

ZYMODEMES OF TRYPANOSOMA CONGOLENSE
AND A PRELIMINARY ASSESSMENT OF THEIR
EPIDEMIOLOGICAL SIGNIFICANCE

by

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TO MY MOTHER AND FATHER

WITH AFFECTION AND GRATITUDE

Zymodemes of *Trypanosoma congolense* and a Preliminary
Assessment of their Epidemiological Significance

by Christina J. Young

ABSTRACT

Throughout this century, considerable confusion has arisen over the specific status of members of the subgenus *Nannomonas*. At the present time, only two species are generally recognized, *T.(N.) congolense* and *T.(N.) simiae*. However, many differences still exist in morphological and behavioural characteristics of *T.congolense* stocks, which led in the past, to numerous designations of species, subspecies and varieties. In an attempt to clarify the problem, 78 stocks of *T.congolense* were characterized intrinsically by enzyme polymorphism.

Conditions devised for thin-layer starch-gel electrophoresis of the isoenzymes of trypanosomes from other subgenera were modified for 14 enzymes: L-threonine 3-dehydrogenase (TDH), purine nucleoside hydrolase (NH), pyruvate kinase (PK), glyceraldehyde phosphate dehydrogenase (GAPDH), aspartate aminotransferase (ASAT), phosphoglucomutase (PGM), alanine aminotransferase (ALAT), malate dehydrogenase (decarboxylating NADP) (ME), malate dehydrogenase (MDH), glucose phosphate isomerase (GPI) and four peptidases using either L-leucylglycylglycine (PEP 1) or L-leucyl-L-alanine (PEP 2) as substrate.

Eight of these enzymes readily distinguished *T.congolense* from representatives of the subgenera *Trypanozoon* and *Duttonella*.

The numbers of different isoenzyme patterns for *T.congolense*, each often with multiple banding, ranged from one for TDH, PK and NH, to ten for GPI. Altogether 71 different isoenzyme bands were seen and from the various combinations of these bands, 75 zymodemes were found among the 78 stocks of *T.congolense* examined. The degrees of relationship between the zymodemes was established by computer analysis. A major dichotomy existed between stocks originating from savannah regions and those from riverine or forest areas. This distinction was most obvious in the GPI, PEP 1 and PEP 2 isoenzyme patterns.

Besides morphological comparisons of these two major zymodemes initial investigations of behavioural characteristics included transmissibility by *Glossina* spp., drug sensitivity, infectivity and susceptibility to disease in different strains of inbred mice. Differences between the two major zymodemes were found in morphology and drug sensitivity; the savannah zymodeme appeared to be more efficiently transmitted than the riverine/forest zymodeme by the savannah tsetse fly, *G.morsitans*. The susceptibility to disease of two strains of inbred mice varied with different trypanosome stocks; there was no correlation with the two major zymodemes.

The relevance of these findings is discussed.

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CHAPTER 1

1. INTRODUCTION

1.1. General Introduction

Trypanosoma (Gruby, 1843) is one of nine genera contained in the family Trypanosomatidae (Doflein, 1901), order Kinetoplastida (Honigberg, 1963). The majority of Trypanosoma are digenetic parasites found in the blood of all major classes of vertebrates and the gut of leeches and arthropods.

Evans (1880) was the first to recognize the pathogenic nature of a trypanosome, later named T.evansi (Balbiani, 1888). This discovery prompted numerous investigations which led to the incrimination of trypanosomes as the causative organisms of many already recognized diseases of man and his domestic animals.

During the first decade of this century, great advances were made in the knowledge of the trypanosomes of mammals. Five species of pathogenic trypanosomes of domestic livestock were reported: T.equiperdum (Doflein, 1901), T.congolense (Broden, 1904), T.vivar (Ziemann, 1905), T.suis (Ochmann, 1905) and T.simias (Bruce et al., 1912). Three species of trypanosome parasitizing man were also identified: T.zambiense (Dutton, 1902), T.cruzi (Chagas, 1909) and T.rhodesiense (Stephens and Fantham, 1910).

In 1964, Hoare reorganized the classification of the mammalian trypanosomes into two groups, Stercoraria and Salivaria, based on the site of production of the metatrypanosomes (= metacytic

trypanosomes) in the insect host and the subsequent method of transmission to the mammalian host. He divided the Stercoraria into three subgenera, Megatrypanum (Hoare, 1964), Herpetosoma (Doflein, 1901) and Schizotrypanum (Chagas, 1909) and the Salivaria into four subgenera, Trypanozoon (Lühe, 1906), Duttonella (Chalmers, 1918), Nannomonas (Hoare, 1964) and Pycnomonas (Hoare, 1964).

Typically, in the salivarian trypanosomes, epimastigote and trypomastigote forms are found in the vector, Glossina spp.; the cycle is completed in the mouthparts or salivary glands, and transmission is by inoculation of infected saliva into the mammalian host, where the trypomastigote forms thrive in the blood, sometimes invading the tissues. Exceptions in the mode of transmission do occur in the Salivaria; T.vivax can be mechanically transmitted by haematophagous insects, T.evansi is always mechanically transmitted by bloodsucking flies other than tsetse flies and T.equiperdum is transmitted during coitus of equines.

The mammalian trypanosomes are described in the exhaustive work of Hoare (1972) and in the collection of reviews edited by Lumsden and Evans (1976). In this thesis, only the subgenus Nannomonas will be considered in detail.

1.2. Subgenus Nannomonas

1.2.1. Recognition

Members of the subgenus Nannomonas are readily distinguished from the other salivarian subgenera by their morphological appearance in Giemsa-stained thin blood films (Plates 1.1 to 1.4). They are small, measuring 8 to 24 μm in total length, and of comparable size to the rare subgenus Pycnomonas. The kinetoplast of Nannomonas

Plates 1.1 to 1.4. Giemsa-stained thin blood films (Scale = 20µm)

Plate 1.1. T.(N.) congolense in mouse blood

Plate 1.2. T.(D.) vivax in cattle blood

Plates 1.3 and 1.4. T.(T.) brucei in mouse blood

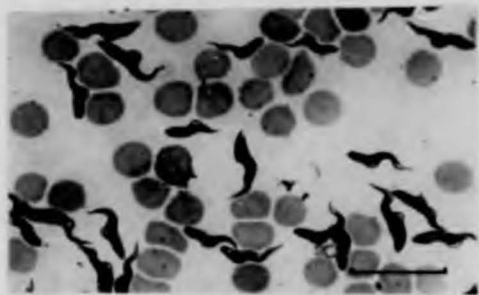


Plate 1.1.

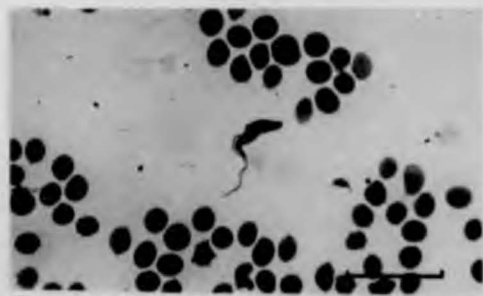


Plate 1.2.

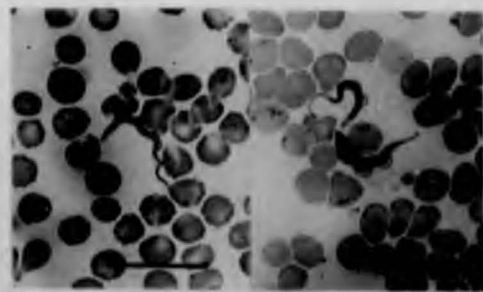


Plate 1.3. Plate 1.4.

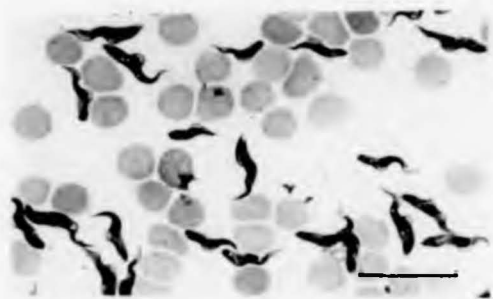


Plate 1.1.

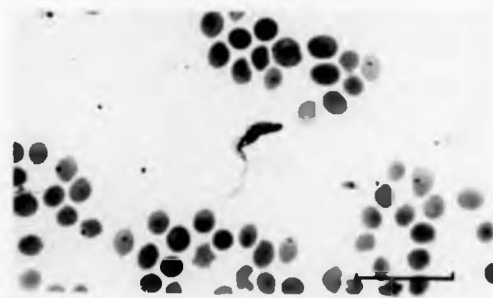


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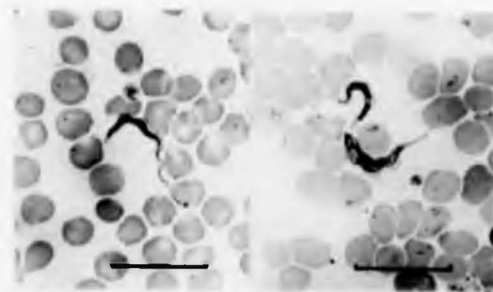


Plate 1.3.

Plate 1.4.

trypanosomes is of medium size (0.7 to 0.8 μm in diameter), smaller than Duttonella and larger than both Trypanozoon and Pycnomonas, and situated in a subterminal marginal position. However, the most distinctive feature is the apparent lack of a free flagellum which is found in many individuals of the other subgenera; in those instances when a free flagellum is observed in a stained blood film of Nannomonas, it is very much shorter than that of the other subgenera.

When transmission is effected by tsetse flies, identification of the subgenus can be made by the position of development of the trypanosomes; Nannomonas develops in the midgut and proboscis, whereas Duttonella develops in the proboscis alone and Trypanozoon and Pycnomonas in the midgut and salivary glands. However, complications can arise from mixed or aberrant infections.

1.2.2. Distribution, transmission and economic importance

Trypanosomes belonging to the subgenus Nannomonas are restricted to the tsetse fly belts of tropical Africa (Fig. 1.1). Survival of the subgenus is largely dependent on cyclical transmission by Glossina spp., although mechanical transmission is believed to occur with T.(N.) simiae (Montgomery and Kinghorn, 1909b).

T.congolense, the principal species in the subgenus Nannomonas, is one of the most important pathogenic parasites affecting livestock in Africa. Hosts include bovines, equines, sheep, goats, camels, pigs and dogs. In the past, it has been regarded as strictly a plasma parasite confined to the circulatory system but recent reports provide conclusive evidence of their occurrence in connective tissue and lymph nodes (Luckins and Gray, 1978, 1979a; Gray and Luckins, 1979), although no involvement of the central nervous system has been found. Manifestation of the disease, while varying with different trypanosome

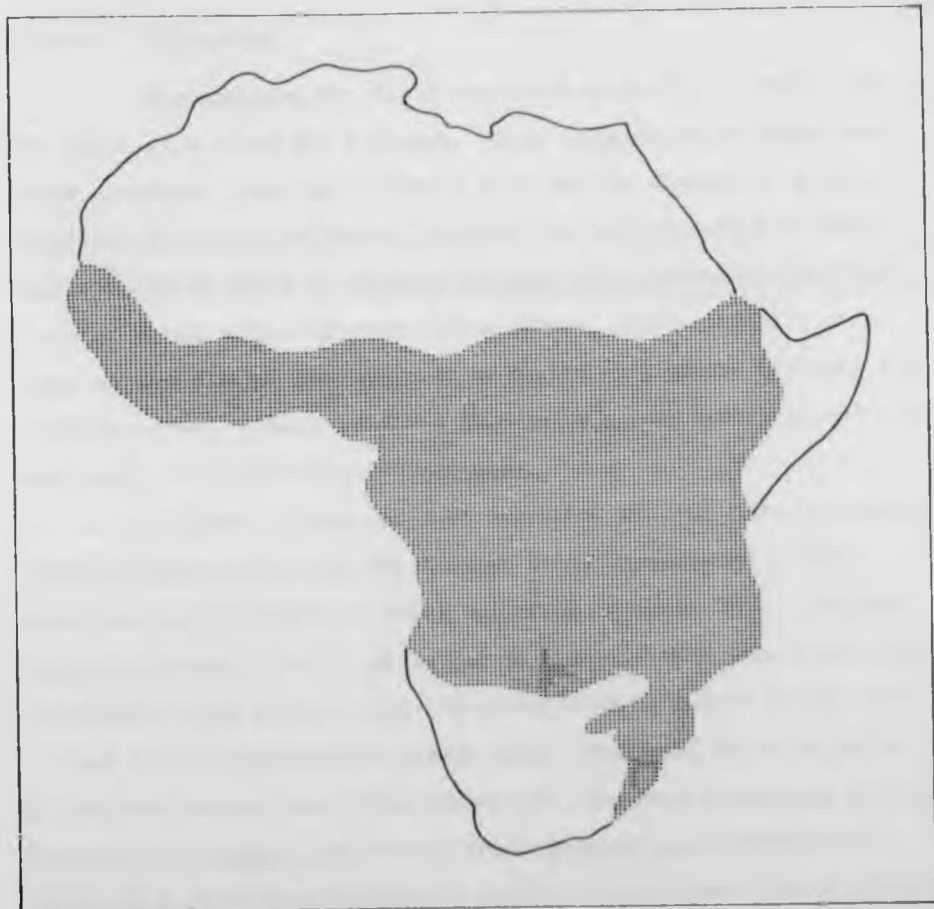


Fig. 1.1. Tsetse fly belt of Africa

stocks and the host, is generally characterized by fever, anaemia and cachexia.

1.2.3. Speciation

1.2.3.1. Historical

T. congolense was first described by Broden in 1904, from the blood of a sheep and a donkey. This trypanosome differed from those previously seen by its small size and the absence of a free flagellum in all individuals. However, an earlier record of this parasite can be found in Bruce's drawings of trypanosomes from the blood of a cow suffering from nagana (Bruce, 1895), the Zulu name given to the disease now known to be caused by T. brucei (Plimmer and Bradford, 1899). Bruce believed this to be a pure infection but one individual is undoubtedly T. congolense.

In 1903, Dutton and Todd described another mixed trypanosome infection under the name 'The Gambian Horse Trypanosome'. This infection was described as being comprised of three forms; 'tadpole forms' measuring 11 to 13 μm by 0.8 to 1.0 μm with a very short free flagellum, 'long forms' measuring 26 to 30 μm by 1.6 to 2.0 μm with a long free flagellum and 'stumpy forms' measuring 16 to 19 μm by 3.5 μm with a very short free flagellum. The long forms were probably T. brucei or T. vivax, the tadpole forms T. congolense (despite the report of a short free flagellum) and the stumpy forms, the trypanosome later named T. montgomeryi (Laveran, 1909). A horse harbouring these trypanosomes was sent to Liverpool, where Thomas and Breinl (1905) found only the tadpole forms. A rat was subsequently infected from this horse and sent to Laveran and Mesnil (1904) who recognized not only the tadpole forms but a longer form without a free flagellum;

they named this dimorphic trypanosome T.dimorphon. The T.vivax or T.brucei infection had probably been lost on passaging into the rat and the morphology of the remaining infection may have altered in the different host giving rise to the longer forms (Godfrey, 1960a).

In 1905, Laveran found a trypanosome, morphologically similar to T.congolense, in cattle in the Sudan. However, he proposed a new name T.nanum, as it appeared to be slightly smaller than T.congolense Broden and was specific to cattle; laboratory animals were resistant to infection (Montgomery and Kinghorn, 1909a).

Meanwhile, the confusion over The Gambian Horse Trypanosome continued; Montgomery and Kinghorn (1909a) rightly alleged that the original infection had been mixed and adopted the name T.dimorphon for the mixed infection and proposed T.confusum for the pure infection seen by Laveran and Mesnil (1904). However, as Laveran and Mesnil commented (Mesnil, 1909; Laveran and Mesnil, 1912), these changes were not justified because firstly, the name T.dimorphon had priority for the pure infection which they had described, secondly, The Gambian Horse Trypanosome was a mixed infection and thirdly, T.confusum was already in use for an avian parasite (Lühe, 1906).

Several trypanosome infections resembling The Gambian Horse Trypanosome, including individuals with a free flagellum, were reported as T.dimorphon, but it is believed that they were mixed infections of T.congolense with T.brucei or T.vivax (Dutton *et al.*, 1907; Montgomery and Kinghorn, 1908, 1909a). Martoglio (1911) described mixed infections of T.congolense and T.brucei from livestock in Somaliland (Somalia) under the names T.somalilense (= T.somaliense) and T.celii. T.dimorphon was also used, mistakenly, to describe some pure infections of T.brucei (Balfour, 1906, 1909; Kopke, 1908;

Hindle, 1909; Mendes et al., 1909). Kinghorn et al. (1913) described a mixed infection, again similar to The Gambian Horse Trypanosome, under the name T.multiforme.

Thus, the early literature on congolense-like trypanosomes contains so many discrepancies and misinterpretations that it is a difficult and perhaps impossible task to resolve the confusion completely. Bruce et al. (1910a) made an attempt and showed T.congolense, T.dimorphon and T.confusum to be morphologically indistinguishable both from one another and from T.pecorum, a trypanosome described by Bruce et al. (1910a) from cattle in Uganda. They proposed to unite these four species under the name T.pecorum, but this was unacceptable, as by the Law of Priority of the International Code of Zoological Nomenclature (1958), T.congolense had precedence. T.nanum maintained its independence due to its host specificity and low pathogenicity to laboratory animals (Bruce et al., 1911c), but in 1913, Blacklock and Yorke found it indistinguishable from T.congolense and suggested, that as the infectivity of the latter also varied widely, these two species should be considered as identical.

In 1909(b), Montgomery and Kinghorn described another congolense-like organism, differing from T.congolense Broden in being broader and possessing a well developed undulating membrane and sometimes a bristle-like free flagellum. Laveran examined this parasite, and proposed a new species, T.montgomeryi (Laveran, 1909). In 1912, Laveran and Mesnil reduced T.montgomeryi from species status on the grounds that it was insufficiently described. Since then, however, numerous reports have been made of this trypanosome both in pure infections and mixed with T.congolense and T.simiae (Kinghorn and Yorke, 1912a; Kinghorn et al., 1913; Weissenborn, 1911; Wenyon, 1926;

Schwetz, 1930, 1934; Bourguignon, 1933, 1935; Lhoverol and Philippe, 1947; Peel and Chardome, 1954a; Chardome and Peel, 1954; Stephen, 1963). A new species, T. frobeniusi, was also created for a similar infection containing congolense- and montgomeryi-like individuals (Weissenborn, 1911).

In 1921, yet another species was named, T. ruandae, for an infection of trypanosomes bearing all the characteristics of T. congolense (van Saceghem, 1921). More recently, Chardome and Peel, working in Burundi, isolated different trypanosome stocks from G. brevipalpis, which differed in minor morphological features and host-parasite relationships. Initially, they regarded these stocks as varieties of T. congolense; T. congolense var. urundiense (Peel and Chardome, 1954b), T. congolense var. berghei (Chardome and Peel, 1954) and T. congolense var. mossoense (Peel and Chardome, 1954c). Later, however, these varieties were accorded the status of species (Chardome and Peel, 1967).

By 1959, many of the early species were no longer recognized but there was still controversy over the separate speciation of T. dimorphon and T. congolense. Hoare (1959) undertook a morphological and biometric study of T. dimorphon and compared his findings with previous work on T. congolense (Bruce et al., 1910a; Blacklock, 1912; Kinghorn et al., 1913; Bruce et al., 1913a). He found two types of monomorphic trypanosomes whose mean lengths were distinct and did not overlap, and therefore concluded that T. congolense and T. dimorphon were, indeed, two separate species. However, Godfrey (1960a) isolated bovine T. congolense stocks from Nigeria with intermediate mean lengths and Fairbairn (1962) confirmed these results in stocks from other parts of Africa. Huisenga (1969), measuring only individuals in the

first stage of division, found no length differentiation between T.dimorphon and T.congolense. Thus, based on their morphology, T.dimorphon was considered to be synonymous with T.congolense, but Godfrey (1961) demonstrated a difference in pathogenicity between dimorphon-type and congolense-type stocks.

During the years of confusion over the nomenclature of T.congolense, discrepancies had arisen over the identification of another member of this subgenus. In 1909(b), Montgomery and Kinghorn working in Zambia, found a pig which suddenly developed severe symptoms of trypanosomiasis in spite of the fact that there were no tsetse flies in the area. The blood of the pig was swarming with trypanosomes and within six hours the animal was dead. Bloodsucking Diptera were common in the area, so it was assumed that they were the transmitters of this virulent disease. Montgomery and Kinghorn (1909b) suggested, tentatively, that the trypanosome was T.nanum.

Bruce et al. (1912) redescribed this parasite from a monkey and a goat which had been infected by wild G.morsitans, caught in Malawi; they named it T.simiae. They stated that 'T.simiae is a well defined species easily separated by its morphology alone from the other trypanosomes which have been described as causing disease among domestic animals'. However, from their description, it closely resembled the then recognized species, T.dimorphon. The main difference between T.simiae and the other congolense-like organisms was behavioural T.simiae displaying an extreme virulence in domestic pigs (Montgomery and Kinghorn, 1909b; Bruce et al., 1913b). A trypanosome, identical to T.simiae, was described a month later under the name T.ignotum and was again isolated from a monkey (Kinghorn and Yorke, 1912b).

The first record of a devastating outbreak of porcine trypanosomiasis was recorded by Lichtenheld (1912) in Tanzania, who stated it was caused by nagana. It has been presumed he meant T. brucei, but this is unlikely as cattle and horses in the vicinity were apparently unaffected. In subsequent years, numerous similar outbreaks were reported especially from Zaire, but the causative organism continued to be disputed. From one outbreak, a new species, T. rodhaini was described (Walravens, 1924), but Hornby (1926) criticised this speciation believing the trypanosome to be T. uniforme, a parasite now classified as belonging to the subgenus Duttonella.

In 1930, an outbreak of porcine trypanosomiasis was recorded near Kisangani, in Zaire, caused by forms resembling T. congolense, T. simiae and T. rodhaini; a new species T. porci was designated for this pleomorphic organism (Schwetz, 1932). Further work at Kisangani and Katanga led Bourguignon (1933) to believe that these infections were produced by a mixture of T. simiae and T. congolense, but later he acknowledged that the organisms were of one species, T. simiae (Bourguignon and Juissant, 1934). In 1936, Hoare undertook morphological and biometric studies using these original preparations of T. simiae, T. rodhaini, T. porci and T. uniforme and concluded that they were all similar and undoubtedly of one species, T. simiae.

1.2.3.2. Present

There is still some uncertainty as to the number of species contained in the subgenus Nannomonas, due to the diversity in morphological and behavioural characteristics of different stocks. However, only two species are still generally recognized, namely T. congolense and T. simiae. Morphological differentiation is difficult and the division is based not only on the predilection of T. simiae

for the Suidae and its virulence and fatality to domestic pigs, but also on its failure to infect cattle and laboratory rodents. However, reports can be found on differences in pathogenicity of T.simiae stocks (Mackenzie and Boyt, 1969) including one, non-lethal in pigs (Killick-Kendrick and Godfrey, 1963); although the identity of this parasite is questionable (Godfrey, 1977). There are also reports of pig restricted T.congolense stocks (Peel and Chardome, 1954b; Chardome and Peel, 1954). Records can be found of T.simiae infecting cattle, a horse and camels, but the true identity of these parasites is again debatable (Culwick and Fairbairn, 1947; Pellegrini, 1948; Wilson, 1958; Killick-Kendrick and Godfrey, 1963). Also, like T.simiae, certain stocks of T.congolense fail to infect laboratory rodents (Blacklock and Yorke, 1913).

Hoare (1972) suggests that this gradation of characteristics may justify the division of one species of Nannomonas into three subspecies; T.congolense congolense, T.congolense dimorphon and T.congolense simiae.

1.2.4. Conclusions

Trypanosomes of the subgenus Nannomonas are easily distinguished from the other salivarian subgenera by their morphological appearance in Giemsa-stained thin blood films; however, further division or speciation within this subgenus is difficult. There is a wide diversity in the behavioural characteristics of Nannomonas trypanosomes including host specificity and pathogenicity. Morphological differences have proved to be inadequate in distinguishing these trypanosomes and some other characterization method is required to provide accurately defined material for research, and to identify 'strains' for epidemiological purposes. With this aim, attention

has been paid to biochemical methods of characterization.

1.3. Characterization by Examination of the Cell Genotype

Since the sequences of bases in nuclear and cytoplasmic DNA determines the characteristics of an organism (except some viruses) (see Newton, 1976), studies of the DNA of organisms, or the genotype, should give valuable information about its phylogeny and evolution. During the last ten years rapid advances have been made in the techniques for the determination of base sequences of relatively small nucleic acid molecules.

The DNA of kinetoplastids has been examined using DNA buoyant densities (Newton and Burnett, 1972; Chance et al., 1974) and preliminary experiments on hybridization of complementary RNA with kinetoplast DNA (k DNA) (Newton et al., 1973; Steinert et al., 1973) and restriction endonuclease analysis (Riou and Yot, 1975; Kleisen and Borst, 1975; Brack et al., 1976) have produced some promising results.

1.3.1. DNA buoyant density

DNA buoyant density values give an assessment of the relative proportions of the base pairs, guanine-cytosine and adenine-thymine in the double-stranded DNA helix.

Subgeneric identification of many kinetoplastids has been achieved by DNA buoyant density measurements, but species recognition was not always apparent. Originally, it was thought that T.gambiense could be distinguished from other members of the T.brucei - complex by the presence of a 'satellite C' band in its DNA buoyant density profile (Newton and Burnett, 1972). However, examination of more

material has failed to reveal this band (Gibson et al., 1978a). T.congolense can be differentiated from all other members of the genus Trypanosoma by the presence of an 'A' component which has only been found in one other kinetoplastid, Crithidia oncopelti (Newton and Burnett, 1972; Chance et al., 1974). However, no record of the numbers of T.congolense stocks examined can be found and no subspecific differentiation was reported. Since the measurement of DNA buoyant density is both costly and time consuming and the same levels of distinction can be readily made from the morphological appearance of trypanosomes, it is unlikely that this technique will be used for routine identification of stocks.

1.3.2. DNA hybridization

Preliminary hybridization experiments have indicated that there are considerable differences in the base sequences of k DNA from different species of kinetoplastids, even when the k DNA's have identical buoyant densities, for example between C.oncopelti and C.fasciculata (Newton et al., 1973; Steinert et al., 1973). Thus, hybridization has proved to be a useful technique for differentiating closely related species. However, unstable hybrids can be formed, whose molecules are only partially united by base sequences and thus the overall sequences may not all be complementary (Newton, 1976).

1.3.3. Restriction endonuclease analysis

Restriction endonucleases recognize specific nucleotide sequences in double-stranded DNA and cleave both strands of the duplex. This technique has been used in the analysis of k DNA of T.cruzi and C.luciliae, providing evidence of sequence heterogeneity in the mini-circular molecules (Riou and Yot, 1975; Kleisen and Borst, 1975).

Brack et al. (1976) succeeded in distinguishing T. brucei from T. rhodesiense but extensive characterization was restricted because of the need to use purified k DNA; the purification process is long and complicated.

1.4. Characterization by Examination of the Cell Phenotype

The DNA of an organism can be studied indirectly by investigation of the phenotypic expression, for example, the nutrition, metabolism, or structure of proteins and other cell constituents.

1.4.1. Nutrition

Nutritional requirements have been widely used for the characterization of bacteria but determination of the nutrients required by the kinetoplastids is apparently proving to be a difficult task (see Newton, 1976). So far, of the trypanosomes, only T. brucei has been grown under defined conditions (Cross and Manning, 1973).

1.4.2. Metabolism

Present knowledge of the metabolism of kinetoplastid flagellates is extensive and differences in the oxidative pathways and consequent end products of metabolism are known between the Trypanosoma subgenera (see Bowman and Flynn, 1976); but differentiation at species level has not yet been found.

1.4.3. Serological comparisons

Any attempt to characterize trypanosomes by immunological means is severely hampered by their ability to change their surface coat antigens, possibly, an infinite number of times. Considerable progress, however, has been made in the development of techniques

for studying the relationships of trypanosomes belonging to the subgenus Trypanozoon, both in the mammalian host (Gray, 1970, 1977; Latif and Adam, 1973; Le Ray et al., 1971; Paris et al., 1976) and the tsetse fly (Jenni, 1977; Le Ray et al., 1977; Le Ray et al., 1978). Using the indirect fluorescent antibody test (IFAT), Latif and Adam (1973) succeeded in differentiating between T.brucei, T.rhodesiense and T.gambiense. Le Ray et al. (1971), studying extracts of soluble antigens from trypanosomes by immunoelectrophoresis and the double diffusion technique, differentiated T.gambiense from T.rhodesiense and T.brucei but failed to distinguish between the last two.

Similar investigations of T.congolense and T.vivax have been limited by lack of suitable material. Nevertheless, work based on cross protection tests has established the existence of up to three different 'strains' of T.congolense (Schilling and Neumann, 1932; Fiennes, 1950a). Examination of 'primary parasitaemic populations' of trypanosomes, developing in animals inoculated with organisms isolated from wild-caught tsetse flies, demonstrated six different 'strains' (Wilson et al., 1973). However, these 'primary parasitaemic populations' may differ antigenically from those isolated from microscopically subpatent infections six to nine days after infecting by tsetse fly bite (Uilenberg and Giret, 1972; Uilenberg et al., 1973). In T.congolense antigenic variation apparently occurs during the long initial microscopically subpatent period. To overcome this problem, recent work by Luckins and Gray (1979b) has produced promising results by using T.congolense isolated from localized skin reactions in rabbits, occurring at the site of an infected tsetse fly bite. However, this is also complicated by evidence suggesting that the metatrypanosomes of T.congolense are antigenically heterogeneous in the tsetse fly (Schlappi and Jenni, 1977) and not all

stocks produce skin reactions (Luckins and Gray, 1979b). Despite these problems, by combining IFAT's, enzyme-linked immunosorbent assays (ELISA) and neutralization tests, Luckins and Gray (1979b) found at least three different 'strains' of T.congolense amongst the four stocks examined.

Cross (1977) tentatively suggested that morphological differences seen between T.brucei, T.vivax and T.congolense might be due in part to differences in surface antigen structure. Indications of species-specific differences in surface antigens have been seen by electron microscopy (Vickerman, 1974) and from charge differences (Lanham and Godfrey, 1971). However, at present, the structure of the amino acid sequences of variant-specific surface antigens has only been examined in T.brucei and T.evansi (Cross, 1977).

Characterization of trypanosome stocks by serological, immunological and immunochemical techniques is only at a preliminary stage and comparison of large numbers of stocks cannot, as yet, be undertaken.

1.4.4. Multiple enzyme forms or isoenzymes

Each enzyme performs a specific biochemical function in the cell. Enzymes are largely protein molecules whose primary structures are genetically determined and a particular enzyme catalysing a certain reaction can occur as several different molecular forms; when these occur in one species they are termed isoenzymes. Isoenzymes have been defined as 'multiple, separable forms of enzymes occurring within the same organism and having similar catalytic activities' (Shaw, 1969). Comparison of the isoenzymes of two organisms allows indirect examination of their genomes and an assessment of their relationship to each other.

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If the different molecular structures of the isoenzymes are associated with a dissimilarity in the net electrical charge, they can be distinguished by their mobilities in an electrical field i.e. by electrophoresis. Separation can be further enhanced by using a molecular sieving supporting medium, such as starch or polyacrylamide, which slows down the movement of the larger molecules. After electrophoresis, the bands of enzyme activity are detected by staining reactions linked to the conversion of the specific substrate. Thus, a small quantity of enzyme is readily detected since one enzyme molecule will catalyse the conversion of many molecules of substrate. There are many enzymes in each cell, probably several thousand, but many are unstable or too weak to detect. Nevertheless, the activities of numerous enzymes can be specifically stained and each enzyme may display several isoenzymes after electrophoresis giving in total, numerous characteristics. Thus, differentiation can be made between protozoal organisms using many characters, as opposed to the relatively few morphological features.

Enzyme electrophoresis provides a quick, simple method for the comparison of many enzyme characteristics of numerous samples and has been widely used in the characterization of protozoa (Table 1.1). Originally, work on each genus was designed to determine differences between stocks, but in many cases this work was expanded in an attempt to find correlations between the enzyme variations and epidemiological or other biological characteristics of the organisms.

The first epidemiological studies were made with Plasmodium falciparum from different geographical locations in Africa (Carter and Voller, 1973; Carter and Voller, 1975). Carter and Voller (1975) examined three enzymes of isolates of P.falciparum from five different

Table 1.1. Parasitic protozoa characterized by isoenzyme electrophoresis

<u>Genus</u>	<u>References</u>
<u>Entamoeba</u>	Reeves and Bischoff, 1968 Sargeaunt and Williams, 1978 Sargeaunt <u>et al.</u> , 1978
<u>Plasmodium</u>	Carter, 1970 Carter and McGregor, 1973 Carter and Voller, 1973, 1975
<u>Leishmania</u>	Gardener and Howells, 1972 Ebert, 1973, 1974 Gardener <u>et al.</u> , 1974 Kilgour <u>et al.</u> , 1974 Peters <u>et al.</u> , 1977 Al-Taqi and Evans, 1978 Brazil, 1978 Chance, 1979 Miles <u>et al.</u> , 1980b Aljeboori and Evans, 1980a, 1980b
<u>Trypanosoma</u>	Kilgour and Godfrey, 1973 Bagster and Parr, 1973 Toyé, 1974 Kilgour <u>et al.</u> , 1975 Godfrey and Kilgour, 1976 Miles <u>et al.</u> , 1977 Miles <u>et al.</u> , 1978 Baker <u>et al.</u> , 1978 Gibson <u>et al.</u> , 1978a Gibson <u>et al.</u> , 1978b Murray, 1979 Letch, 1979 Gibson, 1979 Barrett <u>et al.</u> , 1980 Miles <u>et al.</u> , 1980a Gibson <u>et al.</u> , 1980
<u>Eimeria</u>	Shirley and Rollinson, 1979

regions of tropical Africa and showed that the organisms formed a genetic continuum from East to West Africa, despite reports of differences in drug sensitivity and transmissibility (Garnham, 1966).

Godfrey and Kilgour (1976) separated T.gambiense from T.brucei and T.rhodesiense by characteristic aminotransferase patterns. Subsequently, Gibson et al. (1980) carried out a detailed epidemiological study of 160 stocks belonging to the T.brucei-complex. They correlated the enzyme patterns to geographical distribution showing a major dichotomy between East and West African stocks. During the course of their work they found trypanosome stocks isolated from pigs to be enzymically indistinguishable from T.gambiense of man, thus implicating pigs as reservoirs of gambian trypanosomiasis (Gibson et al., 1978a).

Miles et al. (1977) identified two strain-groups of T.cruzi, the causative organism of Chagas' disease or South American trypanosomiasis, which circulated independently of one another in a sylvatic and domestic cycle, the latter including man. Later, it was shown that the sylvatic cycle also involved man and that three electrophoretically distinct types (zymodemes) of T.cruzi were all found in man in Brazil (Miles et al., 1978). Barrett et al. (1980) expanded this work and found that the three zymodemes were associated with three independent host-vector and transmission cycles and that regional differences in the ecology of two of the zymodemes was apparent. They also incriminated five different vectors of T.cruzi and three reservoir hosts by isoenzyme electrophoresis.

Sargeant et al. (1978) identified four electrophoretically distinct groups of Entamoeba histolytica, and by electrophoresis of two enzymes, differentiated the invasive form causing clinical amoebiasis, from the non-invasive form.

Peters et al. (1977) confirmed the geographical distribution of Leishmania aethiopica by isoenzyme characterization and DNA buoyant density comparisons. They also proved the animal reservoir to be the hyrax and the vector, Phlebotomus pedifer, of L.aethiopica on the slopes of Mount Elgon, Kenya.

Al-Taqi and Evans (1978) working with stocks of L.tropica, isolated in Kuwait, distinguished enzymically between the rural and urban nosodemes, L.t.major and L.t.minor respectively, by comparing stocks with known case histories. This distinction is often impossible from the clinical appearance of the sore, since it may frequently be altered by patients applying antiseptic ointments.

Aljeboori and Evans (1980b) using seven enzymes found Rattus rattus to be harbouring Leishmania which was enzymically indistinguishable from L.t.minor in man, and similarly Rhombomys opimus (the gerbil) to be infected with L.t.major.

Thus, isoenzyme electrophoresis is beginning to prove to be an invaluable tool in epidemiological studies of parasitic protozoa.

It must be appreciated however, that the detection of isoenzymes or multiple bands of enzyme activity after electrophoresis is not proof that an enzyme exists in multiple forms. Harris and Hopkinson (1976) classified isoenzymes into three major groups; those arising from multiple genetic loci coding for functionally the same enzyme, those arising from multiple alleles at a single locus, and those arising from post-translational changes (secondary isoenzymes). These secondary isoenzymes include enzyme aggregates, conformational isomers, enzymes bound to other types of molecules, or enzymes which have suffered proteolytic action or deamination by other enzymes during isolation. The formation of secondary isoenzymes, nevertheless depends on the basic structure of the original enzyme, so can still

provide information about genotype. Even if the preparation procedure changes the enzyme structure, if all the samples are made and treated in exactly the same manner, differences seen will arise from fundamentally different molecular structures.

It must be noted that while detection of differences in isoenzymes can be used to separate two organisms, the occurrence of identical bands does not necessarily mean that two organisms are identical. Electrophoretic differences depend mostly on differences in the net charge of two enzyme molecules, and only one-third of all possible single amino acid substitutions in proteins result in a charge difference (Harris and Hopkinson, 1976). Thus, some molecular differences will not be detected by electrophoresis and if structures are similar no separation may occur even when the matrix has molecular sieving properties. However, if many enzymes are examined in two stocks without any differences being found, it is very likely that the populations are similar; the more enzymes which are examined the greater is the degree of confidence in the closeness of the relationship.

1.5. Conclusions of Characterization Techniques

From this synopsis of characterization techniques currently available or under development, it is evident that isoenzyme electrophoresis provides, at present, the most reliable and practical technique for the characterization of large numbers of trypanosome samples. It is hoped, that this thesis will show the usefulness of isoenzyme electrophoresis in characterizing T.congolense, an organism often shunned by research workers because of its unobliging habits in the laboratory; many stocks of T.congolense have long subpatent periods and may only display low grade parasitaemias throughout the infection.

SECTION A

General Materials and Methods and

Isoenzyme Electrophoresis

CHAPTER 2

2. GENERAL MATERIALS AND METHODS

2.1. Materials

The materials used during this work and their suppliers are listed in Appendix I.

2.2. Buffers

2.2.1. For trypanosome maintenance and separation

Two buffers were constantly in use throughout this work; phosphate-buffered saline (PS) for equilibration of DE 52 (Section 2.3), and phosphate-buffered saline glucose (PSG) for both equilibration of DE 52 (Section 2.3) and for trypanosome separation (Section 2.6.1).

PS - phosphate-buffered saline, pH 8.0

(6:4; Lanham and Godfrey, 1971)

80.88 g	Na ₂ HPO ₄ anhydrous	} made up to	
4.68 g	NaH ₂ PO ₄ ·2H ₂ O		10 litres with
25.50 g	NaCl		distilled water

PSG - phosphate-buffered saline glucose, pH 8.0

(6:4; Lanham and Godfrey, 1971)

(conductivity at 23°C = $1.08 \times 10^4 \text{ ohm}^{-1}$)

600 ml PS

400 ml H₂O

15 g glucose

2.2.2. For electrophoresis

See Tables 3.2 and 3.3.

2.3. Equilibration of DEAE-cellulose for Trypanosome Separation

Two kg of expanded DE 52 were suspended in PS and the pH of the slurry was adjusted to 8.0 using H_3PO_4 or NaOH. The cellulose was allowed to settle and the buffer sucked off with a vacuum pump. The cellulose was given a further three washes with PS and then two with PSG. The pH of the slurry was confirmed to be 8.0 before freezing at $-20^{\circ}C$.

2.4. Experimental Hosts

2.4.1. Rodents

For the work carried out at The London School of Hygiene and Tropical Medicine (LSHTM), adult male and female Tuck's Original (TO) mice and Tuck's Wistar rats were used. Primary isolations were made into a number of different rodents many of unknown strain and some EATRO stocks were isolated into Zebu cattle.

At LSHTM, the animals were maintained on an unlimited supply of food (Dixon's Diet 86) and water.

2.4.2. Tsetse flies

Five species of tsetse fly from two different sources were used: Glossina morsitans morsitans and G.austeni were supplied by Dr. A.M. Jordan of The Tsetse Research Laboratory, Langford, Bristol; G.palpalis gambiensis, G.fuscipes fuscipes and G.tachinoides were supplied by Dr. J. Itard, Institut d'Elevage et de Médecine Vétérinaire

des Pays Tropicaux, Maisons-Alfort, France.

On arrival, the puparia were placed in washed sand in large hatching cages, at a constant temperature of 26°C and relative humidity of 80%. Within 48 hours of eclosing, the flies were separated into batches of approximately 20 per cage and offered blood meals, three times a week, from mice sedated with hypnorm (0.7 ml kg⁻¹ body weight).

2.5. Trypanosome Stocks

2.5.1. Isolation

A list of stocks and their histories is shown in Appendix II.

Wherever possible, stocks which had only been passaged a few times were used, to avoid possible population selection effects and also any previous laboratory cross-contamination. A few rapidly growing laboratory stocks proved useful as easily obtainable standards and for modifying techniques.

The majority of East African samples came from The East African Trypanosomiasis Research Organization (EATRO) cryopreservation bank; samples from Liberia and the Ivory Coast from Dr. D. Mehlitz of the Bernhard-Nocht-Institut, Hamburg; those from The Gambia were collected by Dr. D.G. Godfrey and myself (Section 6.2); the remainder were isolated in south west Nigeria by Miss Jacqueline Townsend.

2.5.2. Maintenance

Mice which had been inoculated with trypanosomes were regularly checked for parasitaemic infections; microscopical examinations, at x400 magnification, were made of wet blood films obtained from the cut tail vein.

When the parasitaemia reached about 10^8 trypanosomes ml^{-1} blood, the mouse was exsanguinated, under anaesthesia, into 10 i.u. of heparin ml^{-1} blood and passaged into further mice or rats by intraperitoneal (i.p.) inoculation.

Some stocks failed to reach a high parasitaemia and these were passaged at their peak through several mice in an attempt to increase the parasitaemic level. Attempts were also made to increase parasitaemia by immunosuppression of the host. X-irradiation at doses of 450, 600 and 800 rads in rats and mice (Balber, 1972) were used; cyclophosphamide was also administered once, 24 hours before infecting at 100 mg kg^{-1} body weight or three times during the infection at 50 mg kg^{-1} , in mice. (K.M. Hudson, personal communication). The resulting immunosuppression caused a synchronization of parasitaemic waves in different animals and a marginally shorter length of time until the first peak, compared with control mice and rats. These slight advantages were outweighed by the lack of availability of the Marconi X-irradiation machine at the Middlesex Hospital along with the problem of transporting large numbers of rats and the possible detrimental effects of cyclophosphamide on the trypanosomes including alteration of the delicate enzyme systems.

2.5.3. Cryopreservation

The method for cryopreservation of stocks was based on Cunningham *et al.* (1963).

When the parasitaemia reached approximately 10^6 trypanosomes ml^{-1} mouse blood, the mouse was exsanguinated into 10 i.u. of heparin ml^{-1} blood, held on ice. The blood was diluted 3:1 with PSG and glycerol was added to give a final concentration of 7%. Glass capillaries were two-thirds filled with diluted blood, heat sealed,

cooled at 1°C a minute in nitrogen vapour for at least 1.5 hours, before storing in liquid nitrogen.

2.5.4. Cloning

Trypanosome infected blood containing about 10^6 trypanosomes ml^{-1} blood was taken from a mouse with a rising parasitaemia and centrifuged at 200 g for five minutes at 4°C. The supernatant was examined and diluted with 5% foetal calf serum in PSG until a small flat drop, approximately 1 mm across, contained only one trypanosome.

Using this diluted solution, small hanging drops were placed on individual alcohol-cleaned coverslips in a well made with plastic strips secured to a microscope slide. Each drop was searched under low power and dark ground microscopy until a drop with only one trypanosome was found. This was checked by a second observer to verify that only one trypanosome was present; the drop was then washed off the coverslip with 5% foetal calf serum in PSG and inoculated i.p. or intravenously (i.v.) into mice; i.v. inoculation gave a higher percentage of parasitaemic mice. LRU TSW 99/77 and Gamb 2 were cloned successfully.

2.5.5. Morphology

Before each rat was exsanguinated, a thin blood film was made from the tail, fixed in methanol, and stained with 10% Giemsa in Sorrenson's buffer pH 7.2 for 40 minutes. The slides were examined microscopically to detect mixed infections of T.brucei and T.congolense.

2.6. Preparation of Samples for Electrophoresis

2.6.1. Separation of trypanosomes from blood

Rats, with approximately 10^8 trypanosomes ml^{-1} blood, were exsanguinated into 10 i.u. of heparin ml^{-1} blood as an anticoagulant. The blood was diluted four times with PSG and kept on ice. The blood was then passed through equilibrated DEAE cellulose (Section 2.3), held in a Büchner funnel with a sintered glass base (Lanham, 1968). The highly negatively charged blood cells were retained on the cellulose while the less negatively charged trypanosomes were washed through with PSG and collected in a vessel held on ice.

2.6.2. Preparation of Lysates

The eluate from the cellulose column was centrifuged at 1500 g for 20 minutes at 4°C . The supernatant was discarded, the packed trypanosomes resuspended in PSG and centrifuged. The supernatant was again removed and the packed trypanosomes resuspended in an equal volume of enzyme stabilizers (ϵ -amino-n-caproic acid, dithiothreitol and EDTA dissolved in distilled water to give a final concentration of 1 mM each in the lysate). The suspension was mixed thoroughly on a vortex mixer and frozen at -20°C .

Within a week, the lysate was thawed and centrifuged at 10,000 g for one hour at 4°C . The supernatant was removed in 15 μl aliquots and dropped into liquid nitrogen. The beads formed were stored in labelled vials under liquid nitrogen until required for electrophoresis.

CHAPTER 3

3. METHODS FOR ISOENZYME CHARACTERIZATION OF T.congolense

3.1. Introduction

Previous work on isoenzyme characterization of protozoa was discussed in Section 1.5.4. Kilgour and Godfrey (1973), published the first report of the separation of isoenzymes of T.congolense by thin-layer starch-gel electrophoresis using two stocks and two enzymes, ALAT and ASAT (see Table 3.1). Soon afterwards, Bagster and Parr (1973) attempted electrophoresis of another three enzymes, GPI, ME and G6PD using one T.congolense stock, but only GPI was successful; ME and G6PD failed, apparently due to low enzyme activity.

Starting from these reports and the numerous other techniques under development in these laboratories for demonstrating enzyme polymorphism in T.brucei, a comprehensive examination of the isoenzymes of T.congolense was undertaken. Although 13 different enzymes were attempted and several different peptidases using different peptides as substrates, only 14 enzymes including four peptidases visible from the addition of two peptide substrates i.e. from two tanks, were eventually used for routine screening because 12 electrophoretic tanks were available. Hence, 12 different enzyme systems could be run at one time using only 45 μ l of lysate which conserved material obtained only with great difficulty.

3.2. Thin-layer Starch-gel Electrophoresis

The method used for thin-layer starch-gel electrophoresis

Table 3.1. Enzyme nomenclature

Enzyme	Abbreviation	Enzyme Number*
L-threonine 3-dehydrogenase	TDH	E.C. 1.1.1.103
Purine nucleoside hydrolase	NH	E.C. 3.2.2.1.
Pyruvate kinase	PK	E.C. 2.7.1.40.
Adenylate kinase	AK	E.C. 2.7.4.3.
Glyceraldehyde phosphate dehydrogenase	GAPDH	E.C. 1.2.1.12.
Aspartate aminotransferase	ASAT	E.C. 2.6.1.1.
Phosphoglucomutase	PGM	E.C. 2.7.5.1.
Alanine aminotransferase	ALAT	E.C. 2.6.1.2.
Malate dehydrogenase (decarboxy- lating NADP) 'Malic enzyme'	ME	E.C. 1.1.1.40.
Malate dehydrogenase	MDH	E.C. 1.1.1.37.
Glucose phosphate isomerase	GPI	E.C. 5.3.1.9.
Peptidases	PEP 1 and 2	E.C. 3.4.11.
Isocitrate dehydrogenase	ICD	E.C. 1.1.1.42.
Glucose-6-phosphate dehydrogenase	G6PD	E.C. 1.1.1.49.

*enzyme numbers according to the Commission on Enzyme Nomenclature (1972).

was based on Wraxall and Culliford (1968).

3.2.1. Preparation of starch gel

Gels were prepared using hydrolysed potato starch at 10% above the manufacturer's recommended concentration. The starch was dissolved in the tank buffer diluted with distilled water (Table 3.2), except in the case of the enzyme purine nucleoside hydrolase when a discontinuous buffering system was used (Table 3.2).

The starch was heated to boiling point with continuous stirring, then evacuated with a vacuum pump to remove air bubbles. The hot solution was poured onto a glass plate with raised plastic edges, spread to form a gel 0.1 x 14.0 x 21.5 cm, covered and left to cool.

3.2.2. Application of samples

Lysate beads were removed from liquid nitrogen and placed in separate wells in a microtitration plate, held on ice. Once the beads were thawed, small lengths of previously boiled and separated Anchor stranded embroidery cotton were dipped in the lysate. Only one end of the cotton was placed in the liquid and the rest wetted by capillary action to prevent over-saturation of the threads and hence streaking over the gel surface. The damp threads were placed in slots, previously made approximately half way along the gel. Between one and four threads were placed in each slot depending on the activity of the enzyme being examined.

Initially, each sample was electrophoresed at least twice, but once the patterns were clearly established samples were examined only once, unless a new pattern was discovered.

LUMP 89 or MIAG 108 Clone were always run as standards on each plate.

Table 3.2. Electrophoretic conditions

ENZYME*	TANK BUFFER (TB)	GEL BUFFER	Volts cm^{-1} across gel	TIME (hours)
TDH	0.015M Tris, 0.007M citric acid pH 9.0 + 40mM KCl in bridge buffer	1 in 10 dilution of Tris-citric acid buffer + 40mM KCl	7	2
NH	0.04M Tris, 0.44M boric acid pH 6.5	0.013M Tris, 0.004M citric acid pH 7.2	17	1.5
PK and AK	0.015M Tris, 0.007M citric acid pH 9.0	1 in 10 dilution of TB + 0.1mM Fructose-1,6-diphosphate	17	2
GAPDH	0.1M KH_2PO_4 -KOH pH 7.0 + 0.1% mercaptoethanol	1 in 10 dilution of KH_2PO_4 -KOH buffer + paper overlay of TB	20	2.5
ASAT	0.15M glycine-NaOH pH 9.5	3 in 40 dilution of TB	40	1.25
PGM	Bridge: 0.10M Tris, 0.10M maleic acid, 0.01M EDTA, 0.01M MgCl_2 (adjust to pH 7.4 with NaOH) Electrode: 0.2M phosphate pH 7.0	1 in 10 dilution of bridge buffer	20	2
ALAT	0.150M Tris, 0.007M citric acid pH 9.0	3 in 40 dilution of TB	40	1.25
ME	0.2M phosphate pH 7.0	3 in 40 dilution of TB	20	3
MDH	0.050M Na_2HPO_4 and 0.007M citric acid pH 7.0	1 in 5 dilution of TB	20	2.5
GPI	0.2M phosphate pH 7.0	3 in 40 dilution of TB	20	3
PEP 1 and 2	0.18M Tris, 0.02M K_2PO_4 (adjust to pH 9.0 with HCl)	1 in 10 dilution of TB	20	2

*see Table 2.1.

3.2.3. Electrophoresis

The tanks were set up as shown in Fig. 3.1. They were wired in series to power packs providing constant voltage with variable current. The buffers, voltages and duration of the runs are shown in Table 3.2.

3.2.4. Staining

After the gel had been electrophoresed, the appropriate developing solution was applied (Table 3.3), either as an agar overlay for formazan and carbazole dye linked reactions, or on filter paper for fluorescent developers.

The developing plates were placed in an incubator at 37°C and inspected about every 10 minutes, for the appearance of bands of enzyme activity. The formazan and carbazole linked reactions were examined under normal light but fluorescent gels were checked under UV light (360 nm).

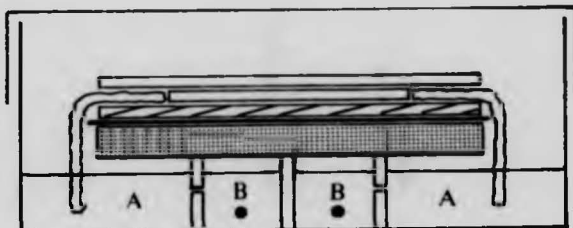
3.2.5. Photography

When the areas of enzyme activity were developed fully (usually within two hours), polaroid photographs were taken of each gel. For fluorescent reactions a no. 8 yellow Wratten filter was used; for carbazole reactions a mercury green filter was found to improve the photograph.

3.3. Origins and Modifications of Electrophoretic Techniques

The basic techniques and final conditions used during this work are described in Section 3.2. Since the majority of methods were based on those devised for T.brucei, modifications had to be made to give the best results for T.congolense. Apart from alterations

Fig. 3.1









-  Glass gel plate, 15 X 23 cm
-  Glass cover plates, 15 X 23 cm
-  Water cooled cooling plate at 8°C
-  Spontex wick
-  Stainless steel electrode wire
-  Melinex sheet
- A Bridge buffer
- B Electrode buffer

Fig. 3.1. Electrophoresis tank

Table 3.3. Enzyme developers for 130 cm² of gel plate

Enzyme	Buffer	Substrates	Coenzyme	Linking enzyme	Additional ions	Dye	Water
TDH	12ml 0.3M Tris/HCl pH 8.0	200mg L-threonine	25mg NAD		80mg KCl	10mg MTT 2mg PMS	3ml
NH	5ml 0.3M Tris/HCl pH 7.4	1mg inosine		1.2 x 10 ⁻² U xanthine oxidase		10mg MTT 2mg PMS	2ml
PK	6.6ml 0.3M Tris/HCl pH 7.4	22mg phosphoenol pyruvate 40mg ADP	6mg NADH	80 U LDH	8.0ml 0.1M MgCl ₂ 220mg KCl		
GAPDH	3ml 0.3M Tris/HCl pH 7.4	120mg fructose-1,6 diphosphate 30 U aldolase	6mg NAD		10mg sodium arsenate	10mg MTT 2mg PMS	
ASAT	6ml 0.1M phosphate pH 7.4	10mg 2-oxoglutaric acid 23mg aspartic acid	3mg NADH	80 U MDH			
PGM	8ml 0.3M Tris/HCl pH 7.4	16mg glucose-1-phosphate with 1% glucose-1,6-diphosphate	10mg NADP	5U glucose-6- phosphate dehydrogenase	1ml 0.5M MgCl ₂	10mg MTT 2mg PMS	7ml
ALAT	6ml 0.1M phosphate pH 7.4	10mg 2-oxoglutaric acid 120mg L-alanine	3mg NADH	80 U LDH			

Table 3.3 continued

Enzyme	Buffer	Substrates	Coenzyme	Linking enzyme	Additional ions	Dye	Water
ME	8.5ml 0.3M Tris/HCl pH 7.4	0.38ml 0.1M DL-malic acid (adjusted to pH 7.0 with NaOH)	2mg NADP		1ml 0.5M MgCl ₂	10mg MTT 2mg PMS	6ml
MDH	6ml 0.3M Tris/HCl pH 7.4	1ml 1.0M DL-malic acid (adjusted to pH 7.0 with NaOH)	3mg NADH			10mg MTT 2mg PMS	7ml
GPI	5ml 0.3M Tris/HCl pH 8.0	1mg fructose-6-phosphate	5mg NADP	50U glucose-6-phosphate dehydrogenase	1ml 0.1M MgCl ₂	10mg MTT 2mg PMS	4ml
PEP 1	2ml 0.1M phosphate pH 7.4	7mg L-leucylglycylglycine		1mg peroxidase 1mg L-amino oxidase	0.2ml 0.1M MnCl ₂	1ml saturated solution of 3-amino- 9-ethyl carbazole in EtOH	5ml
PEP 2	2ml 0.1M phosphate pH 7.4	7mg L-leucyl-L-alanine		1mg peroxidase 1mg L-amino oxidase	0.2ml 0.1M MnCl ₂	1ml saturated solution of 3-amino- 9-ethyl carbazole in EtOH	5ml
ICD	6ml 0.3M Tris/HCl pH 8.0	2mg isocitric acid	5mg NADP		1ml 0.1M MgCl ₂ * or 0.2ml 0.1M MnCl ₂	10mg MTT 2mg PMS	1.5ml

Table 3.3 continued

Enzyme	Buffer	Substrates	Coenzyme	Linking enzyme	Additional ions	Dye	Water
G6PD	8.5ml 0.3M Tris/HCl pH 7.4	5mg glucose-6-phosphate	2mg NADP		0.5ml 0.1M MgCl ₂	10mg MTT 2mg PMS	2.5ml
AK	6.7ml 0.3M Tris/HCl pH 8.0	36mg glucose 54mg fructose-1,6- diphosphate 20mg ADP	1mg NADP	5U hexokinase 5U G6PD	0.7ml 0.3M MgCl ₂ 1.2ml 1.0M KCl	10mg MTT 2mg PMS	3.0ml

To each developer was added an equal volume of agar (12mg ml^{-1}) except for ALAT, ASAT and PK which were applied on a no. 1 Whatman filter paper.

*see Section 3.4.10.

in the buffers, the duration or voltage of the electrophoretic runs often had to be adjusted to give maximum band separation, without the bands running off the gel; the migration of many enzymes differed from those of T.brucei. Some enzymes were tried with several buffers; the ingredients of a buffer, when altered, gives improved band resolution, or the pH may be adjusted to increase or decrease enzyme mobility. If no difference was found between the buffers, for convenience, one already in use for another enzyme was chosen.

Since the running time was dependent on voltage and only four power packs were available for 12 tanks, conditions were also modified to allow 12 enzyme systems to be run concurrently.

3.3.1. L-threonine 3-dehydrogenase

The electrophoretic conditions used for TDH were based on Gibson (1979) from Linstead et al. (1977). The same Tris/citrate buffer pH 9.0 + 40 mM KCl was used but better results were achieved with 40 mM KCl in the gel than the 4 mM used by Gibson (1979).

The voltage was lower than suggested by Gibson et al. (1978a) for T.brucei but higher than used by Gibson (1979) to allow the TDH tank to be run from the same power pack as the PK tank.

The activity of TDH in T.congolense was so low that four threads had to be placed in each slot for every sample.

3.3.2. Purine nucleoside hydrolase

The electrophoretic method for NH was adapted from Harris and Hopkinson (1976) and Gibson et al. (1978a).

Gibson (1979) demonstrated that a discontinuous buffer system gave sharper banding for T.brucei, i.e. different gel and tank buffers. 0.04 M lithium/0.44 M boric acid buffer (Harris and

Hopkinson, 1976) with 0.015 M Tris/0.004M citric acid gel buffer usually gave clear, neat results but due to the cost of lithium hydroxide, a Tris/borate buffer was tested. The 0.04 M Tris/0.44M boric acid buffer gave better results than the lithium/borate which occasionally resulted in bowed banding. The only disadvantage of the Tris/borate buffer was that the solvent front became stained with MTT during development of the enzyme bands, but providing the gel was run for long enough, this did not interfere or affect the banding.

3.3.3. Pyruvate kinase and adenylate kinase

The methods for electrophoresing PK and AK were modified from Lanham and Scott (1976). The same tank buffer, Tris/citrate pH 9.0, was used but the voltage was greatly reduced to allow this enzyme to be run connected to the TDH tank (S.M. Lanham and C.M. Scott, personal communication).

The direct staining method (Table 3.3) gave the best results for both AK and PK. An indirect fluorescent staining method was also tried which develops both AK and PK on the same gel but the banding was not clear (Lanham and Scott, 1976; S.M. Lanham and C.M. Scott, personal communication).

AK was not used routinely, however, as the results were unpredictable, sometimes giving clear banding but at other times only a darkened streak. This did not materially affect the work as 12 enzyme systems had already been modified satisfactorily.

3.3.4. Glyceraldehyde phosphate dehydrogenase

The electrophoretic methods for GAPDH were based on Harris and Hopkinson (1976).

The potassium phosphate buffer first used (M.A. Miles and S.C. Oswald, personal communication), gave good resolution of the bands of enzyme activity. Mercaptoethanol was used to stabilize this enzyme by keeping -SH groups in the reduced state during electrophoresis, otherwise diffuse banding resulted (Gibson, 1979). A 0.1% solution of mercaptoethanol was used in the potassium phosphate tank buffer and a Whatman no. 1 filter paper was dampened with this buffer and placed on the gel. In the past, this overlay has been applied 15 minutes before inserting the threads, but it was found to be equally successful if the overlay was applied for 15 minutes after inserting the threads, which had the advantage of avoiding an evil smelling gel while applying the samples. The overlay paper must only be damp as excess moisture caused diffusion of the samples.

Sodium pyruvate, used by Harris and Hopkinson (1976) during development of human GAPDH, to reduce lactate dehydrogenase activity, was omitted as Nannomonas trypanosomes do not contain this enzyme (S.M. Lanham, personal communication).

The samples were electrophoresed for 2.5 hours instead of 3 hours used for T.brucei (Gibson et al., 1978a).

3.3.5. Aspartate aminotransferase

The electrophoretic methods used for ASAT, were based on Kilgour and Godfrey (1973), Kilgour et al. (1974) and Godfrey and Kilgour (1976).

Four buffers were tested; 0.15 M glycine/NaOH pH 9.5 (Godfrey and Kilgour, 1976), 0.15 M Tris/citrate pH 9.0 (Kilgour and Godfrey, 1973), 0.15 M Tris/citrate pH 8.5 and 0.3 M glycine/NaOH pH 9.5. The first of these buffers gave the best results.

The duration of the run was again reduced. Preincubation of the gel, without L-aspartate, gave much quicker and sharper banding when L-aspartate was added than if the preincubation was omitted. Gibson (1979) reported that preincubation of T. brucei samples was not advantageous.

3.3.6. Phosphoglucomutase

The methods used for electrophoresing PGM were based on Miles et al. (1977). No modification of the Tris/maleate/versene buffering system was necessary as the bands were clear and well separated from one another. However, with this buffer, corrosion of the stainless steel electrodes occurred which was remedied by replacing the buffer in the electrode compartments with 0.2 M phosphate buffer pH 7.0; the holes between the tank compartments were plugged with non-absorbent cotton wool.

The duration of the run was reduced from 2.5 hours used for T. cruzi (Miles et al., 1977) to 2 hours.

3.3.7. Alanine aminotransferase

The method used for the electrophoresis of ALAT was modified from Kilgour and Godfrey (1973) and Kilgour et al. (1974). Variations in buffer pH and molarity were investigated including 0.15 M Tris/citrate, pH 9.0 (Kilgour and Godfrey, 1973), pH 8.0 and pH 8.5 and 0.3 M Tris/citrate pH 9.0, in an attempt to produce more discrete banding. No improvement was seen with the changes in pH and although the increase in molarity produced sharper banding there was such a decrease in band separation as to make differentiation difficult (Plates 3.1 and 3.2). The original buffer, 0.15 M Tris/citrate

The duration of the run was again reduced. Preincubation of the gel, without L-aspartate, gave much quicker and sharper banding when L-aspartate was added than if the preincubation was omitted. Gibson (1979) reported that preincubation of T.brucei samples was not advantageous.

3.3.6. Phosphoglucomutase

The methods used for electrophoresing PGM were based on Miles et al. (1977). No modification of the Tris/maleate/versene buffering system was necessary as the bands were clear and well separated from one another. However, with this buffer, corrosion of the stainless steel electrodes occurred which was remedied by replacing the buffer in the electrode compartments with 0.2 M phosphate buffer pH 7.0; the holes between the tank compartments were plugged with non-absorbent cotton wool.

The duration of the run was reduced from 2.5 hours used for T.cruzi (Miles et al., 1977) to 2 hours.

3.3.7. Alanine aminotransferase

The method used for the electrophoresis of ALAT was modified from Kilgour and Godfrey (1973) and Kilgour et al. (1974). Variations in buffer pH and molarity were investigated including 0.15 M Tris/citrate, pH 9.0 (Kilgour and Godfrey, 1973), pH 8.0 and pH 8.5 and 0.3 M Tris/citrate pH 9.0, in an attempt to produce more discrete banding. No improvement was seen with the changes in pH and although the increase in molarity produced sharper banding there was such a decrease in band separation as to make differentiation difficult (Plates 3.1 and 3.2). The original buffer, 0.15 M Tris/citrate

Plate 3.1. ALAT, 0.015 M Tris/citrate pH 9.0, 580 volts
for 75 minutes

Stocks from left to right

1. 1/148 FLY
2. S104/FLY/BE
3. "
4. LUMP 89
5. "
6. MIAG 108 Clone
7. "

Plate 3.2. ALAT, 0.03M Tris/citrate buffer, pH 9.0, 300 volts
for 2 hours

Stocks from left to right

1. 1/148 FLY
2. S104/FLY/BE
3. "
4. LUMP 89
5. "
6. MIAG 108 Clone
7. "



Plate 3.1.

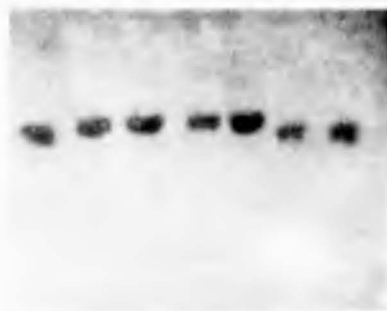


Plate 3.2.

pH 9.0, proved to be the most successful.

The duration of the run had to be decreased as this enzyme from T.congolense migrated much faster than from T.brucei.

As with ASAT, preincubation of the gel without the substrate, L-alanine, before adding the L-alanine gave more discrete banding.

3.3.8. Malate dehydrogenase (decarboxylating NADP) or 'malic enzyme'

The electrophoretic methods used for ME were based on Bagster and Parr (1973) and Miles et al. (1977). The best results were obtained using 0.2 M phosphate pH 7.0 as tank buffer with a 3 in 40 dilution of this buffer in the gel (Miles et al., 1977). Other less successful attempts were made with 0.2 M phosphate pH 7.0, gel dilution 1 in 10; 0.15 M Tris/citrate pH 9.0 + 40 mM KCl, gel dilution 3 in 40; and 0.2 M phosphate pH 8.0, gel dilution 1 in 10 (S.M. Lanham, personal communication).

However, even with 0.2 M phosphate pH 7.0 and 3 in 40 gel dilution the bands were often diffuse making determination of their positions difficult and repetitive runs necessary.

3.3.9. Malate dehydrogenase

No alteration of the buffers used by Gibson et al. (1978a), after Harris and Hopkinson (1976), were necessary for electrophoresis of T.congolense MDH. The duration of the run was reduced by 0.5 hours from the 3 hours used for T.brucei (Gibson et al., 1978a).

The direct staining technique (Gibson et al., 1978a) gave neat distinct bands making trials with the indirect fluorescent method unnecessary (Al-Taqi and Evans, 1978).

3.3.10. Glucose phosphate isomerase

The conditions used for GPI were very similar to those of Miles *et al.* (1977), working with T.cruzi. However, better results were obtained with a phosphate buffer pH 7.0 than 7.4. Bagster and Parr (1973) was also consulted for the developing technique.

3.3.11. Peptidases

The electrophoretic methods used for PEP were modified from Harris and Hopkinson (1976) and Gibson *et al.* (1978a). The same phosphate tank buffer as used by Gibson *et al.* (1978a) for T.brucei proved suitable for T.congolense but the pH was adjusted to 9.0. The duration of the run was 2 hours as opposed to 3 hours generally used for T.brucei.

Ten different peptides were used as substrates with four stocks of T.congolense (Table 4.1), but only two, L-leucylglycylglycine and L-leucyl-L-alanine were used for routine screening.

From this work, an attempt was made to correlate the different peptidases capable of catalysing the breakdown of different substrates with those found in human samples (Harris and Hopkinson, 1976) and Trypanozoon stocks (Gibson and Letch, in press).

3.3.12. Isocitrate dehydrogenase

The techniques used for electrophoresis of ICD were based on Harris and Hopkinson (1976), Gibson *et al.* (1978a) and Gibson (1979). Attempts were made with 0.05 M phosphate/0.007 M citric acid buffer pH 7.0 (Gibson, 1979) with the developing solution used by Gibson *et al.* (1978a) for T.brucei including $MgCl_2$ and also as suggested by Gibson (1979) with $MnCl_2$ instead of $MgCl_2$. No ICD

activity was detected in any of the T.congolense samples tested, while T.brucei samples, run on the same gel, developed.

To determine the level of activity of ICD in T.congolense, enzyme assays were carried out (Section 3.5).

3.3.13. Glucose-6-phosphate dehydrogenase

The electrophoretic and developmental methods were based on Bagster and Parr (1973), Miles et al. (1977) and Gibson (1979).

Four tank and gel buffers were tried, 0.1 M, 0.2 M and 0.3 M phosphate buffers pH 7.0 (Gibson, 1979) and 0.1 M Tris/0.1 M maleic acid/0.01 M MgCl₂/0.01 M EDTA pH 7.4 (Miles et al., 1977), but consistent with the results of Bagster and Parr (1973) no banding was seen for any T.congolense samples tested, while the T.brucei lysates run on the same gel developed.

Enzyme assays were carried out to determine the level of activity of G6PD in T.congolense (Section 3.5).

3.4. Controls

A control plate was run for each of the 14 enzymes, used routinely, by omitting the appropriate specific substrate from each developer (Table 3.3).

Control plates were also run for each enzyme with two different T.brucei stocks isolated in The Gambia, a T.vivax sample and a lysate prepared from rat blood in addition to T.congolense stocks giving different enzyme patterns. Bands on other plates, due to other trypanosome species in mixed infections and/or blood contaminants could be deduced from these controls.

Three stocks of T.congolense. S104/FLY/BE, 1/148 FLY and

MIAG 108 Clone, were harvested at different times during the infection in rats and infections were set up twice from the deep-frozen parent stock. The electrophoretic patterns of these lysates were compared.

3.3. Enzyme Assays

Enzyme assays were carried out for ICD and G6PD using T. congolense stocks LUMP 89, Gamb 2, EATRO 2033 and LRU TSW 4/77.

The reaction mixtures are shown in Table 3.4. G6PD activity cannot be determined directly but from assaying G6PD and 6-phosphogluconate dehydrogenase (6PGD) together and then subtracting the activity of 6PGD alone. The enzyme reactions were monitored at 340 nm and 30°C using a Unicam SP 1700 spectrophotometer. The enzyme activity, E, was calculated as U ml⁻¹ using the following formula with a 1 cm light path length:

$$E = \frac{\Delta A}{\Delta t} \cdot \frac{10^3}{\epsilon} \cdot \frac{V_c}{V_1} \cdot D$$

- where
- $\frac{\Delta A}{\Delta t}$ - change in absorbance per minute
 - ϵ - molar absorption coefficient for NADP
 - V_c - volume of assay mixture in cuvette
 - V_1 - volume of lysate in cuvette
 - D - dilution of lysate

Table 3.4. Enzyme assays

Enzyme	Buffer	Coenzyme	Substrate	Additional ions	Water
ICD	3.35ml 0.3M Tris/HCl pH 8.0	5mg NADP	0.5mg DL-isocitrate	0.1ml 1.0M MgCl ₂	6.55ml
G6PD + 6PGD	1.00ml 0.3M Tris/HCl pH 8.0	1mg NADP	0.75mg 6-phospho-gluconate 0.75mg glucose-6-phosphate	0.3ml 0.1M MgCl ₂	1.7ml
6PGD	1.00ml 0.3M Tris/HCl pH 8.0	1mg NADP	0.75mg 6-phospho-gluconate	0.3ml 0.1M MgCl ₂	1.7ml

CHAPTER 4

4. ELECTROPHORETIC RESULTS

4.1. General

Preliminary work was carried out to ascertain the best conditions for electrophoresis of each of the 14 enzymes chosen for routine screening of stocks of T.congolense. Once the conditions were satisfactorily established, large numbers of stocks were compared; each sample was only run once unless it represented a pattern not previously encountered.

The patterns obtained with stocks of T.congolense for different enzymes are shown diagrammatically in Figs. 4.1 to 4.13. Each enzyme pattern was numbered with a roman numeral, which gives no indication of the numbers of bands in that pattern, but allows rapid comparisons to be made by eye. Comparisons of stocks by direct examination of the zymograms were generally made from the whole pattern, and each band was considered separately only during computer analysis, when every different band received an arabic numeral (Chapter 5). The pattern numbers for each enzyme and stock are listed in Appendix III. The occurrence of each pattern, expressed as a percentage of the 75 zymodemes, found in the 78 stocks, is shown in Table 4.1. A zymodeme is defined as a population characterized by its enzymes and when two populations differ in one or more enzymes they are considered as different zymodemes.

Photographs are also shown demonstrating each enzyme pattern seen, except for PEP 1 and PEP 2 where the patterns were too numerous.

Table 4.1. Percentage frequency of each enzyme pattern in the 75 zymodemes found during this study

Enzyme	Pattern Number																	
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII
NH	100.0																	
PK	100.0																	
TDH	100.0																	
GAPDH	88.0	12.0																
ASAT	30.7	40.0	16.0	13.3														
PGM	57.3	17.3	18.7	6.7														
ALAT	8.0	37.3	18.7	20.0	16.0													
ME	16.0	48.0	12.0	12.0	12.0													
MDH	69.4	17.3	4.0	4.0	1.3	4.0												
GPI	50.6	9.3	2.7	1.3	1.3	2.7	18.7	6.7	4.0	2.7								
PEP 2	19.4	1.3	5.3	13.3	2.6	7.9	14.6	1.3	3.9	15.9	1.3	9.3	2.6	1.3				
PEP 1	21.4	8.0	9.3	12.0	2.7	2.7	8.0	1.3	4.0	2.7	1.3	5.3	4.0	2.7	2.7	9.3	1.3	1.3

Comparisons with the subgenera Trypanozoon and Duttonella were limited to two stocks of T.brucei originating from The Gambia and one stock of T.vivax. Extensive studies have been made into the isoenzyme characterization of the subgenus Trypanozoon (Gibson, 1979; Gibson et al., 1980) and also several stocks of the subgenus Duttonella (Murray, 1979); representatives were included in this study to give an indication of the similarity or dissimilarity of the isoenzymes in the three subgenera. The optimum conditions developed for T.congolense often differed from those devised for T.brucei or T.vivax; thus, the banding produced by T.brucei and T.vivax when electrophoresed under conditions for T.congolense did not always produce the band separation shown by Gibson (1979), Gibson et al. (1980) and Murray (1979).

The patterns obtained from the control rat blood lysate are described under each of the following sections designated to the individual enzymes. In these enzymes (Section 4.2.1 to 4.2.11), all bands migrated towards the anode unless otherwise stated.

4.2. Individual Enzymes

4.2.1. L-threonine 3-dehydrogenase

Representatives of the three subgenera all gave a single band of TDH (Plate 4.1). The two T.congolense samples migrated further than either the T.vivax or T.brucei lysates which moved the same distance as each other. Four threads were inserted for both the T.congolense samples as this enzyme was known to be weak in this species, while two threads were inserted for the other samples. The TDH activity of T.vivax was also low compared with T.brucei (Plate 4.1). No enzyme activity was seen in the rat blood lysate

L-THREONINE DEHYDROGENASE

Fig. 4.1. Diagrammatic representation of T. congolense pattern I

Plate 4.1. Comparison of three species of salivarian trypanosomes
Conditions as in Tables 3.2 and 3.3

Stocks from left to right

- | | | |
|--------------|---|--------------------------|
| 1. LUMP 89 | } | <u>T.(N.) congolense</u> |
| 2. LUMP 89 | | |
| 3. GB 1 | } | <u>T.(T.) brucei</u> |
| 4. GB 2 | | |
| 5. Liverpool | } | <u>T.(D.) vivax</u> |
| 6. Liverpool | | |
| 7. Rat blood | | |

THREONINE DEHYDROGENASE

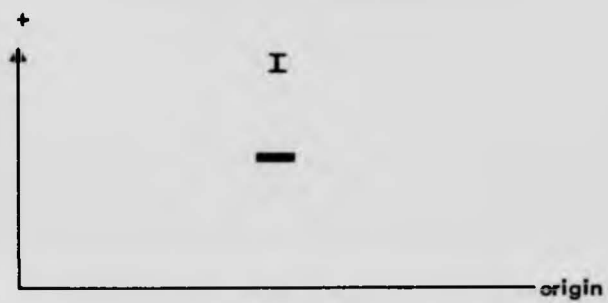


Fig. 4.1.

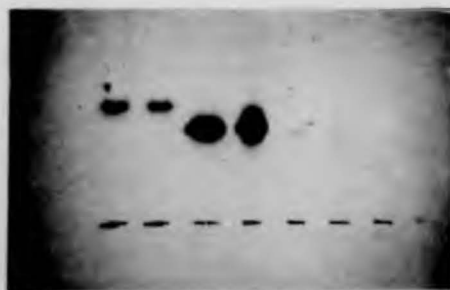


Plate 4.1.

THREONINE DEHYDROGENASE

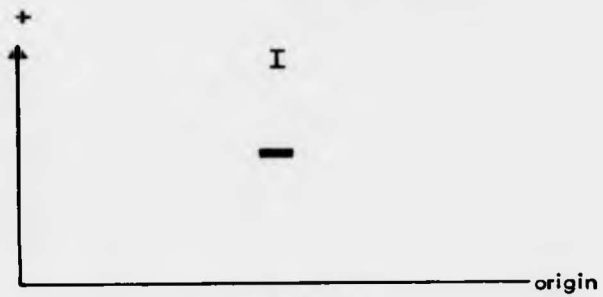


Fig. 4.1.

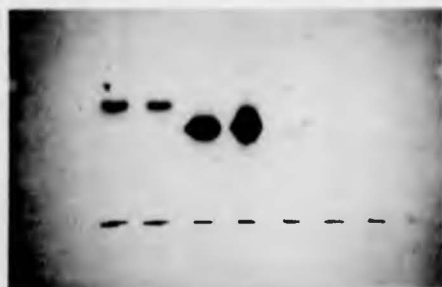


Plate 4.1.

in the area of gel developed.

All the 78 stocks of T.congolense examined had only the one single common band (Fig. 4.1, Plate 4.1).

4.2.2. Purine nucleoside hydrolase

Members of each subgenera could be easily distinguished by their NH patterns (Plate 4.2). The pattern for T.brucei, shown in Plate 4.2, is common to all except three of the numerous stocks examined by Gibson (1979). The T.vivax sample gave one band which migrated the same distance as the slower of the two bands seen for T.brucei. The T.congolense stocks migrated slightly less than the fast T.brucei band. Each sample was applied on two threads and the intensity of staining was similar for all of them, implying that the activity of this enzyme was approximately the same in the three species examined.

A single band of common mobility was found in every T.congolense stock investigated (Fig. 4.2, Plate 4.2).

4.2.3. Pyruvate kinase

The T.congolense samples shown in Plate 4.3 could be clearly distinguished from the T.brucei and T.vivax lysates; the T.congolense PK migrated further than the others. Because all the samples were applied on two threads, it can be deduced that the two T.brucei samples had higher PK activities than either T.congolense or T.vivax, thus, producing streaking over the gel surface due to an excess of enzyme (Plate 4.3). A very weak band developed from the rat blood lysate which migrated the furthest.

In all 78 stocks of T.congolense examined only the one single common band was seen (Fig. 4.3, Plate 4.3).

PURINE NUCLEOSIDE HYDROLASE

Fig. 4.2. Diagrammatic representation of T. congolense pattern I

Plate 4.2. Comparison of three species of salivarian trypanosomes
Conditions as in Tables 3.2 and 3.3

Stocks from left to right

- | | | |
|--------------|---|--------------------------|
| 1. LUMP 89 | } | <u>T.(N.) congolense</u> |
| 2. LUMP 89 | | |
| 3. GB 1 | } | <u>T.(T.) brucei</u> |
| 4. GB 2 | | |
| 5. Liverpool | } | <u>T.(D.) vivax</u> |
| 6. Liverpool | | |
| 7. Rat blood | | |

PURINE NUCLEOSIDE HYDROLASE

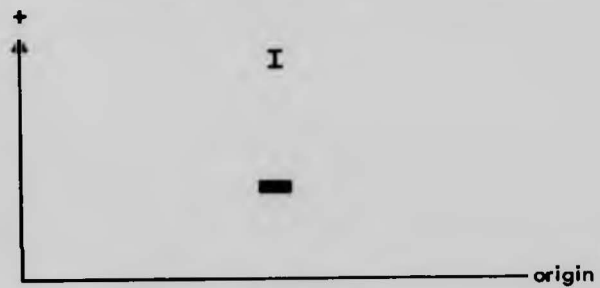


Fig. 4.2.

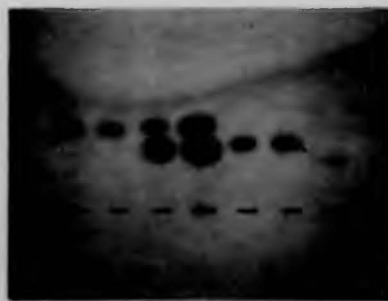


Plate 4.2.

PURINE NUCLEOSIDE HYDROLASE

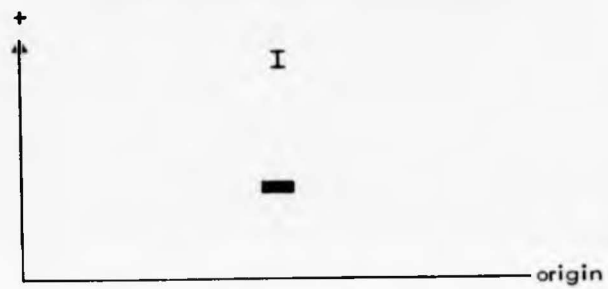


Fig. 4.2.

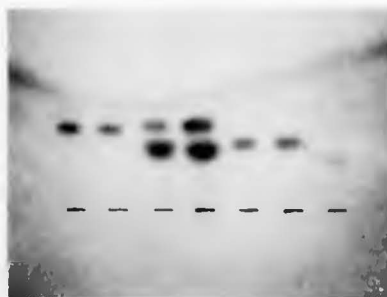


Plate 4.2.

PYRUVATE KINASE

Fig. 4.3. Diagrammatic representation of T. congolense pattern I

Plate 4.3. Comparison of three species of salivarian trypanosomes
Conditions as in Tables 3.2 and 3.3

Stocks from left to right

- | | | |
|--------------|---|--------------------------|
| 1. LUMP 89 | } | <u>T.(N.) congolense</u> |
| 2. LUMP 89 | | |
| 3. GB 1 | } | <u>T.(T.) brucei</u> |
| 4. GB 2 | | |
| 5. Liverpool | } | <u>T.(D.) vivax</u> |
| 6. Liverpool | | |
| 7. Rat blood | | |

PYRUVATE KINASE

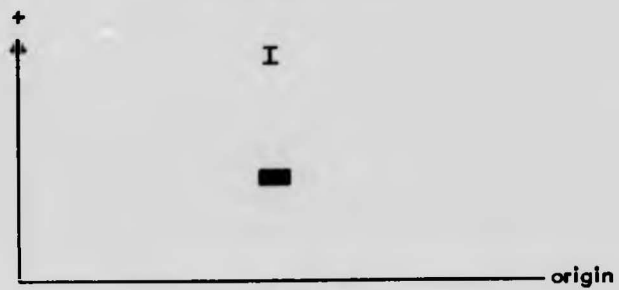


Fig. 4.3.

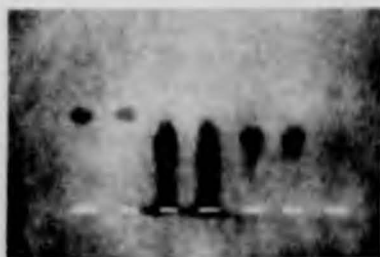


Plate 4.3.



Fig. 4.3.

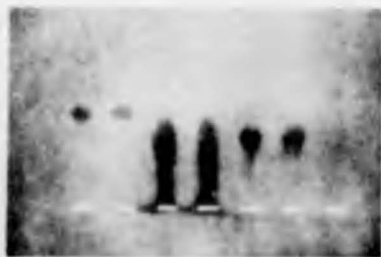


Plate 4.3.

4.2.4. Glyceraldehyde phosphate dehydrogenase

The two T.brucei and one T.vivax samples had identical double-banded GAPDH patterns, consisting of a strong slow-moving component and a weak, slightly faster band (Plate 4.4). One of the T.congolense samples gave a similar double banded pattern but this doublet migrated further than the T.brucei or T.vivax. The five-banded T.congolense pattern II is also represented in Plate 4.5. The rat blood lysate developed as two bands in the region of the upper bands of T.congolense pattern II.

Plate 4.5 shows, more clearly, the two GAPDH patterns found among the 78 T.congolense stocks (Fig. 4.4).

4.2.5. Aspartate aminotransferase

The two T.brucei samples displayed single ASAT bands which migrated a short distance from the origin (Plate 4.6). The T.vivax lysate had two components, one of which remained on the origin. These three samples could be readily distinguished from the cathodically migrating T.congolense bands; this enzyme gave the only cathodic band migration seen in T.congolense. Although not shown on Plate 4.6, the fast moving band from T.congolense could also be identified from the fast T.vivax band as it moved nearly twice as far (Plates 4.6 and 4.7). The rat blood lysate gave a single band which moved further than the fast T.vivax component.

Four patterns were found in the 78 stocks of T.congolense (Fig. 4.5 and Plates 4.6 and 4.7): patterns I, II and III differed from one another by the presence or absence of two bands, one of which migrated anodically and the other cathodically; pattern IV consisted of a single band which migrated a shorter distance towards

GLYCERALDEHYDE PHOSPHATE DEHYDROGENASE

Fig. 4.4. Diagrammatic representation of T. congolense patterns I and II

Plate 4.4. Comparison of three species of salivarian trypanosomes
Conditions as in Tables 3.2 and 3.3

Stocks from left to right

- | | | |
|---------------------------|---|--------------------------|
| 1. LUMP 89, GAPDH I | } | <u>T.(N.) congolense</u> |
| 2. Gamb 18, " II | | |
| 3. IBADAN 44, weak sample | | |
| 4. GB 1 | } | <u>T.(T.) brucei</u> |
| 5. GB 2 | | |
| 6. Liverpool | } | <u>T.(D.) vivax</u> |
| 7. Liverpool | | |
| 8. Rat blood | | |

Plate 4.5. Variation in T. congolense
Conditions as in Tables 3.2 and 3.3

Stocks from left to right

1. Gamb 1, GAPDH I
2. Gamb 12, " I
3. Gamb 16, " II
4. Gamb 19, " II
5. Gamb 21, " I
6. EATRO 1564, " I
7. EATRO 2025, " I
8. EATRO 1755, " I

GLYCERALDEHYDE PHOSPHATE DEHYDROGENASE

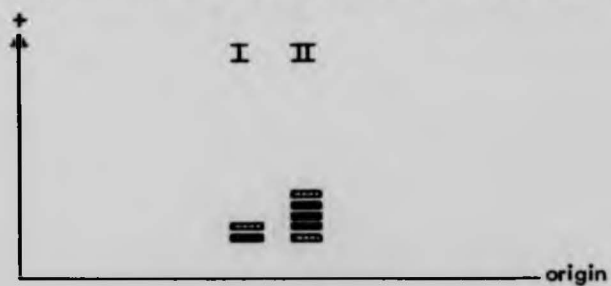


Fig. 4.4.

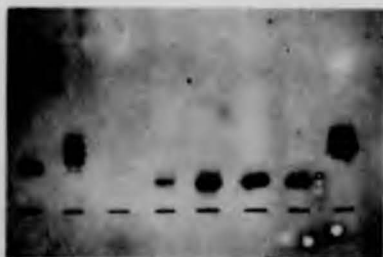


Plate 4.4.

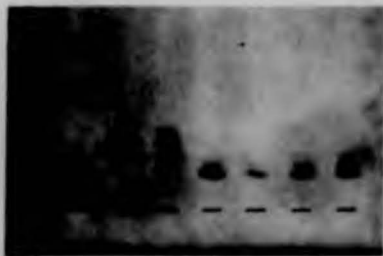


Plate 4.5.

GLYCERALDEHYDE PHOSPHATE DEHYDROGENASE

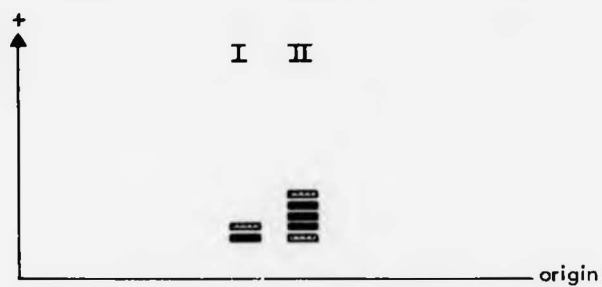


Fig. 4.4.

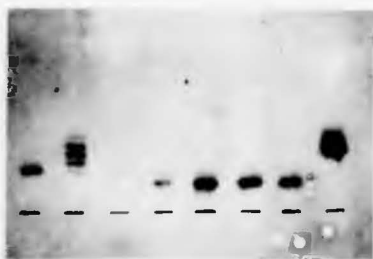


Plate 4.4.

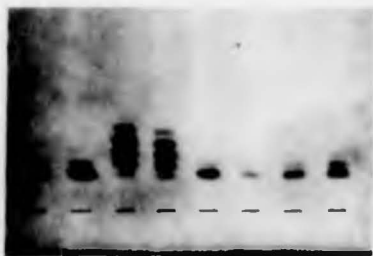


Plate 4.5.

ASPARATE AMINOTRANSFERASE

Fig. 4.5. Diagrammatic representation of T. congolense patterns I to IV

Plate 4.6. Comparison of three species of salivarian trypanosomes
Conditions as in Tables 3.2 and 3.3

Stocks from left to right

- | | | |
|------------------------------------|---|--------------------------|
| 1. LRU TSW 94/77, ASAT IV | } | <u>T.(N.) congolense</u> |
| 2. LUMP 89, weak sample | | |
| 3. EATRO 2027, ASAT I | | |
| 4. Gamb 21, " IV | | |
| 5. GB 1 | } | <u>T.(T.) brucei</u> |
| 6. GB 2 | | |
| 7. Liverpool - <u>T.(D.) vivax</u> | | |
| 8. Rat blood | | |

Plate 4.7. Variation in T. congolense

Conditions as in Tables 3.2 and 3.3

Stocks from left to right

1. Gamb 11, " II
2. Gamb 13B, " I
3. Gamb 5, " III
4. Gamb 2, " I
5. Gamb 22, " I
6. Gamb 17, " I
7. Gamb 9, " III

ASPARTATE AMINOTRANSFERASE

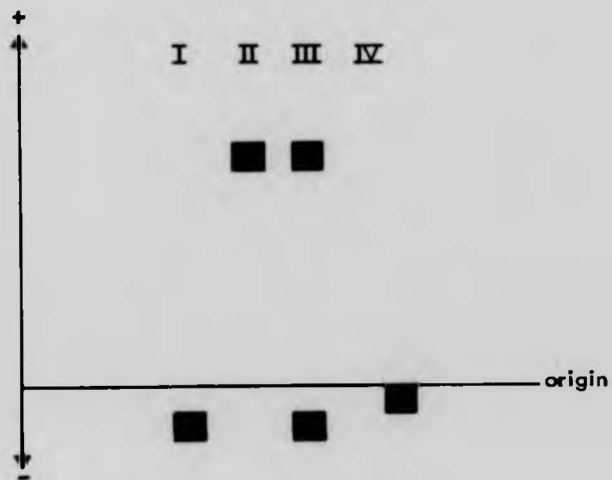


Fig. 4.5.

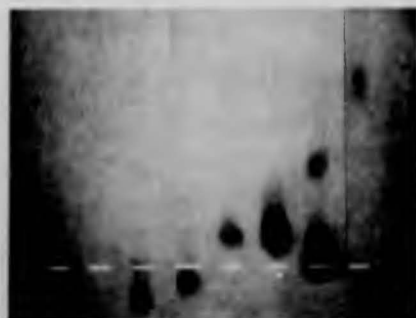


Plate 4.6.

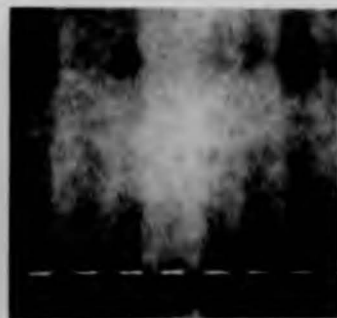


Plate 4.7.

ASPARTATE AMINOTRANSFERASE

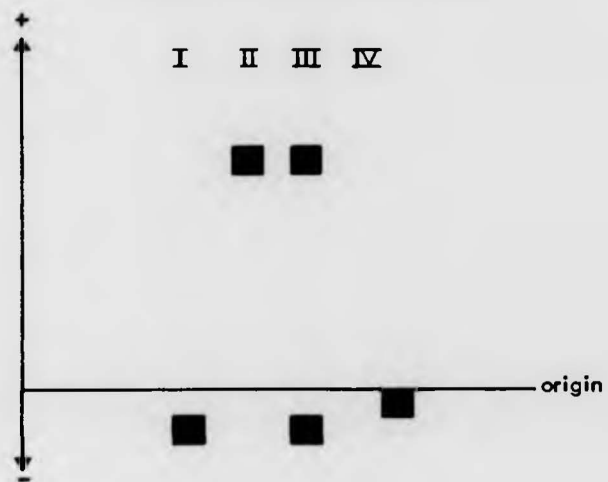


Fig. 4.5.



Plate 4.6.

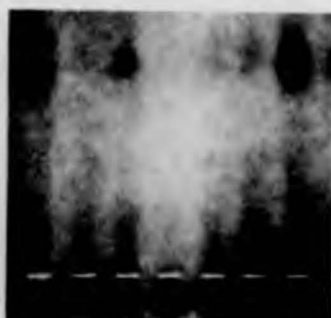


Plate 4.7.

the cathode. In all the patterns, a band occasionally appeared which had migrated a short distance towards the anode but only after prolonged development; it is visible in Plate 4.7 sample number 1, but did not always develop in this sample. Due to its inconsistency, this band was not used for stock characterization.

4.2.6. Phosphoglucomutase

The PGM of T.congolense migrated further than the T.brucei, T.vivax or the rat blood lysate (Plate 4.6).

Four patterns were obtained for PGM with the 78 T.congolense stocks, with one band common to each pattern (Fig. 4.8, Plates 4.9 and 4.10). Patterns I and II had the same two bands but with different relative activities; in pattern I the slower band was strongly active with only a weak fast band, whereas in pattern II, the two components were of similar activity.

4.2.7. Alanine aminotransferase

The T.congolense ALAT moved approximately twice as far as the T.brucei samples and the T.vivax was even slower (Plate 4.11). No enzyme activity in the rat blood lysate was detected. The banding in Plate 4.11 is not clear probably because placing 12 samples on one gel meant that the band widths were too narrow for clear interpretation.

Five ALAT patterns were found among 78 T.congolense stocks, made up of various combinations of five bands (Fig. 4.7, Plates 4.11 to 4.13).

PHOSPHOGLUCOMUTASE

Fig. 4.6. Diagrammatic representation of T. congolense patterns I to IV

Plate 4.8. Comparison of three species of salivarian trypanosomes
Conditions as in Tables 3.2 and 3.3

Stocks from left to right

- | | | |
|----------------------------|---|--------------------------|
| 1. Gamb 18, PGM II | } | <u>T.(N.) congolense</u> |
| 2. LUMP 89, " I | | |
| 3. TDRN 9, " IV | | |
| 4. EATRO 1564, weak sample | | |
| 5. GB 1 | } | <u>T.(T.) brucei</u> |
| 6. GB 2 | | |
| 7. Liverpool | } | <u>T.(D.) vivax</u> |
| 8. Liverpool | | |
| 9. Rat blood | | |

Plate 4.9. Variation in
T. congolense

Conditions as in Tables 3.2 and 3.3.

Stocks from left to right

1. S104/FLY/BE, PGM I
2. Gamb 1, " II
3. Gamb 12, " II
4. Gamb 16, " II
5. Gamb 19, " I

Plate 4.10. Variation in
T. congolense

Conditions as in Tables 3.2 and 3.3.

Stocks from left to right

1. LRU TSW 115/77, PGM III
2. IBADAN 44, " I
3. IBADAN 69, " IV
4. MIAG 108 Clone, " III

PHOSPHOGLUCOMUTASE

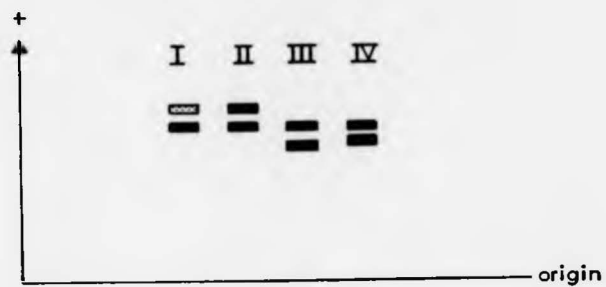


Fig. 4.6.

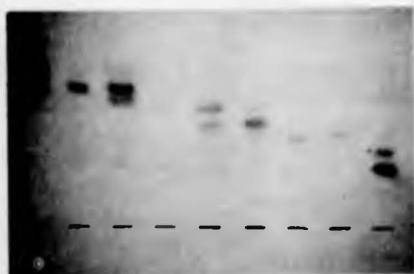


Plate 4.8.

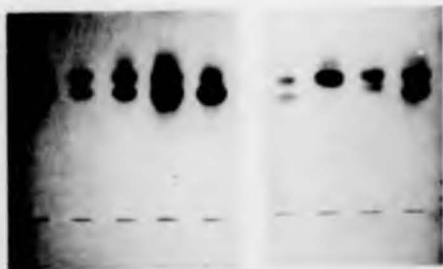


Plate 4.9.

Plate 4.10.

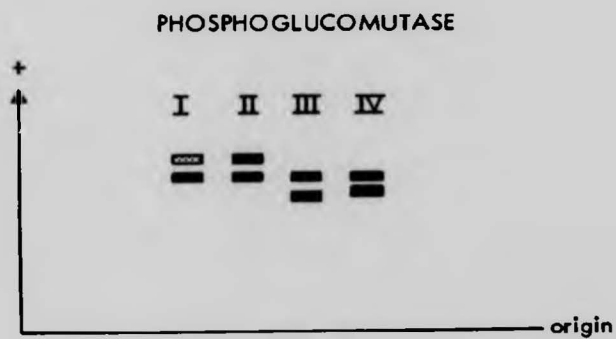


Fig. 4.6.

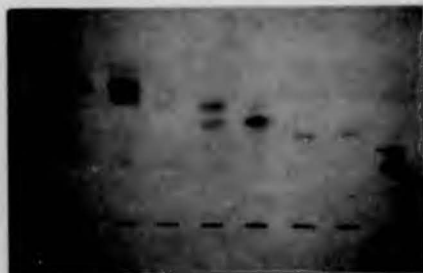


Plate 4.8.



Plate 4.9.



Plate 4.10.

ALANINE AMINOTRANSFERASE

Fig. 4.7. Diagrammatic representation of T. congolense patterns I to V

Plate 4.11. Comparison of three species of salivarian trypanosomes
Conditions as in Tables 3.2 and 3.3

Stocks from left to right

- | | |
|----------------------------|----------------------------|
| 1. EATRO 2027, weak sample | |
| 2. Gamb 13Y, ALAT II | } <u>T.(N.) congolense</u> |
| 3. Gamb 3, " III | |
| 4. EATRO 1967, weak sample | |
| 5. Gamb 2, ALAT III | |
| 6. Gamb 18, " V | |
| 7. TSW 25/78E " IV | |
| 8. GB 1 | |
| 9. GB 2 | |
| 10. Liverpool | } <u>T.(D.) vivax</u> |
| 11. Liverpool | |
| 12. Rat blood | |

Plate 4.12. Variations in
T. congolense

Conditions as in Tables 3.2 and
3.3

Stocks from left to right

1. Gamb 5, ALAT V
2. Gamb 2, " III
3. Gamb 22, " V
4. Gamb 17, " II
5. Gamb 9, " III
6. Gamb 14, " III
7. MIAG 108 Clone, " II

Plate 4.13. Variations in
T. congolense

Conditions as in Tables 3.2 and
3.3

Stocks from left to right

1. S104/FLY/BE, ALAT I
2. EATRO 2084, " III
3. EATRO 2033, " II
4. EATRO 1582, " II

ALANINE AMINOTRANSFERASE

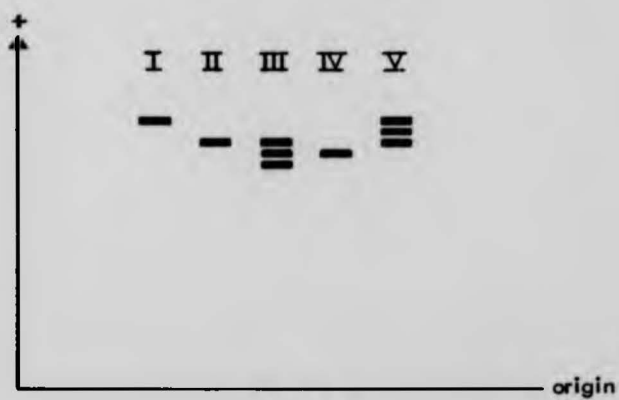


Fig. 4.7.



Plate 4.11.



Plate 4.12.



Plate 4.13.

ALANINE AMINOTRANSFERASE

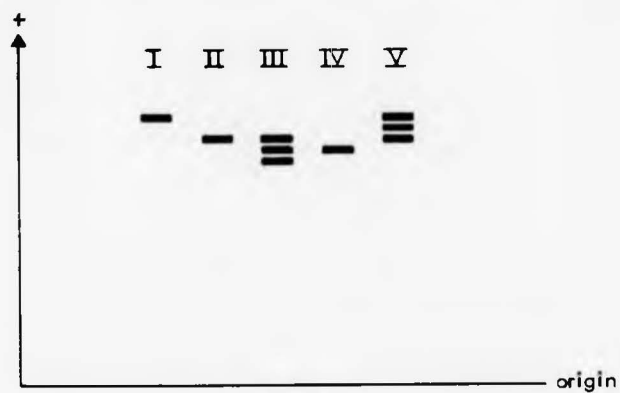


Fig. 4.7.



Plate 4.11.

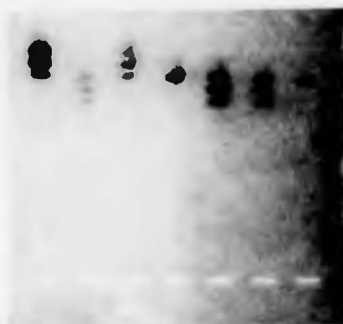


Plate 4.12.



Plate 4.13.

4.2.8. Malate dehydrogenase (decarboxylating NADP) or 'malic enzyme'

The two T. brucei and the T. vivax ME migrated further than that of T. congolense, although in both, faint banding was seen in the region of the fast T. congolense components (Plate 4.14). No rat blood activity could be detected in the developed area.

From 78 stocks of T. congolense, five ME patterns were found made up of different combinations of a possible five bands (Fig. 4.8, Plates 4.15 and 4.16). Consistent results were the most difficult to obtain with this enzyme, discrete banding rarely occurring and samples usually had to be electrophoresed several times before a clear result was obtained.

4.2.9. Malate dehydrogenase

The two T. brucei samples had an identical single-banded MDH pattern to T. congolense pattern I (Plate 4.17); the T. vivax sample had two bands, one of which was common with a component of T. congolense pattern II. The rat blood lysate gave a single component which migrated further than any trypanosome band.

Six patterns consisting of various combinations of eight possible bands were found for MDH in the 78 T. congolense stocks (Fig. 4.9, Plates 4.18 and 4.19). Pattern I generally appeared as a single band (Plate 4.18), but if the concentration of the sample was increased, two faster moving weaker bands became visible (Plate 4.18). This band triplet was at an identical position to that of pattern VI but the intensity of the three bands in the latter was the same.

'MALIC ENZYME'

Fig. 4.8. Diagrammatic representation of T. congolense patterns I to V

Plate 4.14. Comparison of three species of salivarian trypanosomes.
Conditions as in Tables 3.2 and 3.3.

Stocks from left to right

- | | | |
|----------------------------|---|--------------------------|
| 1. Gamb 20, ME IV | } | <u>T.(N.) congolense</u> |
| 2. Gamb 15, " III | | |
| 3. LUMP 89, " I | | |
| 4. EATRO 1564, weak sample | | |
| 5. Gamb 18, ME III | | |
| 6. Gamb 5, " IV | | |
| 7. Gamb 3, " IV | | |
| 8. GB 1 | } | <u>T.(T.) brucei</u> |
| 9. GB 2 | | |
| 10. Liverpool | } | <u>T.(D.) vivax</u> |
| 11. Liverpool | | |
| 12. Rat blood | | |

Plate 4.15 Variations in
T. congolense.

Conditions as Tables 3.2 and 3.3.

Stocks from left to right

1. Gamb 21, ME IV
2. Gamb 16, " V
3. Gamb 20, " IV
4. Gamb 18, " III
5. Gamb 1, weak sample
6. Gamb 12, ME II
7. SUBAKIA, " III
8. MIAG 108 Clone, ME II
9. EATRO 2025, ME II
10. Gamb 2, ME II

Plate 4.16 Variations in
T. congolense

Conditions as in Tables 3.2 and
3.3

1. Gamb 22, ME II
2. Gamb 17, " II
3. Gamb 19, " II
4. LUMP 89, " I

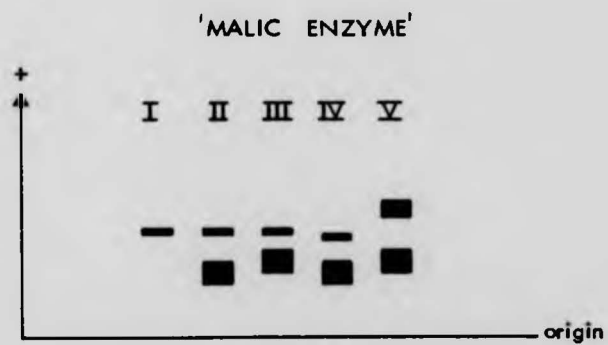


Fig. 4.8.

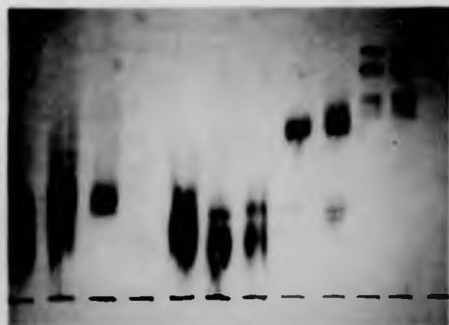


Plate 4.14.

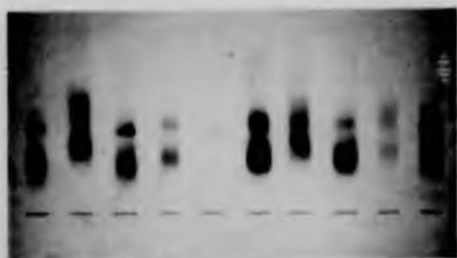


Plate 4.15.



Plate 4.16.

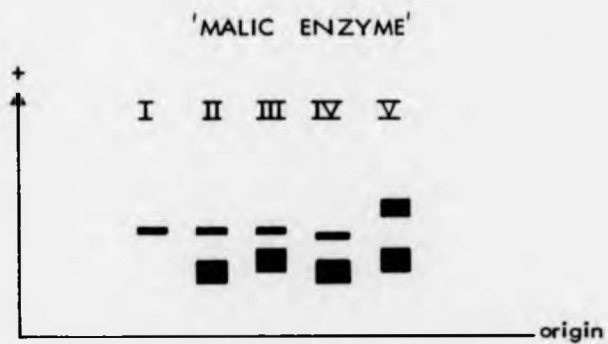


Fig. 4.8.

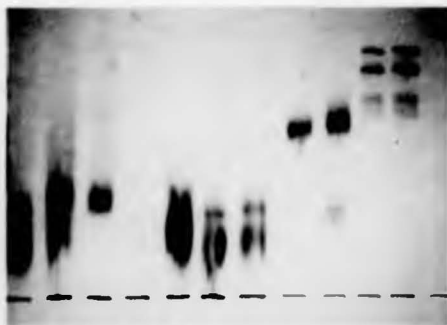


Plate 4.14.



Plate 4.15.

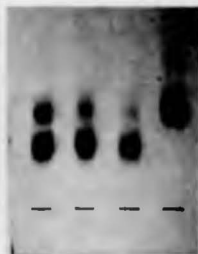


Plate 4.16.

MALATE DEHYDROGENASE

Fig. 4.9. Diagrammatic representation of T. congolense patterns I to VI

Plate 4.17. Comparison of three species of salivarian trypanosomes
Conditions as in Tables 3.2 and 3.3

Stocks from left to right

- | | | | |
|------------------|-------|----------------------|--------------------------|
| 1. LUMP 89, | MDH I | } | <u>T.(N.) congolense</u> |
| 2. IBADAN 44, | " II | | |
| 3. LRU TSW 4/77, | " III | | |
| 4. Gamb 18, | " I | | |
| 5. Gamb 22, | " VI | | |
| 6. Gamb 13Y, | " V | | |
| 7. GB 1 | } | <u>T.(T.) brucei</u> | |
| 8. GB 2 | | | |
| 9. Liverpool | - | <u>T.(D.) vivax</u> | |
| 10. Rat blood | | | |

Plate 4.18. Variations in
T. congolense

Conditions as in Tables 3.2 and 3.3

Stocks from left to right

1. S104/FLY/BE, MDH I
2. LRU TSW 108/77, " II
3. LRU TSW 114/77, " I
4. LRU TSW 4/77, " III
5. LRU TSW 78/77, " II
6. LRU TSW 6/77, " III
7. LRU TSW 115/77, " I
8. IBADAN 44, " II
9. IBADAN 69, " I
10. MIAG 108 Clone, " I

Plate 4.19. Variations in
T. congolense

Conditions as in Tables 3.2 and 3.3

Stocks from left to right

1. S104/FLY/BE, MDH I
2. Gamb 6, " I
3. Gamb 7, " IV
4. Gamb 10, " I
5. Gamb 13Y, " V
6. Gamb 15, " I
7. Gamb 18, " I
8. Gamb 23, " I
9. Gamb 27, " VI
10. MIAG 108 Clone, " I

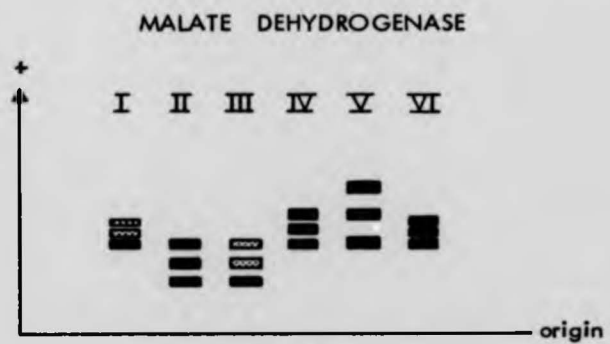


Fig. 4.9.

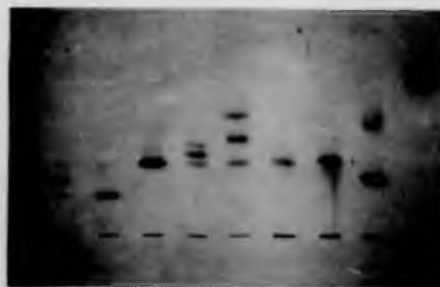


Plate 4.17.

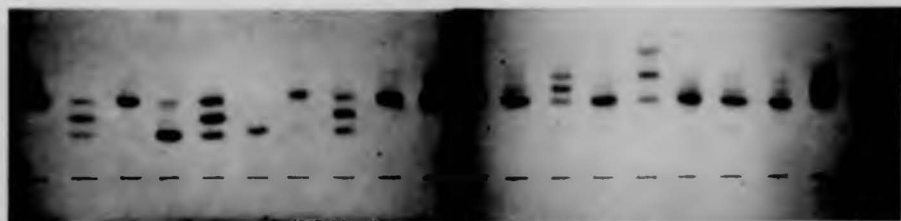


Plate 4.18.

Plate 4.19.

MALATE DEHYDROGENASE

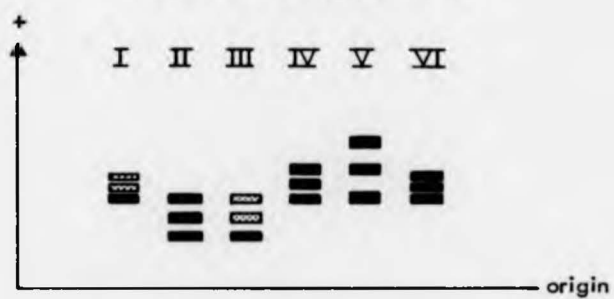


Fig. 4.9.

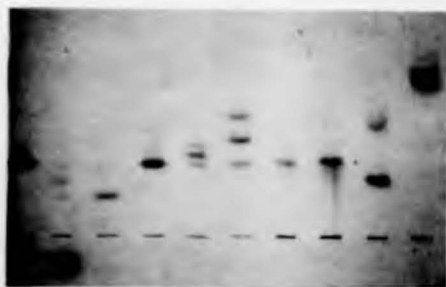


Plate 4.17.

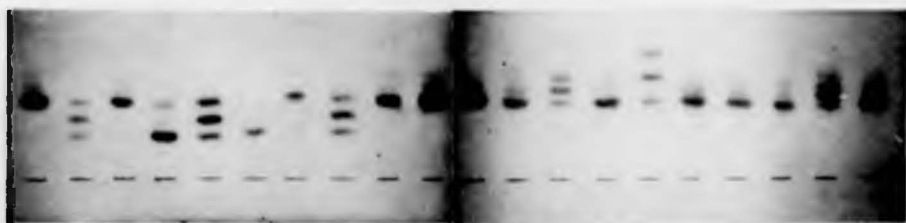


Plate 4.18.

Plate 4.19.

4.2.10. Glucose phosphate isomerase

The T.congolense GPI migrated further than the T.brucei or rat blood lysates, the latter produced faint bands on and either side of the origin (Plate 4.20); the T.vivax lysate migrated cathodically.

Ten GPI patterns were seen in the 78 T.congolense stocks, made up of combinations of 13 possible major bands (Fig. 4.10, Plates 4.21 to 4.23). Patterns I and III consisted of identical triplets of bands but the enzyme activities of each component differed consistently; patterns VIII and X were also triplets which differed only in the enzyme activities of the bands. Patterns VII to X migrated much further than patterns I to VI.

4.2.11. Peptidases

When T.congolense, T.brucei and T.vivax samples were electrophoresed on the same gel, and stained for PEP 1 or PEP 2, the banding appeared in the same area for all the samples making recognition of the subgenera difficult (Plates 4.24 and 4.27); the rat blood lysate could be identified as it migrated further than the trypanosome PEP bands.

18 patterns were found using L-leucylglycylglycine as the substrate, for PEP 1 and 14 patterns with L-leucyl-L-alanine as the substrate for PEP 2 (Figs. 4.11 and 4.12, Plates 4.24 to 4.28).

For PEP 1, all the bands seen are shown in Fig. 4.12. PEP 2 was found to be a combination of the strong staining bands seen in PEP 1 with the addition of slower moving bands; the weaker bands seen in PEP 1 occasionally appeared in PEP 2, but they were found to be very concentration-dependent and are not included in Fig. 4.11. The stocks which gave slow moving patterns in PEP 1 also gave

GLUCOSE PHOSPHATE ISOMERASE

Fig. 4.10. Diagrammatic representation of T. congolense patterns I to X

Plate 4.20. Comparison of three species of salivarian trypanosomes
Conditions as in Tables 3.2 and 3.3
Stocks from left to right

- | | |
|---------------------------------|----------------------------|
| 1. LRU TSW 4/77, GPI VII | } <u>T.(N.) congolense</u> |
| 2. LRU TSW 108/77, " VIII | |
| 3. LRU TSW 78/77, " IX | |
| 4. EATRO 1582, " III | |
| 5. LUMP 89, " II | |
| 6. Gamb 19, " I | |
| 7. GB 1, } <u>T.(T.) brucei</u> | |
| 8. GB 2, } <u>T.(D.) vivax</u> | |
| 9. Liverpool | |
| 10. Liverpool | |
| 11. Rat blood | |

Plate 4.21. Variations in T. congolense
Conditions as in Tables 3.2 and 3.3, but
time reduced to 2.5 hours
Stocks from left to right

1. 1/148 FLY, GPI IV
2. EATRO 1564, weak sample
3. EATRO 1755, GPI II
4. EATRO 1968, " I
5. MIAG 108 Clone " II
6. EATRO 2027, " I
7. EATRO 1582, " III
8. EATRO 2025, " V
9. EATRO 1712, " II

Plate 4.22. Variations in T. congolense

Conditions as in Tables 3.2 and 3.3
Stocks from left to right

1. TD 378/78E, GPI X
2. TSW 25/78E, " X
3. TSW 152/78E, " VII
4. LRU TSW 114/77, " VII
5. TD 28/78E, " VIII
6. LRU TG14/78, " VII
7. LRU TSW 99/77, " IX
8. Gamb 19, " I
9. LUMP 89, " II

Plate 4.23. Variations in T. congolense

Conditions as in Tables 3.2 and 3.3
Stocks from left to right

1. S104/FLY/BE, GPI II
2. Gamb 28, " I
3. Gamb 29, " I
4. LRU TSW 13, " VI
5. TRU TSW 29, " VI
6. Gamb 1, " I
7. TDRN 9, " I
8. LRU TSW 103/77b, " VII
9. LRU TSW 243/77, " VIII

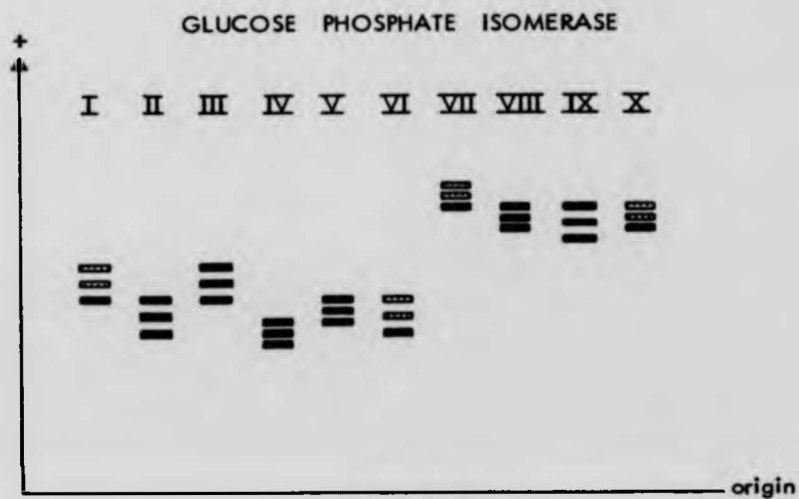


Fig. 4.10.



Plate 4.20.

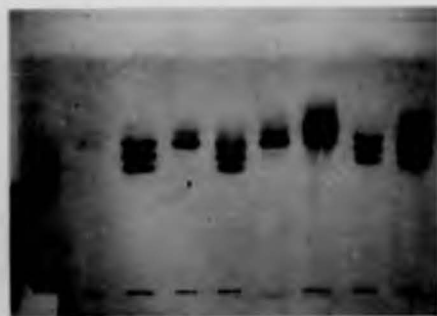


Plate 4.21.

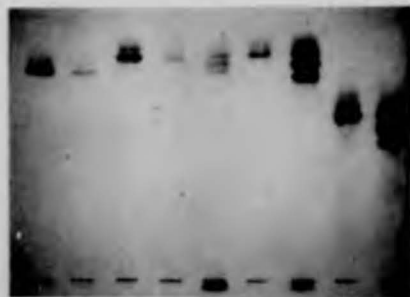


Plate 4.22.

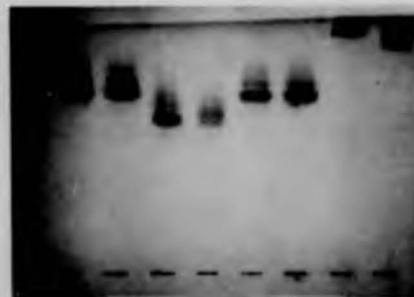


Plate 4.23.

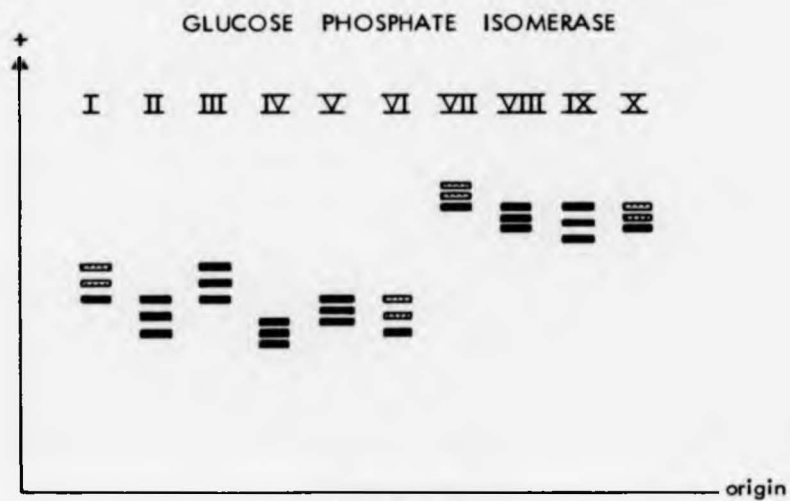


Fig. 4.10.

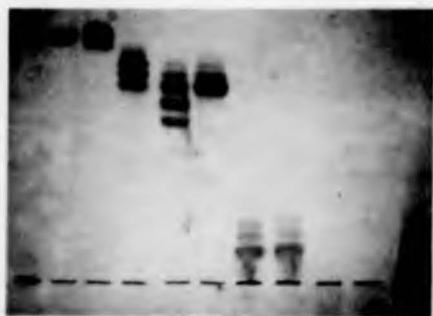


Plate 4.20.

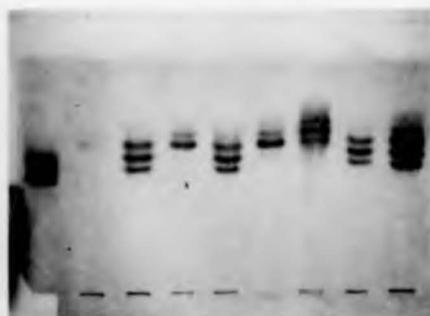


Plate 4.21.

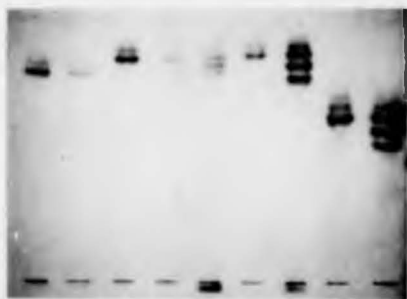


Plate 4.22.

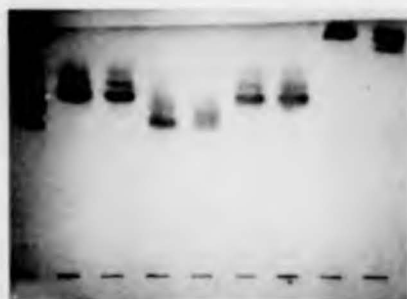


Plate 4.23.

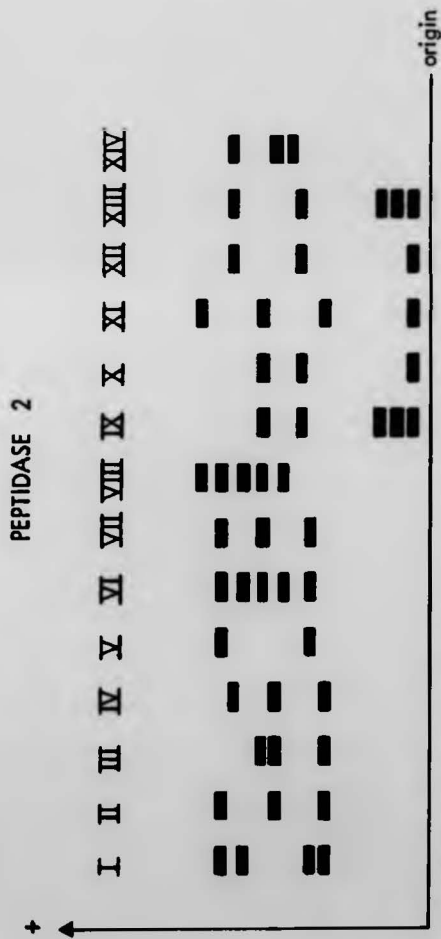


Fig. 4.11. Diagrammatic representation of T. congolense patterns I to XIV

PEPTIDASE 2

Plate 4.24. Comparison of three species of salivarian trypanosomes
Conditions as in Tables 3.2 and 3.3

Stocks from left to right

- | | | |
|--------------------------------|---|--------------------------|
| 1. EATRO 1582, PEP 2 VI | } | <u>T.(N.) congolense</u> |
| 2. Gamb 20, " VII | | |
| 3. IBADAN 44, weak sample | | |
| 4. LUMP 89, PEP 2 VI | | |
| 5. LRU TSW 103/77b, PEP 2 XIII | | |
| 6. Gamb 18, " I | | |
| 7. GB 1 | } | <u>T.(T.) brucei</u> |
| 8. GB 2 | | |
| 9. Liverpool | } | <u>T.(D.) vivax</u> |
| 10. Liverpool | | |
| 11. Rat blood | | |

Plate 4.25. Variations in
T.congolense
Conditions as in Tables 3.2 and
3.3

Stocks from left to right

1. Gamb 29, PEP 2 III
2. LRU TSW 13, PEP 2 IV
3. LRU TSW 29, weak sample
4. Gamb 1, PEP 2 III
5. TDRN 9, " II

Plate 4.26. Variations in
T.congolense
Conditions as in Tables 3.2 and
3.3

Stocks from left to right

1. LRU TSW 108/77 PEP 2 XII
2. LRU TSW 114/77 " XII
3. LRU TSW 4/77, " XII
4. LRU TSW 78/77 " XII
5. LRU TSW 6/77 " XII
6. LRU TSW 115/77 " IX
7. IBADAN 44 " X
8. IBADAN 69 " XII
9. MIAG 108 Clone " VII



Plate 4.24.

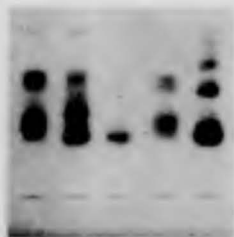


Plate 4.25.

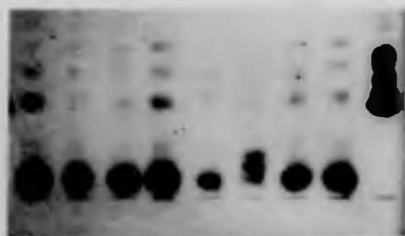


Plate 4.26.



Plate 4.24.

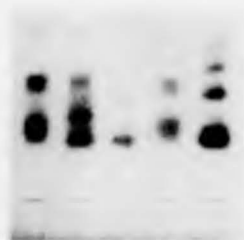


Plate 4.25.

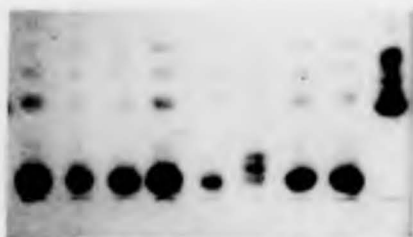


Plate 4.26.

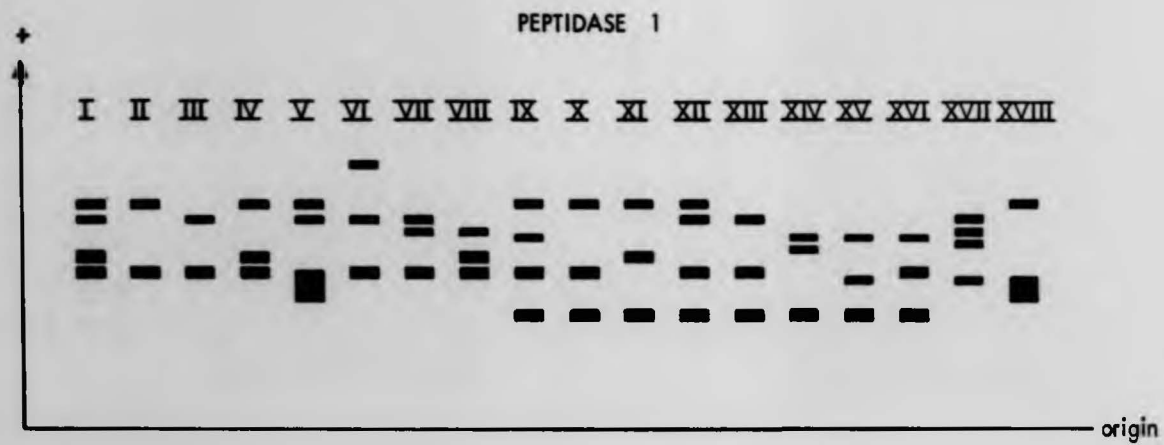


Fig. 4.12. Diagrammatic representation of T. congolense patterns I to XVIII

PEPTIDASE 1

Plate 4.27. Comparison of three species of salivarian trypanosomes
Conditions as in Tables 3.2 and 3.3

Stocks from left to right

- | | | |
|--------------------------------|---|--------------------------|
| 1. LUMP 89, PEP 1 III | } | <u>T.(H.) congolense</u> |
| 2. IBADAN 44, weak sample | | |
| 3. TDRN 9, PEP 1 XVII | | |
| 4. LRU TSW 108/77, weak sample | | |
| 5. LRU TSW 103/77b, PEP 1 XVI | | |
| 6. EATRO 1599, " VII | | |
| 7. EATRO 1582, " I | | |
| 8. GB 1 | } | <u>T.(T.) brucei</u> |
| 9. GB 2 | | |
| 10. Liverpool | } | <u>T.(D.) vivax</u> |
| 11. Liverpool | | |
| 12. Rat blood | | |

Plate 4.28. Variations in T. congolense
Conditions as in Tables 3.2 and 3.3

Stocks from left to right

1. S104/FLY/BE, PEP 1 III
2. EATRO 2084, " V
3. Gamb 4, " XVII
4. EATRO 1582, " I
5. Gamb 26, " IV
6. Gamb 20, " IV
7. IBADAN 34, " XVI
8. IBADAN 35, " XV
9. Gamb 3, " IV
10. MIAG 108 Clone " VII



Plate 4.27.

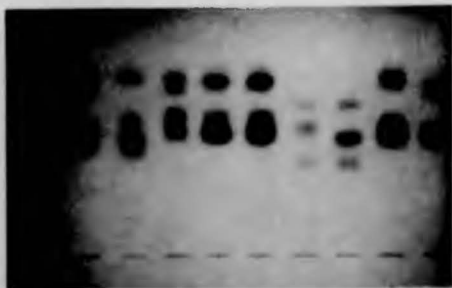


Plate 4.28.

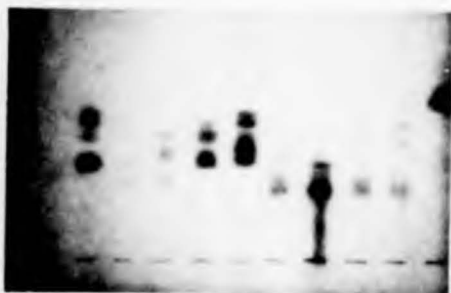


Plate 4.27.



Plate 4.28.

the slow moving patterns of PEP 2.

The results obtained with 10 different peptides as substrate are shown in Plates 4.29 to 4.37. The samples were all duplicated on the gel, the right hand side was developed using L-leucylglycylglycine and the left side with one of the other peptides as substrate. The L-leucylglycylglycine side of Plates 4.36 is only faintly stained due to a limited amount of substrate available. One sample, EATRO 1564, was weak and did not always stain.

The peptidase banding of T.congolense obtained with these different peptide substrates was compared with the banding seen with human peptidases (Rapley et al., 1971) and with trypanosomes of the subgenus Trypanozoon (Gibson and Letch, in press) (Table 4.2). The letters designated to each band were assigned in order of discovery of the bands by Rapley et al. (1971) as they used different peptides as substrates. Many of the peptides can be catalysed by more than one peptidase giving multiple banding on the gel; Rapley et al. (1971) published a table giving an indication of which peptidase obtained from human tissue could utilize which particular substrate. From these results, correlations were made with T.congolense and members of the subgenus Trypanozoon (Letch and Gibson, in press). Seven human peptidases are reported (Rapley et al., 1971), and six in Trypanozoon (Gibson and Letch, in press) while five were found in T.congolense (Table 4.2). With the T.congolense samples, if two or more bands always appeared together they were considered as two or more isoenzymes of one peptidase rather than separate peptidases, but this assumption may be incorrect. No equivalent of the human C band was found in either of the trypanosome species investigated and no F band was seen in T.congolense. The banding is shown

Plates 4.29 to 4.34. Zymograms of T. congolense peptidases
using different peptides as substrate

Conditions as for PEP 1 and 2 in Tables 3.2 and 3.3

Stocks from left to right

1. EATRO 1564, weak sample
2. IBADAN 69
3. LRU TSW 103/77b
4. MIAG 108 Clone
5. EATRO 1564, weak sample
6. IBADAN 69
7. LRU TSW 103/77b
8. MIAG 108 Clone

Samples 1 to 4 developed with L-leucylglycylglycine
as substrate

Samples 5 to 8 developed with:-

Plate 4.29. L-valyl-L-leucine

Plate 4.30. L-valyl-L-alanine

Plate 4.31. Glycyl-L-leucine

Plate 4.32. L-leucylglycine

Plate 4.33. L-leucyl-L-alanine

Plate 4.34. L-lysyl-L-leucine

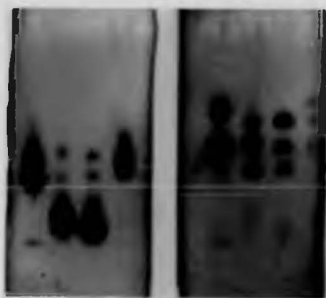


Plate 4.29.

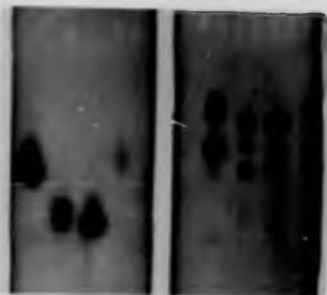


Plate 4.30.

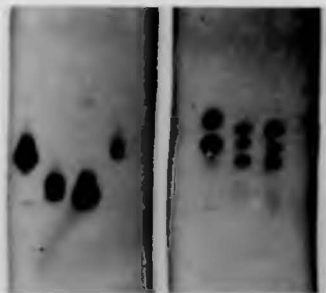


Plate 4.31.

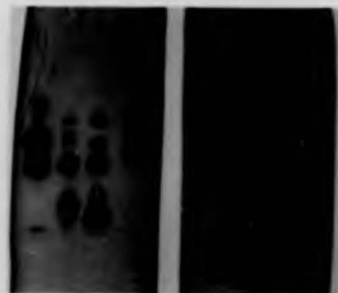


Plate 4.32.

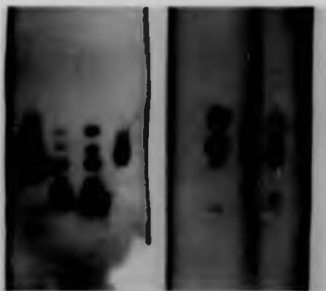


Plate 4.33.

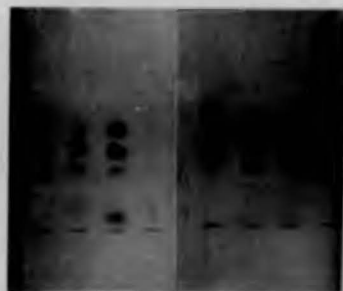


Plate 4.34.

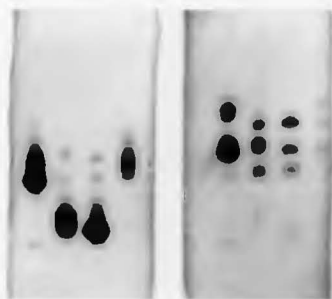


Plate 4.29.

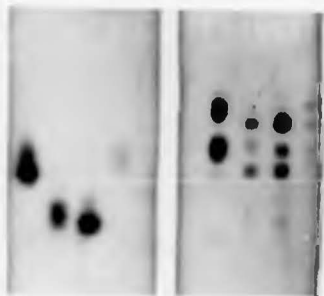


Plate 4.30.

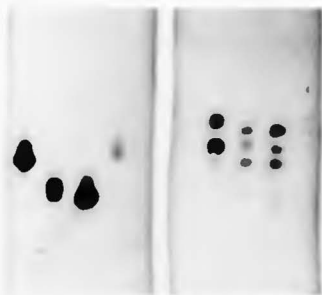


Plate 4.31.

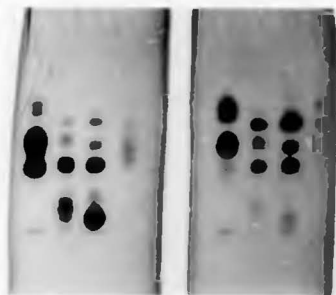


Plate 4.32.

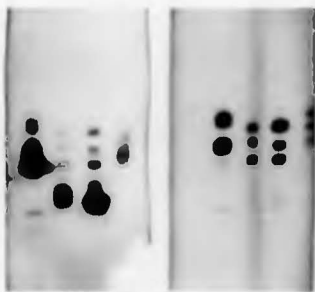


Plate 4.33.

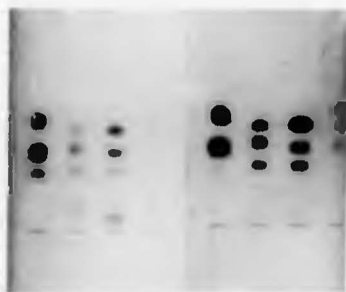


Plate 4.34.

Plates 4.35 to 4.37. Zymograms of T.congolense peptidases
using different peptides as substrate

Conditions as for PEP 1 and 2 in Tables 3.2 and 3.3

Stocks from left to right

1. EATRO 1564, weak sample
2. IBADAN 69
3. LRU TSW 103/77b
4. MIAG 108 Clone
5. EATRO 1564, weak sample
6. IBADAN 69
7. LRU TSW 103/77b
8. MIAG 108 Clone

Samples 1 to 4 developed with L-leucylglycylglycine
as substrate

Samples 5 to 8 developed with:-

Plate 4.35. L-leucyl-L-leucyl-L-leucine

Plate 4.36. L-leucyl- ρ -nitroanilide

Plate 4.37. L-isoleucyl-L-proline

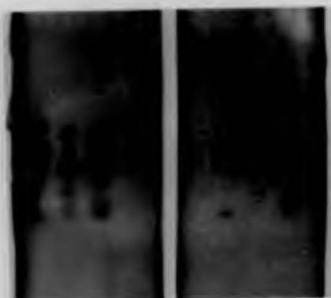


Plate 4.35.



Plate 4.36.

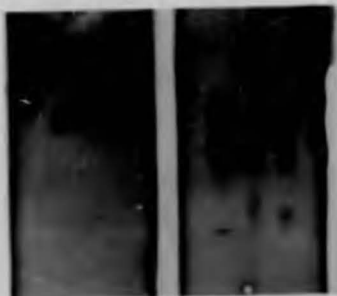


Plate 4.37.

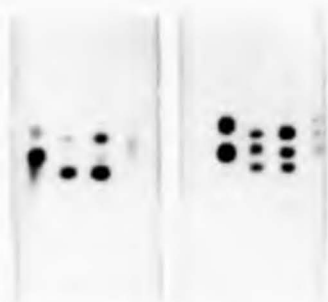


Plate 4.35



Plate 4.36



Plate 4.37

Table 4.2. Peptidase banding with different peptides as substrate

Peptide Substrate	Human*	<u>Trypanozoon**</u>	<u>T. congolense</u>
L-valyl-L-leucine	SA	SA	SAB
L-valyl-L-alanine	SA	-	A
Glycyl-L-leucine	AC	A	A
L-leucylglycine	SAC	SA	SAB
L-leucyl-L-alanine	SAC	SA	SABE
L-lysyl-L-leucine	SABCE	SBE	SABE
L-leucylglycylglycine	SBE	SBE	SBE
L-leucyl-L-leucyl-L-leucine	SBEF	SBEF	SBE
L-leucyl-p-nitroanilide	E	E	E
L-isoleucyl-L-proline	D	D	D

* Rapley et al. (1971)

** Letch and Gibson (in press)

diagrammatically for three samples of T.congolense in Fig. 4.13.

Using L-leucyl-L-alanine, as substrate, four of the five T.congolense peptidases could be detected but the E band was often very weak and increasing the concentration of the sample prevented band separation of the S, A and B bands. With L-leucylglycylglycine, as substrate, the S, B and E bands could all be seen clearly.

4.2.12. Isocitrate dehydrogenase

No ICD activity was detected in T.congolense after electrophoresing. Enzyme assays on four samples, LUMP 89, Gamb 2, EATRO 2033 and LRU TSW 4/77 showed that there was only a very low ICD activity between 0 and 0.045 U ml^{-1} with an average of 0.019 U ml^{-1} in the four samples, each run twice. This activity is about 50 times too low to be detected by the staining of a starch gel.

4.2.13. Glucose-6-phosphate dehydrogenase

No G6PD activity was detected in T.congolense either during electrophoresis or enzyme assays of four stocks LUMP 89, Gamb 2, EATRO 2033 and LRU TSW 4/77.

4.3. Controls

No change in electrophoretic patterns for the 14 enzymes occurred in samples of 1/148 FLY, S104/FLY/BE and MIAG 108 Clone, either when harvested at different times during the infection in rats or when re-examined in fresh infections derived from deep-frozen stabilates of the parent stocks.

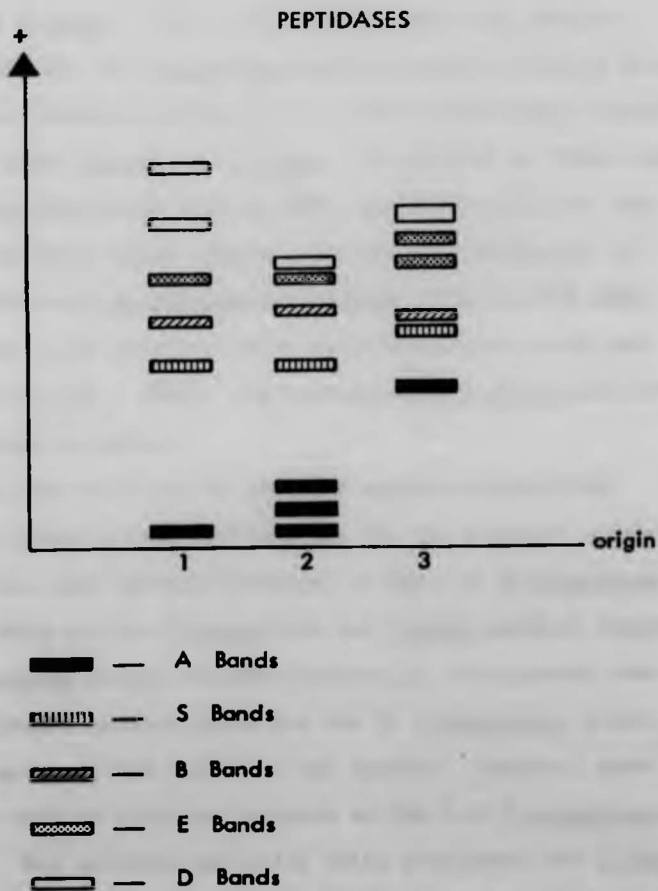


Fig. 4.13. Diagrammatic representation of the isoenzyme bands of five peptidases of three stocks of T. congolense

4.4. Discussion

Eight of the 14 enzymes used for the routine screening of T.congolense stocks, readily distinguished this species of trypanosome from T.brucei or T.vivax. PK and NH both gave only one, single-banded pattern for all 78 T.congolense stocks examined, making them clear markers for identifying this species even among mixed infections of T.congolense with T.brucei or T.vivax. In addition to these eight enzymes, the four peptidases seen in PEP 1 and PEP 2 could be used for separation of these three species, but the wide diversity of patterns exhibited by T.congolense and T.brucei often in the same region of the gel, made identification difficult (Plates 4.24 and 4.27) (see Gibson *et al.*, 1980); the peptidases of T.vivax have not as yet been studied in detail.

MDH and TDH could not be used for species distinction. With MDH, the T.vivax pattern differed but the two T.brucei samples both displayed the same pattern identical to MDH I of T.congolense (Plate 4.17). From the two T.brucei and one T.vivax samples compared with two T.congolense stocks for TDH (Plate 4.1), it appeared that the one single-banded pattern found for the 78 T.congolense stocks could be distinguished from the other two species. However, some T.brucei stocks have an identical pattern to TDH I of T.congolense (Gibson, 1979). New patterns are still being discovered for T.brucei which may prove to be the same as those of T.congolense and more extensive work on T.vivax could also reveal identical banding.

The apparent lack of G6PD activity in T.congolense both from electrophoresis and assaying should be regarded with caution. It is extremely unlikely that T.congolense has dispensed with the

pentose phosphate pathway whose initial reaction, the dehydrogenation of glucose-6-phosphate to 6-phosphoglucone-5-lactone, is catalysed by G6PD. G6PD may be insoluble in water and was therefore not contained in these lysates made as described in Section 2.6.2, or its apparent absence may be due to its failure to be released from a membrane bound body in which it is believed to exist (A. Fairlamb, personal communication). The freeze thawing process may fail to rupture this membrane and thus the enzyme could not be released into the lysate. If this enzyme is membrane bound its release could possibly be brought about by freeze thawing in a high concentration of K or Na ions (A. Fairlamb, personal communication).

The same reasons may account for the low activity of ICD, but it is possible that this is a true representation of its activity since the tricarboxylic acid cycle may operate at an insignificant rate as in T. rhodesiense (Flynn and Bowman, 1973). The major end products of metabolism in T. congolense, acetate, succinate, glycerol and CO₂ (see Bowman and Flynn, 1976) can be reached without this enzyme.

T. congolense was found to be enzymically heterogeneous giving 71 different isoenzyme bands with 14 enzymes among 78 stocks. Combinations of these isoenzymes gave 75 distinct zymodemes. A similar polymorphism was seen with L. tropica (Al-Taqi and Evans, 1978), and the trypanosomes of bats, subgenus Schizotrypanum, (Baker *et al.*, 1978). In contrast, only three major zymodemes have been found among 250 stocks of T. cruzi (Miles *et al.*, 1980a).

25 of the 71 different isoenzymes of T. congolense were found with PEP 1 and PEP 2. This high degree of polymorphism arises because the isoenzymes of four different peptidases are in fact being

examined (Section 4.2.11). It would probably have been preferable to examine each peptidase separately, by providing a peptide substrate specific to each peptidase. However, by using L-leucylglycylglycine and L-leucyl-L-alanine, the four peptidases A,B,E and S could all be investigated with the minimum amount of lysate; only peptidase D was not revealed with these two peptides (Section 4.2.11).

GPI gave ten patterns and there was a striking difference in the mobilities of patterns I to VI compared with VII to X (Fig. 4.10). Some of the fast and slow patterns displayed the same relative configurations i.e. II and V with VIII and IX (Fig. 4.10). There may be a more negatively charged carrier protein attached to the faster moving patterns, which thus greatly increases their mobility. In addition to the 15 isoenzymes of GPI shown in Fig. 4.10, were numerous weaker bands which appeared inconsistently, perhaps indicating instability; these weak bands were considered unreliable and were not used for characterization.

Direct examination of GPI, PEP 1 and PEP 2 zymograms revealed a division of the 78 stocks of T.congolense into two groups. The division could be directly correlated with the type of vegetation present in their area of origin, except for the two stocks LRU TSW 13 and TSW 29/77. 52 stocks came from savannah regions of East and West Africa and from such diverse hosts as cattle, G.pallidipes, gazelle (Gazella granti) and a lion; all gave slow moving GPI patterns (I to VI) and the fast moving PEP 1 patterns (I to VIII, XVII and XVIII) and PEP 2 patterns (I to VIII and XIV). The remaining 26 were all isolated in West Africa from cattle, pigs, dogs, a sheep and a goat, in riverine or forest areas; these stocks (except LUR TSW 13 and TSW 29/77 which gave savannah patterns) each displayed

fast moving GPI patterns (VII to X) and slow moving peptidase patterns (PEP 1, IX to XVI and PEP 2, IX to XIII), particularly visible with peptidase A (Section 4.2.11).

The two exceptions to this vegetational division, LRU TSW 13 and TSW 29/77, were isolated from pigs in nearby villages in the Ganta area of Liberia (Fig. 4.14). However, these villages are within 20 km of a major road junction; one road originates from further north in Guinea and it is possible that these infections were introduced by animals trekked or transported from the savannah regions of Guinea. Tsetse flies infected with a savannah zymodeme could also have been brought into the area in vehicles. There is evidence to suggest that this zymodeme would not have been successfully transmitted by the local tsetse fly population (Chapter 7) and as TSW 29/77 and LRU TSW 13 were isolated two years apart it appears that savannah zymodemes are continually being introduced into the area.

The two major zymodemes found in the different vegetational regions must normally be transmitted by species of Glossina which prefer that particular habitat. Tsetse flies belonging to the morsitans- group are generally restricted to savannah regions, fusca- group flies to the forest and palpalis- group flies to riverine galleries and swamps. Therefore, the savannah group of stocks might be expected to be transmitted primarily by members of the morsitans- group and the remaining stocks by palpalis- or fusca- group flies.

The riverine/forest stocks originated from Liberia, southwestern Nigeria and the south of the Ivory Coast in regions where only palpalis- or fusca- group flies are believed to exist

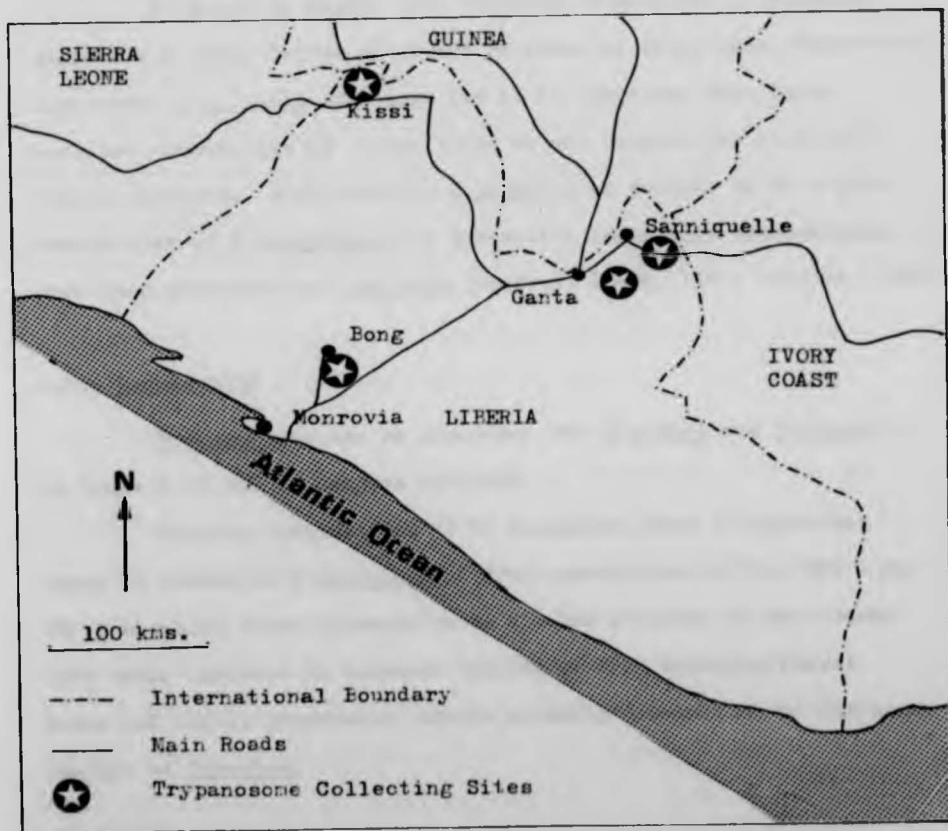


Fig. 4.14. Isolation sites of Liberian trypanosome stocks

(Bequaert, 1946; Hutchinson et al., 1964; Ford, 1970, 1971). The savannah stocks originated from areas where morsitans-group flies are predominant and palpalis- group flies occur in the riverine galleries (Ford, 1970, 1971).

It might be argued that livestock maintained in savannah regions are often driven to rivers or lakes to drink where they would come under G.palpalis challenge but it is suggested that these isolated communities of tsetse flies do not harbour the riverine/forest zymodeme. Additionally, G.palpalis is thought to be a poor transmitter of T.congolense; no successful laboratory transmissions have been effected by G.palpalis (Godfrey, 1960b, 1964; Stephen, 1962).

4.5. Conclusions

T.congolense can be separated from T.brucei and T.vivax by at least 8 of the 14 enzymes examined.

Various combinations of 71 isoenzymes gave 75 zymodemes among 78 stocks of T.congolense. From examination of GPI, PEP 1 and PEP 2 patterns there appeared to be a major division of the stocks into those isolated in savannah regions or from riverine/forest areas and hence, presumably, stocks normally transmitted by different species of Glossina.

CHAPTER 5

5. COMPUTER ANALYSIS OF ISOENZYME PATTERNS

5.1. Introduction

Since interpretation of the results by direct examination of the zymograms was unduly complex, the information was prepared for computer analysis.

5.2. Materials and Methods

Each isoenzyme band was treated separately i.e. was a 'unit character' (Sneath and Sokal, 1973), and given an arabic number. (The roman numerals (Section 4.1) were used for combinations of bands). Every stock was coded as to which bands it possessed. For example, consider two theoretical zymodemes A and B for three enzymes PK, ASAT and PGM: with PGM there is a possibility of four different bands and with ASAT, three and with PK, one. This is represented diagrammatically in Figs. 5.1 to 5.3. Supposing zymodeme A gave PK I, ASAT IV and PGM III, while zymodeme B displayed PK I, ASAT III and PGM IV. The computer coding for these two stocks is shown in Table 5.1. It should be noted however, that at least 60 units are required for a valid computer analysis (Sneath and Sokal, 1973).

This information for each stock was introduced into the computer programme GENSTAT V, mark 4.01, Lawes Agricultural Trust, originating from Rothamsted Experimental Station.

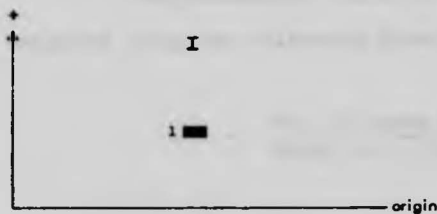
Fig. 5.1. Diagrammatic representation of PK isoenzyme bands of T.congolense

Fig. 5.2. Diagrammatic representation of PGM isoenzyme bands of T.congolense

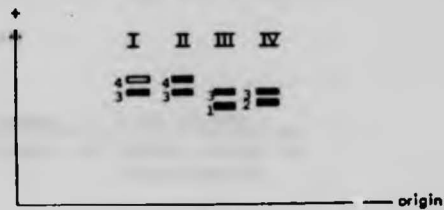
Fig. 5.3. Diagrammatic representation of ASAT isoenzyme bands of T.congolense

Table 5.1. Preparation of isoenzyme data of two theoretical stocks of T.congolense for computer analysis

PYRUVATE KINASE



PHOSPHOGLUCOMUTASE



ASPARTATE AMINOTRANSFERASE

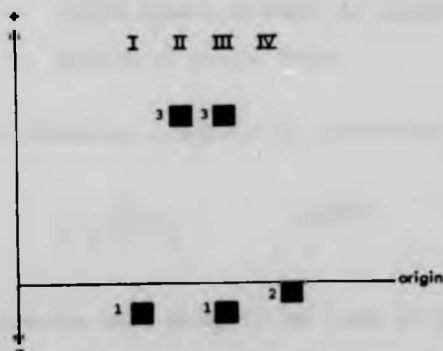


Table 5.1.

Enzyme	PK				ASAT				PGM			
Band no.	1	2	3	4	1	2	3	4	1	2	3	4
'Unit' no.	1	2	3	4	5	6	7	8	9	10	11	12
Stock A	+								+			+
Stock B					+							+

The similarity index (S) between every two stocks was computed using the following formula,

$$s = \frac{\text{No. of bands common to both stocks}}{\text{Total no. of common and other bands in both stocks}}$$

$$\text{i.e. } s = \frac{z}{a + b - z}$$

where, a = total number of bands in zymodeme A
b = total number of bands in zymodeme B
z = number of shared bands

Thus, for our two zymodemes A and B, the similarity index would be:-

$$\frac{2}{4 + 5 - 2} = 0.2857$$

These results were given in the form of a similarity matrix, readily convertible to a percentage similarity. The percentage similarity of the two theoretical zymodemes A and B would be 57.14%.

The input information was then subjected to Furthest Neighbour Hierarchical Cluster Analysis; this analysis examines the degree of similarity between stocks, from the similarity level 95% to 25% in 5% steps. A cluster, consists of a group of similar stocks with the most similar in the centre of the cluster and the least similar on the outside. Two stocks with high similarities to one another for example 95%, would form the centre of the cluster and they could be joined by another stock showing 90% similarity to both the other two stocks already in the cluster. These stocks would subsequently be joined by stocks showing 85%, 80% etc. similarities

to all the stocks already in the cluster.

This computer programme was run twice, once with the information from all 14 enzymes and once for only 10, omitting the 4 peptidases, because the peptidases displayed so many different bands making the interpretation of the zymograms difficult and subject to error. Further, some of the enzyme bands were identical in PEP 1 and 2 (Section 4.2.11) and so were included twice in the computer analysis possibly weighting the results.

5.3. Results

The percentage similarities of 78 stocks of T.congolense examined with 14 enzymes varied from 22% to 100% (Appendix IV).

The numerical results of the Furthest Neighbour Hierarchical Cluster Analysis, with 14 enzymes, is shown in Appendix V. A visual interpretation of this clustering is given as dendrograms for both 14 and 10 enzymes (Figs. 5.4 and 5.5).

5.4. Discussion

A numerical approach to the analysis of the isoenzymes of trypanosomes has previously been made by Ready and Miles (1980), Gibson (1979) and Gibson et al. (1980). Ready and Miles (1980) confirmed the presence of three major zymodemes previously seen by direct examination of the zymograms of 18 stocks of T.cruzi. Gibson (1979) and Gibson et al. (1980) after computer analysis of the isoenzyme data of 160 Trypanozoon stocks found a major dichotomy between stocks originating from East and West Africa. This division could also be seen, with the majority of stocks, by examination of ICD and PGM zymograms. However these numerical analyses should not be regarded



* stock numbers - see Appendix III

Fig. 5.4. Dendrogram showing the results of hierarchical cluster analysis with 71 isoenzyme characters of 78 stocks of *T. congolense*



* stock numbers - see Appendix III

Fig. 5.5. Dendrogram showing the results of hierarchical cluster analysis with 46 isoenzyme characters of 78 stocks of *T. congolense*

as conclusive as in both cases fewer characters were used than the 60 suggested as necessary for accurate analysis (Sneath and Sokal, 1973).

Unlike Trypanozoon stocks, no geographical distinction was seen for T.congolense; Gamb 16 from West Africa and Subakia from East Africa showed an 82% similarity in their enzymes. Trypanozoon stocks isolated in the same area had high percentage similarities to one another (Gibson, 1979). This was not seen for T.congolense; stocks isolated from the same herd of cattle had similarities as low as 43% (Gamb 7 and Gamb 4) while Gamb 7 and Gamb 16 both isolated in The Gambia but from villages 52 km apart had only a 33% similarity. However, each of the three pairs of identical stocks found, did come from the same country: Gamb 28 and Gamb 29 originated from the same herd of cattle in The Gambia; EATRO 1599 and EATRO 1751 from G.pallidipes caught in the same village in Kenya; TREU 1290 and TREU 1095 originated in Nigeria although the origin of TREU 1095 may not be Ibadan, where it was isolated, as many cattle are trekked there and few are actually kept in the area.

Computer analysis of the 71 isoenzyme characters of T.congolense confirmed the important finding of a major dichotomy of stocks between those originating from savannah or riverine/forest areas. However, besides the two expected exceptions LRU TSW 13 and TSW 29/77 (Section 4.4), LRU TSW 251/77, isolated in the Kissi area of Liberia (Fig. 4.14), appeared in the savannah branch of the dendrogram (Fig. 5.4). At first this would seem correct as Kissi is derived savannah but, as yet, no morsitans- group flies have colonized the area (Bequaert, 1946; Hutchinson et al., 1964; Ford, 1970, 1971). The highest percentage similarity displayed by LRU TSW 251/77 to any other stock was only

63% and it gave a unique PEP 2 pattern (XI). No explanation can be found for its uniqueness as other stocks isolated in the same area conformed with riverine/forest patterns recorded elsewhere.

The major division of T.congolense stocks was also seen when computer analysis was run using 10 enzymes (Section 5.2). However, the distinction was not so clear and anomalies occurred in both branches (Fig. 5.5). A small group of savannah stocks, isolated from The Gambia, appeared in the riverine/forest branch and a small central branch arose from the savannah division and contained a mixture of riverine/forest and savannah stocks including LRU TSW 13 and TSW 29/77; LRU TSW 251/77 was found in the riverine/forest branch. Thus, the number of unit characters considered has altered the grouping and shape of the dendrogram. This instability of the clusters on addition or removal of information is perhaps not surprising when this organism has shown such an enzymic diversity. Presumably the more data used the more stable the groupings will become. However, instability was also seen in the homogeneous Trypanozoon subgenus (Gibson et al., 1980) suggesting that it may be due to an inherent fault in the clustering method. As described in Section 5.2 the clustering method employed in this programme greatly restricts the way in which later zymodemes group, and the inclusion or exclusion of a zymodeme greatly alters the shape of the dendrogram. A different clustering method could possibly overcome this problem, such as a single-cluster formation technique in which each cluster initiated is completed before another is begun, but the disadvantage of this method is in the formation of overlapping clusters (Cormack, 1971). It would be interesting to carry out the computer analysis used during this work with the removal of each isoenzyme character, alone or in

63% and it gave a unique PEP 2 pattern (XI). No explanation can be found for its uniqueness as other stocks isolated in the same area conformed with riverine/forest patterns recorded elsewhere.

The major division of T. congolense stocks was also seen when computer analysis was run using 10 enzymes (Section 5.2). However, the distinction was not so clear and anomalies occurred in both branches (Fig. 5.5). A small group of savannah stocks, isolated from The Gambia, appeared in the riverine/forest branch and a small central branch arose from the savannah division and contained a mixture of riverine/forest and savannah stocks including LRU TSW 13 and TSW 29/77; LRU TSW 251/77 was found in the riverine/forest branch. Thus, the number of unit characters considered has altered the grouping and shape of the dendrogram. This instability of the clusters on addition or removal of information is perhaps not surprising when this organism has shown such an enzymic diversity. Presumably the more data used the more stable the groupings will become. However, instability was also seen in the homogeneous Trypanozoon subgenus (Gibson et al., 1980) suggesting that it may be due to an inherent fault in the clustering method. As described in Section 5.2 the clustering method employed in this programme greatly restricts the way in which later zymodemes group, and the inclusion or exclusion of a zymodeme greatly alters the shape of the dendrogram. A different clustering method could possibly overcome this problem, such as a single-cluster formation technique in which each cluster initiated is completed before another is begun, but the disadvantage of this method is in the formation of overlapping clusters (Cormack, 1971). It would be interesting to carry out the computer analysis used during this work with the removal of each isoenzyme character, alone or in

combination with others; the results may illustrate the relative significance of each enzyme character and provide information to improve the screening process. This could be particularly important in examining stocks where little material is available due to such factors as, inability to infect rodents or the production of low grade parasitaemias.

With the computer analysis a major problem arose in preparing the input information. This form of analysis or numerical taxonomy is based on the Adansonian principles (Adanson, 1763) revised by Sokal and Sneath (1963):

1. All characters are of equal importance in creating natural groups.
2. These groups should be based on as many features as possible.
3. The relationship between the groups is a function of the similarities of the characters which are being compared.

The problem arose when there were two patterns with bands of identical mobilities but consistently different activities and if principle 1 was obeyed the two patterns could not be entered into the computer as different. For example, PGM I and PGM II each consisted of two bands with the same mobilities but in pattern II the bands were of equal intensity and in pattern I, the slower band was denser than the fast band (Fig. 4.6). These two patterns were considered as different but even if principle 1 was ignored there was no means of weighting characters in the computer programme, Genstat V. To overcome this problem, the less dense band was omitted, for example the fast band of PGM I, but it was realized that this would lower the degree of relationship of stocks below their true similarity level.

The four stocks TREU 1291, TREU 1290, TREU 1095 and TREU 1381 (= TREU 1173) have also been compared using serological and immunological techniques (Luckins and Gray, 1979b). Although isoenzyme electrophoresis showed TREU 1290 and TREU 1095 to be identical in 14 enzymes, they have been found to be antigenically distinct from one another (Luckins and Gray, 1979b). Additionally TREU 1290 produced skin reactions in rabbits after infecting by tsetse fly bite while TREU 1095 did not (Luckins and Gray, 1979b).

Two stocks, S104/FLY/BE and TREU 1381, obtained from different sources were found to have originated from the same isolate. They were not however, enzymically identical but differed in their PEP 1 patterns which reduced their similarity level to 85%. S104/FLY/BE was obtained after tsetse fly transmission of S104 which had previously been subpassaged numerous times. TREU 1381 was the product of at least four stabilizations of the original stock S104, and each time the stock had received a different name and number. Therefore, the peptidase isoenzymes altered either during repeated subpassages, in which case neither S104/FLY/BE or TREU 1381 may display the original peptidase patterns, or on transmission through tsetse flies either by selection of a certain trypanosome population, a mutation, or a genetic 'switching' on and off of certain peptidase isoenzymes. This illustrates the importance of using stocks after as few passages as possible, as each passage in an abnormal host could be a selective process. Population selection could be overcome by cloning each stock, but preparation of large numbers of clones of T. congolense would be difficult, tedious and time consuming. Moreover, the two cloned stocks of Camb 2 and TSW 99/77 were identical to their parent stocks and if T. congolense has a tendency to 'switch' isoenzymes

on and off or for mutations to occur, such changes would also be expected to result from repeated subpassages of a cloned population.

The common origin of S104/FLY/BE and TREU 1381, illustrates the confusion and unnecessary work entailed in tracing a stock which results from repeated renaming. While it may be useful for each laboratory to have its own reference number for their stabilates, each stock should always retain its original name, given at the time of isolation, in the stock history (Anon., 1978).

Despite the high degree of polymorphism seen in the enzymes of T.congolense, isoenzyme electrophoresis divided the species into two major groups. Some behavioural and morphological characteristics of these two zymodemes were investigated and are reported in Section B.

5.5. Conclusions

Computer analysis of 71 isoenzymes found among 78 stocks of T.congolense divided the stocks into two groups corresponding to those found by direct examination of GPI, PEP 1 and PEP 2 zymograms. This major dichotomy could be correlated with the different types of vegetation in the area of origin of the stocks.

CHAPTER 6

6. BOVINE TRYPANOSOMIASIS IN THE GAMBIA

6.1. Introduction

The Gambia consists of an area of 11,162 km² covered mainly by savannah open woodlands, grazed by a cattle population of between 300,000 and 500,000 (Clifford and Sanyang, in press; McIntyre et al., in press). The cattle are generally considered to be N'dama, but most are of mixed breed with Zebu characteristics clearly visible from the dewlaps, long legs and the occasional hump. The N'dama (Hamitic or Fouta Longhorn) are a breed of cattle originating from the Fouta Djallon plateau in French Guinea, which has been imported into many West African countries because of its 'trypanotolerance' or resistance to the effects of infection with African pathogenic trypanosomes. Zebu cattle are preferred by African pastoralists because of their larger size, higher milk yields and better herd instincts (MacLennan, 1970a). Therefore, interbreeding of N'dama with Zebu cattle has been practised to produce animals with some of the better Zebu qualities but at the expense of reduced trypanotolerance (Chandler, 1952).

Despite a reputed resistance, many of the Gambian cattle display symptoms of trypanosomiasis including stunting, wasting, infertility, abortion and persistent low grade anaemias (McIntyre et al., in press).

In 1977, a visit was made to The Gambia primarily to

isolate new stocks of T. congolense for isoenzyme characterization, but also to make an epidemiological study of this trypanosome in the cattle, in an attempt to find correlations with the zymodemes found.

6.2. Materials and Methods

6.2.1. Collection of blood samples

Herds of N'dama X Zebu cattle were sampled throughout the length of The Gambia, on both the north and south banks of the river (Fig. 6.1). During the dry season, the cattle roam, often unattended, but at night they are tethered on the outskirts of the villages (Plate 6.1). For convenience, the herds were visited soon after sunrise before their release.

From each herd, which contained about 30 to 80 cattle, 25 individuals were selected as a representative cross section regarding age, sex and condition. Each animal was restrained by placing one foreleg over its neck (Plate 6.2), before bleeding from the jugular vein using a Vacutainer (Plate 6.3). Approximately 7ml of blood was taken into 70i.u of heparin as an anticoagulant and the samples, whenever possible, were placed on ice in an insulated cold box. The age, sex and condition of each animal was recorded and the species of tsetse fly present in the area was identified where possible or assessed according to the vegetation.

584 blood samples were obtained, including samples from an entire herd of 77 cattle at Keneba, bled twice at a one-month interval. 47 additional trypanosome infected blood samples were collected with the help of Mr. D.J. Clifford, a Veterinary Officer, during routine examinations of village cattle. Two horses and two

Key to trypanosome collection sites in The Gambia (Fig. 6.1)

1. Essau
2. Yallol
3. Fuga
4. Yorobawol
5. Tamba San Sang
6. Mansajang Kunda
7. Sololo Fula
8. Sololo Mandinka
9. Willingara Illo
10. Fula Kunda
11. Madina
12. Dutabulu
13. Kantong Kunda
14. Keneba
15. Juoli
16. Lamin

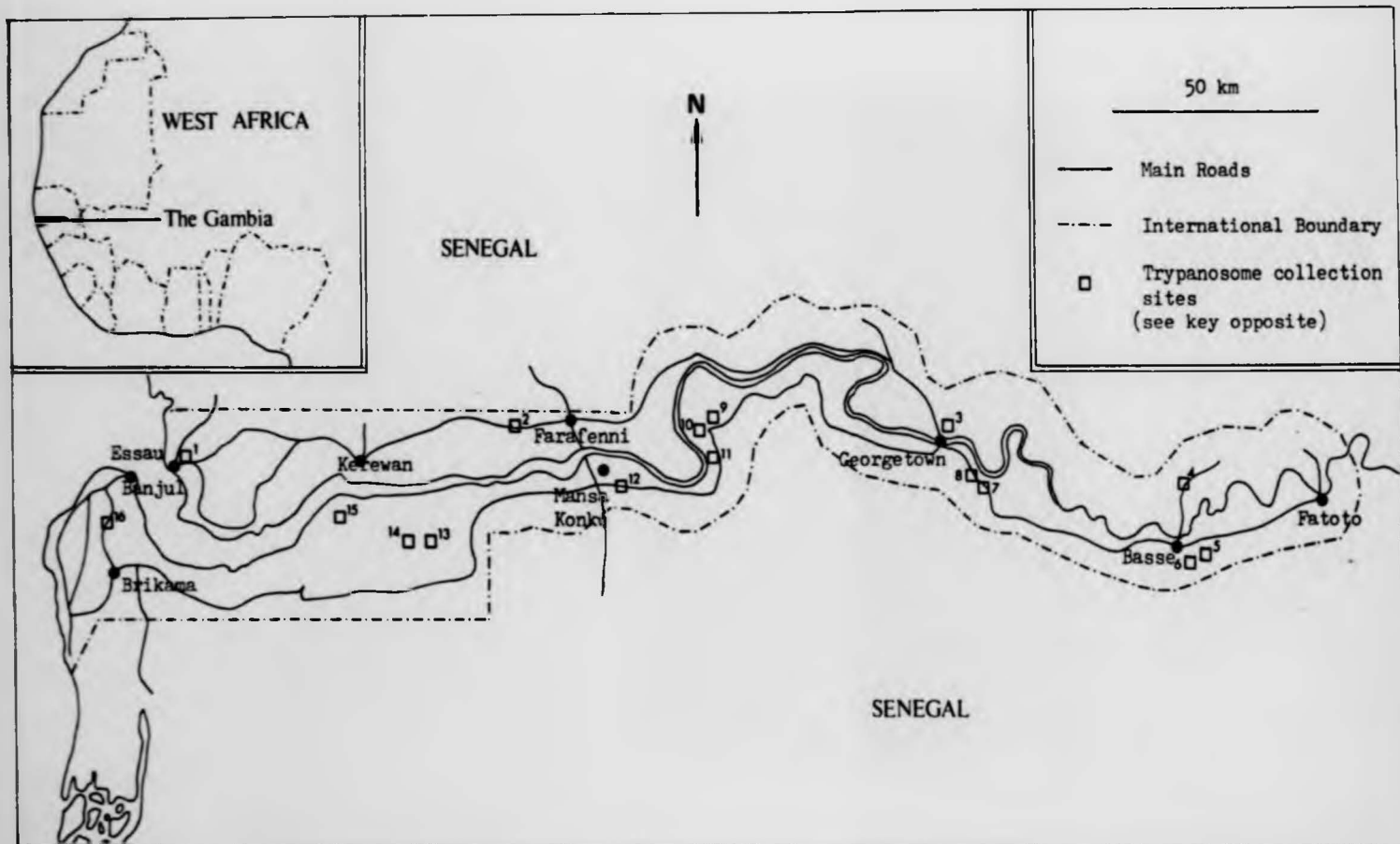


Fig. 6.1. Trypanosome isolation sites in The Gambia

Plate 6.1. Herd of N'dama x Zebu cattle tethered on the outskirts of a Gambian village



Plate 6.1.

Plate 6.2. Restrained Gambian cow being bled from the jugular vein

Plate 6.3. Bleeding a Gambian cow from the jugular vein using a Vacutainer



Plate 6.2.



Plate 6.3.

donkeys were also bled with Vacutainers after being hobbled and seven pigs were bled with syringes from the jugular, making a total of 642 samples.

6.2.2. Examination of blood samples

Batches of samples were taken to the nearest electricity source, up to two hours away, and examined by modification of the Woo haematocrit technique (Woo, 1969). After centrifugation of blood-filled capillaries and evaluation of the packed cell volumes (PCV's), the buffy coat and underlying red cells were examined on a microscope slide by dark ground microscopy for the presence of trypanosomes (Murray *et al.*, 1977). Even after a two hour journey the trypanosomes were still active.

6.2.3. Isolation of trypanosome stocks

0.5 ml of each blood sample was inoculated i.p. into a mouse, or when unavailable, a rat. The mice were checked at least once a week for the presence of trypanosomes in a wet blood film (Section 2.5.2). When the parasitaemia reached about 10^6 trypanosomes ml^{-1} blood, the stocks were cryopreserved in liquid nitrogen as described in Section 2.5.3, but the cooling rate of 1°C min^{-1} was achieved by placing the capillaries in a polystyrene box fitted with a metal conductor rod in a -80°C deep freeze. Nine samples which had only displayed transient or low grade parasitaemias were also stabilised.

6.2.4. Electrophoresis

In London, the stabilates were inoculated into mice, then rats and trypanosome lysates were prepared (Section 2.6). Enzyme

electrophoresis was carried out as described in Section 3.2.

6.3. Results

33 stabilates of T.congolense were prepared. 10 stabilates, including 9 made from chronic or transient infections, failed to reinfect mice. The histories of these 23 samples are shown in Appendix II.

The incidence of infection varied from 0 to 42.2% in different villages (Table 6.1). The herd at Keneba was bled on three different dates; at the first visit the herd consisted of 57 cattle. At the second visit, one month later, 56 of these cattle were still alive and were bled; in addition two calves had been born and 10 Zebu and 9 pure N'dama had been introduced into the herd. The third visit, again one month later, was to bleed only the 19 newly purchased Zebu and N'dama.

The highest numbers of infections were found in cattle eight to nine years old (Fig. 6.2) with PCV's in the 16 to 20 range (Fig. 6.3). The PCV's of all the cattle are shown in Fig. 6.4. The Keneba cattle are not included in Figs. 6.2 and 6.3 since they were an experimental herd (Section 6.4).

The majority of herds sampled were under G.morsitans challenge. Only one herd, at Lamin, was in an area frequented solely by G.palpalis (D.J. Clifford, personal communication). Willingara Illo, Fula Kunda, Mansajang Kunda and Tamba San Sang all had both G.morsitans and G.palpalis in or near the village.

Out of 62 T.congolense infections detected by haematocrit centrifugation and dark ground microscopy, 26 became parasitaemic in mice or rats; four were T.brucei and 22, T.congolense of which

Table 6.1. Percentage of cattle infected with trypanosomes in villages throughout The Gambia

Village	No. of cattle bled	% of cattle with trypanosomiasis				
		<u>T. congolense</u>	<u>T. vivax</u>	<u>T. brucei</u>	<u>Mixed</u>	<u>Total</u>
Lamin	40	0.0	0.0	2.5	0.0	2.5
Kantong Kunda	21	4.8	4.8	9.6	0.0	19.2
Willingara Illo	30	3.3	0.0	0.0	0.0	3.3
Fula Kunda	27	0.0	0.0	0.0	0.0	0.0
Madina	22	0.0	0.0	4.5	0.0	4.5
Sololo Fula	25	0.0	4.0	0.0	8.0	12.0
Sololo Mandinka	31	0.0	3.2	0.0	3.2	6.4
Fuga	30	6.6	3.3	0.0	0.0	9.9
Manajang Kunda	30	3.3	3.3	3.3	3.3	13.2
Yorobawol	21	0.0	0.0	0.0	0.0	0.0
Tamba San Sang	32	0.0	0.0	3.1	3.1	6.2
Essau	48	16.7	2.1	0.0	0.0	18.8
Yallol	30	0.0	0.0	0.0	0.0	0.0
Dutabulu	30	16.7	0.0	0.0	0.0	16.7
Jouli	14	7.1	7.1	7.1	0.0	21.3
Keneba 1*	57	21.1	3.5	0.0	0.0	24.6
Keneba 2*	77	10.4	10.4	1.3	0.0	22.1
Keneba 3*	19	0.0	21.1	21.1	0.0	42.2

*same herd visited on three occasions (see Section 6.3).

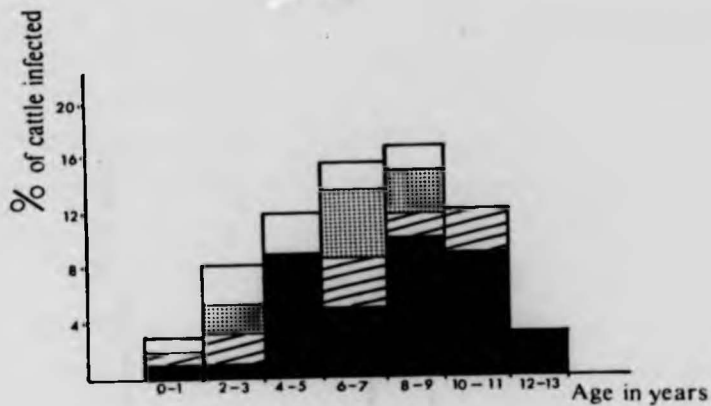


Fig. 6.2. Percentage of trypanosome infected cattle in each age group

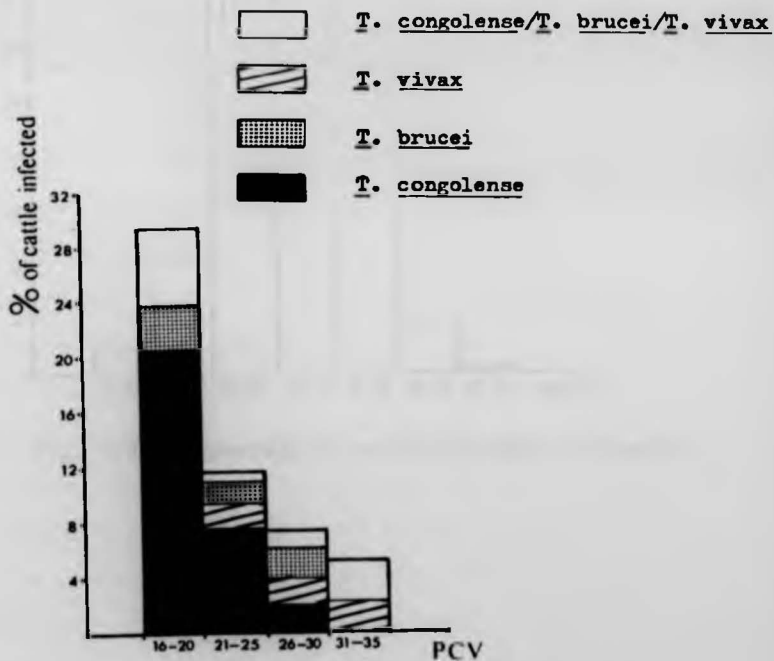


Fig. 6.3. Percentage of trypanosome infected cattle in each PCV group

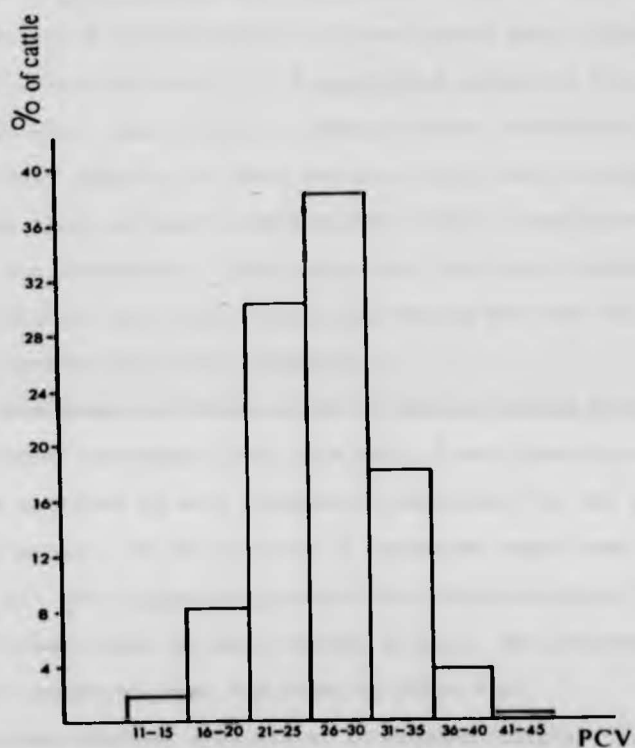


Fig. 6.4. Percentage of cattle in each PCV group

six showed only transient infections. A further 13 rodents became parasitaemic with T.congolense after inoculation of cattle blood containing undetected trypanosomes, but three showed only transient parasitaemias. Blood infected with T.congolense collected from a horse, a donkey and a pig, failed to produce patent infections in mice. It was only possible to check the mice about once a week as the rest of the time was spent sampling other herds, usually some distance from the laboratory. This meant that some mice displaying parasitaemias too low for stabilating, died during the week while they remained unattended at the laboratory.

26 zymodemes were found in the 27 Gambian stocks finally examined for their isoenzymes (Figs. 5.4 and 5.5 and Appendices III to V); Gamb 28 and Gamb 29 were enzymically identical for the 14 enzymes investigated. Of the possible 71 isoenzyme bands seen for 14 enzymes in all the T.congolense stocks from Africa examined in this study, 42 were found in these Gambian stocks. The percentage frequencies of enzyme patterns are shown in Table 6.2.

From the computer analysis of 14 enzymes (Chapter 5), all the Gambian stocks gave 'savannah' patterns. However, when only 10 enzymes were examined seven of these stocks were grouped, inexplicably, in the riverine/forest branch. On the dendrograms (Figs. 5.4 and 5.5), these stocks are represented by numbers 1 to 27. Using 14 enzymes, 1 to 8 formed a distinct group, as did 15 to 27 and 10 to 12. 1 to 8 all originated from Keneba but no such correlation was found in the other groups. Zymodemes 9, 13 and 14 each showed a greater similarity to stocks from different parts of Africa than to other Gambian stocks (Fig. 5.4).

No correlations could be found between these minor

Table 6.2. Percentage frequency of each enzyme pattern in the 26 zymodemes found in The Gambia

Enzyme	Pattern Number																	
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII
NH	100.0																	
PK	100.0																	
TDH	100.0																	
GAPDH	76.9	23.1																
ASAT	30.8	38.5	26.9	3.8														
PGM	69.2	30.8	0.0	0.0														
ALAT	0.0	38.5	38.5	0.0	23.0													
ME	3.8	50.0	11.6	30.8	3.8													
MDH	76.9	0.0	0.0	11.6	3.8	7.7												
GPI	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0								
PEP 2	53.8	0.0	11.6	19.2	0.0	3.8	11.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0				
PEP 1	38.4	11.6	0.0	34.6	3.8	0.0	11.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

*The limits of the figures (including zero) show the numbers of patterns found for a particular enzyme throughout this whole study.

zymodeme groupings and the values in the cattle for PCV, parasitaemia, condition and age or for the infectivity of the trypanosomes to mice; the infectivity was not measured quantitatively.

6.4. Discussion

The main purpose of the visit to The Gambia was to isolate stocks of T.congolense for isoenzyme characterization but some of the incidental information collected during the survey produced interesting results.

8.9% of the cattle surveyed were found to have trypanosomiasis, 43.8% of which were attributed to T.congolense (Table 6.1). Only the infections which produced patent parasitaemias in rodents were identified by Giemsa-stained thin blood films. Those identified by dark ground microscopy after haematocrit centrifugation were distinguished by their movements; T.vivax moves rapidly across the field of vision, T.brucei translocates at a slower rate than T.vivax. T.congolense is sluggish and rarely leaves the field of vision. Although the microscopists were confident of their identifications, this method cannot be considered totally reliable especially when mixed infections occur. Therefore, the percentages of T.vivax, T.brucei and T.congolense reported should only be considered as approximations.

The percentage of infected cattle is probably far higher than 8.9%, due to individuals harbouring subpatent infections which failed to infect rodents. Indeed, McIntyre et al. (in press) estimated that 50% of the anaemias seen in these cattle were caused by trypanosomiasis. The average PCV of N'dama and Zebu cattle is about 34 (Dargie et al., 1979) and in this survey 77.4% of the cattle had PCV's below

31 and 39.3% below 26 (Fig. 6.3), so if 50% of these cattle were harbouring trypanosomes the infection rate was between 19.7 and 39.3%, rather than 8.9%.

Anaemia has long since been recognized as a major symptom of bovine trypanosomiasis (Hornby, 1921) and, as expected, the highest percentage infection rates were found with the lowest PCV values (Fig. 6.3).

The steady increase in the percentage of infected cattle as the animals aged, up to eight to nine years (Fig. 6.2), was probably due to their receiving a greater number of bites by infected tsetse flies. Although the calves suckle for up to two years (Rains, 1975) it is unlikely that they receive much protection after colostrum production ceases i.e. only for one week after birth. It is suggested that the decrease in the percentage infected from 10 to 13 years may be due to these cattle having acquired a high degree of immunity from repeated light infections allowing these older cattle to eliminate or reduce subsequent infections. The inaccuracies of ageing the cattle must be borne in mind. A reasonably accurate estimate could be made up to five years old from the number of teeth, but after this age, the state of deterioration of the teeth and the advice of the herdsmen were combined to give an approximate age.

The percentages of cattle infected and the comparisons of age and PCV do not include the results obtained from Keneba. This was an experimental herd maintained in the same way as the village herds but 14 of the cattle were pure Zebu and about the same number were pure N'dama. These pure bred cattle had been brought from Senegal, beyond the northern limit of the tsetse fly belt or from

a tsetse free area in the west of The Gambia. Some of the N'dama x Zebu had also been brought, as calves, from other areas of The Gambia (D.J. Clifford, personal communication). Even the pure N'damas could be highly susceptible to trypanosome stocks circulating at Keneba, as it is believed that although they possess an intrinsic resistance to trypanosomiasis it must be augmented by previous infection of the cattle or their dams (Roberts and Gray, 1973). If the animals have never received a trypanosome challenge or are infected with different 'strains', and if the challenge is high, the animals may succumb to the infection (MacLennan, 1970a); it is not known how antigenically distinct a stock must be for the animal's immunity to fail. Even if the results, obtained in this survey, of these imported cattle were excluded and only those originating at Keneba considered, the high parasitaemias in the introduced cattle could cause an increase in the chance of transmission among the herd, both cyclically by tsetse flies and mechanically by tsetse or other haematophagous insects.

The highest infection rates were found at Keneba (Table 6.1), followed by Juoli and Kantong Kunda, two villages both within 5 km of Keneba. Possibly the infection rates in the tsetse flies were particularly high in these areas from the herd at Keneba acting as the source of infected flies. However, infection rates at Essau and Databulu were of similar magnitude and no explanation could be found for this or the other differences in infection rates found among the herds.

Over half of the trypanosome infections detected in cattle blood failed to infect mice and rats. Differences in responses of rodents to inoculation of T. congolense stocks have been recognized

for many years (e.g. Blacklock and Yorke, 1913, see Section 9.1) and the susceptibility of individual animals may vary (Browning et al., 1934; Binns, 1938; Jennings et al., 1978). Recent work has shown that certain strains of inbred mice are more susceptible to T.congolense than others (Morrison et al., 1978; Jennings et al., 1978) but, as described in Chapter 9, susceptibility may be dependent on the nature of the infecting stock. Inoculation of stocks, producing transient parasitaemias, into different strains of mice may facilitate isolation but some strains of mice may not be suitable for isolating certain zymodemes. Therefore, isolation of stocks by inoculation into a single strain of rodent must remain a highly selective process.

The isoenzyme patterns of stocks of T.congolense from The Gambia have already been discussed with respect to stocks from other parts of Africa (Section 5.4). Considering the stocks from The Gambia alone, no correlation could be found between the enzyme patterns and the different herds, degrees of anaemia or parasitaemia in the cattle. The 100% similarity of Gamb 28 and Gamb 29 isolated from the same herd was not surprising, indeed, it was the great diversity among other stocks which was unexpected. The enzymic distinction of stocks Gamb 6 and Gamb 26, isolated from the same cow one month apart, could be due to the co-existence of two stocks, reaching peak parasitaemias at different times or reinfection by a new stock which became dominant during that month.

Comparing the frequencies of enzyme patterns found in The Gambia (Table 6.2) with those seen in a total of 75 zymodemes for T.congolense (Table 4.2), all the known patterns were seen in GAPDE, ASAT and ME and of course in the three enzymes NH, PK and

TDH which have not so far been found to be polymorphic in T.congolense. Of the remaining six enzymes 10 to 67% of the total number of patterns were seen in these Gambian stocks. The most striking uniformity occurred in GPI where all the stocks gave pattern I out of a total of ten patterns found among T.congolense stocks from outside The Gambia. A greater homogeneity was also found for PEP 2, only 5 of a possible 18 patterns being represented in The Gambia.

All the stocks gave 'savannah' patterns for GPI, PEP 1 and PEP 2 (Section 5.4) and appeared in the savannah branch of the dendrogram when all 14 enzymes were considered (Fig. 5.4). However, seven Gambian stocks were found, inexplicably, grouped together in the riverine/forest branch of the dendrogram when only 10 enzymes were considered (Fig. 5.5).

Although The Gambia is primarily covered in savannah woodland, the habitat of G.morsitans, mangroves and oil palms abound along the river banks and bolons (creeks), and are the haunt of G.palpalis. G.longipalpis has also been reported from a strictly localized area near the Casamance border (Hutchinson, 1953). The majority of herds examined were located in areas where only G.morsitans is found and these cattle did not enter areas frequented by G.palpalis, as they were either too far from the river to be driven there for watering or the water is too saline for drinking up to 280 km inland. The Lamin herd was believed to be solely under G.palpalis challenge, and four herds at Willingara Illo, Fula Kunda, Mansajang Kunda and Tamba San Sang were within 1.5 km of the River Gambia or swamps where G.palpalis are known to exist. Cattle from these four herds were driven to the river to drink and would undoubtedly have come under G.palpalis challenge as well as G.morsitans. Only one stock was

isolated from any G.palpalis area, from Willingara Illo, and this too gave savannah patterns. The possibility of the riverine/forest zymodeme being present cannot be excluded and more stocks from G.palpalis areas should be examined enzymically. From the results obtained during this survey, it appears that the majority, if not all, of the T.congolense stocks found in The Gambia belong to the 'savannah' zymodeme and as there is evidence to suggest that G.palpalis may be a poor vector of this zymodeme (Chapter 7); G.morsitans is probably the major vector of T.congolense in The Gambia.

6.5. Conclusions

At least 8.9% of the N'dama x Zebu cattle examined in The Gambia were infected with trypanosomiasis and 43.8% of the infections were attributed to T.congolense. The highest infection rates were found in cattle between eight and nine years old and in those with low PCV's. 50% of the detected T.congolense infections failed to infect laboratory rodents, making the isolation of stocks by rodent inoculation a highly selective process. 26 zymodemes were found in 27 stocks, each displaying savannah enzyme characteristics. No correlations of epidemiological data with enzyme variation was seen with these Gambian stocks.

SECTION B

**Behavioural and Morphological Characteristics
of Two Major Zymodemes**

CHAPTER 7

7. TSETSE FLY TRANSMISSION

7.1. Introduction

T. (Nannomonas) congolense is capable of developing in many species of Glossina. The majority of vectors are in the morsitans-group, but members of the palpalis- and fusca- groups have also been incriminated (see Hoare, 1972).

Development of Nannomonas trypanosomes takes place in the midgut and proboscis and complete development of T. congolense in a number of species including G. morsitans, G. palpalis, G. fuscipes, G. brevipalpis and G. tachinoides has been described (Duke, 1912; Fraser and Duke, 1912; Robertson, 1913; Bruce et al., 1914a, 1914b; Lloyd and Johnson, 1924; Roubaud, 1935). When the tsetse fly takes a bloodmeal, trypanosomes are ingested and development commences in the endoperitrophic space of the midgut, where they transform to elongated trypomastigotes. The flagellates quickly multiply, and it has been generally accepted, though unproven, that they escaped via the open end of the peritrophic membrane into the ectoperitrophic space. Recently, however, Evans et al. (1979) have shown that they reach the ectoperitrophic space by direct penetration of the membrane. They migrate forwards, again penetrating the peritrophic membrane, into the endoperitrophic space of the cardia (= proventriculus); during their sojourn in the cardia, the trypomastigotes elongate to proventricular forms. The forward progression continues up the

oesophagus to the proboscis where the flagellates attach to the labrum walls and transform into epimastigotes. Finally, the epimastigotes migrate to the hypopharynx and develop into infective metatrypanosomes. This entire developmental cycle takes from 19 to 53 days (Bruce et al., 1914b); B.J. Elce (personal communication) investigating the transmission of a few trypanosome stocks found that each stock had a consistently different length of developmental cycle if the tsetse flies were maintained under constant conditions.

The following work was carried out to investigate the relationship between Glossina spp. and two major zymodemes of T. congolense (Section 4.4). These two zymodemes originated from different vegetational regions, namely savannah (S) and riverine or forest (RF) areas. As the species of Glossina differs with the vegetation, the natural vector of these two major zymodemes must also differ and it was thought that a vector specificity or preference might exist. Comparisons were made on the length of development cycle, and the infectivity of these two zymodemes to tsetse flies; flies from both the morsitans- and palpalis- groups were investigated but fusca- group tsetse flies were unobtainable. The electrophoretic patterns of stocks after laboratory transmission by tsetse flies were also examined.

7.2. Length of Developmental Cycle

7.2.1. Materials and methods

Transmission of T. congolense stocks, representing the savannah and riverine/forest zymodemes, were attempted through five species of tsetse fly; G. morsitans morsitans, G. austeni, G. tachinoides,

G.fuscipes fuscipes and G.palpalis gambiensis (Table 7.1).

The basic techniques for the routine maintenance of the flies are described in Section 2.4.2. Within 48 hours of eclosing, the flies were offered a bloodmeal from a sedated mouse with a high, rising parasitaemia of trypanosomes. At this stage of the infection, large numbers of trypomastigotes were present and it has been shown, with the subgenus Trypanozoon, that at this time, they are at their most infective (Robertson, 1912; Wijers and Willett, 1960). The numbers of fed and unfed flies were noted; the seemingly unfed flies were not removed as an infection could be established from a very small undetectable meal. The flies were subsequently fed three times a week for one month; a different mouse was used for each cage and feed. After one month, few flies were still alive and the majority of infections should have been mature (Nantulya et al., 1978a). Each mouse was checked weekly, for one month after being bitten, for the presence of trypanosomes by examination of a wet blood film (Section 2.5.2). Parasitaemias were monitored until they reached a suitable level for stabilating (Section 2.5.3).

Initially, no flies were dissected, but four G.morsitans originally fed on TSW 99/77-infected blood were dissected 30 days after the initial feed and examined as described in Section 7.3.1.

7.2.2. Results

The times taken for the development to infective metatrypanosomes varied from 11 to 17 days (Table 7.1). Three out of six of the attempted transmissions of savannah stocks (S) through G.morsitans were successful compared with three out of ten riverine/forest (RF) stocks (Table 7.1).

Table 7.1. Transmission of T.congolense through Glossina spp.

Species of tsetse fly	Stock	No. offered meal	No.fed	Length of cycle in days
<u>Savannah</u>				
<u>G.morsitans</u>	Gamb 19	10	6	11
<u>G.morsitans</u>	Gamb 21	22	16	-
<u>G.morsitans</u>	Gamb 10	20	15	-
<u>G.morsitans</u>	Gamb 6	40	25	14
<u>G.austeni</u>	Gamb 6	40	28	16
<u>G.morsitans</u>	IBADAN 44	20	15	-
<u>G.morsitans</u>	EATRO 2033	40	30	-
<u>G.austeni</u>	EATRO 2033	20	18	-
<u>Riverine/Forest</u>				
<u>G.morsitans</u>	TSW 94/77	60	49	17
<u>G.morsitans</u>	TSW 4/77	20	16	-
<u>G.morsitans</u>	TSW 6/77	40	36	-
<u>G.morsitans</u>	TSW 103/77b	20	13	-
<u>G.morsitans</u>	TD 28/78E	40	18	-
<u>G.morsitans</u>	TSW 25/78E	20	10	11
<u>G.morsitans</u>	TD 56/78E	25	13	11
<u>G.morsitans</u>	TSW 152/78E	20	15	-
<u>G.morsitans</u>	TSW 3/78E	20	13	-
<u>G.morsitans</u>	TSW 99/77	104	99	-
<u>G.tachinoides</u>	TSW 99/77	83	79	-
<u>G.palpalis</u>	TSW 99/77	65	46	-
<u>G.fuscipes</u>	TSW 99/77	66	6	-

A high mortality rate occurred in G. tachinoides, G. fuscipes and G. palpalis after the infected bloodmeal containing TSW 99/77 (RF); 8 out of 79 G. tachinoides survived to the second feed and only 12 of the 46 G. palpalis and 1 of the 6 G. fuscipes survived for more than 10 days. Greater success was achieved with G. morsitans and 4 out of 99 survived to day 30. On dissecting these four G. morsitans, one had a patchy midgut infection, a few parasites in the pharynx and five in the hypopharynx. No infection resulted in mice fed on by this fly despite the virulence of the stock after direct blood inoculation (Chapter 9).

7.3. Infection Rates

7.3.1. Materials and methods

Mice infected with Gamb 6 (S), originating from a savannah region or TSW 99/77 (RF), from a forest region were offered as the initial bloodmeal to newly eclosed G. morsitans. The flies were maintained as described in Sections 2.4.2 and 7.2.1, except that any flies which did not take a detectable first meal were discarded. The flies were fed for 16 days. By day 18 when 76% of the infected flies should have had mature infections (Nantulya et al., 1978a), only 14 of the 41 flies fed with Gamb 6 (S) were still alive and 12 of the 49 fed with TSW 99/77 (RF).

These 26 flies were sexed, the head removed with the salivary glands attached and the complete gut dissected out into PSG. The entire gut and salivary glands were examined by phase contrast microscopy and the position of trypanosomes noted. The pharynx and mouth parts were removed from the rest of the head and similarly examined. Giemsa-stained smears were made of any infections

found, as described in Section 2.5.5, but the staining time was reduced to 30 minutes.

7.3.2. Results

The results are summarized in Table 7.2. The infections were scored on a system of plusses, + for a light infection to ++++ for a heavy infection. 21.4% of the dissected flies originally fed on Gamb 6 (S) were infected and 8.3% of those fed on TSW 99/77 (RF). Heavy head infections were produced by Gamb 6 (S) in three flies and these were accompanied by massive midgut and cardia infections, especially in fly no. 1 (Table 7.2), whose cardia contained a solid mass of writhing trypanosomes. The trypanosomes at the posterior end of the hypopharynx were found singly or in small groups, either attached to the wall or free in the lumen (Plate 7.2). Unlike the flexible forms seen at the posterior end of the hypopharynx, the trypanosomes at the anterior end appeared to be more rigid with limited movement and congregated in large clumps attached to the hypopharynx walls (Plates 7.3 and 7.4).

In contrast to the heavy infections of Gamb 6 (S), TSW 99/77 (RF) produced a light infection in one fly no. 22 (Table 7.2) and only in the head. Five flexible trypanosomes were seen in the hypopharynx, the biggest clump consisted of three (Plate 7.1). As expected no trypanosomes were seen in the salivary glands of any of these 26 tsetse flies.

Giemsa-stained smears of trypanosomes from the heads and guts of Gamb 6 (S)-infected flies showed a wide variety of forms including trypomastigotes, epimastigotes and metatrypanosomes (Plates 7.5 to 7.14).

Table 7.2. Summary of trypanosome infections in G.morsitans

No.	Infecting Stock	Sex M or F	Gut		Head		Other Notes
			Mid	Cardia	Pharynx	Hypo- Pharynx	
<u>Savannah</u>							
1	Gamb 6	F	++++	++++	++++	++++	clumping in hypopharynx
2	Gamb 6	M	-	-	-	-	
3	Gamb 6	M	-	-	-	-	
4	Gamb 6	F	++	++	++	++	bacterial infection in gut; clumping in hypopharynx
*							
5	Gamb 6	F	-	-	-	-	
6	Gamb 6	M	+++	+++	++	++	clumping in hypopharynx
7	Gamb 6	M	-	-	-	-	
8	Gamb 6	M	-	-	-	-	
9	Gamb 6	F	-	-	-	-	
10	Gamb 6	M	-	-	-	-	
11	Gamb 6	M	-	-	-	-	
12	Gamb 6	F	-	-	-	-	
13	Gamb 6	F	-	-	-	-	
14	Gamb 6	F	-	-	-	-	
<u>Riverine/Forest</u>							
15	TSW 99/77	M	-	-	-	-	
16	TSW 99/77	F	-	-	-	-	gravid
17	TSW 99/77	F	-	-	-	-	
18	TSW 99/77	M	-	-	-	-	
19	TSW 99/77	M	-	-	-	-	
20	TSW 99/77	F	-	-	-	-	
21	TSW 99/77	F	-	-	-	-	
22	TSW 99/77	F	-	-	+	+	very scanty head infection
23	TSW 99/77	F	-	-	-	-	
24	TSW 99/77	M	-	-	-	-	
25	TSW 99/77	F	-	-	-	-	
26	TSW 99/77	F	-	-	-	-	

*Horizontal lines indicate separate cages

Plates 7.1 to 7.4. Phase contrast micrographs of T.congolense
in the hypopharynx of G.morsitans.
(Scale = 40µm)

Plate 7.1. One T.congolense, TSW 99/77 (RF), trypanosome in
the hypopharynx of a G.morsitans

Plate 7.2. Posterior end of hypopharynx of G.morsitans
infected with T.congolense, Gamb 6 (S)

Plates 7.3 and 7.4. Clumps of T.congolense, Gamb 6 (S), in
the anterior end of the hypopharynx of
G.morsitans

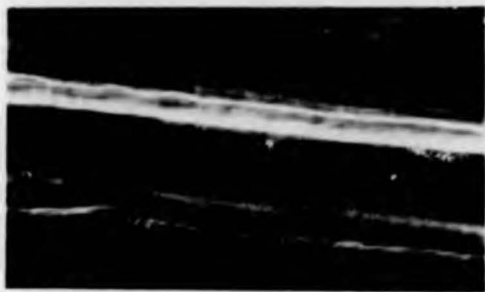


Plate 71.

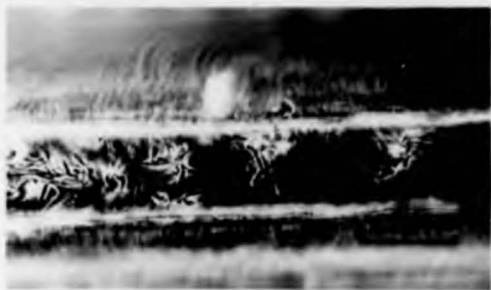


Plate 72.

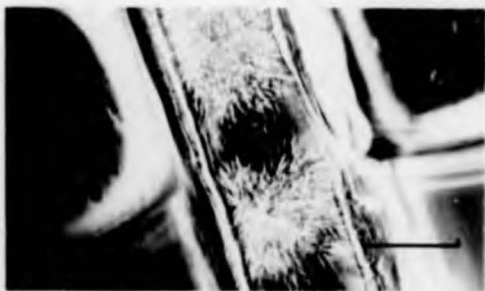


Plate 73.

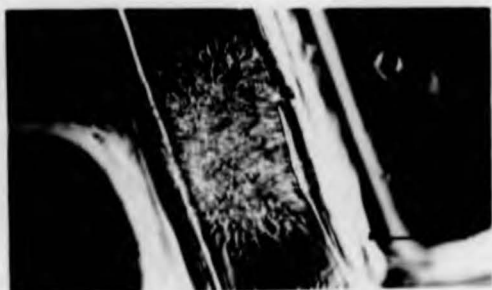


Plate 7.4.

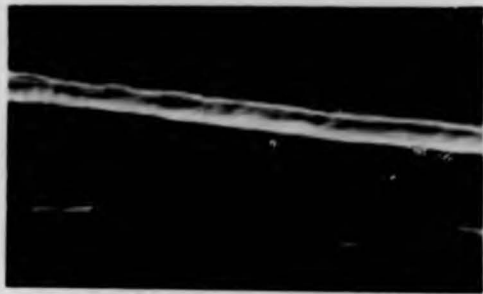


Plate 71.



Plate 72.

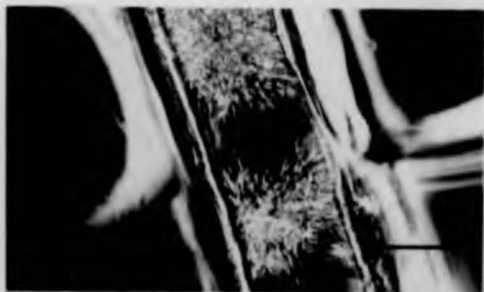


Plate 73.

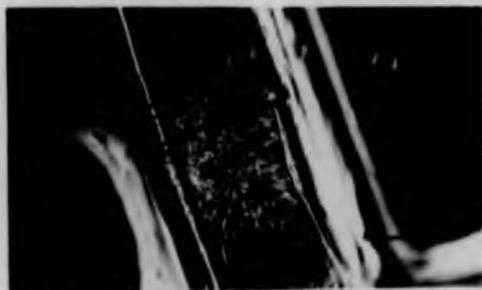


Plate 7.4.

Plates 7.5 to 7.9. Giemsa-stained smears of T. congolense,
Gamb 6 (S) in G. morsitans. (Scale = 20 μ m)

Plates 7.5 and 7.6. Trypomastigotes from the midgut

Plate 7.7. Slender proventricular trypomastigote and
bacterial infection from the cardia

Plate 7.8. Slender proventricular trypomastigote
from the cardia

Plate 7.9. Slender proventricular trypomastigote from
the cardia and epimastigote from the food
canal

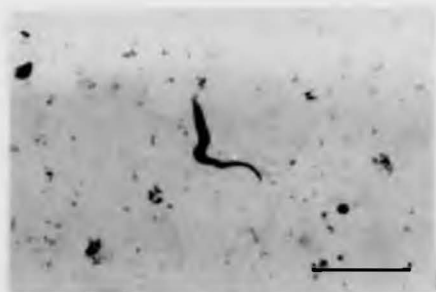


Plate 7.5.

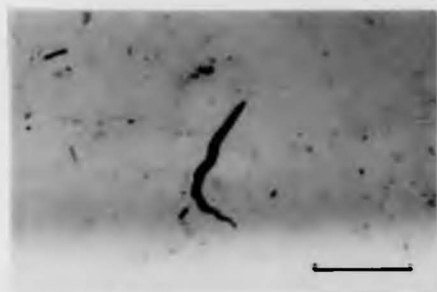


Plate 7.6.



Plate 7.7.

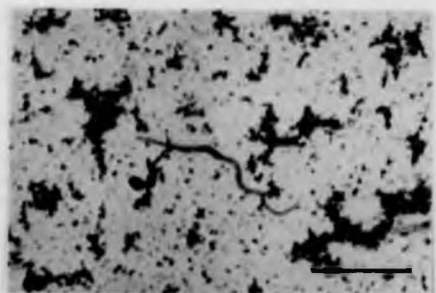


Plate 7.8.

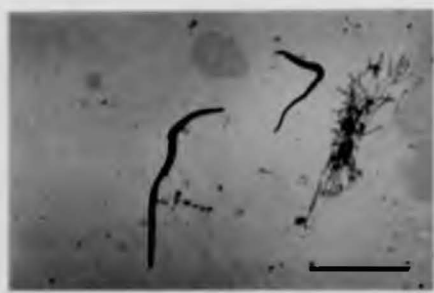


Plate 7.9.



Plate 7.5.

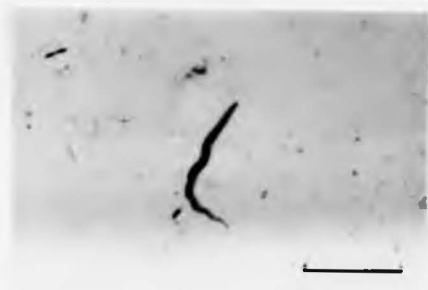


Plate 7.6.



Plate 7.7.

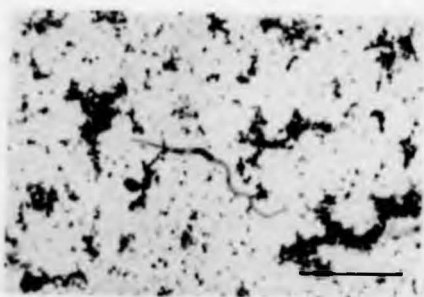


Plate 7.8.

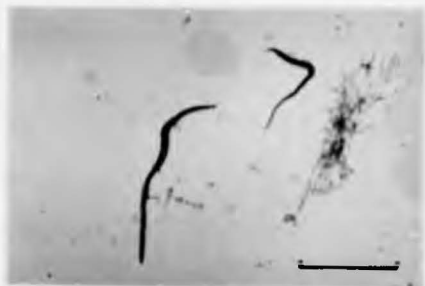


Plate 7.9.

Plates 7.10 to 7.14. Giemsa-stained smears of T. congolense.
Camb 6 (S) in G. morsitans.
(Scale = 20 μ m)

Plates 7.10 - 7.13. Epimastigotes from the food canal

Plate 7.14. Metatrypanosome from the hypopharynx



Plate 7.10.



Plate 7.11.



Plate 7.12.



Plate 7.13.

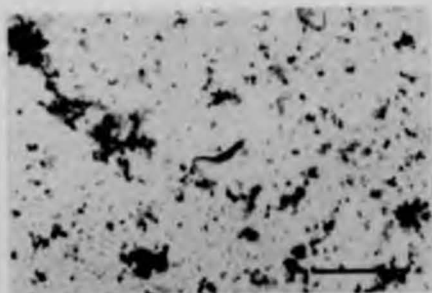


Plate 7.14.

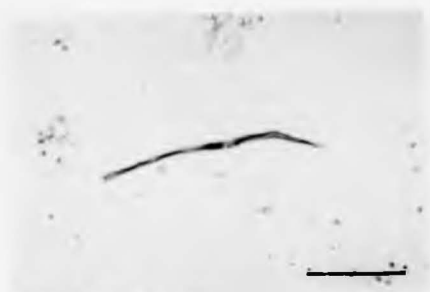


Plate 7.10.



Plate 7.11.



Plate 7.12.



Plate 7.13.

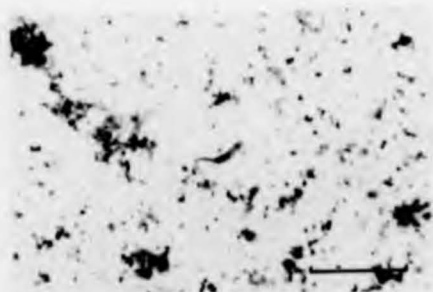


Plate 7.14.

No stained trypanosomes were obtained from the head of fly no. 22, the only fly infected with TSW 99/77 (RF) during this experiment. The few that were successfully separated from the head were lost from the slide during fixation and staining, but some were observed in Giemsa-stained films made from a G.morsitans with a 30 day infection of the same stock in a previous experiment (Section 7.2.1) (Plates 7.15 and 7.16). Unlike the Gamb 6 (S) infection, no long slender proventricular forms were found and only one epimastigote was seen. About 50% of the TSW 99/77 (RF) were vacuolated and of unhealthy appearance.

7.4. Discussion

From the initial experiments on transmission of different stocks of T.congolense through G.morsitans, this species of Glossina appeared to be a better vector of stocks originating from savannah regions (50% transmitted) than those from riverine or forest areas (30% transmitted).

The length of complete development of different stocks of T.congolense varied from 11 to 27 days with no clear demarcation between savannah or riverine/forest stocks. It has been suggested that a stock completes its cycle in the tsetse fly in a constant number of days when maintained under constant conditions (B.J. Elce, personal communication), but Nantulya et al. (1978a) found that with three stocks of T.congolense, development proceeded at different rates in individual flies. Therefore, the times reported in Table 7.1 are the shortest cycle length for each stock. Bruce et al. (1914b) reported that cyclical transmission could not occur until day 19 but Nantulya et al. (1978a) achieved transmission 7 days after the

Plates 7.15 and 7.16. Giemsa-stained smears of T. congolense,
TSW 99/77 (RF), in G. morsitans
(Scale = 20 μ m). Vacuolated trypano-
mastigotes from the midgut.



Plate 7.15.



Plate 7.16.

infective feed and by day 12, 45% of the flies had mature infections and 76% by day 18. The shorter cycle lengths found in this study and by Nantulya et al. (1978a) may be due to different stocks, dissimilar hosts providing the bloodmeals, incubation of the puparia at varying temperatures or differences in the tsetse fly populations.

Attempts to transmit the riverine/forest stock, TSW 99/77 (RF), through different species of tsetse flies from the morsitans- and palpalis- groups, were prevented by the high mortality of the flies. Only a few G.fuscipes could be induced to feed on mice suggesting a strong host preference. A characteristic of most riverine tsetse flies is their adaptation to feed on any potential host which strays into their habitat, but G.fuscipes is thought to be more host specific, feeding largely on aquatic reptiles (Weitz, 1970). Although G.palpalis and G.tachinoides fed readily on mice, a high mortality rate still occurred. Initially it was thought that this was caused by bacterial infections, as in fly no.4 (Plate 7.7) but Giemsa-stained smears of the guts of these tsetse flies made soon after death showed that uncontaminated flies were also dying. Transmission electron microscopy of the guts of two G.tachinoides and two G.palpalis revealed the cause of death to be disintegration of the gut wall, even in flies which had been interrupted during feeding after only taking a small amount of blood (D.S. Ellis, personal communication). Since both these species of flies came from viable laboratory colonies, it seems unlikely that this was a genetic defect. It is suggested that the development of the gut was interrupted at a crucial time by the cold weather encountered by the puparia while in transit from France.

The gradual mortality seen with G.morsitans is thought to be caused by the detrimental effects of mouse blood forming a black

deposit in the hindgut of the fly which apparently could not be excreted. Langford Laboratories, Bristol, now rear all their G.morsitans by membrane feeding with defibrinated pig blood and their findings suggest that better survival rates could be achieved by giving an infective feed of mouse blood followed by feeds of pig blood, through a membrane. Membrane feeding would also reduce the bacterial infections contracted by the flies. However, since certain T.congolense stocks will infect mice but not pigs (Godfrey, 1958a) the pig blood in tsetse meals might affect the cyclical development of such stocks.

The infection rates of G.morsitans, determined from dissected flies, was 21.4% for Gamb 6 (S) and 8.3% for TSW 99/77 (RF) and the magnitude of the infections produced also suggest a more efficient transmission of the savannah stock by this species of tsetse fly. Bruce et al. (1915) obtained an experimental infection rate of only 10%, possibly because some of these flies were maintained at mean temperatures of 18.3°C to 28.8°C. According to Burt (1946), the receptivity of tsetse flies can be increased by raising the temperature at which the puparia are incubated. Godfrey (1958b) obtained an infection rate of 35% with T.congolense in G.morsitans by raising the temperature of puparia incubation to 28°C. Attempts at laboratory transmission of T.congolense through G.palpalis have been unsuccessful (Godfrey, 1960b, 1964; Stephen, 1962); possibly, success would have been achieved using a riverine stock. Reports of infection rates in wild caught G.morsitans are as high as 29.2% in south west Nigeria (Baldry, 1969) but only 0.27% in G.palpalis and 3.8% in G.fusca from Liberia (Foster, 1963), and 0.5% in G.tachinoides from northern Nigeria (Roberts and Gray, 1972).

In this work, there was a difference in infection rates between individual cages (Table 7.2), cage 1, 50%, cage 2, 33% and cage 3, 0%. The only variable factor between these cages was that the infected feed was taken from different mice, so these differences may be due to the effect of different mice on the trypanosome (Chapter 9). A high rising parasitaemia was always used but the numbers of peak parasitaemias that the infection had been through was not taken into account and possibly the age of the infection affects infectivity.

The heavy infections produced by Gamb 6 (S) in G.morsitans showed that the infection was firmly established whereas the sparse proboscis infection of TSW 99/77 (RF) indicated a regression of the infection possibly due to a failure of the flagellates to multiply in the midgut. During the first transmission experiments, one G.morsitans was dissected with a 30 day TSW 99/77 (RF) infection. This fly similarly had only a few parasites in the hypopharynx and a few in the midgut and no infection resulted in mice bitten by this fly.

From the Giemsa-stained smears made from the infected flies, the TSW 99/77 (RF) midgut infection appeared to have regressed, whereas the diverse trypanosome forms seen in Gamb 6 (S)-infected flies implied healthy established infections. Peel (1962) suggested that regression of midgut infections was caused by bacterial infections but no such contaminants were found in these TSW 99/77 (RF)-infected flies. In a Giemsa-stained smear of a 30 day old midgut infection of TSW 99/77 (RF), no slender proventricular forms were seen, suggesting that no forward migration was in progress and, furthermore, 50% of the trypanosomes were of unhealthy appearance. These trypanosomes may have failed to establish an infection and multiply while

the few in the head region had established themselves directly. A more likely explanation is that some parasites had achieved the forward migration to the head initially, but possibly with a failure to multiply and replenish the numbers of flagellates in the midgut. Further work is required to determine the correct explanation. Dissection of flies soon after the infective feed would show whether the midgut infections were healthy initially and if flagellates could establish themselves directly in the proboscis before they could have migrated forward from the midgut.

In the past, knowledge that the different trypanosome subgenera develop in different parts of the tsetse fly has facilitated their identification in naturally infected specimens (Buxton, 1955). T.vivax undergoes its cycle in the proboscis only (Bruce et al., 1910b); T.brucei in the midgut and salivary glands with subsequent migration of metatrypanosomes to the proboscis (Bruce et al., 1911a; Robertson, 1913) and as described in Section 7.1, T.congolense develops in the midgut and proboscis. Misidentifications could easily occur using this technique. For example in fly no. 22 infected with TSW 99/77 (RF), parasites were only seen in the hypopharynx; had this been a wild caught fly, it would have been identified as harbouring T.vivax. Godfrey (1964) found that 20% of G.morsitans experimentally infected with T.congolense had proboscis infections alone. Roubaud (1935) observed a wild caught G.longipalpis with a T.congolense infection confined to the proboscis and he suggested that T.congolense could establish itself directly in the proboscis like T.vivax; however, a secondary regression of the intestinal infection cannot be excluded. Deviations from the classical cycle of Trypanozoon trypanosomes in tsetse flies are also reported

(Duke, 1930; Duke, 1933; Burt, 1953; Peel, 1962) and these too can lead to faulty diagnosis. Another source of error could be mixed infections occurring in the same fly (Peel, 1962). With a mature T.vivax infection in the proboscis and an early T.brucei infection in the midgut, the diagnosis of T.congolense would be incorrect.

Thus, identification of trypanosomes by their position in the tsetse fly should be regarded with caution and conclusions from field observations can be erroneous. It can be useful in giving an immediate tentative result in the field, but some additional assessment is required. Inoculation of infected probosces into laboratory animals or feeding of wild caught flies on animals appears to be unsuccessful (Godfrey, 1964). At present, diagnosis can only be confirmed by the preparation and laborious examination of Giemsa-stained smears; in the case of low infections the trypanosomes may be lost from the slide during the staining process. Adaptation of electrophoretic, serological or immunological techniques to a microscale could prove useful.

More attempts at transmissions of numerous stocks representing these two major zymodemes and some of the minor ones should be undertaken, to confirm the indications of a close vector/zymodeme relationship seen from these preliminary experiments; especially, transmission experiments should be conducted through forest and riverine flies.

7.5. Electrophoresis

7.5.1. Materials and methods

T.congolense stocks were transmitted through G.morsitans as described in Section 7.2.1. When the infected mice were exsanguinated for stabilisation of the infection, some of this blood was

inoculated i.p. into two mice whose subsequent infections were passaged into rats; lysates were made (Section 2.6) and electrophoresis carried out (Section 3.2).

7.5.2. Results

The electrophoretic patterns of one tsetse fly-transmitted infection of Gamb 6 (S), Gamb 19 (S) and TSW 99/77 (RF) and three transmitted infections of TD 56/78E (RF) and TSW 25/78E (RF) were determined. They all gave identical patterns in 14 enzymes to the parent stock except for Gamb 6 (S) TFT (Tsetse Fly Transmitted) which showed a variation in two enzymes, PGM and GAPDH. In PGM, the pattern altered from I to II and with GAPDH from II to I (Sections 4.2.4 and 4.2.6).

7.5.3. Discussion

Of the five T.congolense stocks examined by enzyme electrophoresis before and after transmission by G.morsitans in the laboratory, electrophoretic pattern changes occurred in only one. This change may have been due to the original Gamb 6 (S) stock consisting of two enzymically distinct trypanosome populations and on development in the tsetse fly the original undetected population became dominant to be transmitted when the infection matured. With members of the subgenus Trypanozoon, if several populations are fed to the tsetse fly in equal proportions one will always become dominant (C.A. Letch, personal communication). Enzyme electrophoresis only detects the majority of trypanosomes present. Therefore if several populations are present reaching their peak parasitaemias at different times it will depend on the time of preparation of the lysate or in this case which population was predominant when the blood was used as an

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infective meal. This problem could be eliminated by using cloned stocks. However, if there was only one population in the original Gamb 6 (S) stock, the enzyme change must have occurred by mutation or the 'switching' on and off of particular isoenzymes by the trypanosomes. Isoenzyme changes have also been seen after prolonged subculturing of T. cruzi (Romanha et al., 1979) and the weakening and eventual disappearance of an ALAT isoenzyme after innumerable syringe passages of a T. brucei stock through laboratory rodents (Kilgour, 1976); this latter case cannot be accounted for by population selection or genetic exchange.

7.6. Conclusions

Results from these preliminary transmission experiments indicated that the two major zymodemes of T. congolense may be more efficiently transmitted by the species of Glossina normally associated with the same kind of vegetational zone from which the stocks originated.

CHAPTER 8

a. DRUG SENSITIVITY

8.1. Introduction

Numerous reports exist of salivarian trypanosome stocks resistant to commonly used trypanocidal drugs (see Williamson, 1970; MacLennan, 1970b). These resistant trypanosomes apparently retain this property after transmission through the tsetse fly (van Hove and Grainge, 1966; Gray and Roberts, 1968, 1971). The resistance may be an inherent property of a particular 'strain', which may have arisen by adaptation, or from the selection of drug resistant individuals from a mixed population. It may however, simply be the appearance of individual organisms that originated from 'occult' or tissue forms which escaped the action of the drug (Jennings et al., 1977). This latter explanation is feasible in the T. brucei-complex trypanosomes as they are known to invade the tissues but until recently T. congolense has been considered as a strictly blood plasma parasite. However, there have been, in the past, numerous reports of tissue forms of T. congolense (Schwetz, 1928; Schwetz and Fomara, 1929; Fiennes, 1950a, 1950b) and recently, more conclusive evidence has been produced (Luckins and Gray, 1978, 1979a; Gray and Luckins, 1979). In 1955, Goodwin and Rollo suggested that these occult forms of T. congolense, like those of T. brucei, could escape the action of drugs. However, there is often no evidence that resistant stocks have been previously exposed to drugs, so there may also be an inherent variation in the sensitivity of different stocks to trypanocides.

The following work was carried out to investigate the sensitivity of four stocks of T. congolense, representing the two major zymodemes, defined in Section 4.4, to Ethidium bromide and Berenil.

8.2. Materials and Methods

Gamb 6 (S) and Gamb 19 (S) represented the savannah zymodeme and TSW 103/77b (RF) and TSW 99/77 (RF), the riverine/forest zymodeme.

180 TO mice were divided into 36 batches of comparable weight. 10^6 trypanosomes were inoculated i.p. into 45 mice and the parasitaemia monitored until the first peak was reached six to eleven days later (Section 5.2.5). Groups of five mice for each stock were inoculated i.p. with Ethidium bromide at 10.0, 5.0, 2.5 and 1.25 mg kg⁻¹ body weight or Berenil at 40.0, 30.0, 20.0 and 10.0 mg kg⁻¹ body weight. The highest doses were taken from Jennings et al. (1977) working with T. brucei; lower doses were also used to allow a greater chance of relapse or a failure of initial clearance of detectable trypanosomes from the bloodstream. Five mice infected with each stock were left untreated as controls.

Wet blood films were examined daily until trypanosomes completely disappeared from the bloodstream, and then weekly for nine weeks or until a relapse was detected. To prevent cross-contamination from relapsed mice, the scissors used for tail snipping and each cut tail after taking blood, were swabbed with 70% alcohol; any mice with patent relapses were destroyed.

8.3. Results

Initially, there was a total clearance of detectable trypanosomes from the bloodstream of all drug-treated mice two to four days after administering the drugs; trypanosomes were present in control mice on most days of examination. More mice infected with the riverine/forest zymodeme relapsed than those infected with the savannah zymodeme (Table 8.1). The earliest relapses for each stock treated with the two drugs varied from 5 to 27 days with the earliest relapses occurring with the lowest drug doses (Table 8.2); a similar trend can be seen for the average number of days taken by the five mice in each group to relapse (Table 8.2).

8.4. Discussion

The two savannah stocks were more sensitive to Berenil and Ethidium bromide than the riverine/forest stocks. It appears then, that this is an inherent difference between the stocks. There is field evidence of drug resistance in T.congolense, both to Berenil (MacLennan, 1970b) and Ethidium bromide (Jones-Davies and Folkes, 1966), but these drugs are not used extensively in The Gambia (D.J. Clifford, personal communication) and not at all in Liberia (D.G. Godfrey, personal communication), the countries of origin of these four stocks. However, since only four stocks were investigated, the observations may not necessarily hold true for all representatives of each zymodeme; many more stocks need to be examined. Larger numbers of mice should also be used as differences between stocks on five mice at each dose was not always distinct, for example, Gamb 19 (S) and TSW 103/77b(RF) at 1.25 mg kg^{-1} Ethidium bromide. Moreover, the two savannah stocks were isolated from cattle and the two riverine/forest

Table 8.1. Numbers of mice relapsing after drug treatment

Stock	Berenil mg kg ⁻¹				Ethidium bromide mg kg ⁻¹			
	40.0	30.0	20.0	10.0	10.0	5.0	2.5	1.25
<u>Savannah</u>								
Gamb 19	2/5	2/5	4/5	5/5	0/5	0/5	1/5	0/5
Gamb 6	0/5	0/5	4/5	5/5	0/5	0/5	0/5	0/5
<u>Riverine/forest</u>								
TSW 103/77b	5/5	5/5	5/5	5/5	0/5	1/5	1/5	1/5
TSW 99/77	5/5	5/5	5/5	5/5	1/5	1/5	5/5	5/5

Table 8.2. Number of days after drug treatment for a patent parasitaemia to occur

Stock	Berenil mg kg ⁻¹								Ethidium bromide mg kg ⁻¹							
	40.0		30.0		20.0		10.0		10.0		5.0		2.5		1.25	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
<u>Savannah</u>																
Gamb 19	27->63	49	27->63	49	12->63	21	10-15	11	>63	>63	>63	>63	10->63	52	>63	>63
Gamb 6	63	63	63	63	12- 63	33	12-12	12	63	63	63	63	63	63	63	63
<u>Riverine/Forest</u>																
TSW 103/77b	11	11	11	11	11	11	7-14	11	>63	>63	25->63	55	21->63	55	21->63	33
TSW 99/77	11-23	13	11	11	11	11	5-11	9	19->63	55	17->63	57	19-35	28	12-26	13

stocks from pigs, so the sensitivity differences may be associated with the predilection of certain stocks for different hosts. Additionally, a purely geographical differentiation cannot be excluded and stocks from East Africa should also be compared; it has been suggested that East African T. congolense stocks are more susceptible to trypanocides than those from West Africa (Williamson, 1970). Unfortunately, time did not allow further extension of this work, although work carried out by a colleague has since confirmed these initial findings (P. Dukes, personal communication).

Jennings et al. (1977) found that if mice were treated within three days of infecting with T. brucei, or within two weeks for T. congolense, no relapses occurred. However, during this present work all mice were treated by day 11 and relapses still resulted.

Assuming that these four stocks of T. congolense were unlikely to have been previously exposed to Ethidium bromide or Berenil, individuals in the population were probably not innately resistant to such high doses of the drugs as 40 mg kg^{-1} Berenil and 10.0 mg kg^{-1} Ethidium bromide. This indicates that some individuals were able to escape from the drugs, thus lending support to the idea of an occult or tissue form which could subsequently re-invade the bloodstream resulting in a relapse (Goodwin and Rollo, 1955; Jennings et al., 1977). Of course, such trypanosomes would not themselves be drug resistant.

Support could also be lent to this hypothesis by the increase in subpatent period seen with an increase in the drug dose (Table 8.2). The higher the dose, the longer the drug would remain in the circulation and be able to destroy trypanosomes released from the tissue forms. The relatively longer subpatent period seen after treatment with Ethidium bromide than Berenil may be due to the rapid

excretion of Berenil from the mouse, within 24 hours, except at high doses when residual amounts can be detected for up to three weeks (van Hoeve et al., 1965). By contrast, Ethidium bromide is retained for a longer period of time, only 50% being excreted during the first 24 hours (Kandaswamy and Henderson, 1963).

It may be that the greater ability of the riverine/forest stocks to relapse after drug treatment is due to their production of a larger number of tissue forms than the savannah stocks. Although a direct association may exist with the different isoenzymes found in the different zymodemes and the susceptibility to trypanocides, this is unlikely since so few enzymes were examined compared with the several thousand in a cell. Other concomitant properties may contribute to the observed differences, such as the permeability of membranes to drugs varying in the different stocks or zymodemes.

8.5. Conclusions

Two stocks of T. congolense originating from cattle in The Gambia, a savannah region, exhibited a higher sensitivity to Berenil and Ethidium bromide than two enzymically distinct stocks originating from pigs in Liberia, a riverine/forest region. It is suggested that the increased tendency of the riverine/forest stocks to relapse may be associated with a greater ability to produce tissue or occult forms which can escape the action of the drugs.

CHAPTER 9

9. EXPERIMENTAL INFECTIONS IN INBRED AND OUTBRED MICE

9.1. Introduction

Inoculation of rodents with natural infections of T.congolense results in one of several possible responses; no infection, transient infection, chronic infection with no signs of disease or death, and chronic or acute infection terminating in death (Montgomery and Kinghorn, 1909c; Bevan, 1910; Jowett, 1910; Bruce et al., 1911b; Blacklock and Yorke, 1913; Bruce et al., 1913a; Yorke and Blacklock, 1915; Binns, 1938; Godfrey, 1961). Obviously an important factor is the host variability to infection and recent work has shown that certain strains of inbred mice are more susceptible to disease caused by T.congolense than others (Jennings et al., 1978; Morrison et al., 1978) i.e. T.congolense is more pathogenic to certain strains of mice, while the infectivity remains the same (Morrison et al., 1978).

The following experiments were carried out to investigate the infectivity of four stocks of T.congolense representing two major zymodemes, and the susceptibility to disease of two strains of inbred mice to these stocks.

9.2. General Materials and Methods

Four stocks of T.congolense, Camb 6 (S) and Camb 19 (S), TSW 103/77b (RF) and TSW 99/77 (RF), were used as representatives of two major zymodemes defined in Section 4.4.

For each experiment, stabilates of the four stocks were inoculated i.p. into four TO outbred mice and the infections were monitored until parasitaemias reached about 10^8 trypanosomes ml^{-1} blood (Section 2.5.2). One mouse for each stock, was exsanguinated into 10 i.u. of heparin ml^{-1} blood, as an anticoagulant, held on ice. The numbers of trypanosomes ml^{-1} blood were determined, after diluting with PSG, on a haemocytometer.

The levels of parasitaemia in mice subsequently infected, were recorded on a scale of plusses or at low levels the numbers of trypanosomes per field at x400 total magnification.

+	= 20-50 trypanosomes field ⁻¹	$\approx 10^5$ - 10^6	trypanosomes ml^{-1} blood
++	= 50-100 " "	$\approx 10^6$ - 10^7	" "
+++	= >100 " "	$\approx 10^7$ - 10^8	" "
++++	= as many trypanosomes as red blood cells	$\approx 10^8$	" "

When a mouse was scored as negative, 20 fields had been examined without detecting a trypanosome but the mouse may still have been harbouring a subpatent undetectable infection.

9.3. Infectivity Titrations

9.3.1. Materials and Methods

The method was based on Lumsden *et al.* (1963). The heparinized mouse blood was diluted with ice-cold PSG to give solutions containing 10^4 , 10^3 , 10^2 , 10^1 , 10^0 and 10^{-1} trypanosomes ml^{-1} , for each stock. 0.1 ml of each solution was inoculated i.p. into six TO outbred mice; the mice were all female and had previously been divided on weight to give a range of 25 to 35g in each batch. The mice were checked for the presence of trypanosomes by microscopical

examination of 20 fields of a wet blood film (Section 2.5.2), three times a week for two weeks and then weekly for a further month. Precautions were taken to avoid contamination of uninfected mice from parasitaemic ones; the scissors used for snipping the tail were swabbed with 70% alcohol between each mouse and parasitaemic mice were destroyed.

9.3.2. Results

In all four stocks of T.congolense examined, no infections resulted in mice inoculated with solutions theoretically containing 10^{-1} or 10^{-2} trypanosomes, and one mouse became infected, with each stock, when inoculated with one trypanosome. The numbers of mice which became parasitaemic from inoculation of larger numbers of trypanosomes, were similar in the four stocks (Table 9.1).

9.4. Infectivity of Stocks and Susceptibility to Disease of Inbred Mice

9.4.1. Materials and methods

The heparinized blood was diluted with ice-cold PSG to give 10^2 trypanosomes ml^{-1} . 0.1 ml of this solution, i.e. 10 trypanosomes, was inoculated i.p. into five C57 Black (C57Bl) inbred mice, five Ajax (AJ) inbred mice and five TO outbred mice; 10 trypanosomes were inoculated as this number infected about 80% of TO mice (Section 9.3.2) and it was hoped to combine the infectivity of stocks and the susceptibility to disease of C57Bl and AJ inbred mice into one experiment. The mice were all female and weighed between 25 and 35 g. The mice were checked three times a week for 60 days and then weekly for a further 30 days, for the presence of trypanosomes (Section 2.5.2). Precautions were again taken to

Table 9.1. Number of TO mice infected after i.p. inoculation of trypanosome suspensions.

Stock	Number of trypanosomes inoculated					
	10^3	10^2	10^1	10^0	10^{-1}	10^{-2}
<u>Savannah</u>						
Gamb 19	5/6	6/6	4/6	1/6	0/6	0/6
Gamb 6	6/6	6/6	5/6	1/6	0/6	0/6
<u>Riverine/Forest</u>						
TSW 103/77b	6/6	4/6	4/6	1/6	0/6	0/6
TSW 99/77	6/6	6/6	4/6	1/6	0/6	0/6

prevent contamination of uninfected mice from infected ones; the scissors were swabbed with 70% alcohol between each tail snip and every tail was also swabbed with 70% alcohol after taking the blood.

9.4.2. Results

The infectivity of Gamb 19 (S) was identical in all three strains of mice, C57Bl, AJ and TO, and similar for TSW 103/77b (RF) and Gamb 6 (S). With TSW 99/77 (RF), three of the five AJ mice became infected but no C57Bl mice (Table 9.2).

The 12 mice which became infected with Gamb 19 (S) all displayed high parasitaemias (Figs. 9.1 to 9.3). Of the five mice which showed parasitaemias with Gamb 6 (S), two TO mice displayed repeatedly high parasitaemic peaks every 7 to 11 days, one infected AJ mouse only showed a low parasitaemia on one day (day 11), and the surviving C57Bl mouse had a high parasitaemia for 60 days (Figs. 9.4 to 9.6). With TSW 103/77b (RF) infections the five AJ and four TO mice had high parasitaemias whereas the one C57Bl which survived the initial high parasitaemic peak, maintained a fluctuating parasitaemia which was rarely high (Figs. 9.7 to 9.9). The two TO mice infected with TSW 99/77 (RF) displayed fluctuating parasitaemias peaking about every 7 days (Fig. 9.11); the three AJ mice displayed an initial high parasitaemic peak (Fig. 9.10). No C57Bl mice became infected with TSW 99/77 (RF).

Considering the survival of the mice, all except one of the Gamb 19 (S)-infected mice died at the first parasitaemic peak (Figs. 9.1 to 9.3). Of the mice which became parasitaemic with Gamb 6 (S), one C57Bl died at the first peak while the other survived for 60 days, the two TO mice died on day 64 and 84 and the AJ mouse was still alive on day 90 after being negative for 76 days (Figs. 9.4

Table 9.2. Number of mice infected after i.p. inoculation of 10^1 trypanosomes

Stock	Mouse strain		
	C57Bl	AJ	TO
<u>Savannah</u>			
Gamb 19	4/5	4/5	4/5
Gamb 6	2/5	1/5	2/5
<u>Riverine/Forest</u>			
TSW 103/77b	4/5	5/5	4/5
TSW 99/77	0/5	3/5	2/5

Table 9.3. Survival times of mice infected with 10^1 trypanosomes

Stock	Mouse strain					
	C57Bl		AJ		TO	
	Range(days)	Mean	Range(days)	Mean	Range(days)	Mean
<u>Savannah</u>						
Gamb 19	11	11	11-30	16	11-37	22
Gamb 6	18-58	38	>90	>90	64-84	74
<u>Riverine/Forest</u>						
TSW 103/77b	>90	>90	10-12	11	10-17	14
TSW 99/77	*-	-	17-24	21	>90	>90

*no C57Bl mice became parasitaemic with TSW 99/77 (RF)

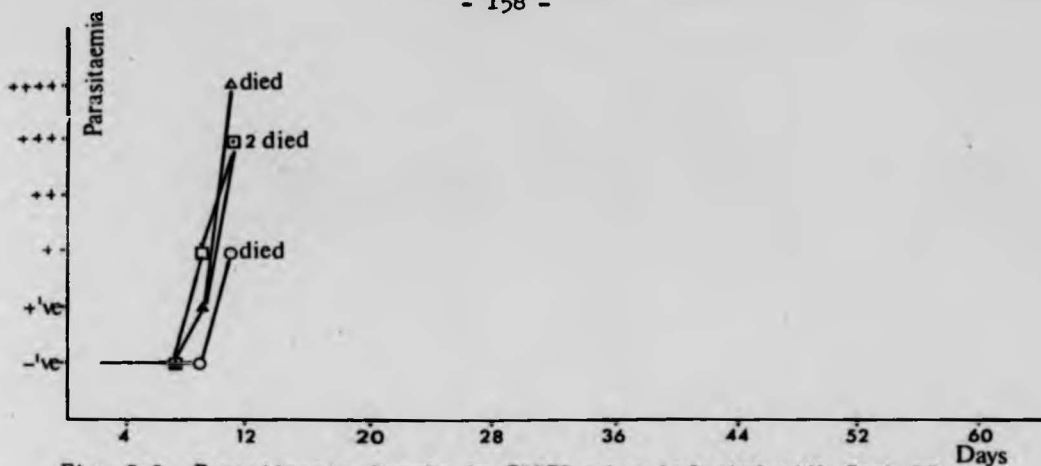


Fig. 9.1. Parasitaemic levels in C57Bl mice infected with Gamb 19

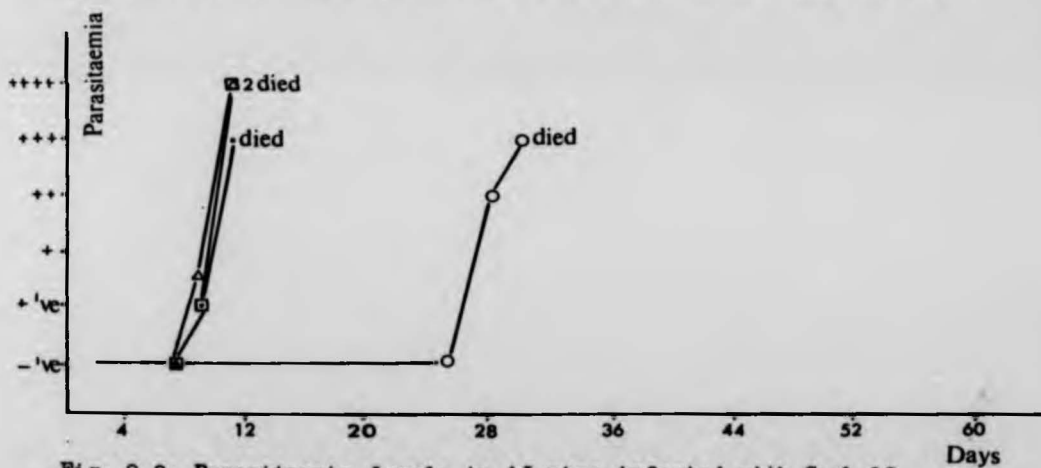


Fig. 9.2. Parasitaemic levels in AJ mice infected with Gamb 19

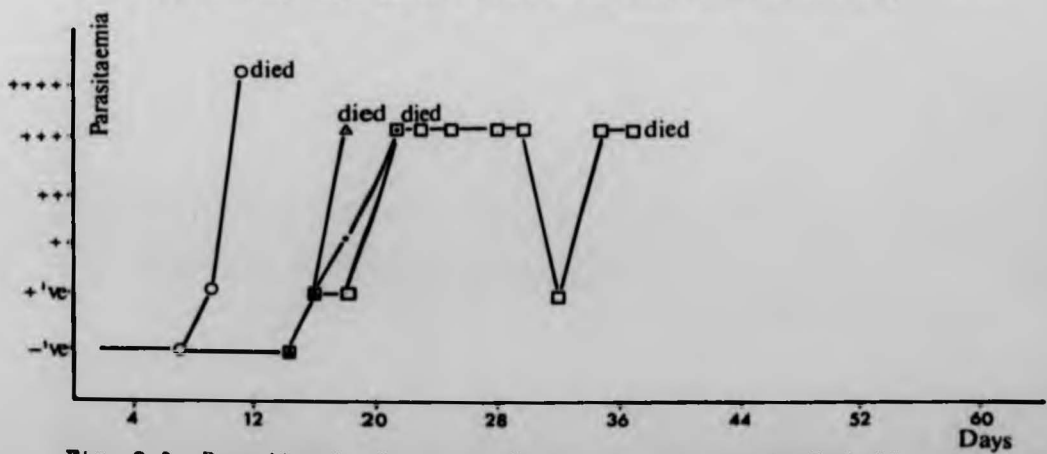


Fig. 9.3. Parasitaemic levels in TO mice infected with Gamb 19

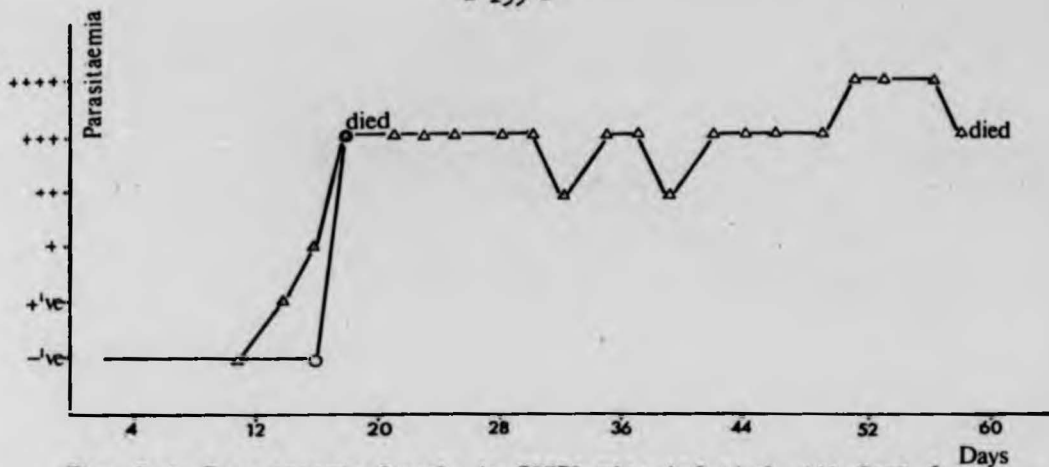


Fig. 9.4. Parasitaemic levels in C57Bl mice infected with Gamb 6

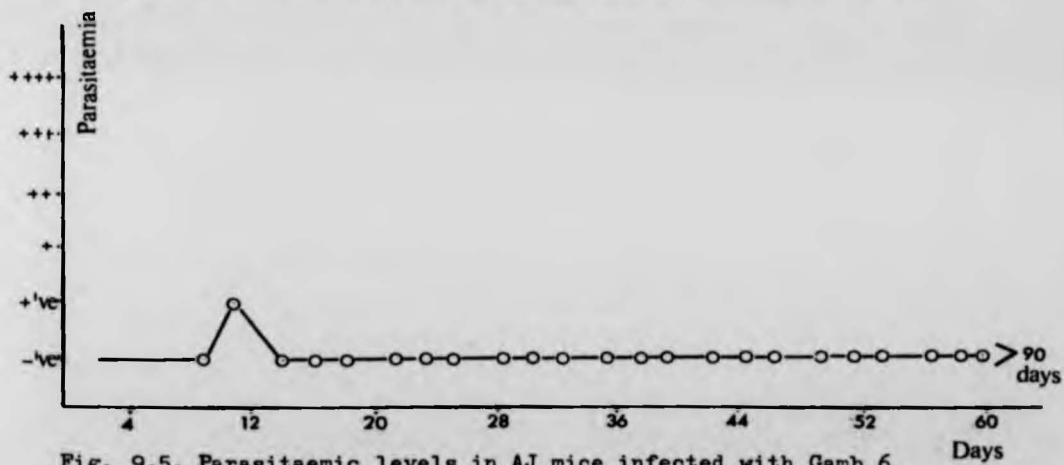


Fig. 9.5. Parasitaemic levels in AJ mice infected with Gamb 6

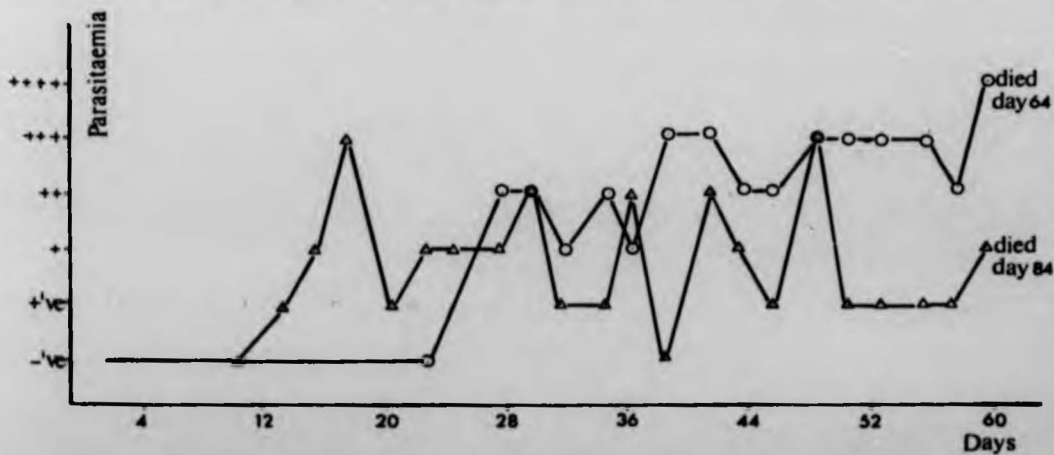


Fig. 9.6. Parasitaemic levels in TC mice infected with Gamb 6

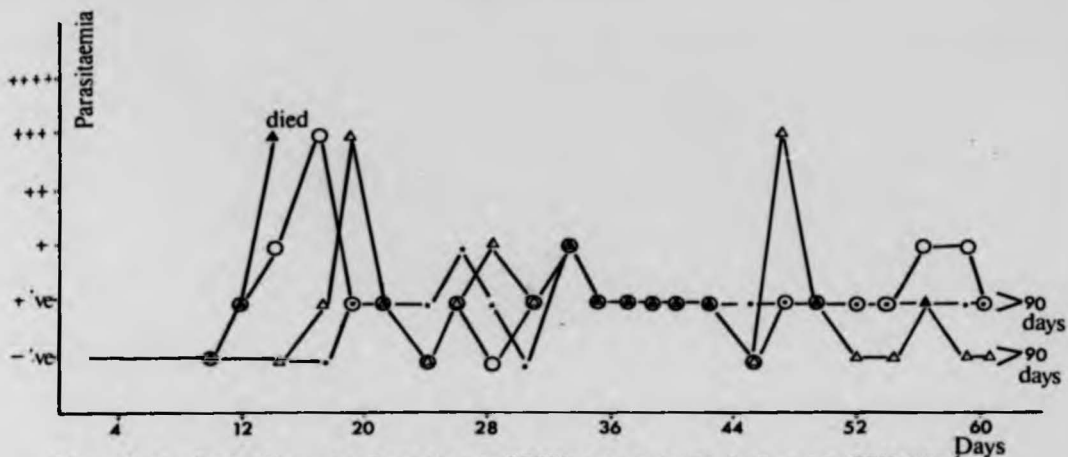


Fig. 9.7. Parasitaemic levels in C57Bl mice infected with TSW 103/77b

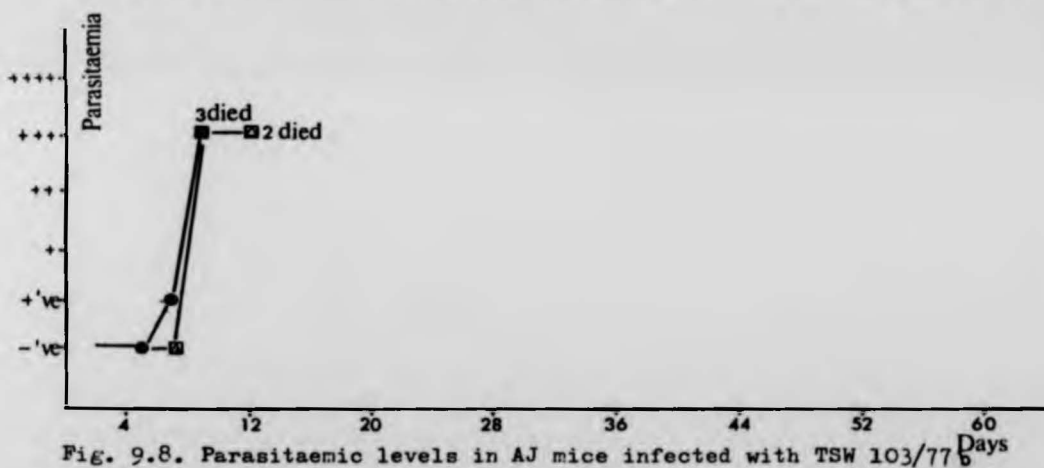


Fig. 9.8. Parasitaemic levels in AJ mice infected with TSW 103/77b

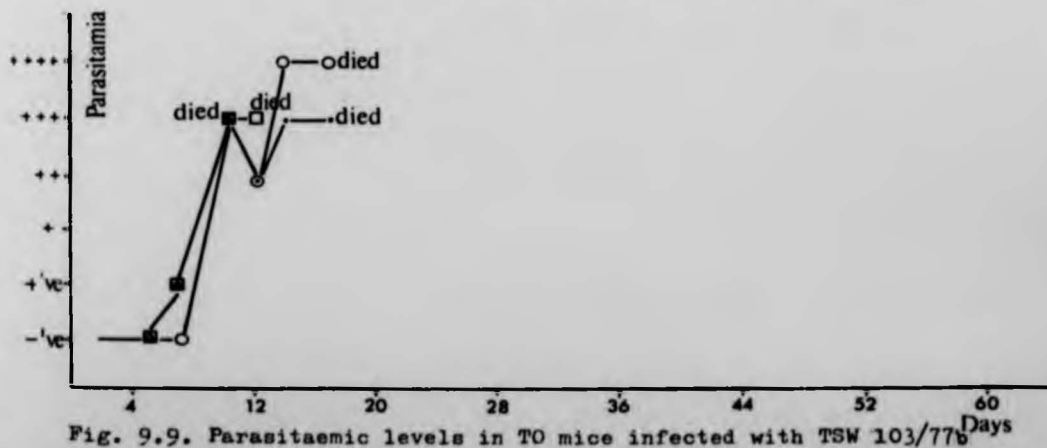


Fig. 9.9. Parasitaemic levels in TO mice infected with TSW 103/77b

No C57B1 mice became parasitaemic with TSW 99/77

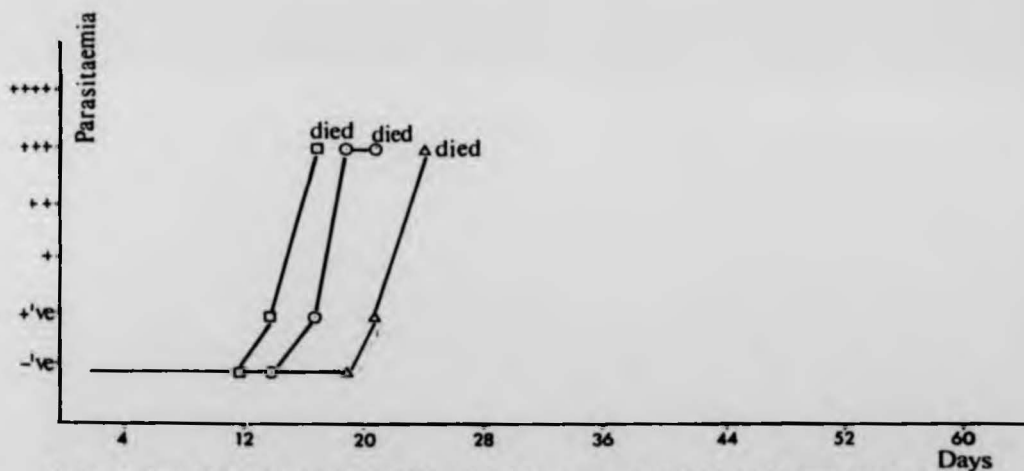


Fig. 9.10. Parasitaemic levels in AJ mice infected with TSW 99/77

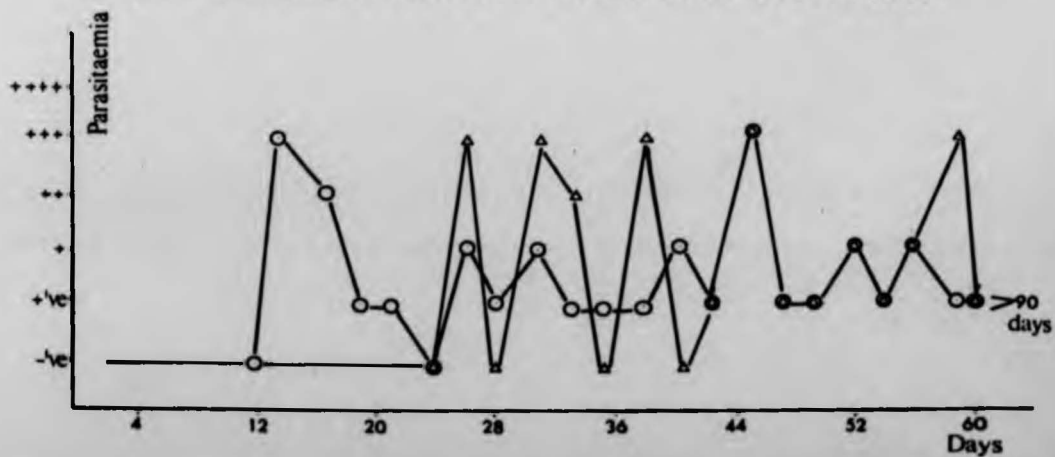


Fig. 9.11. Parasitaemic levels in TO mice infected with TSW 99/77

No C57Bl mice became parasitaemic with TSW 99/77

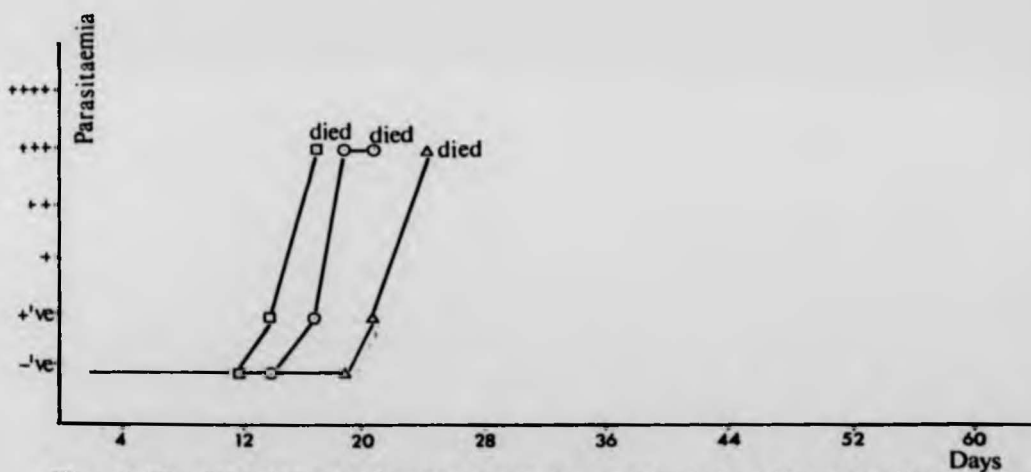


Fig. 9.10. Parasitaemic levels in AJ mice infected with TSW 99/77

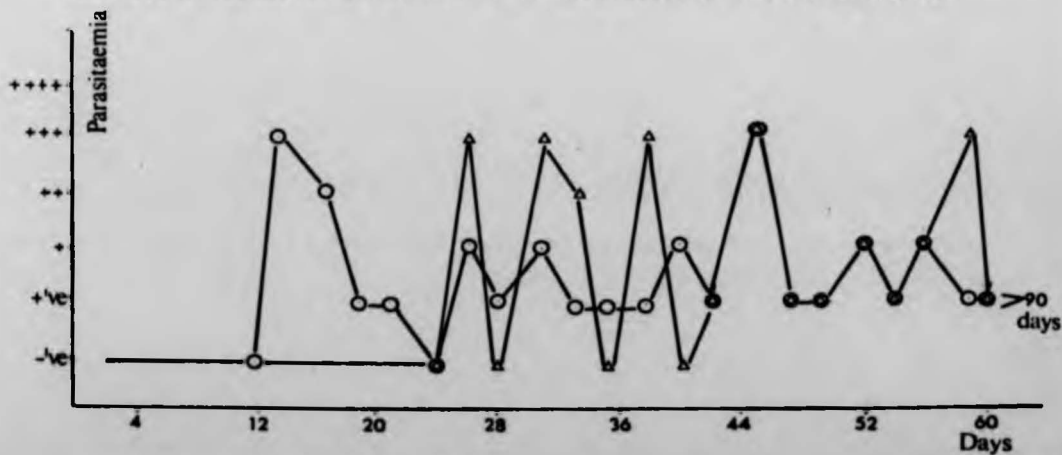


Fig. 9.11. Parasitaemic levels in TO mice infected with TSW 99/77

to 9.6). The TO and AJ mice infected with TSW 103/77b (RF) all died at the first or second parasitaemic peak whereas three of the four infected C57Bl mice lived for more than 90 days (Figs. 9.7 to 9.9). Infections of TSW 99/77 (RF) killed all three AJ mice at the first parasitaemic peak but the two TO mice were still alive on day 90 (Figs. 9.10 to 9.11). A summary of survival times is shown in Table 9.3.

9.5. Discussion

No differences were found in the infectivity of the four stocks of T.congolense to TO outbred mice. It is unlikely that this was due to the stocks becoming rodent-adapted as none had experienced many rodent passages.

AJ and C57Bl inbred mice were used for the susceptibility tests as Morrison et al. (1978) found AJ mice to be the most susceptible to disease with T.congolense and C57Bl mice the most resistant, out of the eight strains of mice they investigated. The TO outbred mice were used in the present work to see if this susceptibility varied more with the individual animal in outbred than inbred strains, although this did not appear to be the case.

Morrison et al. (1978) found a strong correlation between the level of parasitaemia at the first peak and the subsequent susceptibility or resistance of the mice to the infection. They found that initial peak levels of parasitaemia of 10^5 organisms μl^{-1} resulted in death, whereas in less susceptible strains the level was usually lower than 10^4 organisms μl^{-1} (10^5 organisms μl^{-1} corresponds to about a +++ parasitaemia). With TSW 103/77b (RF), the initial peak parasitaemic level in C57Bl and AJ mice was

similar (+++) but the C57Bl appeared to be able to tolerate and reduce the high parasitaemia while the AJ mice were overcome. Subsequent parasitaemic peaks in the C57Bl mice were generally at a low level. The prepatent period of individual animals varied even in the inbred strains.

The difference in susceptibility to disease seen by Morrison et al. (1978) was clearly demonstrated with TSW 103/77b (RF) (Figs. 9.7 to 9.9). All five infected AJ mice were dead by day 13, whereas three of the four C57Bl mice were still alive by day 90. This difference was not found, however, with Gamb 19 (S) (Figs. 9.4 to 9.6).

It is realized that the low numbers of mice used during this work cannot be compared with the large numbers used by Morrison et al. (1978) or Jennings et al. (1978); the high cost of these inbred mice and lack of time prevented further experimentation. Additionally, the infecting dose was much lower for this work than that used by Morrison et al. (1978) or Jennings et al. (1978) and this may affect the course of the infection although in TO mice this was not found to be the case.

Morrison et al. (1978) working with three stocks including one recently isolated, only found a marked difference in susceptibility in one of the stocks, and although the same trend was followed by the other two it was not so pronounced; none of these stocks were defined. From the present work, it appears that with certain stocks, AJ mice are the most susceptible to disease and C57Bl the most resistant (TSW 103/77b (RF)); with others the susceptibility appears to be the same (Gamb 19 (S)), or else the susceptibility of the inbred strains appears to be reversed (Gamb 6 (S)). No distinct demarcation

was seen between the two major zymodemes.

More extensive work should be carried out on both the infectivity of many different T.congolense stocks and the susceptibility to disease of different inbred mice strains. It is important to use defined, recently isolated stocks of T.congolense before they become rodent-adapted.

The aim of both Morrison et al. (1978) and Jennings et al. (1978) was to find a suitable mouse strain for the study of trypanotolerance as found in N'dama cattle. However, current work, carried out in conjunction with Mrs. D. Swyndercombe-Court and Miss P. Smith at the Haematology Department of the London Hospital, indicated that rabbits may provide a better, though more expensive model, than inbred mice. This work was initiated to investigate the haematological responses in rabbits produced by two enzymically distinct stocks of T.congolense. The two stocks, TSW 99/77 (RF) and Gamb 19 (S) caused different effects on different strains of inbred mice (Section 9.4.2). However, in rabbits, after an initial parasitaemia of 144 trypanosomes in 30 fields with TSW 99/77 (RF) and only 16 in 30 fields with Gamb 19 (S), both parasitaemias fell. From two to six months after infecting, no trypanosomes were seen in the weekly microscopical examinations of wet blood films, but occasionally trypanosomes were encountered during reticulocyte counts made from stained blood films. It appears that although the initial infection differed, both between the two stocks and also in individual rabbits, each animal succeeded in reducing the parasitaemia to a level which it could tolerate. Other haematological investigations included red cell life span using Cr 51, reticulocyte counts, total bilirubins levels, plasma ion levels, white cell counts, red cell

counts, haemaglobin levels, packed cell volumes, differential white cell counts, the damage of red cells and platelets and more recently the effect of splenectomy. It appears that a greater response was produced by TSW 99/77 (RF) than Gamb 19 (S), for example, a greater increase in reticulocyte production, but once the initial parasitaemic peak was reduced, the responses were similar in the two stocks. This work is still in progress and results are incomplete; it will be reported in full following the termination of the experiment.

9.6. Conclusions

No difference was seen in the infectivity of four stocks of T.congolense to TO outbred mice. The infectivity of the four stocks differed for AJ and C57Bl inbred mice and the susceptibility to disease of the inbred mice also varied with the stock. No clear distinctions could be correlated with the division of stocks into two major zymodemes. Although, the reputed increased susceptibility to disease of one strain of inbred mice to T.congolense was confirmed with one stock, it did not hold true for other stocks. Apparently, infectivity and susceptibility depend on the strains of both mouse and trypanosome.

CHAPTER 10

10. MORPHOLOGY

10.1. Introduction

T. (Nannomonas) congolense is one of the smallest salivarian trypanosomes, measuring 8 to 24 μm in length with means of 11.2 to 17.6 μm (Hoare, 1959; Godfrey, 1960a; Fairbairn, 1962). Typically, it is devoid of a free flagellum in all stages of development both in the mammalian and insect hosts, though in some individuals a minute free portion is sometimes visible (Schwetz, 1931; Peel and Chardome, 1954c; Stephen, 1963; Hoare, 1972). In the blood of mammalian hosts, it is generally monomorphic (Hoare, 1972) and in fresh blood preparations its movements are sluggish and departure from the microscope field is rare. The kinetoplast is of intermediate size between that of trypanosomes in the subgenera Duttonella and Trypanozoon, measuring 0.7 to 0.8 μm and is situated usually in a subterminal, marginal position. In bloodstream forms, the nucleus is in the centre of the body, and the undulating membrane is relatively inconspicuous compared with the other salivarian subgenera.

Until 1960, it was believed that T. dimorphon and T. congolense were two distinct species based on the differences in their lengths: T. congolense (Broden, 1904) measuring 8 to 19.5 μm with mean lengths of 12.2 to 14.4 μm (Hoare, 1959; Bruce et al., 1910a; Kinghorn et al., 1913; Bruce et al., 1913a); T. dimorphon (Laveran and Mesnil, 1904) measuring 11.0 to 24.0 μm with mean lengths of 15.3 to 17.6 μm (Hoare, 1959). In 1960(a), Godfrey described stocks from Nigeria with intermediate mean lengths

of 12.5 to 13.9 μm , while Fairbairn (1962) reported similar intermediate stocks from other parts of Africa, with mean lengths of 14.0 to 15.0 μm . From these investigations and those of Huisenga (1969), it became evident that the speciation of T.congolense and T.dimorphon, at least on differences in mean lengths, was not valid.

T.congolense includes at least three different types distinguishable on certain morphological characteristics shown in Table 10.1 (Godfrey, 1960a). The differences in mean lengths, shown in this table, obtained by a) Godfrey (1960a) and b) Fairbairn (1962), are possibly due to different hosts. Recently, Nantulya *et al.* (1978b) put forward the view that these different types were a manifestation of the parasitaemic level; the validity of their findings is discussed in Section 10.4.

Controversy has also arisen over the status of another trypanosome with a similar morphological appearance to T.congolense. In 1909(b), Montgomery and Kinghorn isolated a peculiar trypanosome from a cow on the shores of Lake Tanganyika in Zambia. This trypanosome resembled the short or 'tadpole' forms seen in T.congolense except that it was much broader (ratio of 4:1, length:breadth), with a better developed undulating membrane and sometimes a bristle-like free flagellum. Successful subinoculations were made into two sheep, a guinea pig, a goat and an ox. The unusual morphology was maintained in the goat but the trypanosomes subsequently seen in the sheep were morphologically identical to the tadpole forms of T.congolense. The authors suggested that this may have been due to a natural change in morphology or to a secondary infection acquired during transportation of the host, although every precaution had been taken to prevent this. Montgomery and Kinghorn (1909b) tentatively assigned the 'Ninamwenda' trypanosome to T.dimorphon. Laveran received specimens

Table 10.1. Main diagnostic features of three morphological types of T. congolense
(Godfrey, 1960; Fairbairn, 1962)

Characteristic	Morphological type		
	Short	Transitional	Long
1. Shape of posterior end	rounded	bluntly pointed	sharply pointed
2. Distance of kinetoplast from posterior end	sub-terminal	about 1 μ m	about 2 μ m
3. Undulating membrane	indistinct	thrown into 1-2 weak folds	thrown into 2-3 well developed folds
4. Mean length of trypanosome μ m	a) 11.20 - 13.80	12.45 - 13.85	13.75 - 15.68
	b) 12.50 - 13.80	14.11 - 15.01	14.85 - 15.61

of this trypanosome and Ronald Ross, in an editorial footnote to Montgomery and Kinghorn's paper, quoted from Laveran that this trypanosome was similar to T. congolense but broader and 'ce qui permet de croire qu'il agit (encore!) d'une espèce nouvelle'. If further investigations confirmed a new species, Laveran proposed to call it T. montgomeryi. However, three years later the status was still unresolved and Laveran and Mesnil (1912) listed it under 'espèces douteuses ou insuffisamment connues'.

In the same year, Kinghorn and Yorke (1912a) described a similar trypanosome from a dog in Zambia, while a year later Kinghorn et al. (1913) found them in the same host on the Zambia-Malawi border. The ratio of length to breadth (4.8:1) was slightly greater than seen in the 'Ninamwenda' trypanosome (4:1).

Weissenborn (1911) described, under the name T. frobeniusi, trypanosomes found in the blood of a horse in Togo. This was undoubtedly a T. congolense infection containing some montgomeryi-type individuals. Trypanosomes of the montgomeryi-type have continued to be reported in infections of T. congolense and T. simiae in pigs, sheep, dogs and goats (Wenyon, 1926; Schwetz, 1930, 1934; Bourguignon, 1933, 1935; Lhouverol and Philippe, 1947; Peel and Chardome, 1954a; Chardome and Peel, 1954; Stephen, 1963). Stephen (1963) concludes that 'T. montgomeryi is an atypical form or variety found in members of the T. congolense group. Sometimes it occurs in apparently true form in individual animals, but it has not maintained its morphology through successive subinoculations to susceptible animals. It cannot therefore be considered as a valid independent species, although later work may show that it is capable of 'breeding true' in equally susceptible animals, and that there is enough genetic variation to warrant its classification as a true variety or

subspecies of T. congolense'.

In view of the past confusion on morphological differences between the various reputed species, it was decided to ascertain whether examples of the two major zymodemes (Section 4.4) differed morphologically and whether any variation corresponded to the previous morphological records; in particular, the occurrence of montgomeryi-forms was investigated. The disputed presence of free flagella was examined by scanning electron microscopy (SEM).

10.2. Materials and Methods

10.2.1. Light microscopy

Four stocks of T. congolense were examined morphologically, TSW 99/77 Clone and TSW 103/77b originating from riverine or forest areas (RF) and Gamb 19 and Gamb 6 isolated from savannah regions (S). Eight TO mice, two for each stock, were inoculated i.p. with 10^6 trypanosomes and examined daily for the presence of trypanosomes in wet blood films (Section 2.5.2). Once trypanosomes were detected by searching five fields, Giemsa-stained thin blood films (Section 2.5.5) were made from the mice every 12 hours until one complete parasitaemic wave (defined as the cycle of minimal parasitaemia through maximal and back to minimal) had passed. The series of films made from one mouse was chosen to represent each stock according to parasitaemic levels and survival (some mice died or failed to reach a high parasitaemia at the first peak).

One hundred trypanosomes were measured from blood films made of each stock at the following numbers of trypanosomes ml^{-1} blood; 10^6 in a rising parasitaemia, approximately 10^8 at the peak and 10^6 in a falling parasitaemia. The outline of the trypanosome, the

flagellum, nucleus and kinetoplast were drawn with the aid of a camera lucida, set at x1500. Fifty individuals were drawn, where possible from consecutive fields and the remaining fifty from consecutive fields in another area of the blood film; damaged or dividing forms were not included. A scale was marked on each set of drawings from part of the scale of a stage micrometer, and was subsequently used to calibrate a pair of callipers with their points set to the equivalent of 1 μm apart. The trypanosome lengths were measured by walking the callipers down the midline of the organism, and the free flagellum when present. The breadth was similarly measured across the trypanosome through the centre of the nucleus. The above methods were also applied to an earlier (rising) phase of the TSW 99/77 Clone (RF) parasitaemia i.e. 10^5 trypanosomes ml^{-1} blood and to a rising parasitaemia, 10^6 trypanosomes ml^{-1} blood, of Gamb 19(S) in a rat.

The percentage of morphological types (Godfrey, 1960a) present at each level was determined using the criteria 1 to 3 in Table 10.1. The mean length of the trypanosomes was not used as a criterion for differentiation because the length alters with the host (Godfrey, 1960a), so the lengths reported by Godfrey (1960a) and Fairbairn (1962), in different hosts, could not be used.

10.2.2. Scanning electron microscopy

0.5 ml of eluate of T. congolense, stock TSW 99/77 Clone (RF), from a DEAE-cellulose column (Section 2.6.1) were passaged through a 0.6 μm pore-size polycarbonate Nucleopore filter held in a syringe filter head. The trypanosomes were fixed in 3% gluteraldehyde in S-collidine buffer for one hour, washed in distilled water and rapidly dehydrated through a graded acetone series. The

filter head was transferred to a trough of acetone in a Polaron critical point dryer and the acetone exchanged with liquid CO₂ which was subsequently vented off.

The filter was mounted on a stub, coated with gold in an Edwards sputter coater S150 and examined under a Joel T20 scanning electron microscope.

10.3. Results

The savannah stocks (S), Gamb 6 (S) and Gamb 19 (S), were longer and narrower than the riverine or forest stocks (RF), TSW 99/77 Clone (RF) and TSW 103/77b (RF), at the three parasitaemic levels examined (Tables 10.2 and 10.3). Unlike Gamb 19 (S), TSW 103/77b (RF) and Gamb 6 (S) showed a direct correlation between change in length and the numbers of trypanosomes circulating in the bloodstream. Initially, it appeared that a similar result was emerging for TSW 99/77 Clone (RF), but examination of a lower parasitaemia (10⁵ trypanosomes ml⁻¹ blood) contradicted this trend. Gamb 19 (S) was longer and narrower in rats than in mice.

A graphical representation of measurements of the four stocks, each at three parasitaemic levels, is given in the modified Dice-Leraas diagrams (Dice and Leraas, 1936) (Figs. 10.1 and 10.2). The horizontal line, which is the range of measurements, represents the extreme limits of the sample. The arithmetical mean is represented by the crossbar on the horizontal line. The shaded rectangle is the standard deviation of the series, plotted on either side and from, the crossbar; assuming normal distribution 68% of the population will lie between the outer limits of this rectangle. If the shaded rectangles do not overlap it can be considered that the two populations

Table 10.2. Lengths of four stocks of T.congolense representing two major zymodemes

Stock	Host	Trypanosomes ml ⁻¹ blood	Parasitaemic phase	Mean µm	Range µm	Standard deviation	Standard error x 2
<u>Savannah</u>							
Gamb 19	mouse	10 ⁶	rising	13.75	10.0-18.9	1.38	0.276
Gamb 19	mouse	10 ⁸	peak	13.61	10.0-17.9	1.45	0.290
Gamb 19	mouse	10 ⁶	falling	13.76	10.5-16.3	1.36	0.272
Gamb 19	rat	10 ⁶	rising	14.30	11.1-18.9	1.52	0.304
Gamb 6	mouse	10 ⁶	rising	13.22	9.8-17.9	1.53	0.306
Gamb 6	mouse	10 ⁸	peak	13.47	10.0-17.4	1.71	0.342
Gamb 6	mouse	10 ⁶	falling	13.16	9.7-16.8	1.61	0.322
<u>Riverine/forest</u>							
TSW 103/77b	mouse	10 ⁶	rising	11.73	9.2-13.8	1.10	0.220
TSW 103/77b	mouse	10 ⁸	peak	12.33	9.9-18.0	1.34	0.268
TSW 103/77b	mouse	10 ⁶	falling	12.10	9.2-16.0	0.86	0.172
TSW 99/77C1	mouse	10 ⁶	rising	12.11	9.9-14.8	1.27	0.254
TSW 99/77C1	mouse	10 ⁸	peak	12.63	9.0-16.0	1.40	0.280
TSW 99/77C1	mouse	10 ⁶	falling	12.54	9.1-15.0	1.38	0.276
TSW 99/77C1	mouse	10 ⁵	rising	12.57	9.0-15.8	1.25	0.250

Table 10.3. Breadths of four stocks of T. congolense representing two major zymodemes

Stock	Host	Trypanosomes ml ⁻¹ blood	Parasitaemic phase	Mean µm	Range µm	Standard deviation	Standard error x 2
<u>Savannah</u>							
Gamb 19	mouse	10 ⁶	rising	1.46	0.8-2.4	0.36	0.072
Gamb 19	mouse	10 ⁸	peak	1.56	0.8-2.2	0.37	0.074
Gamb 19	mouse	10 ⁶	falling	1.45	0.9-2.1	0.34	0.068
Gamb 19	rat	10 ⁶	rising	1.26	0.8-2.0	0.34	0.068
Gamb 6	mouse	10 ⁶	rising	1.75	1.0-2.8	0.31	0.062
Gamb 6	mouse	10 ⁸	peak	1.72	1.0-2.5	0.32	0.064
Gamb 6	mouse	10 ⁶	falling	1.75	0.9-2.7	0.40	0.080
<u>Riverine/forest</u>							
TSW 103/77b	mouse	10 ⁶	rising	1.99	1.1-2.8	0.31	0.062
TSW 103/77b	mouse	10 ⁸	peak	2.30	1.6-3.2	0.39	0.078
TSW 103/77b	mouse	10 ⁶	falling	1.87	1.1-2.8	0.32	0.064
TSW 99/77C1	mouse	10 ⁶	rising	1.80	1.0-2.4	0.32	0.064
TSW 99/77C1	mouse	10 ⁸	peak	2.33	1.8-3.5	0.43	0.086
TSW 99/77C1	mouse	10 ⁶	falling	1.95	1.0-2.5	0.30	0.060
TSW 99/77C1	mouse	10 ⁵	rising	1.90	1.1-3.0	0.36	0.072

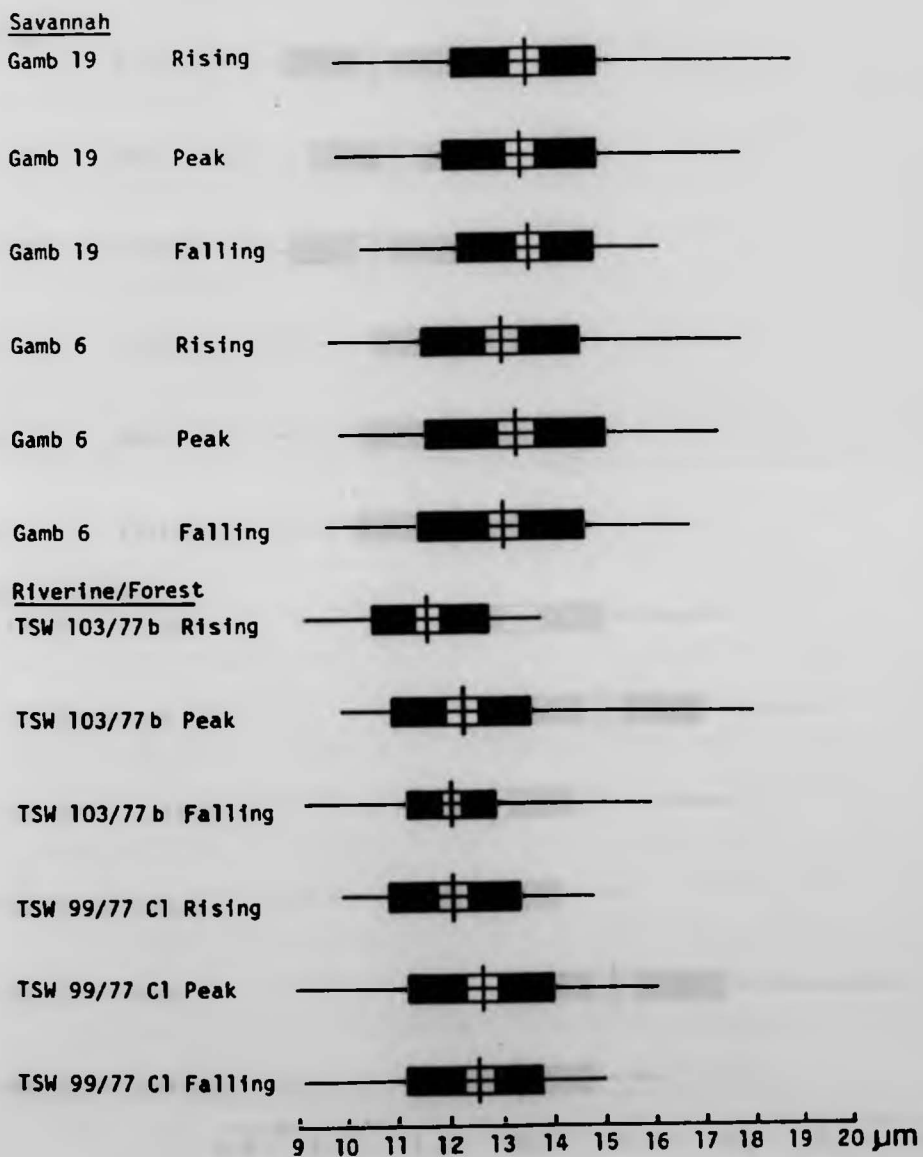


Fig. 10.1.

Dice - Leraas diagrams of lengths of four stocks of T. congolense, throughout one parasitaemic wave.

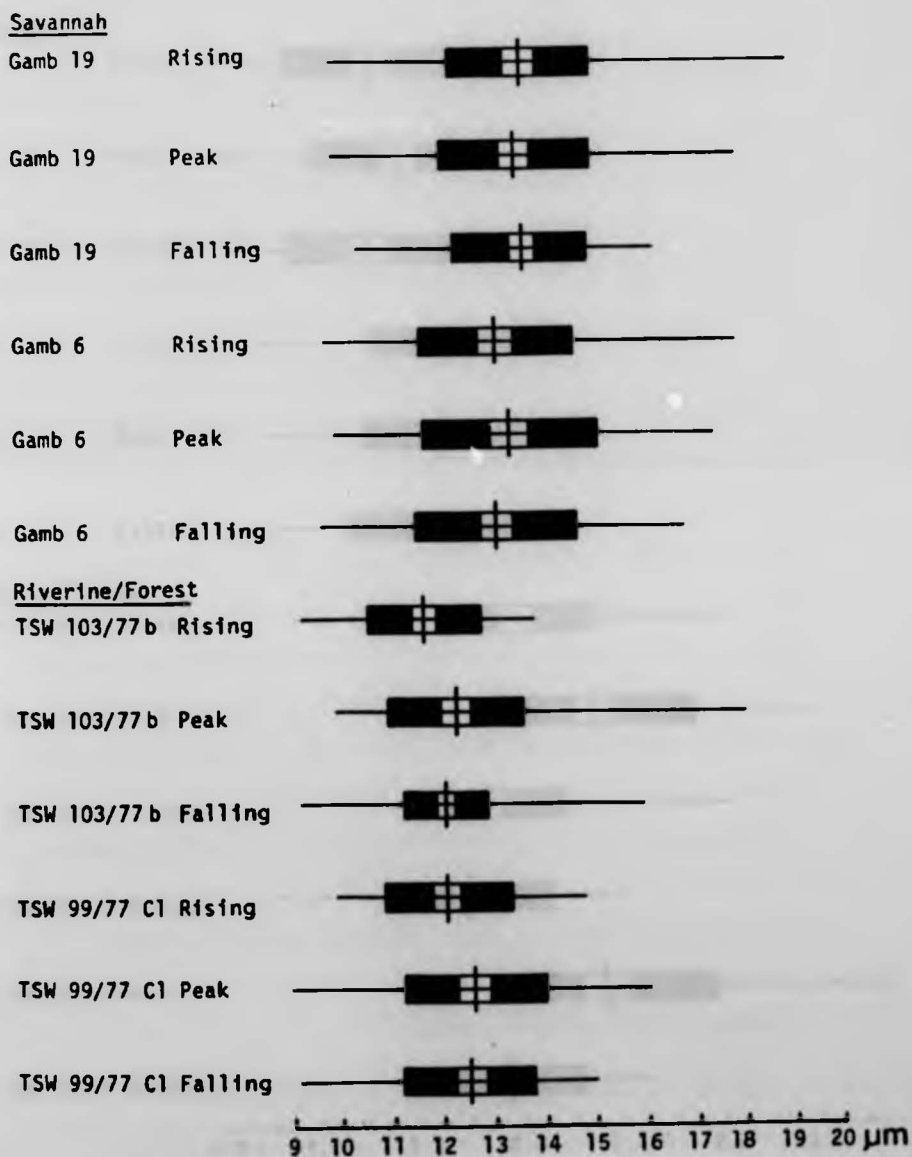


Fig. 10.1.

Dice - Leraas diagrams of lengths of four stocks of T.congolense, throughout one parasitaemic wave.

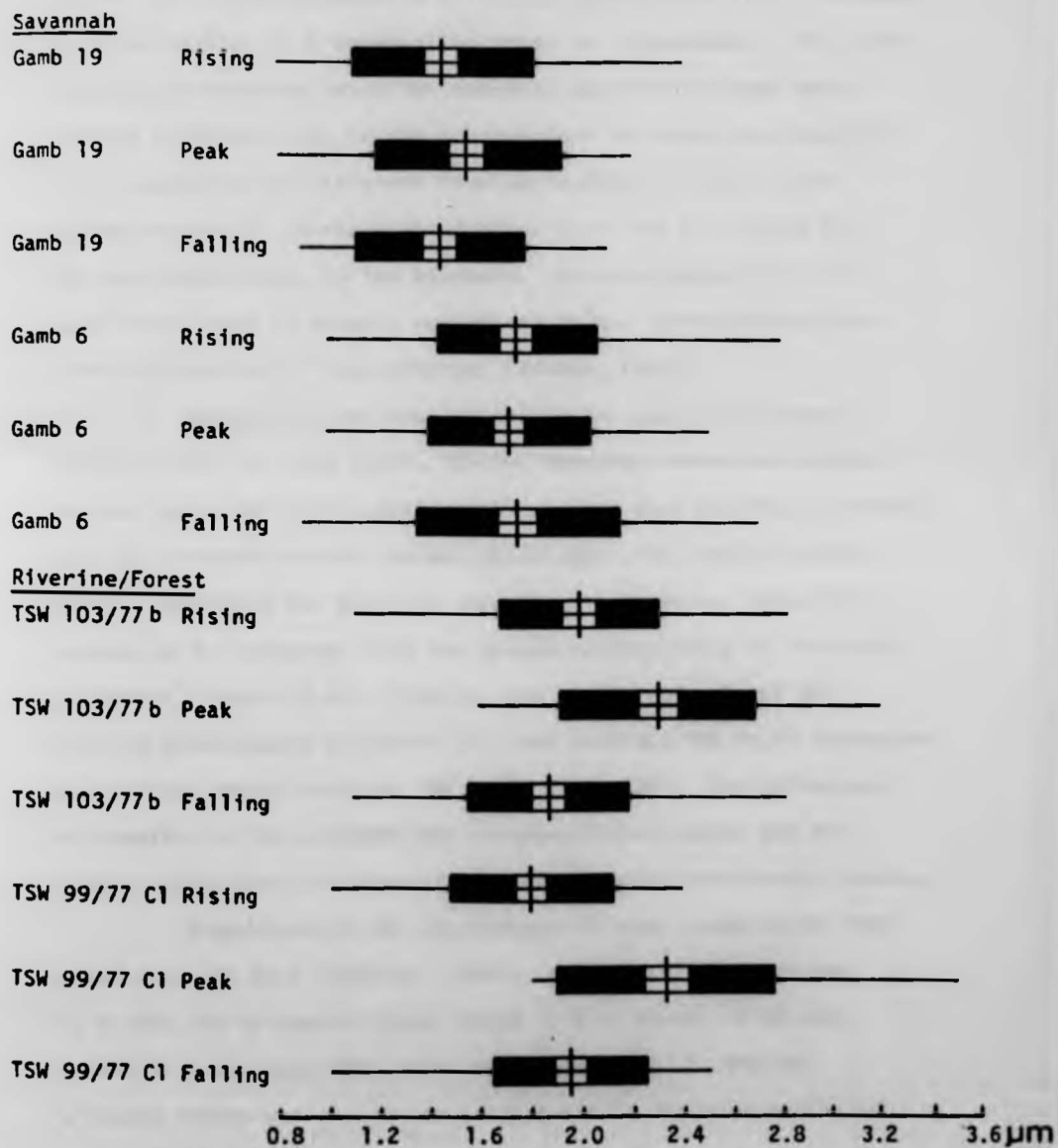


Fig. 10.2.

Dice - Leras diagrams of breadths of four stocks of *T. congolense*, throughout one parasitaemic wave.

are at least 84% separable, a difference which Hoare (1959) accepted as a distinction of a subspecific order in trypanosomes. The white rectangles represent twice the standard error of the mean again plotted on either side of the crossbar and the means are considered to be significantly different from one another if these white rectangles do not overlap; the further apart the rectangles lie, the more significant is the distance. In this simple test, the confidence limit is roughly equivalent to the corresponding limit $P = 0.05$ used for χ^2 distribution (Fisher, 1946).

Considering the four stocks at the same parasitaemic levels (Figs. 10.1 and 10.2), the two savannah stocks are similar to each other but have significantly greater mean lengths ($P = 0.05$) than the riverine/forest stocks, which again are similar to each other. Providing the level of parasitaemia is known, these four stocks can be separated into two groups corresponding to two major zymodemes (Section 4.4). However, the white rectangle of the falling parasitaemia of Gamb 6 (S) just overlaps the white rectangle of the peak parasitaemia of TSW 99/77 Clone (RF). The difference in breadths of the savannah and riverine/forest stocks was not always statistically different even at the same parasitaemic levels.

Comparison of the percentages of long, transitional and short forms in mice (Godfrey, 1960a), showed a high percentage, 74 to 90%, to be transitional forms in each stock. A greater number of long forms than short forms were found in the two savannah stocks and the converse was found in the riverine/forest stocks (Table 10.4).

Montgomeryi-type forms were seen in the riverine/forest stocks but not in the savannah ones (Table 10.5). These montgomeryi-

Table 10.4. Percentage of different morphological types of T.congolense in four stocks representing two major zymodemes

Stock	Host	Trypanosomes ml ⁻¹ blood	Parasitaemic phase	Morphological type		
				Long	Transitional	Short
<u>Savannah</u>						
Gamb 19	mouse	10 ⁶	rising	18	86	2
Gamb 19	mouse	10 ⁸	peak	21	78	1
Gamb 19	mouse	10 ⁶	falling	25	74	1
Gamb 19	rat	10 ⁶	rising	27	68	5
Gamb 6	mouse	10 ⁶	rising	11	87	2
Gamb 6	mouse	10 ⁸	peak	19	78	3
Gamb 6	mouse	10 ⁶	falling	13	87	0
<u>Riverine/forest</u>						
TSW 103/77b	mouse	10 ⁶	rising	4	83	13
TSW 103/77b	mouse	10 ⁸	peak	3	88	9
TSW 103/77b	mouse	10 ⁶	falling	4	87	9
TSW 99/77C1	mouse	10 ⁶	rising	2	90	8
TSW 99/77C1	mouse	10 ⁸	peak	3	90	7
TSW 99/77C1	mouse	10 ⁶	falling	4	84	7
TSW 99/77C1	mouse	10 ⁵	rising	3	89	8

Table 10.5. Percentage of montgomeryi-forms found in four stocks of T.congolense representing two major zymodemes

Stock	Parasitaemic phase		
	Rising	Peak	Falling
<u>Savannah</u>			
Gamb 19	0	0	0
Gamb 6	0	0	0
<u>Riverine/forest</u>			
TSW 103/77b	14	32	2
TSW 99/77c1	0	19	2

forms should not be confused with the swollen or damaged individuals often encountered at the edges of Giemsa-stained thin blood films (Plate 10.2). The cytoplasm of these individuals is faintly stained compared to healthy specimens. Plates 10.1 and 10.2 were both taken from the same Giemsa-stained thin blood films of TSW 99/77 Clone (RF).

8 to 69% of the trypanosomes in the four stocks appeared to possess a free flagellum on examination by light microscopy (Table 10.6). The lowest number of individuals possessing a free flagellum was found in Gamb 19 (S) and the highest number in TSW 99/77 Clone (RF), which exhibited 69% at the parasitaemic peak by light microscopy and 53% by SEM. By light microscopy, flagella up to 2.0 μm were found, but by SEM, judged from pore-size, the maximum was about 1.0 μm (Plates 10.5 to 10.8).

10.4. Discussion

The two savannah stocks were generally longer and narrower than riverine/forest stocks. The Dice-Leraas diagrams allow easy visual confirmation of these results and also a statistical comparison. This method has previously been used for biometrical studies of trypanosomes by Davis (1952, 1969), Hoare (1956, 1959), Diamond (1965), Keymer (1969) and Letch (1979).

unc X The differences between lengths of the savannah and riverine/forest stocks was only statistically significant when comparing each parasitaemic level separately; the mean lengths of Gamb 6 (S) at a falling parasitaemia were not significantly different from the length of TSW 99/77 Clone (RF) at its peak parasitaemia. Thus, biometrical studies of these stocks without reference to the parasitaemic level cannot be used for differentiation. This could possibly be overcome

Plates 10.1. to 10.4. Giemsa-stained thin blood films of
T. congolense

(Scale = 20 μ m)

Plate 10.1. Diverse forms of T. congolense TSW 99/77 Clone (RF)
in mouse blood

- A - short form
- B - transitional form
- C - montgomeryi form

Plate 10.2. Swollen and damaged forms at the edges of a blood
film of TSW 99/77 (RF) Clone

Plates 10.3. and 10.4. T. congolense, Gamb 19 (S), in rat blood

- D - long form

Plates 10.1. to 10.4. Giemsa-stained thin blood films of
T. congolense

(Scale = 20 μ m)

Plate 10.1. Diverse forms of T. congolense TSW 99/77 Clone (RF)
in mouse blood

- A - short form
- B - transitional form
- C - montgomeryi form

Plate 10.2. Swollen and damaged forms at the edges of a blood
film of TSW 99/77 (RF) Clone

Plates 10.3. and 10.4. T. congolense, Gamb 19 (S), in rat blood

- D - long form

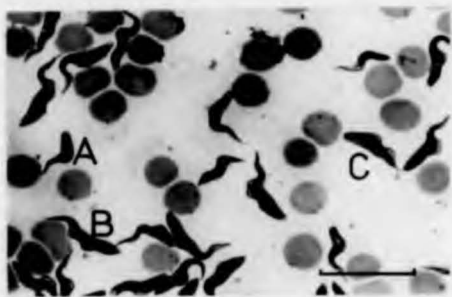


Plate 10.1.

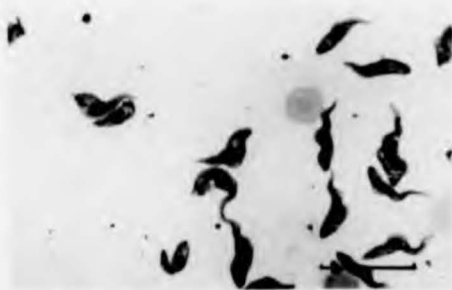


Plate 10.2.

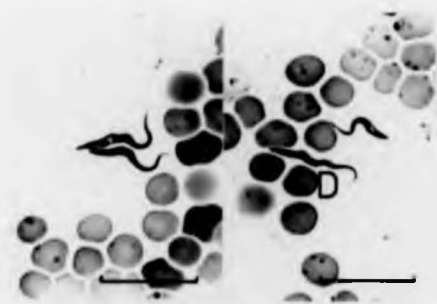


Plate 10.3. Plate 10.4.

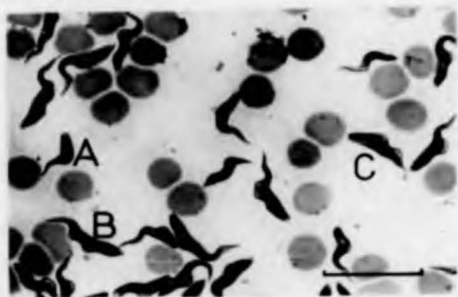


Plate 10.1.

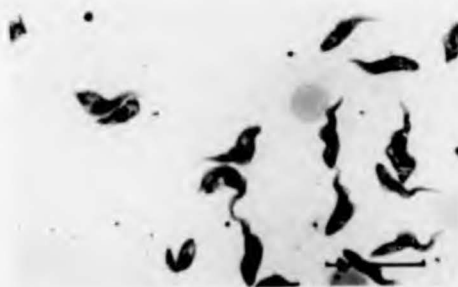


Plate 10.2.



Plate 10.3. Plate 10.4.

Table 10.6. Percentage frequency and lengths of free flagella in four stocks of T. congolense representing two major zymodemes

Stock	Trypanosomes ml ⁻¹ blood	Parasitaemic phase	Mean µm	Range µm	%
<u>Savannah</u>					
Gamb 19	10 ⁶	rising	0.94	0.4-2.0	19
Gamb 19	10 ⁸	peak	0.83	0.5-1.2	16
Gamb 19	10 ⁶	falling	0.74	0.4-2.0	10
Gamb 6	10 ⁶	rising	0.87	0.5-2.0	25
Gamb 6	10 ⁸	peak	0.68	0.2-1.1	34
Gamb 6	10 ⁶	falling	0.96	0.5-1.8	16
<u>Riverine/forest</u>					
TSW 103/77b	10 ⁶	rising	0.92	0.7-1.5	13
TSW 103/77b	10 ⁸	peak	0.99	0.5-1.9	41
TSW 103/77b	10 ⁶	falling	0.78	0.4-1.0	8
TSW 99/77C1	10 ⁶	rising	0.96	0.5-1.8	42
TSW 99/77C1	10 ⁸	peak	0.92	0.2-2.0	69
TSW 99/77C1	10 ⁶	falling	0.88	0.5-1.5	15

Plates 10.5. and 10.6. Scanning electron micrographs of
T. congolense. TSW 99/77 Clone

Plate 10.5. Trypanosome showing free flagellum x 10,000

Plate 10.6. Trypanosome showing free flagellum x 20,000

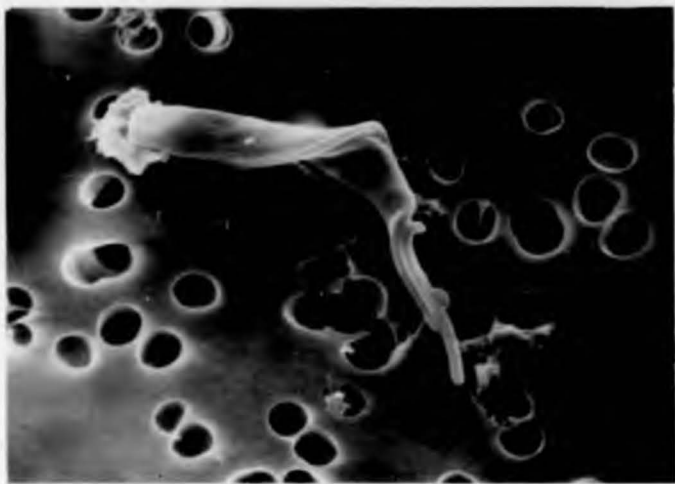


Plate 10.5.



Plate 10.6.

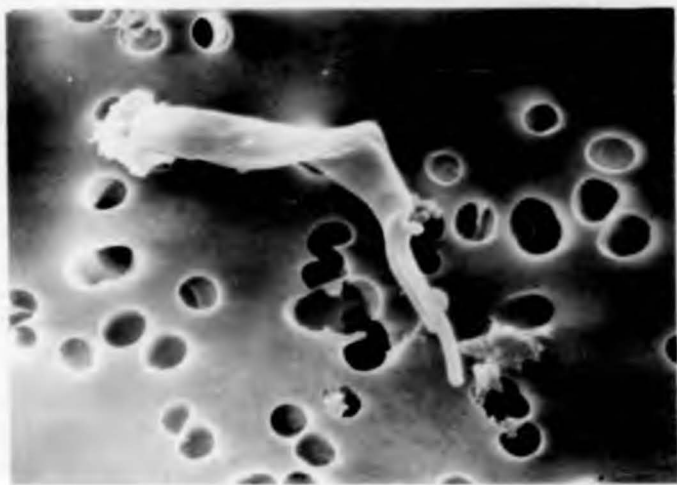


Plate 10.5.



Plate 10.6.

Plates 10.7. and 10.8. Scanning electron micrographs of
T. congolense. TSW 99/77 Clone

Plate 10.7. Anterior end of two trypanosomes showing free
flagella x 30,000

Plate 10.8. Trypanosome showing free flagellum x 20,000

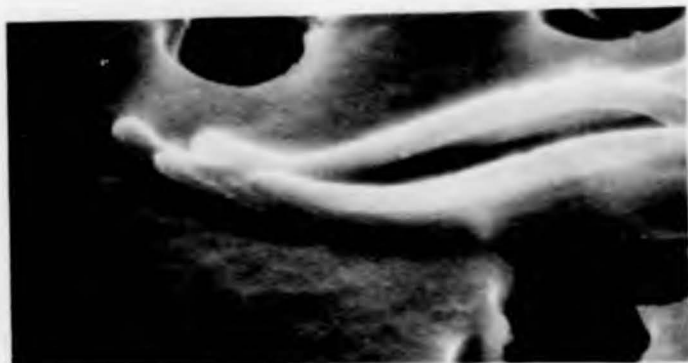


Plate 10.7.



Plate 10.8.



Plate 10.7.

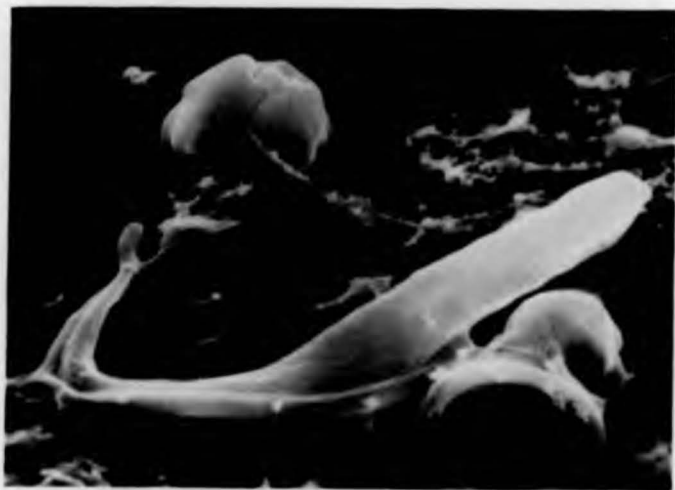


Plate 10.8.

Plates 10.9 and 10.10 Scanning electron micrographs of
T.congolense, TSW 99/77 Clone

Plate 10.9. Dividing trypanosome with no free flagellum
x 10,000

Plate 10.10. Anterior end of trypanosome with no free flagellum
x 30,000



Plate 10.9.



Plate 10.10.



Plate 10.9.



Plate 10.10.

by measuring trypanosomes only when in the first stage of division as suggested by Huisenga (1969), but this would limit the investigation to an increasing population.

The riverine/forest stocks were generally broader than the savannah stocks; however, even at the same parasitaemic levels this distinction could not always be seen. It was most marked at the parasitaemic peak where there was a broadening of the riverine/forest stocks to a much greater extent than in the savannah ones. 32% of the trypanosomes examined at a parasitaemic peak of TSW 103/77b (RF) conformed to the early descriptions of T. montgomeryi with length to breadth ratios of 4.8:1 or less. The appearance of these forms as 19% of a cloned population (TSW 99/77 Clone (RF)) demonstrates that these forms do not multiply as a separate entity; no such form was seen in the savannah stocks. It seems incorrect to regard montgomeryi-forms as 'atypical' (Stephen, 1963) when, during this present work, they were found to constitute such a large proportion of the population, or indeed as apparently pure infections described by earlier workers (Montgomery and Kinghorn, 1909b; Kinghorn and Yorke, 1912a; Kinghorn et al., 1913; Gillian, 1937). The higher percentage of these forms found at the peak parasitaemia than at other times, in the riverine/forest stocks, upholds Gillian's observations (1937) that they appear in the congolense-group when the host is overcoming the illness or dying.

From the earliest reports of T. congolense-like organisms, differences in behavioural characteristics of various stocks were recorded. Morphological studies have been made of these behaviourally different stocks in an attempt to classify or characterize them. Laveran and Mesnil (1912) reported that stocks of T. congolense with

short mean lengths were more host restricted and less pathogenic than stocks with longer mean lengths; this difference in pathogenicity was confirmed by Godfrey (1961). Chardome and Peel reported forms of intermediate length which infected only pigs (Chardome and Peel, 1954; Peel and Chardome, 1954b). From these morphological investigations arose innumerable species based on minor variations but examination, in this study, of a cloned population (TSW 99/77 Clone (RF)) reveals numerous different forms (Plate 10.1); this diversity was seen in every stock examined, for example Gamb 19 (Plates 10.3 and 10.4). From this work, each stock, especially those originating from riverine/forest regions, could have been placed in different species if examined at different phases of the parasitaemia.

Investigations into the proportions of long, transitional and short forms (Godfrey, 1960a) of the four stocks, indicated that in mice, the majority of trypanosomes were transitional forms. The savannah stocks displayed more long forms than short and the converse was found with the riverine/forest stocks. Godfrey (1960a), investigating the different forms in cattle, sheep, goats and dogs, suggested three types of T. congolense based on the percentages of long, transitional and short forms; congolense-type, intermediate-type and dimorphon-type. The four stocks used in this study did not fall into any of these categories having too many transitional forms, except for Gamb 19 (S) in a rat which placed this stock in the dimorphon-type category. The differences in ratios of long to short forms were constant in the four stocks; the high numbers of transitional forms may be due to the host which differed from those investigated by Godfrey (1960a) or, since great difficulty was experienced in distinguishing between the forms, many may have been scored as transitional, whereas Godfrey (1960a) would have recorded them as

one of the extreme forms.

Nantulya et al. (1978b) in a similar morphological investigation of T.congolense suggested that these forms occur in different proportions during the parasitaemic wave. So, depending on the parasitaemic phase at which the trypanosome was examined it would be termed as congolense-, intermediate- or dimorphon- type. Nantulya et al. (1978b) also suggested that the mean length of the population depended on the predominant morphological type and this varied significantly during the course of each parasitaemic wave. They add further evidence to this idea from Hoare's (1959) earlier work on a T.congolense infection in a rat, the mean length of the trypanosome population altered on successive days. In Gamb 6 (S) and TSW 103/77b (RF) there appeared to be an increase in the mean length of the trypanosomes with a rise in parasitaemia but it could not be correlated with any change in the forms present.

Further confusion arises over the results of Nantulya et al. (1978b) in that the morphology of their stocks could easily be altered by rapid syringe passaging. They state that, 'a rapid syringe subpassage at 3-4 day intervals of such a dimorphic trypanosome population in mice resulted into a shift to the short T.congolense-type appearance.' From other results they suggest that at low parasitaemias the overall impression was always congolense-type. Possibly, they are implying that these stocks lost their pleomorphism with rapid and prolonged subpassaging as seen in the T.brucei-complex (Murgatroyd et al., 1937; Fairbairn and Culwick, 1947). No indication is given of the number of rapid subpassages needed to reduce the pleomorphism in these T.congolense stocks. Additionally, it is felt that the concentration of trypanosomes from low parasitaemias by haematocrit centrifugation, as used by Nantulya et al. (1978b), could affect the morphological

appearance of these delicate flagellates.

Despite numerous reports of minute free flagella in trypanosome stocks, now believed to be T.congolense (Schwetz, 1931; Chardome and Peel, 1954; Stephen, 1963; Hoare, 1972), it is generally regarded as being devoid of a free flagellum. In 1909(d), Montgomery and Kinghorn suggested that the cytoplasm at the anterior end of the body extended further than was visible under the light microscope. This view is still held by many modern workers and coupled with a possible shrinkage of the body cytoplasm during methanol fixation, it is thought that the free flagellum of T.congolense could be an artifact. Recently, free portions of flagella have been seen by transmission electron microscopy (D.S. Ellis, personal communication) but this may be due to damage or tearing during the preparation of the specimen. In this study, SEM of TSW 99/77 Clone (RF) showed that in some individuals the flagellum extended for a short distance past the end of the body cytoplasm (Plates 10.5 to 10.8) while in others they terminated simultaneously (Plate 10.9 and 10.10). As the results were found in a cloned stock it further illustrates the pleomorphism of T.congolense. Again, it could be said that a greater shrinkage of the body cytoplasm compared with the relatively rigid flagellum structure could have occurred. However, the narrowing anterior end of the body possesses a high proportion of subpellicular microtubules to body volume making such shrinkage unlikely (R.E. Sinden, personal communication). Also, no sign of damage was seen on the flagellum sheath or body wall of these trypanosomes which would undoubtedly have been visible had uneven shrinkage and subsequent tearing occurred.

A higher proportion of free flagella was found from

examination by light microscopy than by SEM in TSW 99/77 Clone (RF) suggesting that in some cases the apparent free flagella seen by light microscopy were accompanied by a thin strip of cytoplasm too fine to be detected. The differences in length of free flagellum seen by light microscopy and SEM indicated that the cytoplasm usually extended at least a short distance further than could be detected by light microscopy. However, the light microscopy work was carried out on trypanosomes in mouse blood whereas the trypanosomes used for the SEM were harvested from rat blood. Thus, this discrepancy could be due to a change in morphology in different hosts (Godfrey, 1960a).

Morphological examination of these four stocks of T.congolense indicated a difference between those originating from savannah regions and those from riverine/forest areas. This differentiation could be seen from the length and breadth of stocks and from the percentages of long to short forms. However, discrepancies may arise from the use of different hosts and workers. It must also be noted that these stocks originated not only from different vegetational regions, but different geographical locations and from different hosts. Further work should be carried out on stocks originating from different vegetational zones but similar geographical regions and perhaps most important of all, from the same species of host.

The subgenus Nannomonas is generally regarded as containing two species, T.simias and T.congolense, separated mainly on host specificity, the predilection of T.simias for the Suidae and its fatality in domestic pigs; further features are the lack of infectivity of T.simias to cattle and laboratory rodents, and its reputed pleomorphism. However, deviations of the normally accepted behaviour

of T. simiae can be found in the literature. Reports have been made of T. simiae infections in cattle, a horse, a sheep and camels (Culwick and Fairbairn, 1947; Pellegrini, 1948; Wilson, 1958; Killick-Kendrick and Godfrey, 1963; Stephen, 1963). As suggested by Godfrey (1960a), T. congolense should no longer be regarded as a monomorphic trypanosome. Even in a cloned stock, pleomorphism is pronounced (Plate 10.1). Thus, both T. simiae and T. congolense are pleomorphic.

Doubts on the validity of this speciation were first expressed by Roberts (1971) and later, by Godfrey (1977, 1979). Both behavioural and morphological characteristics seem inadequate for this speciation as there are too many exceptions to every criterion put forward.

It had been hoped that isoenzyme characterization of T. simiae could have been included in this work, but no lysates were available and importation of pig blood stabilates infected with trypanosomes into Britain is not permitted by the Ministry of Agriculture and Fisheries. As yet, the question of the relationship of T. congolense to T. simiae must remain contentious.

10.5. Conclusions

Morphological differences were displayed between the two major zymodemes of T. congolense, each represented by two stocks. Montgomeryi-forms appeared in the two riverine/forest stocks, one of which was cloned, and were considered as normal forms in these stocks.

From the diverse morphology seen in all four stocks, T. congolense should be regarded as a pleomorphic trypanosome.

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Morphological characteristics can give an indication of the stock type but it cannot be used as a reliable criterion for characterization since it is influenced by too many factors.

CHAPTER 11

11. GENERAL DISCUSSION AND SUMMARY OF RESULTS

11.1. Discussion

11.1.1. Isoenzyme polymorphism

T.(N.) congolense has long since been recognized as displaying a wide diversity of morphological and behavioural characteristics, so it was perhaps not surprising that this species should show such a high degree of polymorphism in 14 enzymes. 71 different isoenzymes were found in different combinations giving 75 zymodemes among the 78 stocks of T.congolense examined.

Three enzymes, NH, PK and TDH, each consisted of a single enzyme band in all the stocks (Figs. 4.1 to 4.3), and the single band of TDH was also common with some T.brucei stocks (Section 4.2.1). In contrast, GPI displayed 15 different isoenzymes (Fig. 4.10). Why then, is there such a high degree of polymorphism in many of the enzymes of T.congolense? Isoenzymes are presumed to have arisen in evolution by the process of gene duplication, or through random genetic events, both point mutations and chromosomal events (Shaw, 1969). However, the reasons for retention of 'new' isoenzymes are in dispute. One explanation is simply genetic drift when these new isoenzymes are neither advantageous nor disadvantageous to the organism (Yamazaki and Maruyama, 1975). The other explanation is natural selection of advantageous mutations permitting optimum survival of the organism under certain conditions (Powell, 1975;

Godfrey, 1979). Natural selection is well known in morphological and anatomical modifications of higher organisms. Additionally, genetic drift would not account for the genetic continuum found in P.falciparum (Carter and Voller, 1975). Genetic drift may however, occur in certain enzymes but not in them all. For example, some selection pressure must be maintaining the apparent single structure of TDH, NH and PK. Despite the extreme polymorphism of some enzymes, certain bands are found common to many of the patterns suggesting that they are important to the organism and maintained by selection pressure. The extreme diversity of the other bands perhaps reflects genetic drift. For example, 15 isoenzymes were found with GPI giving 10 different patterns but one of three isoenzyme bands was present in each pattern (Fig. 4.10). Similarly, MDH displayed 7 patterns with 8 isoenzymes but each pattern contained a common isoenzyme band. Further work should be undertaken to determine the importance of these common bands in characterizing T.congolense.

T.congolense displayed a greater enzyme polymorphism than either T.cruzi (Miles et al., 1980) or members of the subgenus Trypanozoon (Gibson et al., 1980). Is there some pressure being exerted on T.congolense to produce a period of rapid evolution? T.congolense, while being harmful to cattle, is generally believed to produce no ill effects in game animals, indicating that game animals are the natural hosts. However, in recent years, especially in West Africa, there has been a large reduction in the numbers of game animals due to extensive cultivation of the land and, to a lesser extent, their slaughtering for food and skins. Possibly, T.congolense has been forced to enter this domestic cycle to a much greater extent; a cycle to which it is not completely adapted.

If this were the case, the evolutionary pressure on T.congolense would be much greater than on T.brucei, which, in comparison, is relatively harmless to domestic livestock. However, this disappearance of game animals is only very recent in evolutionary terms.

Even if T.congolense is undergoing a period of rapid evolution, why then is there such a variation between every stock? It is extremely unlikely that 75 of the 78 stocks examined had evolved independently, occurring in just one animal in a herd. It is therefore suggested that each stock has the ability to produce numerous isoenzymes which they can 'switch' on and off. Adams and Rosso (1966) working with α -ketoglutaric dehydrogenase of Pseudomonas found that one form of the enzyme was constitutive while the other was inducible and only appeared when the appropriate substrates were present. However, all the samples of T.congolense were grown in the same strain of rat kept under the same conditions and any 'switching' on and off could be expected to be the same. If this phenomenon of 'switching' does occur, this again indicates that the common isoenzymes in the patterns may be more important than those which rarely occur.

Multiple banding could also result from the examination of enzymically mixed populations. Many natural trypanosome infections probably consist of several populations who reach their parasitaemic peaks at different times. Isolation of a population into rodents would depend on which population was predominant at that time. However, it does not appear that the mixed population is maintained in the rodent, as trypanosomes harvested at different times during an infection are enzymically identical (Section 3.4). Additionally, the two cloned stocks of T.congolense produced identical multiple banding to their parent stocks.

From the complexity of isoenzymes, seen in T.congolense, emerged two distinct zymodemes whose separation could be correlated with the vegetation in the area of origin i.e. riverine/forest or savannah. This distinction could be readily seen from GPI, PEP 1 and PEP 2, as well as from computer analysis of 14 enzymes. However, a purely geographical division cannot be ruled out as all the riverine/forest stocks originated from West Africa. But, a geographic rather than vegetational distinction seems unlikely, since savannah stocks from East and West Africa were grouped together while stocks from savannah and riverine regions of Nigeria gave the expected enzyme distinctions.

11.1.2. Zymodeme/Glossina spp. relationships

Godfrey (1979) suggested that 'enzyme modification may allow a trypanosome to be more suited to existence in a particular vertebrate host and vector, and thus ensure survival of the parasite'. Results of preliminary tsetse fly transmission experiments indicated that the two major zymodemes of T.congolense are adapted to transmission by the species of Glossina found in their area of origin (Chapter 7).

Hoare (1972) suggests that salivarian trypanosomes are imperfectly adapted to their vectors which is reflected in the relative ease with which they can lose the ability to develop in the tsetse fly. T.congolense can be repeatedly and successfully syringe passaged but unlike members of the subgenus Trypanozoon it does not appear to lose its ability to infect tsetse flies (Murgatroyd and Yorke, 1937). For example, T.congolense stock S104 was syringe passaged innumerable times over a period of five years in this laboratory and yet was successfully transmitted through G.morsitans

(B.J. Elce, personal communication). In addition, members of the subgenus Trypanozoon and Duttonella have both become established outside the tsetse belts of tropical Africa, relying on mechanical transmission by haematophagous insects other than tsetse flies. Although there are suggestions of T.(N.) simiae being mechanically transmitted in areas where no tsetse flies were believed to occur (Montgomery and Kinghorn, 1909b), neither T.congolense nor T.simiae has become established outside the tsetse belts, suggesting a necessary relationship with Glossina. This relationship seems to have developed further to produce better transmission of a T.congolense stock by the species of Glossina present in the area of isolation of the stock (Chapter 7).

11.1.3. Morphology

The two riverine/forest stocks could be distinguished by their morphological appearance from two savannah stocks especially at the peak parasitaemias. Montgomeryi-type individuals were found in both the riverine/forest stocks but not in the savannah isolates.

During the morphological examinations of these stocks the presence of a free flagellum was noted in many individuals, which was confirmed by scanning electron microscopy.

What is the purpose of a free flagellum and undulating membrane seen in many of the salivarian trypanosomes? Léger (1904) believed the formation of the undulating membrane to be an adaptation to life in the bloodstream. Although, these flagellates do not need a form of locomotion in the bloodstream unless they are to remain stationary. However, if trypanosomes move with the flow of blood they will also move with their metabolic waste products so possibly

the motion of the undulating membrane and free flagellum creates small eddies which remove the waste products from their immediate vicinity. If this is the case, how does T.congolense manage with its lack of a long free flagellum or well developed undulating membrane? T.congolense tends to congregate in the capillaries where it binds to the capillary walls (Banks, 1978) and thus the flow of blood will remove the waste products.

This creation of currents round the body may be of particular importance when trypanosomes invade the tissues. T.congolense, until recently, was generally regarded as strictly a blood plasma parasite despite occasional reports of occult or tissue forms (Schwetz, 1928; Schwetz and Fomara, 1929; Fiennes, 1950a, 1950b) but conclusive evidence of their existence in connective tissue at the site of an infected bite and in lymph nodes has been produced (Luckins and Gray, 1978, 1979a; Gray and Luckins, 1979). Thus certain forms, newly released from the connective tissues or those ready to re-invade the tissues, may possess a short free flagellum and better developed undulating membrane to cope with the removal of metabolic waste products.

11.1.4. Drug Sensitivity

The two riverine/forest stocks were less sensitive to Ethidium bromide and Berenil than two savannah isolates. It has been suggested that T.congolense stocks may vary in their ability to produce tissue forms which is reflected in their sensitivity to drugs (see Chapter 8). The stocks which are less sensitive to Ethidium bromide and Berenil also had a greater tendency to possess free flagella, particularly at the parasitaemic peaks; possibly it is these forms which invade the tissues.

This distinction in drug sensitivity may be of considerable practical importance as the less sensitive stocks will need higher drug doses to prevent breakthrough of the infection. Also, if low doses are repeatedly administered, truly resistant forms may emerge.

This difference in drug sensitivity illustrates the need to examine representatives of the two major zymodemes, if not some minor zymodemes as well, before generalizations to the species can be made.

11.1.5. Infectivity of stocks and susceptibility of mice strains to disease

The enzymic distinction of T.congolense could not be correlated with the infectivity of stocks to mice or their susceptibility to disease caused by T.congolense.

Jennings et al. (1978) and Morrison et al. (1978) both found C57Bl inbred mice to be one of the most resistant mouse strains to disease caused by T.congolense. This result was only found to be true for one of three stocks examined during this work (Chapter 9). The four stocks used by Jennings et al. (1978) and Morrison et al. (1978) were all undefined and it was presumably by chance that they each produced a similar response. Jennings et al. (1978) state that, 'in general, stabilates of T.congolense tend to be more pathogenic [than T.brucei] to the mouse resulting in death within three weeks of infection which is often too short a time for many observations'. However, extreme pathogenicity and fatality of T.congolense was not generally found to be the case during the course of this work; many stocks took several months to reach a high parasitaemia, indeed a few stocks never produced parasitaemias high enough to allow separation of the trypanosome from the blood; some stocks of

T.congolense failed to infect mice at all (Chapter 6). Many workers have demonstrated differences in pathogenicity or the course of infection with T.congolense including chronic low grade infections (Montgomery and Kinghorn, 1909c; Bevan, 1910; Jowett, 1910; Bruce et al., 1911b; Blacklock and Yorke, 1913; Bruce et al., 1913a; Yorke and Blacklock, 1915; Binns, 1938; Godfrey, 1961). It is suggested that the lack of chronic laboratory strains arises from the preference of research workers to choose fast growing stocks both at the time of isolation and for future work rather than its being a characteristic of T.congolense. Thus, stocks are often selected by research workers for a particular characteristic, rapid growth, which is then enhanced by repeated syringe passaging, resulting in an abnormal, but easy to handle, stock.

The different results obtained in inbred mice during this work again illustrates the need to use numerous recently isolated defined stocks before generalizations to the species can be made.

Jennings et al. (1978) and Morrison et al. (1978), investigating the responses of different strains of inbred mice, were attempting to find a suitable laboratory host for the study of trypanotolerance as displayed by certain breeds of cattle, for example, N'dama and Mutura. During this work however, it was shown that the infecting trypanosome stock affected the susceptibility to disease of these inbred mice (Chapter 9); the response varied with different trypanosome stocks. Thus, one may speculate that certain stocks of T.congolense may be fatal even to N'dama and Mutura cattle. These cattle are believed to have an innate ability to acquire immunity to trypanosomiasis, but it must be augmented by challenge when the animal is young (Roberts and Gray, 1973). If these cattle are moved and experience a new challenge, they may become infected

and succumb to the disease. Possibly their immunity cannot overcome the new stocks or it may be the presence of these 'fatal' stocks in the new environment.

11.1.6. Future work

The two major zymodemes appeared to be correlated with different vegetational regions and hence different species of Glossina. However, a purely geographical distinction, although unlikely, cannot as yet be ruled out and many more stocks need to be examined, especially those isolated from riverine/forest regions of East Africa. With the addition of more information the hypothesis of a vegetation/Glossina spp./zymodeme relationship may have to be modified.

The significance of the different minor zymodemes is still unclear and requires further investigation. Also there are many more enzymes which with the development of new staining systems should be examined. Certain isoenzymes may prove to be of greater significance than others, but will only be distinguished after examination of more stocks and perhaps with the aid of computer analysis.

11.2. Summary of Results

1. 71 isoenzymes giving 75 zymodemes were found among 78 stocks of T. congolense examined with 14 enzymes by thin-layer starch-gel electrophoresis.
2. Each enzyme differed in its degree of polymorphism. Only 1 isoenzyme band was seen for TDH, NH and PK; 5 different isoenzyme bands and 2 patterns for GAPDH; 4 isoenzyme bands and 4 patterns for PGM; 3 isoenzyme bands and 4 patterns for

ALAT; 5 isoenzyme bands and 5 patterns for ME; 8 isoenzyme bands and 6 patterns for MDH; 15 isoenzyme bands and 10 patterns for GPI; 14 isoenzyme bands and 14 patterns for PEP 2; 11 isoenzyme bands and 18 patterns for PEP 1. PEP 1 included peptidases S, B and E and PEP 2, peptidases S, B and A.

3. 8 of the 14 enzymes examined readily distinguished T. congolense from representatives of the subgenera Duttonella and Trypanozoon. The distinguishing enzymes were PK, NH, GAPDH, ASAT, PGM, ME, ALAT and GPI.
4. From computer analysis of the isoenzyme data, the percentage similarities of stocks and Furthest Neighbour Hierarchical Cluster Analysis demonstrated a major dichotomy between stocks originating from savannah or riverine and forest areas. This distinction could be readily seen from GPI, PEP 1 and PEP 2 zymograms.
5. There was an indication that the savannah stocks were transmitted more efficiently than riverine/forest stocks by the savannah tsetse fly G. morsitans, suggesting a relationship between the zymodeme and the species of Glossina present in the same area.
6. Two savannah stocks were more sensitive to Berenil and Ethidium bromide than two riverine/forest stocks.
7. The infectivity to outbred TO mice of representatives of the two major zymodemes was similar.
8. The susceptibility to disease of two strains of inbred mice, AJ and C57Bl, and one outbred strain, TO, varied with the infecting trypanosome stock; there was no correlation with the two major zymodemes.

9. There was a morphological distinction between four stocks of T.congolense representing the two major zymodemes. Montgomeryi-forms were found only in the riverine/forest stocks, one of which was a cloned population. The presence of a short free flagellum in some individuals of a cloned T.congolense stock was verified by scanning electron microscopy.

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APPENDIX I

LIST OF MATERIALS

Suppliers:

B	-	Boehringer Mannheim GmbH, Biochemica
BD	-	Beckton and Dickinson
BDH	-	BDH Chemicals
C	-	Cryoservice Limited
Ca	-	CalBioChem
Co	-	Connaught Laboratories
D	-	Distillers Company
E	-	Evans
EM	-	EMscope
FH	-	Farbwerke Hoechst AG
G	-	Gibco Biocult Diagnostic
HW	-	Hopkins and Williamson
J	-	Jannesson Pharmaceutical Company
K	-	Koch-Light
S	-	Sigma Chemical Company
W	-	Wessex Chemicals (now supplied through Sigma)
Wh	-	Whatman

<u>Material</u>	<u>Supplier</u>
Acetone (AnalaR)	BDH
Adenosine-5-diphosphate (ADP) (equine muscle, Na Salt)	S
Agar, L28	BDH
L-alanine	S
Aldolase (rabbit muscle)	S
L-amino acid oxidase (crude <u>Crotalus adamanteus</u> venom)	S
ϵ -amino-n-caproic acid	BDH
3-amino-9-ethyl carbazole	S
L-aspartic acid	S
Berenil (diamidine aceturate)	FH
Boric acid (H_3BO_3) (AnalaR)	BDH
Carbon dioxide, liquid	D
Citric acid AnalaR)	BDH
S-collidine	EM
Cyclophosphamide (Endoxana)	K
Diethylaminoethyl (DEAE) cellulose (DE 52)	Wh
DL-dithiothreitol	S
Ethidium bromide	S
Ethylene diaminetetra-acetic acid (EDTA) (AnalaR)	BDH
Foetal calf serum	G
Fructose-6-phosphate (di Na salt)	S
Fructose-1,6-diphosphate (tri Na salt)	W
Giensa stain	HW
Glucose (AnalaR)	BDH
Glucose-1-phosphate with 1% W/W glucose-1,6-diphosphate	W
Glucose-6-phosphate (mono Na salt)	S
Glucose-6-phosphate dehydrogenase (Baker's yeast, type XV)	S

<u>Material</u>	<u>Supplier</u>
Gluteraldehyde (25%)	EM
Glycerol (AnalaR)	BDE
Glycine (AnalaR)	BDE
Glycyl-L-leucine	S
Heparin	E
Hexokinase	S
Hydrochloric acid (HCl) (AnalaR)	BDE
Hypnorm	J
Inosine	B
DL-isocitric acid (tri Na salt)	S
L-isoleucyl-L-proline	Ca
Lactate dehydrogenase (hog muscle, 50% glycerol solution)	B
L-leucyl-L-alanine	S
L-leucylglycine	S
L-leucylglycylglycine	S
L-leucyl-L-leucyl-L-leucine	S
L-leucyl- ρ -nitroanilide	S
Lithium hydroxide (LiOH) (AnalaR)	BDE
L-lysyl-L-leucine	S
Magnesium chloride (MgCl ₂) (AnalaR)	BDE
Malate dehydrogenase (pig heart, 50% glycerol solution)	B
Maleic acid	BDE
DL-malic acid	S
Manganese chloride (MnCl ₂) (AnalaR)	BDE
2-mercaptoethanol	S
Methanol (AnalaR)	BDE
MTT (tetrazolium)	S

<u>Material</u>	<u>Supplier</u>
NAD (nicotinamide adenine dinucleotide)	S
NADH (di Na salt)	B
NADP (mono Na salt)	W
Nitrogen, liquid	C
2-oxoglutaric acid (= α -ketoglutarate) (mono K salt)	S
Peroxidase (horseradish)	S
Phenazine methosulphate (PMS)	S
Phosphoenol pyruvic acid (monocyclohexylamine salt)	S
Phosphogluconic acid (tri Na salt)	W
Phosphoric acid (H_3PO_4) (AnalaR)	BDE
Potassium chloride (AnalaR)	BDE
Potassium dihydrogen phosphate (KH_2PO_4) (AnalaR)	BDE
Potassium hydroxide (AnalaR)	BDE
Sodium arsenate (AnalaR)	HW
Sodium chloride (NaCl) (AnalaR)	BDE
Sodium dihydrogen phosphate (NaH_2PO_4) (AnalaR)	BDE
di Sodium hydrogen phosphate (Na_2HPO_4) (AnalaR)	BDE
Sodium hydroxide (NaOH) (AnalaR)	BDE
Starch (hydrolysed potato)	Co
L-threonine	S
Tris (hydroxymethyl) methylamine (AnalaR)	BDE
L-valyl-L-alanine	S
L-valyl-L-leucine	S
Vacutainer system	BD
Xanthine oxidase (milk)	S

APPENDIX II

STOCK HISTORIES

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Notes

- No. - stock number corresponding to computer number in Appendices IV and V
- Year - year of isolation
- p - number of passages before enzyme characterization
? - number unknown, probably large.

No.	Stock	Host	Country	Locality	Year	P
<u>West Africa</u>						
1	Gamb 1	Bovine	The Gambia	Keneba	1977	4
2	Gamb 2	"	"	"	"	4
3	Gamb 3	"	"	"	"	5
4	Gamb 4	"	"	Kantong Kunda	"	4
5	Gamb 5	"	"	Keneba	"	5
6	Gamb 6	"	"	"	"	6
7	Gamb 7	"	"	"	"	4
8	Gamb 9	"	"	"	"	3
9	Gamb 10	"	"	"	"	4
10	Gamb 11	"	"	Dankunku	"	3
11	Gamb 12	"	"	Keneba	"	4
12	Gamb 13B	"	"	"	"	4
13	Gamb 13Y	"	"	Juoli	"	5
14	Gamb 14	"	"	Essau	"	4
15	Gamb 15	"	"	Fuga	"	6
16	Gamb 16	"	"	Dutabulu	"	4
17	Gamb 17	"	"	"	"	4
18	Gamb 18	"	"	"	"	4
19	Gamb 19	"	"	Essau	"	4
20	Gamb 20	"	"	"	"	4
21	Gamb 21	"	"	"	"	5
22	Gamb 22	"	"	"	"	3
23	Gamb 23	"	"	Keneba	"	4
24	Gamb 26	"	"	"	"	4
25	Gamb 27	"	"	Essau	"	5
26	Gamb 28	"	"	Keneba	"	3
27	Gamb 29	"	"	"	"	5
74	LUMP 89	"	Nigeria	Zaria	?	?
77	1/148 FLY	"	"	nr. River Donga	1960	?
70	TREU 1290 WA 1	"	"	Zaria	1967	?
73	TREU 1095 WA 2	"	"	Ibadan	1971	?
52	IBADAN 34	"	"	Egun	1977	6
53	IBADAN 35	"	"	Hansa	"	4
54	IBADAN 44	"	"	"	"	4
55	IBADAN 69	"	"	Obanigbe	"	5
33	LRU TSW 13	Pig	Liberia	Ganta	1975	11
37	LRU TSW 29	"	"	"	"	7.9
31	LRU TSW 4/77	"	"	Bong	1977	5
32	LRU TSW 6/77	"	"	"	"	5.9
34	LRU TG 14/78	Goat	"	Tonpayah	1978	6

No.	Stock	Host	Country	Locality	Year	P
39	LRU TSW 78/77	Pig	Liberia	Ganta	1977	5,6
40	LRU TSW 94/77	"	"	"	"	7
41	LRU TSW 99/77	"	"	"	"	7
42	LRU TSW 103/77b	"	"	Sanniquelle	"	6
43	LRU TSW 108/77	"	"	"	"	7
44	LRU TSW 114/77	"	"	"	"	4,5
45	LRU TSW 115/77	"	"	"	"	7
49	LRU TSW 243/77	"	"	Kissi	"	4
50	LRU TSW 251/77	"	"	"	"	4
30	TSW 3/78E(020)	"	Ivory Coast	Koudougou	1978	4
35	TSW 25/78E(020)	"	"	"	"	4,7
36	TD 28/78E(020)	Dog	"	"	"	2,7
38	TD 56/78E(021)	"	"	Sietlinfla	"	4,6
46	TSW117/78E(026)	Pig	"	Koudougou	"	4
47	TSW152/78E(027)	"	"	Koetinga	"	4
48	TSW153/78E(027)	"	"	"	"	5
50	TD378/78E(037)	Dog	"	Kowesi	"	5
			<u>East Africa</u>			
56	EATRO 1564	<u>G. pallidipes</u>	Uganda	Lugala	1970	6
57	EATRO 1582	"	"	"	"	5
58	EATRO 1599	"	Kenya	Aitong	"	4
59	EATRO 1712	"	"	"	"	3
60	EATRO 1751	"	"	"	"	3
61	EATRO 1755	"	"	"	"	5
62	EATRO 1816	Bovine	Tanzania	Serengeti	"	4
63	EATRO 1841	<u>Gazella granti</u>	"	"	"	6
64	EATRO 1967	Bovine	Uganda	Lugala	1971	4
65	EATRO 1968	"	"	"	"	4
66	EATRO 2025	"	Tanzania	Ikoma	1972	4
67	EATRO 2027	"	"	"	"	5
68	EATRO 2033	"	"	"	"	6
69	EATRO 2084	"	Uganda	Bukedi	1973	7
71	TREU 1291 EA 1	<u>G. pallidipes</u>	Kenya	Yimbo	1961	?
72	TREU 1381 EA 2	<u>Gazella granti</u>	Tanzania	Serengeti	1966	?
75	MIAG 108 Clone	Lion	"	"	1971	?
76	S104/FLY/BE	<u>Gazella granti</u>	"	"	1966	?
78	SUBAKIA	?	Kenya	Nairobi	1961	?
28	TDRN 9	Impala	Zambia	Luangwa Valley	1977	?
29	TDRN 14	Kuolu	"	"	"	?

APPENDIX III

ENZYME PATTERNS IN 78 STOCKS

NE, TDE and PK gave only one pattern for all 78 stocks

No.	Stock	GAPDH	ASAT	PGM	ALAT	ME	MDH	GPI	PEP 1	PEP 2
1	Gamb 1	I	I	II	II	IV	I	I	I	III
2	Gamb 2	I	I	I	III	II	I	I	I	IV
3	Gamb 3	I	III	I	III	IV	I	I	IV	III
4	Gamb 4	I	III	I	II	II	I	I	VII	I
5	Gamb 5	I	III	I	V	IV	IV	I	IV	I
6	Gamb 6	II	II	I	II	IV	I	I	I	VII
7	Gamb 7	I	II	I	V	IV	IV	I	II	IV
8	Gamb 9	I	III	I	III	II	I	I	VII	I
9	Gamb 10	I	I	II	III	IV	I	I	II	IV
10	Gamb 11	I	II	I	III	II	I	I	VII	I
11	Gamb 12	I	III	II	III	II	I	I	I	I
12	Gamb 13B	I	I	I	III	II	I	I	IV	I
13	Gamb 13Y	I	I	I	II	I	V	I	V	I
14	Gamb 14	I	II	I	III	II	I	I	II	I
15	Gamb 15	I	II	I	II	III	I	I	I	IV
16	Gamb 16	II	II	II	III	V	I	I	I	VI
17	Gamb 17	I	I	I	II	II	I	I	IV	I
18	Gamb 18	II	II	II	V	III	I	I	I	I
19	Gamb 19	II	III	I	II	II	IV	I	IV	VII
20	Gamb 20	II	III	I	II	IV	I	I	IV	VII
21	Gamb 21	I	IV	I	V	IV	I	I	IV	I
22	Gamb 22	I	I	I	V	II	VI	I	IV	I
23	Gamb 23	II	I	II	II	II	I	I	I	IV
24	Gamb 26	I	II	II	III	II	I	I	IV	I
25	Gamb 27	I	II	I	V	III	VI	I	I	I
26	Gamb 28	I	II	II	II	II	I	I	I	III
27	Gamb 29	I	II	II	II	II	I	I	I	III

No.	Stock	GAPDH	ASAT	PGM	ALAT	ME	MDH	GPI	PEP 1	PEP 2
28	TDRN 9	I	I	IV	V	I	I	I	XVII	II
29	TDRN 14	I	I	III	V	V	I	II	I	V
30	TSW 3/78E (020)	I	II	III	IV	II	II	VII	XIII	X
31	LRU TSW 4/77	I	I	I	IV	II	III	VII	XIV	XII
32	LRU TSW 6/77	I	II	III	IV	II	III	VII	XVI	XII
33	LRU TSW 13	I	III	I	II	II	I	VI	II	IV
34	LRU TG 14/78	I	II	I	IV	V	I	VII	X	IX
35	TSW 25/78E (020)	I	IV	I	IV	II	II	X	X	X
36	TD 28/78E (020)	I	IV	I	IV	V	II	VIII	XII	X
37	LRU TSW 29	I	III	I	II	V	I	VI	II	IV
38	TD 56/78E (021)	I	IV	III	IV	II	II	VII	XII	X
39	LRU TSW 78/77	I	I	IV	II	III	II	IX	XVI	XII
40	LRU TSW 94/77	I	IV	III	II	III	I	VIII	IX	X
41	LRU TSW 99/77	I	IV	III	III	V	II	IX	IX	X
42	LRU TSW 103/77b	I	I	III	IV	II	I	VII	XVI	XIII
43	LRU TSW 108/77	I	I	I	IV	II	II	VIII	XVI	XII
44	LRU TSW 114/77	I	I	I	IV	II	I	VII	XVI	XII
45	LRU TSW 115/77	I	I	III	II	III	I	VII	XIV	IX
46	TSW 117/78E (026)	I	I	II	II	V	II	VIII	XIII	X
47	TSW 152/78E (027)	I	IV	III	IV	V	II	VII	XI	X
48	TSW 153/78E (027)	I	I	III	IV	II	III	VII	XIII	X
49	LRU TSW 243/77	I	I	I	II	II	I	VIII	IX	X
50	LRU TSW 251/77	I	III	I	V	IV	II	IX	XII	XI
51	TD 378/78E (037)	I	IV	II	IV	V	II	X	XII	X
52	IBADAN 34	I	IV	IV	III	II	I	VII	XVI	XII
53	IBADAN 35	I	IV	I	II	III	I	VII	XV	IX
54	IBADAN 44	I	I	I	IV	II	II	VII	XV	X
55	IBADAN 69	I	I	IV	IV	II	I	VII	XVI	XII
56	EATRO 1564	I	II	III	II	II	II	I	VI	XIV
57	EATRO 1582	I	II	IV	II	I	I	III	I	VI
58	EATRO 1599	I	II	I	II	I	I	I	VII	VI
59	EATRO 1712	I	II	I	III	II	I	II	I	I
60	EATRO 1751	I	II	I	II	I	I	I	VII	VI

No.	Stock	GAPDH	ASAT	PGM	ALAT ME	MDH	GPI	PEP 1	PEP 2
61	EATRO 1755	I	II	III	V	I	I	II	III VII
62	EATRO 1816	I	II	I	I	I	VI	I	III VII
63	EATRO 1841	I	II	III	II	I	I	I	III VIII
64	EATRO 1967	I	II	II	I	II	I	I	I VI
65	EATRO 1968	I	II	II	I	II	I	I	II IV
66	EATRO 2025	I	II	I	V	II	I	V	I VII
67	EATRO 2027	I	I	I	V	I	I	I	III V
68	EATRO 2033	I	II	I	II	II	I	I	XVIII VII
69	EATRO 2084	II	III	I	III	III	I	I	V III
70	TREU 1290 WA 1	I	II	I	II	II	I	I	VII IV
71	TREU 1291 EA 1	I	II	II	II	II	I	III	III VII
72	TREU 1381 EA 2	I	II	I	I	I	I	II	VIII VII
73	TREU 1095 WA 2	I	II	I	II	II	I	I	VII IV
74	LUMP 89	I	II	I	I	I	I	II	III VI
75	MIAG 108 Clone	I	I	III	II	II	I	II	VII VII
76	S104/FLY/BE	I	II	I	I	I	I	II	III VII
77	1/148 FLY	I	III	I	II	I	I	IV	VI IV
78	SUBAKIA	II	II	I	II	III	I	I	I VI

24	56	65	65	68	64	57	48	76	62	79	92	87	56	91
25	47	50	45	63	66	53	50	59	38	61	66	61	57	63
26	82	71	71	60	52	62	58	56	60	58	69	58	48	60
28	46	50	40	58	45	34	45	54	41	50	45	45	52	41
29	48	47	42	48	57	50	42	45	39	42	52	52	48	43
30	37	40	40	41	29	39	31	43	32	45	41	35	28	41
31	31	44	34	36	27	25	30	38	41	34	35	39	27	36
32	26	38	33	34	26	28	33	37	34	38	34	33	22	39
33	58	70	63	58	50	43	63	54	65	50	56	56	46	58
34	33	32	41	29	29	35	32	31	33	32	33	32	25	38
35	38	41	41	33	29	35	32	35	38	37	38	41	29	43
36	34	33	33	26	24	32	26	29	30	29	31	29	26	30
37	52	56	56	46	45	38	56	43	58	39	45	44	41	46
38	40	43	39	35	28	38	30	38	35	39	39	39	31	40
39	30	38	29	34	27	25	29	32	34	29	31	33	30	30
40	35	34	34	31	28	33	30	29	31	30	31	34	31	35
41	31	34	38	24	25	30	27	30	35	31	32	34	24	35
42	32	45	35	37	29	26	31	39	41	35	36	40	28	37
43	31	43	34	35	28	26	30	38	40	34	35	39	27	35
44	36	50	39	41	31	29	34	43	46	39	40	44	31	41
45	37	35	35	37	29	30	27	34	32	31	32	35	32	32
46	43	33	33	34	27	32	26	32	34	29	34	29	30	26
47	32	31	35	21	25	30	24	23	28	24	29	31	24	28
48	39	43	38	39	26	32	25	41	34	38	39	38	30	34
49	46	45	45	41	36	39	35	39	41	45	41	45	37	41
50	52	41	50	42	50	44	50	40	42	37	42	37	34	38
	1	2	3	4	5	6	7	8	9	10	11	12	13	14

51	40	34	34	27	24	33	26	29	35	30	35	30	27	31
52	32	50	40	37	29	30	35	43	46	45	41	45	28	46
53	29	28	28	29	22	27	24	27	25	28	26	28	29	29
54	33	37	37	33	26	27	24	35	33	32	33	37	29	33
55	34	48	38	39	30	28	33	41	44	38	39	43	30	39
56	46	56	45	52	36	43	50	48	46	50	45	40	37	46
57	48	47	47	59	52	61	38	55	34	57	62	57	54	59
58	44	43	43	77	48	58	38	71	34	74	59	54	56	63
59	47	60	55	62	55	69	41	69	47	71	76	71	52	74
61	41	40	40	58	50	64	45	54	32	56	50	45	46	52
62	41	39	39	58	45	54	39	54	31	56	50	44	46	52
63	44	43	43	63	43	52	38	58	34	60	54	44	50	56
64	56	48	48	62	59	63	43	57	40	59	70	59	50	62
65	58	63	56	52	50	43	70	48	65	50	56	50	41	58
66	54	52	52	59	62	67	52	55	39	57	62	57	48	59
67	48	52	41	68	58	50	46	63	42	58	58	58	61	54
68	48	46	52	61	52	63	46	56	42	58	58	58	61	68
69	52	61	61	47	37	53	41	53	52	50	55	50	52	52
70	58	70	56	73	45	48	63	67	58	70	56	50	46	58
71	54	46	46	67	47	62	41	62	43	64	63	52	48	60
72	39	38	43	56	48	52	38	52	30	54	48	48	39	50
74	38	37	37	60	47	50	37	56	29	58	52	46	48	54
75	52	50	44	71	45	54	35	65	41	62	55	56	52	52
76	41	39	39	58	45	54	39	54	31	56	50	44	46	52
77	48	58	46	54	38	35	46	50	48	46	47	41	43	43
78	47	45	45	57	50	77	36	53	33	55	60	55	52	57
	1	2	3	4	5	6	7	8	9	10	11	12	13	14

16	48																			
17	58	48																		
18	61	71	61																	
19	48	59	65	61																
20	46	63	64	60	84															
21	48	45	74	62	50	59														
22	50	43	83	63	57	55	76													
23	68	52	62	63	62	61	47	53												
24	58	63	78	66	59	57	65	67	55											
25	65	55	65	79	50	48	67	81	47	64										
26	77	52	63	59	57	56	52	54	72	68	57									
28	48	30	48	47	36	34	45	52	47	39	50	46								
29	50	52	56	58	47	50	57	59	34	45	62	43	47							
30	38	33	33	31	36	34	27	29	33	39	32	46	27	29						
31	32	22	37	22	31	29	26	32	37	33	34	31	30	24						
32	36	24	31	25	30	28	25	27	31	37	26	34	21	24						
33	68	32	61	42	50	48	44	52	64	54	45	65	44	41						
34	39	42	30	31	33	35	28	26	26	35	33	38	21	34						
35	34	30	39	28	38	35	37	34	34	40	29	43	24	26						
36	35	39	27	29	27	29	29	24	28	29	31	34	22	35						
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41	32	39	29	27	28	30	31	26	26	33	28	31	21	36						
42	33	23	38	24	32	30	27	33	38	34	25	32	27	29						
43	32	23	37	23	31	29	26	32	36	33	24	31	26	25						
44	37	25	42	26	35	33	30	37	41	38	27	36	30	28						
45	38	30	38	31	36	34	27	33	33	30	32	37	35	38						
46	35	39	31	32	31	32	26	28	35	29	30	39	29	39						

15 16 17 18 19 20 21 22 23 24 25 26 28 29

47	33	37	29	27	29	30	31	26	26	30	29	32	20	38
48	31	28	36	25	34	32	25	31	35	32	26	39	29	31
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51	37	44	28	33	28	29	30	25	32	33	31	40	23	36
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53	34	27	30	28	29	27	28	26	26	27	29	33	32	26
54	26	24	34	21	33	31	24	30	30	31	22	33	32	23
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57	56	61	62	58	57	55	52	53	44	61	62	59	42	48
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63	52	53	52	50	48	46	43	45	40	52	54	56	43	44
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74	44	52	50	53	47	45	46	48	34	50	57	48	41	54
75	43	41	60	42	55	54	45	52	47	48	45	52	50	63
76	48	45	48	51	50	48	44	46	37	48	56	52	44	58
77	63	31	44	39	38	35	32	39	54	40	42	54	46	39
78	59	82	59	79	71	70	50	52	57	59	66	57	36	52

15 16 17 18 19 20 21 22 23 24 25 26 28 29

54	71	73	63	36	48	67	54	27	68	59	50	53	64	75
55	60	76	73	48	50	56	45	37	58	68	52	45	82	78
56	56	39	48	56	28	41	33	44	48	42	34	34	40	43
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58	43	28	31	42	34	34	31	37	37	31	37	29	29	28
59	37	29	31	50	31	34	28	41	36	25	29	29	30	29
61	45	26	33	44	32	32	29	39	39	29	39	31	31	26
62	44	29	32	38	36	36	32	33	38	28	33	26	30	29
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64	43	29	32	43	35	40	32	33	42	25	33	26	30	29
65	39	38	42	71	36	41	28	57	38	32	33	26	39	38
66	42	28	31	46	34	39	31	37	41	28	36	29	29	29
67	36	35	29	46	28	32	29	40	34	38	34	26	36	34
68	41	30	33	52	37	42	29	40	39	29	39	30	31	30
69	36	27	26	50	38	33	34	50	35	31	35	35	29	28
70	44	38	42	71	31	36	28	57	38	37	33	26	39	38
71	46	31	34	46	33	38	30	36	40	30	35	28	32	31
72	38	28	31	42	34	34	27	37	32	27	32	25	29	28
74	41	27	30	41	33	33	30	36	35	26	31	24	28	27
75	45	34	33	50	28	37	29	39	43	33	39	31	40	34
76	44	29	32	44	56	36	32	38	38	28	33	26	30	29
77	37	36	34	73	29	29	26	65	31	39	31	24	37	35
78	36	24	26	40	38	33	34	40	35	31	39	32	25	24

30 31 32 33 34 35 36 37 38 39 40 41 42 43

45	56																		
46	46	57																	
47	44	50	57																
48	68	60	56	54															
49	70	62	63	45	60														
50	41	41	52	41	48	50													
51	43	43	73	72	52	48	44												
52	77	50	42	45	54	62	37	43											
53	52	78	48	52	44	58	34	45	58										
54	72	58	54	58	70	64	47	50	58	60									
55	95	54	44	43	65	67	39	41	82	50	70								
56	39	35	37	31	43	35	41	34	40	28	37	38							
57	28	33	31	29	31	38	35	32	33	30	26	31	42						
58	32	38	35	25	36	38	35	32	33	34	30	31	48	75					
59	32	26	25	26	31	33	35	29	37	24	27	31	41	56					
61	30	40	33	27	38	35	41	30	31	32	28	29	50	57					
62	33	34	32	26	37	34	36	33	30	31	31	32	44	58					
63	32	43	35	29	41	38	39	32	33	34	30	31	54	68					
64	33	30	32	30	37	39	40	38	30	27	31	32	43	73					
65	44	30	28	26	32	39	41	33	39	27	31	42	56	41					
66	32	33	31	29	35	42	47	32	33	30	30	31	47	64					
67	40	41	38	23	38	41	42	30	36	32	32	38	46	54					
68	35	36	29	31	33	46	36	30	36	37	37	33	46	60					
69	31	36	34	32	34	36	38	35	32	33	33	30	41	42					
70	44	34	32	22	37	39	41	29	44	31	31	42	70	46					
71	36	37	39	24	39	41	38	35	37	33	33	34	52	71					
72	32	33	27	29	31	33	31	28	29	30	30	31	38	56					
74	31	32	30	24	34	32	34	31	28	29	29	30	41	65					
75	39	45	38	27	48	45	37	30	35	32	37	38	50	52					
76	33	34	32	26	37	34	36	33	30	31	31	32	44	58					
77	41	37	34	21	34	37	38	27	37	29	29	39	59	39					
78	27	36	34	32	30	36	34	35	29	33	26	26	41	74					

44 45 46 47 48 49 50 51 52 53 54 55 56 57

45	56																	
46	46	57																
47	44	50	57															
48	68	60	56	54														
49	70	62	63	45	60													
50	41	41	52	41	48	50												
51	43	43	73	72	52	48	44											
52	77	50	42	45	54	62	37	43										
53	52	78	48	52	44	58	34	45	58									
54	72	58	54	58	70	64	47	50	58	60								
55	95	54	44	43	65	67	39	41	82	50	70							
56	39	35	37	31	43	35	41	34	40	28	37	38						
57	28	33	31	29	31	38	35	32	33	30	26	31	42					
58	32	38	35	25	36	38	35	32	33	34	30	31	48	75				
59	32	26	25	26	31	33	35	29	37	24	27	31	41	56				
61	30	40	33	27	38	35	41	30	31	32	28	29	50	57				
62	33	34	32	26	37	34	36	33	30	31	31	32	44	58				
63	32	43	35	29	41	38	39	32	33	34	30	31	54	68				
64	33	30	32	30	37	39	40	38	30	27	31	32	43	73				
65	44	30	28	26	32	39	41	33	39	27	31	42	56	41				
66	32	33	31	29	35	42	47	32	33	30	30	31	47	64				
67	40	41	38	23	38	41	42	30	36	32	32	38	46	54				
68	35	36	29	31	33	46	36	30	36	37	37	33	46	60				
69	31	36	34	32	34	36	38	35	32	33	33	30	41	42				
70	44	34	32	22	37	39	41	29	44	31	31	42	70	46				
71	36	37	39	24	39	41	38	35	37	33	33	34	52	71				
72	32	33	27	29	31	33	31	28	29	30	30	31	38	56				
74	31	32	30	24	34	32	34	31	28	29	29	30	41	65				
75	39	45	38	27	48	45	37	30	35	32	37	38	50	52				
76	33	34	32	26	37	34	36	33	30	31	31	32	44	58				
77	41	37	34	21	34	37	38	27	37	29	29	39	59	39				
78	27	36	34	32	30	36	34	35	29	33	26	26	41	74				

44 45 46 47 48 49 50 51 52 53 54 55 56 57

59	53																			
61	67	55																		
62	68	45	70																	
63	81	48	67	61																
64	71	58	59	67	64															
65	42	45	44	50	42	60														
66	62	56	69	64	56	73	52													
67	64	47	73	67	57	56	46	67												
68	64	52	58	59	57	63	52	67	55											
69	43	59	41	40	43	39	45	42	41	52										
70	71	50	50	50	54	48	71	52	52	52	50									
71	70	52	64	65	63	68	52	65	61	68	42	58								
72	65	53	74	68	52	64	48	62	57	57	34	48	56							
74	77	67	78	73	70	75	46	59	61	54	38	46	60	70						
75	67	55	75	56	60	54	39	57	65	68	41	56	64	67	60					
76	68	55	86	80	61	67	50	64	67	59	40	50	65	85	90	70				
77	44	42	46	41	44	35	52	43	48	37	47	65	43	39	43	46	46			
78	72	70	55	56	65	70	40	62	52	58	60	45	57	54	63	50	56	38		

58 59 61 62 63 64 65 66 67 68 69 70 71 72 74 75 76 77

APPENDIX V

HIERARCHIAL CLUSTER ANALYSIS

Stock numbers as in Appendix III

<u>Level of similarity</u>			80%	1	26	27		
				33	37			
				70	73			
95%	26	27		6	20			
	70	73		16	78			
	8	10		4	8	10		
	58	60		11	24	14		
				12	17			
90%	26	27		5	21			
	70	73		22	25			
	6	20		74	76			
	8	10		58	60	63		
	11	24		30	38			
	12	17		36	51			
	74	76		42	44	55		
	58	60						
	44	55	75%	1	26	27		
				2	23			
				3	9			
85%	26	27		33	37			
	70	73		15	70	73		
	6	20		6	20	19		
	4	8	10	16	78			
	11	24		4	8	10		
	12	17		11	24	14	12	17
	74	76		5	21			
	58	60		22	25			
	30	38		61	74	76		
	36	51		58	60	63		
	44	55		30	38	48		
				43	54			
				36	51			
				45	53			
				40	49			
				31	32			
				42	44	55		

Level of similarity

70%

1	26	27	2	23
3	9			
33	37			
15	70	73		
6	20	19		
16	78			
18	59			
4	8	10		
11	24	14	12	17
5	21			
22	25			
57	71			
64	66			
61	74	76	72	
58	60	63		
30	38	48		
43	54			
36	51	46		
41	47			
45	53			
40	49			
31	32			
42	44	55		

Level of similarity

65%

1	26	27	2	23				
3	9							
7	65							
33	37	77						
15	70	73						
6	20	19						
16	78	18	59					
4	8	10	11	24	14	12	17	
5	21	22	25					
57	71							
64	66							
61	74	76	72	62				
58	60	63						
29	67							
30	38	48						
35	43	54						
36	51	46						
41	47							
45	53							
40	49							
31	32	42	44	55				

60%

1	26	27	2	23				
3	9							
7	65							
33	37	77						
15	70	73	56					
6	20	19	68					
16	78	18	59					
4	8	10	11	24	14	12	17	
5	21	22	25					
57	71	64	66					
61	74	76	72	62				
58	60	63						
29	67	75						
30	38	48	35	43	54			
36	51	46						
41	47							
34	45	53						
40	49							
31	32	42	44	55				
39	52							

Level of similarity

55%

1	26	27	2	23	3	9			
7	65								
33	37	77							
15	70	73	56						
6	20	19	68						
16	78	18	59	69					
4	8	10	11	24	14	12	17	13	
5	21	22	25						
57	71	64	66	61	74	76	72	62	
58	60	63							
29	67	75							
30	38	48	35	43	54				
36	51	46	41	47					
34	45	53							
40	49								
31	32	42	44	55	39	52			

50%

1	26	27	2	23	3	9	7	65	
33	37	77							
15	70	73	56						
6	20	19	68						
16	78	18	59	69					
4	8	10	11	24	14	12	17	13	5
21	22	25							
57	71	64	66	61	74	76	72	62	58
60	63								
29	67	75							
30	38	48	35	43	54				
36	51	46	41	47					
34	45	53	40	49					
31	32	42	44	55	39	52			

Level of similarity

45%

1	26	27	2	23	3	9	7	65	33
37	77								
15	70	73	56						
6	20	19	68	16	78	18	59	69	
4	8	10	11	24	14	12	17	13	5
21	22	25							
57	71	64	66	61	74	76	72	62	58
60	63								
28	29	67	75						
30	38	48	35	43	54	36	51	46	41
47									
34	45	53	40	49					
31	32	42	44	55	39	52			

40%

1	26	27	2	23	3	9	7	65	33
37	77	15	70	73	56				
6	20	19	68	16	78	18	59	69	
4	8	10	11	24	14	12	17	13	5
21	22	25							
57	71	64	66	61	74	76	72	62	58
60	63								
28	29	67	75						
30	38	48	35	43	54	36	51	46	41
47	34	45	53	40	49				
31	32	42	44	55	39	52			

Level of similarity

45%	1	26	27	2	23	3	9	7	65	33
	37	77								
	15	70	73	56						
	6	20	19	68	16	78	18	59	69	
	4	8	10	11	24	14	12	17	13	5
	21	22	25							
	57	71	64	66	61	74	76	72	62	58
	60	63								
	28	29	67	75						
	30	38	48	35	43	54	36	51	46	41
	47									
	34	45	53	40	49					
	31	32	42	44	55	39	52			

40%	1	26	27	2	23	3	9	7	65	33
	37	77	15	70	73	56				
	6	20	19	68	16	78	18	59	69	
	4	8	10	11	24	14	12	17	13	5
	21	22	25							
	57	71	64	66	61	74	76	72	62	58
	60	63								
	28	29	67	75						
	30	38	48	35	43	54	36	51	46	41
	47	34	45	53	40	49				
	31	32	42	44	55	39	52			

Level of similarity

35%

1	26	27	2	23	3	9	7	65	33
37	77	15	70	73	56	50			
6	20	19	68	16	78	18	59	69	
4	8	10	11	24	14	12	17	13	5
21	22	25	57	71	64	66	61	74	76
72	62	58	60	63	28	29	67	75	
30	38	48	35	43	54	36	51	46	41
47	34	45	53	40	49	31	32	42	44
55	39	52							

30%

1	26	27	2	23	3	9	7	65	33
37	77	15	70	73	56	50	6	20	19
68	16	78	18	59	69				
4	8	10	11	24	14	12	17	13	5
21	22	25	57	71	64	66	61	74	76
72	62	58	60	63	28	29	67	75	
30	38	48	35	43	54	36	51	46	41
47	34	45	53	40	49	31	32	42	44
55	39	52							

25%

1	26	27	2	23	3	9	7	65	33
37	77	15	70	73	56	50	6	20	19
68	16	78	18	59	69	4	8	10	11
24	14	12	17	13	5	21	22	25	57
71	64	66	61	74	76	72	62	58	60
63	28	29	67	75					
30	38	48	35	43	54	36	51	46	41
47	34	45	53	40	49	31	32	42	44
55	39	52							

20%

1	26	27	2	23	3	9	7	65	33
37	77	15	70	73	56	50	6	20	19
68	16	78	18	59	69	4	8	10	11
24	14	12	17	13	5	21	22	25	57
71	64	66	61	74	76	72	62	58	60
63	28	29	67	75	30	38	48	35	43
54	36	51	46	41	47	34	45	53	40
49	31	32	42	44	55	39	52		

Fructose in wild-caught *Phlebotomus ariasi* and the possible relevance of sugars taken by sandflies to the transmission of leishmaniasis*

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Summary

Cold, acidified anthrone was used to test 200 wild-caught sandflies (*Phlebotomus ariasi* ♀♀) for the presence of fructose. This sugar, or others with a fructose moiety, were demonstrable in 157 (79%) of the flies; proportions of flies positive in different tests ranged from 44 to 91%. Higher proportions of flies gave positive results when tested less than one hour after capture than when kept in the laboratory for more than 12 hours before testing. Similarly high percentages of flies were positive in samples collected coming to engorge on dogs and in those caught in CDC miniature light traps. These results, together with published reports of the demonstration of sugars in 25 individually tested sandflies of other species, confirm that, like other biting flies, sugar is an important part of the diet of sandflies. Natural sugars may influence the development of leishmaniasis in sandflies and their subsequent transmission. The anthrone test was found to be simple, rapid and suitable for field use; it is a valuable addition to indirect techniques for the study of the behaviour of sandflies.

Introduction

With few notable exceptions, attempts to transmit leishmaniasis to human volunteers or experimental animals in the laboratory by the bite of phlebotomine sandflies have met with little success (see review by KILLICK-KENDRICK, 1979). ADLER *et al.* (1938) pointed out that transmission in nature is, without doubt, much more efficient than it is in the laboratory. This view is supported by recent field work in the south of France in which the development of *Leishmania donovani infantum* in *Phlebotomus ariasi*, a natural vector, was studied in flies which, having taken an infecting meal, were marked, released, recaptured and dissected: infections in recaptured sandflies were noticeably heavier than in flies maintained in the laboratory (RIOUX *et al.*, in press).

One explanation is that substances taken by sandflies in nature, but denied them in the laboratory, may contain factors which stimulate the development of the parasite in the fly and facilitate transmission by bite (JOHNSON & HERTIG, 1970). Sugars are a natural food of sandflies (KIRK & LEWIS, 1951; LEWIS & DOMONEY, 1966) and there is evidence that, when given to sandflies in the laboratory, they enhance the chances of infected flies transmitting leishmaniasis to susceptible

vertebrates (SMITH *et al.*, 1940, 1941; SWAMINATH *et al.*, 1942; SHORTT, 1945; see also review by KILLICK-KENDRICK, 1979). Possibly sugars are an important nutrient for leishmaniasis in the foregut of the fly (SHERLOCK & SHERLOCK, 1961; KILLICK-KENDRICK *et al.*, 1977). Parasites in the oesophagus, pharynx, cibarium and proboscis presumably have little to sustain them; they cannot benefit directly from the products of digestion of the bloodmeal and, as in other haematophagous insects (RICHARDS, 1975), the foregut of the sandfly is lined by a relatively impervious cuticular intima. In this comparatively barren environment, sugars passing from the mouthparts of the fly to the diverticulum, which opens from the oesophagus, are the only obvious nutrients available to the parasites.

As part of a current study of the dynamics of the transmission of *L. d. infantum* in the south of France, we have examined wild-caught *P. ariasi* for fructose with a simple test devised by VAN HANDEL (1972) for the detection of sugar in mosquitoes. Positive results were obtained with a high proportion of sandflies and the test appears to be a valuable addition to the techniques used in studies on the biology of sandflies. Our observations are reported in this paper.

Materials and Methods

During July and August, 1977, fructose tests were made on 200 female sandflies collected at Laumède, a small hamlet in the department of Gard, in the Cévennes mountains, southern France (see map in RIOUX *et al.*, in press, a). None of the flies examined was engorged; 58 were caught in CDC miniature light traps; 126 were caught with aspirators as they alighted to feed on dogs; and 16 were collected at rest on a wall of a farmhouse. All the flies were assumed to be *P. ariasi* Tonnoir, 1921, by far the most prevalent species of *Phlebotomus* encountered at Laumède. However, of 420 female flies identified in other studies at Laumède in 1977, two (0.5%) were *P. mascittii* Grassi, 1908, and it is possible therefore, that a few of the 200 flies tested for fructose were this species.

Positive and negative controls for the tests were provided by other flies which were caught on dogs

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Table. Results of anthrone tests for fructose on 200 wild-caught *P. ariasi* according to the method of collection and the delay between collection and test (totals and subtotals in bold type)

Method of collection	Delay before test	Number tested	Number positive for fructose	% with fructose
Aspirated at rest on wall	> 12 h	16	12	75
CDC miniature light trap	> 12 h	10	5	50
	< 2 h	16	7	44
	< 1 h	32	29	91
		58	41	71
Aspirated alighting on dogs to engorge	> 12 h	24	16	67
	< 2 h	24	18	75
	< 1 h	78	70	90
		126	104	83
Totals		200	157	79

or on a wall and were kept in gauze covered cages, in plastic bags, at ambient temperatures for several days before the tests. Flies for positive controls were given access to aqueous solutions of fructose or sucrose on cotton wool; flies for negative controls were given water on cotton wool and had to be kept without sugar for as long as four days to be sure that none gave a positive result.

After preliminary trials, the following procedure was used to test for the presence of fructose (VAN HANDEL, 1972). Flies were narcotized with carbon dioxide from a hand dispenser and placed individually in wells of disposable plastic micro-titration plates. The flies were then crushed with a stainless steel rod, with due care to avoid contamination of one specimen by another. 0.16 ml of a 0.1% (w/v) solution of anthrone in 72% (v/v) of sulphuric acid was added to each well, and the plates were kept at room temperature ($23 \pm 3^\circ\text{C}$). Fructose and fructose-yielding sugars are the only carbohydrates which react quickly with cold acidified anthrone to form a green or blue colour (VAN HANDEL, 1967, 1972). At room temperature, tests should be read 60 min from the time the reagent is added, after which sugars other than those containing fructose may give positive results (VAN HANDEL, 1972). Our tests were read on a subjective scale of -, +, ++, and +++ at intervals of 15, 30, 45, 60 and 120 min, or at 30 and 60 min after adding the reagent.

With mosquitoes, the sensitivity of the test can be increased by treating the insects with a drop of a mixture of chloroform and methanol (1:1) instead of crushing them (VAN HANDEL, 1972). With sandflies, however, we found that the formation of the colour was delayed for two hours or longer when the flies were treated with the solvent mixture without first crushing and all the tests were, therefore, done on crushed sandflies.

VAN HANDEL (1972) tested mosquitoes individually in test-tubes. We found microtitration plates

better than test-tubes, because it was easier both to crush the flies and to read the results in the wells of the plates.

Results

There was some difficulty in deciding the best time at which to read the tests. The speed at which the positive blue colour developed is illustrated by the combined results of two tests, each of 40 flies, read at six different times. Percentages positive were 4 at 5 min, 48 at 15 min, 58 at 30 min, 64 at 45 min, 66 at 60 min, and 75 at 120 min. In accordance with VAN HANDEL's (1972) method, positives appearing after 60 min were ignored, but it is possible that this gives a slight underestimate of the true proportion of sandflies with fructose.

The results of tests on 200 individual sandflies, all or most believed to have been *P. ariasi*, are shown in the Table. The highest proportions of flies reacting positively (90 and 91%) were recorded when tests were made within an hour of collection; it is probable that flies caught with only small reserves of sugar exhausted these between collection and testing. With flies maintained on water as negative controls it was found, however, that some, presumably those with large sugar reserves, reacted positively as late as three days after collection. In comparative observations between different species of sandflies, it would clearly be necessary to standardize the delay, and to keep it as short as possible.

When tested within one hour of collection, there were no differences in the proportions of flies with demonstrable sugar between those taken alighting to feed on dogs and those from light traps. It is possible that the trapped flies may have been dispersing in search of a bloodmeal, and we cannot assume there were differences in the physiological state of flies in the two groups.

Discussion

From laboratory experiments (e.g. CHANIOTIS, 1974), and since nematoceros flies generally take

sugars in nature (DOWNES, 1958), it can be assumed that sandflies require carbohydrate in some form. There are, however, published reports of tests for sugars on only 25 individual sandflies, in all of which sugars were identified. Fowler (in KIRK & LEWIS, 1951) identified glucose and sucrose in the diverticula of all of seven specimens of *P. papatasi* from the Sudan, and fructose in one. LEWIS & DOMONEY (1966) found traces of sugar in the crops of all of 18 individually tested flies of four species of *Lutzomyia* from Belize; in descending order, the prevalence of six sugars was fructose (16), maltose (10), glucose (6), sucrose (4), raffinose (4) and melibiose (2). Glucose, sucrose and fructose were also identified in a pooled sample of the diverticula of five other sandflies.

LEWIS & DOMONEY (1966) used thin-layer chromatography to identify the sugars. This is a much more precise method than the anthrone test, but the latter has the advantage of simplicity, speed and suitability for field use. Cold anthrone reacts only with fructose, or carbohydrates with a fructose moiety, among which are two sugars recorded by Lewis & Domoney (sucrose and raffinose). Unless heated or left at room temperature for several hours, there is no colour reaction between anthrone and sugars which have no fructose moiety (VAN HANDEL, 1967, 1972). An added refinement of the test, not yet exploited, is the determination of the ratio of fructose and fructose-yielding carbohydrates to other carbohydrates in the crops of insects by comparison of the colour produced at room temperature with that which appears after heating (VAN HANDEL, 1967).

In our tests, neither glycogen, the principal sugar reserve of dipteran insects, nor trehalose, the sugar of the haemolymph of insects, would have reacted with anthrone since neither has a fructose moiety. The results show that most female *P. ariasi* contained either fructose or carbohydrates with a fructose moiety (or both) probably in their diverticula (see CHANIOTIS, 1974; READY, 1978) whether taken while seeking a bloodmeal, at rest or by light traps. Our samples were, however, too small to relate the presence of sugar to the physiological state of the flies.

Two important questions remain unanswered. Firstly, what are the sources and full compositions of sugars taken by sandflies in nature? Secondly, do the sugars affect the development of leishmaniae in the sandfly or have any relevance to transmission by bite? KILLICK-KENDRICK (1979) argues that, for different reasons, it seems unlikely that the sugars in sandflies come directly from flowers, fruit or sap and postulates that, as with some mosquitoes (NIELSEN & GREVE, 1950), a possible source is the honeydew of aphids or coccids, insects which are present in most, if not all, the habitats of sandflies. Fructose is a common constituent of honeydews, which also contain other sugars and significant amounts of many amino-acids (AUCLAIR, 1963). Honeydew would thus be a better nutrient for both sandfly and parasite than are the single sucrose solutions normally offered to experimentally infected sandflies. This could be particularly important after promastigotes had moved forward into the head of the fly, where there is presumably little else to

nourish them. The comparatively poor nutritional value of sucrose usually given to experimental sandflies may explain, in part, why the complete life-cycles of some leishmaniae are so difficult to demonstrate in the laboratory, and why experimental transmission by the bite of sandflies is so unpredictable.

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Écologie des Leishmanioses dans le sud de la France

II. La Leishmaniose viscérale canine : succès de la transmission expérimentale " Chien → Phlébotome → Chien " par la piqûre de *Phlebotomus ariasi* Tonnoir, 1921

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Résumé.

Poursuivant leur programme d'étude sur la leishmaniose viscérale en Cévennes, les auteurs réalisent avec succès le cycle parasitaire complet « Chien → Vecteur → Chien ». Le rôle de *Phlebotomus ariasi* Tonnoir, 1921, suspecté depuis les premières enquêtes, est confirmé. Vingt individus femelles, contaminés sur un chien malade 23 ± 2 jours auparavant, sont placés en contact avec un chien sain. Celui-ci réalise, 15 mois après, une forme viscéro-cutanée évolutive. La dissection des Phlébotomes, effectuée immédiatement après le contact infestant, montre qu'un seul d'entre eux s'est réellement gorgé. Les 17 exemplaires disséqués présentent des promastigotes dans l'intestin moyen, 10 d'entre eux renferment également des formes immobiles dans la cavité pharyngienne. L'exemplaire gorgé montre au surplus des parasites dans la trompe, occurrence considérée par plusieurs auteurs comme liée nécessairement au pouvoir infestant.

Summary.

Ecology of leishmaniasis in the south of France. II. Canine leishmaniasis : successful experimental transmission from dog to dog by the bite of *Phlebotomus ariasi* Tonnoir, 1921.

As part of a study of visceral leishmaniasis in the Cévennes in southern France, an infection was transmitted from dog to dog by the bite of a single sandfly, *Phlebotomus ariasi*

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Tonnoir, 1921. The role of this species as a vector, suspected from earlier studies, is, therefore, confirmed. Twenty female sandflies, which had engorged on a naturally infected dog 23 ± 2 days previously were put with a healthy dog which, after an incubation period of 15 months, developed viscerocutaneous leishmaniasis. In dissections of the sandflies immediately after contact with the experimental dog, it was found that only one had engorged. All of 17 sandflies dissected had midgut infections: 10 of these also had infections in the pharynx. The engorged specimen additionally had parasites in the proboscis, a condition thought by many workers to be necessary for the parasite to be transmitted by bite.

Depuis 1961, date de nos préenquêtes sur la transmission de la leishmaniose viscérale dans les Cévennes (11), les preuves, directes et indirectes, impliquant *Phlebotomus ariasi* Tonnoir, 1921 n'ont cessé de se renforcer. Parmi les arguments indirects, d'ordre écologique, s'inscrivait, dès l'abord, l'affirmation du caractère cynoanthropophile de l'espèce (9). Plus tard, la mise en évidence d'une forte corrélation entre les densités vectorielles et les prévalences enzootiques venait appuyer les premières hypothèses (6, 8). Les arguments directs confirmaient peu à peu les précédents. En laboratoire, l'infestation vectorielle était obtenue sans difficulté à partir de chiens atteints de formes cutanéoviscérales (10, 12, 13). *In natura*, quelques individus prélevés dans une ferme du Lodévois, au voisinage d'un chien infesté, se révélaient positifs (14). Plus tard, le comportement intravectoriel du parasite était décrit et interprété: la découverte de formes paramastigotes et promastigotes dans le pharynx et de formes paramastigotes dans la trompe accréditait le rôle vecteur de cette espèce (4).

Restait à « boucler » le cycle épidémiologique en provoquant la contamination d'un chien sain par la piqûre de *Phlebotomus ariasi* expérimentalement infesté. Cette étape est aujourd'hui franchie.

Matériel et méthode

Divers arguments, autécologiques et éco-physiologiques, nous ont amenés à réaliser notre étude au sein même du foyer cévenol. Mentionnons, en particulier, la nécessité d'expérimenter sur des populations naturelles et les difficultés, bien connues des spécialistes, de maintenir *in vivo* les Phlébotomes femelles ayant accompli en laboratoire un cycle gonotrophique complet.

Le site choisi correspondait à ces impératifs: il s'agit d'un hameau de quelques maisons, bâti à mi-pente (500 m) sur la rive droite de la moyenne vallée de l'Hérault (Laumède, Gard). La station proprement dite est constituée de deux maisons contiguës construites en pierres sèches selon la tradition cévenole. L'une sert d'hébergement et de laboratoire, l'autre de point de capture. L'ensemble comporte de

vastes caves et de nombreuses dépendances, utilisées jadis comme resserres et bergeries.

Les *Phlébotomes* sont très abondants dans la station : lors des soirées calmes et chaudes du plein été, la chasse à l'aspirateur-nasse (9, 10) permet en quelques heures de récolter plusieurs centaines d'exemplaires par piégeur. Il s'agit, en très grande majorité, de *Phlebotomus ariasi*. Les femelles capturées sont pures ou nullipares. Les ovocytes ne dépassent pas le stade II. En début d'expérience, un prélèvement témoin permet de confirmer l'absence d'infestation leishmanienne.

Le chien contaminant utilisé dans la présente étude est un Epagneul français ♀ de deux ans (Mirka). Il accuse une leishmaniose viscéro-cutanée évolutive avec amaigrissement, asthénie, dépilation et furfur. Les parasites sont présents dans les ganglions et la peau. Les anticorps fluorescents sont au 1/1280.

Le chien sain est un petit Epagneul breton de trois mois (Djinn) provenant d'un chenil des environs de Montpellier (Pérois). Initialement, il est dépourvu d'anticorps antileishmaniens. Durant la période passée à Laumède, Djinn sera protégé toutes les nuits par une moustiquaire à mailles fines (phlébotomaire).



Fig. 1. Chien Djinn. Leishmaniose viscérale au stade d'invasion cutanée. Noter l'œdème et la dépilation périoculaire

La contamination du vecteur est réalisée selon la technique de la phlébotomie (9). Du 6 au 14 août (tableau 1), le chien infesté est introduit dans le dispositif avec quelques centaines de *Phlebotomus ariasi*. Les matins suivants, les individus gorgés sont récupérés, marqués à l'aide de la poudre fluorescente (3) et relâchés peu après sur le lieu de prélèvement. Les recaptures sont réalisées du 28 août au 11 septembre à l'aide d'une lampe U.V. L'intervalle moyen depuis l'infestation est de 23 ± 2 jours. Durant cette période, 20 individus sont placés en contact avec Djinn soit directement à l'aide d'un tube (10 exemplaires), soit à l'aide d'une phlébotomie (10 exemplaires). Après l'expérience les individus sont disséqués; le tube digestif est examiné dans son intégralité. A la fin de l'expérience le chien est ramené à Montpellier et confié à l'un d'entre nous.

Résultats

Sur les 20 Phlébotomes récupérés, un seul est gorgé. Dix-sept d'entre eux, dont ce dernier, sont disséqués, (*Phlebotomus ariasi*). Tous présentent des promastigotes dans l'intestin moyen. Dix d'entre eux renferment en outre des parasites dans le pharynx. Un seul, l'exemplaire gorgé, montre également des formes mobiles dans la trompe (tableau 1).

Le chien Djinn, quant à lui, reste en bonne santé apparente jusqu'au 27 décembre 1978. En quelques jours se déclare alors un syndrome infectieux subaigu fait de fièvre, d'anorexie, d'amaigrissement et de diarrhée sanglante. L'examen cutané permet également de déceler une discrète dépilation périoculaire, symétrique (fig. 1). Le 8 janvier 1979, le parasite est observé dans le ganglion poplité (examen direct et culture). Le taux d'anticorps fluorescents est au 1/1280 le 3 janvier et au 1/2560 le 8 janvier. Un traitement anti-leishmanien (*Glucanthime*®, *Lomidine*®) est instauré le 20 janvier 1979, amenant une régression rapide des symptômes généraux et cutanés. La guérison se maintient 10 mois après.

Discussion et conclusion

Ainsi, l'infestation d'un chien sain a pu être réalisée par la piqûre d'un seul *Phlebotomus ariasi* ? gorgé, 23 jours auparavant, sur un chien leishmanien. Il s'agit effectivement d'une contamination par piqûre et non par ingestion, puisque la totalité du lot mis en contact avec l'animal a pu être récupéré. Par ailleurs, la protection nocturne du chien sain durant le séjour en zone endémique permet d'éliminer une contamination d'autre origine. Enfin, pendant la période prépatente, l'animal a vécu aux environs de Montpellier, dans le petit village de Grabels où aucun cas de leishmaniose canine n'a été signalé. Ce village est lui-même situé dans une strate noso-écologique à risque réduit (8).

Tableau I. — Cycle de *Leishmania donovani* « Chien → *Phlebotomus ariasi* → Chien ». Les 20 *Phlébotomes* utilisés dans l'expérience ont été capturés et recapturés à des dates s'étendant du 6 août au 11 septembre 1977. La durée moyenne de l'infestation des vecteurs depuis leur contact avec le chien leishmanien (Mirka) jusqu'à leur recapture est de $22,65 \pm 1,98$ jours. Tous les exemplaires disséqués contiennent des parasites dans l'intestin moyen et le cardia. Une assez grande proportion en présente également dans le pharynx. Un seul d'entre-eux (n° 990) contient des formes promastigotes dans la trompe. Il s'agit précisément de l'unique exemplaire gorgé.

N°	Dates de l'infestation du vecteur	Dates du contact avec le chien sain	Jours écoulés depuis l'infestation	Sang dans le tube digestif	Parasite			
					Intestin moyen	Cardia	Pharynx	Trompe
984 ...	10-8-77	29-8-77	19	abs.	+++	++++	+	—
972 ...	9-8-77	29-8-77	20	abs.	+++	+++	—	—
973 ...	9-8-77	29-8-77	20	abs.	++++	++++	++	—
...	10-8-77	31-8-77	21	abs.	non disséqué			
953 ...	6-8-77	28-8-77	22	abs.	++++	++++	—	—
956 ...	6-8-77	28-8-77	22	abs.	++++	++++	—	—
986 ...	11-8-77	2-9-77	22	abs.	+++	+++	—	—
988 ...	11-8-77	2-9-77	22	abs. (1)	+++	++++	+	—
989 ...	11-8-77	2-9-77	22	abs.	+++	++++	+	—
990 ...	11-8-77	2-9-77	22	présence	+++	++++	++	+
985 ...	10-8-77	2-9-77	23	abs.	+++	++++	+	—
...	10-8-77	2-8-77	23	abs. \	non disséqué			
1003 ...	14-8-77	6-9-77	23	abs.	++++	++++	+	—
1004 ...	14-8-77	6-9-77	23	abs.	++++	++++	non disséqué	
950 ...	6-8-77	30-8-77	24	abs.	++++	++++	—	—
952 ...	6-8-77	30-8-77	24	abs.	++++	++++	—	—
...	8-8-77	1-9-77	24	abs.	non disséqué			
987 ...	11-8-77	4-9-77	24	abs.	+++	++++	+++	—
1001 ...	14-8-77	8-9-77	25	abs.	+++	++++	+	—
1002 ...	14-8-77	11-9-77	28	abs.	++++	++++	++	—

(1) Cet exemplaire a essayé sans succès de se gorgier.

Au plan de la transmission, deux points méritent d'être soulignés : le fort pourcentage d'infestation pharyngienne (10/17) et la présence de formes promastigotes dans la trompe de l'unique exemplaire gorgé. Cette occurrence, considérée comme déterminante (1) dans les processus d'infestation (1, 2, 16), est à mettre sur le compte du délai relativement long séparant l'infestation de la recapture. Il est vraisemblable que deux cycles gonotrophiques ont eu lieu pendant ce laps de temps (5).

Quoi qu'il en soit, le cycle parasitaire « Chien → Phlébotome → Chien » est « bouclé » avec *Phlebotomus ariasi* comme vecteur. Ce faisant, pour la première fois, la leishmaniose viscérale méditerranéenne est transmise expérimentalement par piqûre, au réservoir habituel : le Chien (2).

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(1) « ... sandflies can deposit flagellates into any object on which they feed if, and only if, the distal part of the epipharynx is infected » (S. Adler, 1940, p. 420).

(2) La transmission expérimentale de *Leishmania donovani* par la piqûre de Phlébotome a été réalisée en Chine, en Inde et au Brésil avec *Phlebotomus argentipes* (Homme), Hamster chinois, Hamster doré, Souris blanche), avec *Phlebotomus chinensis* (Hamster chinois), avec *Lutzomyia longipalpis* (Hamster doré) (Cf. R. Killick-Kendrick : *Biology of Leishmania* in phlebotomine sandflies. In : *Biology of Kinetoplastida*. Vol. 2, 1979, Acad. Press, Londres, p. 397).

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