in maxi-circle genes essential for mitochondrial protein synthesis; the I^- population of T. brucei used in this study and T. brucei strain 31 [17] could possibly belong in this grouping.

Our results suggest a possible correlation between pleomorphism in the I^+ trypanosomes and the ability to make functional mitochondria. However, earlier observations [28] on the SAK stock of T. evansi have shown that pleomorphism may occur in I^- populations, incapable of activating normal mitochondrial activities and lacking kDNA [29]. Also certain highly monomorphic and virulent syringe-passaged lines of T. brucei (e.g. 427-60) retain the I^+ phenotype, the potential to produce active mitochondria and an intact maxi-circle. The selection of our I^- population by long-term syringe passaging of the I^+ population in mammals might have caused the accumulation of various mutations in this population. These mutations may be completely unrelated to the I^- phenotype or the ability to produce functional mitochondria and might result in the differences observed in pleomorphism and virulence of the I^+ and I^- populations.

If there were no selective advantage in the mammalian host for retaining the ability to make functional mitochondria, then it might be expected that all syringe-passaged

laboratory lines would be I^-, but this is not the case. Also if the kDNA in I^- populations is completely non-functional, then it might be expected that the kDNA would eventually be completely lost in all I^- lines; again this is not the case. This suggests that the presence of an intact mini-circle network is of advantage to trypanosomes, even in I^- stocks (cf. ref. 10). The nature of this advantage remains unclear.

The establishment of I^- monomorphic T. brucei populations from defined I^+ populations which have only minor mutations in their genomes, whether mitochondrial or nuclear, are likely to be useful not only in defining the factors responsible for transmissability by the tsetse fly, but also in the study of the differentiation processes in the life cycle of trypanosomes.

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