STUDIES ON THE PIG PARASITE TRYPANOSOMA (NANNOMONAS) SIMIAE

By

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DECLARATION

I, Zipporah Waithera Njagu, hereby do declare that this thesis is my original work and has not been presented for a degree in any other University.

13th June '87

Zipporah W. Njagu

This thesis has been submitted with our approval as the University Supervisors.

1. 11 June 1987

Professor G.K. Kinoti

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Dr. J.M. Mumo

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A B S T R A C T

STUDIES ON THE PIG PARASITE TRYPANOSOMA (NANNOMONAS) SIMIAE

The pig parasite Trypanosoma (Nannomonas) simiae is morphologically indistinguishable from Trypanosoma (Nannomonas) congolense. The main distinction between the two species is that in the domestic pig the former causes a highly virulent and rapidly fatal disease while the latter causes a more chronic disease. It is not clear whether the two parasites are distinct species, or subspecies or strains of one species. The main aim of the study was to help establish the identity of Trypanosoma (N.) simiae using biochemical and immunological techniques.

Isoenzyme pattern analysis using thin-layer starch gel electrophoresis of the enzymes malate dehydrogenase (MDH), phosphoglucoisomerase (PGI), alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), phosphoglucomutase (PGM), malic enzyme (ME), peptidase I (PEPI) and peptidase II (PEP II), has been carried out on isolates of T. congolense and T. simiae. The results show that T. simiae is distinct from its close relative T. congolense. However, intraspecific variations in some enzymes were found among isolates of T. simiae.

Antigenic variation has been investigated in an isolate of T. simiae using two serological techniques, namely agglutination and lysis. Results obtained from these studies have shown the occurrence of antigenic variation in T. (N.) simiae. In cross-reaction experiments T. simiae did not agglutinate in T. congolense antisera. Similarly T. congolense did not agglutinate in T. simiae

antisera. The results suggest that these two parasites are antigenically distinct from each other.

The end-products of carbohydrate metabolism have been investigated and enzyme assays of trypanosome homogenates carried out. Results from these experiments strongly suggest that glycolysis is as important in T. simiae as it is in other trypanosome species. The major end-products of aerobic glucose metabolism were acetate and pyruvate. Succinate, glycerol and glycerol-phosphate were also present. These results suggest that these organisms have a partial Tri-carboxylic acid cycle (TCA) or an alternative pathway with TCA-cycle intermediates as the end-products. The enzymes hexokinase (HK), pyruvate kinase (PK) and phosphoglucoisomerase (PGI) had the highest levels whereas lactate dehydrogenase (LDH) was present in trace amounts.

CHAPTER I

GENERAL INTRODUCTION AND LITERATURE REVIEW

1. GENERAL INTRODUCTION

1.1.1 Trypanosomiasis

Trypanosomes are tiny flagellate protozoa but those that are pathogenic in man and his domestic animals have a powerful hold on the future of Africa. African trypanosomes include Trypanosoma brucei gambiense and Trypanosoma brucei rhodesiense which cause Gambian and Rhodesian sleeping sickness in man, and T. brucei brucei, T. congolense, T. vivax and T. simiae which cause nagana or animal trypanosomiasis in tropical Africa between latitudes 14°N and 30°S. This area is referred to as the tsetse belt. Beyond this belt in and outside Africa, animal trypanosomiasis occurs in the form of Surra caused by T. evansi, dourine caused by T. equiperdum and Chaga's disease due to T. cruzi. In addition, mechanically transmitted T. vivax infections occur in livestock in the Caribbean islands and South America. The presence of trypanosomes in the lands south of the Sahara is a major obstacle to the utilization of grasslands for animal production. Native vegetation on these lands could be converted by ruminants to high quality protein. Livestock rearing is however minimized if not prevented by trypanosomiasis in these places. The occurrence and spread of trypanosomiasis in African livestock is determined primarily by the degree of contact between the domestic animals and the tsetsefly (Glossina) which is the vector of trypanosomiasis. There are 22 species of Glossina infesting four million square miles of tropical Africa and hence this vast area has been rendered unsuitable for livestock development.

Animal trypanosomiasis is the greatest obstacle to livestock production in tropical Africa and the disease in ruminants is of far greater economic and social importance than in other species. The annual loss of meat production alone in the region is estimated to be US $5 billion.

Sleeping sickness is a severe, often fatal disease that occurs widely in the sub-Saharan region of the continent. There are two varieties; one, caused by Trypanosoma brucei rhodesiense, is found in East Africa; the other, by T. b. gambiense, occurs in West and Central Africa. About 50 million people in 34 African countries are at risk of developing the disease and of these, only 5 to 10 million have access to some form of protection or treatment. The incidence of reported cases is currently 20,000 a year but many more cases go undetected (TDR Report 1984).

1.1.2. Taxonomy and morphology of trypanosomes

The systematic position of the trypanosomes is as follows:

Phylum:	Protozoa
Subphylum:	Sarcomastigophora
Superclass:	Mastigophora
Class:	Zoomastigophora
Order:	Kinetoplastida
Suborder:	Trypanosomastina
Family:	Trypanosomatidae
Genus:	Trypanosoma

Those trypanosomes that infect mammals may be broadly divided into two major sections based on the course of development

in their insect vectors (Hoare, 1970). In the group salivaria are included those trypanosomes whose development is completed in the mouthparts or salivary glands of the vector. Transmission of this group of parasites from the vector to the mammalian host is inoculative. All African trypanosomes of medical and veterinary importance belong to the salivaria. Four subgenera, namely Trypanozoon, Nannomonas, Duttonella and Pcynomonas constitute the salivaria group. Representatives of the subgenus Trypanozoon include T. (T). brucei, T. (T). brucei gambiense, T. (T). brucei rhodesiense, T. (T). evansi, T. (T). equinum and T. (T). equiperdum. Subgenus Nannomonas is represented by T. (N). congolense and T. (N). simiae Bruce et al; 1912. Subgenus Duttonella is represented by T. (D). vivax, and subgenus Pcynomonas is represented by T. (P). suis.

In the section stercoraria developmental cycle in the insect vector is completed in the hindgut with metacylic forms being present in faeces and transmission is contaminative. This group contains T. lewisi, T. theileri and T. cruzi. With the exception of T. cruzi trypanosomes in this section are typically not pathogenic.

The chief diagnostic characters of mammalian trypanosomes are based on the morphology of the stained blood-stream forms. The trypomastigote bloodstream forms of mammalian trypanosomes are basically lanceloate in shape, their body having the form of an elongated flattened blade, which is elliptical or oval in transverse section while its ends taper to a point.

Various species of trypanosomes may differ in size and shape of the body, in the position of the nucleus and kinetoplast, as

well as in the degree of development of the undulating membrane and flagellum. Mammalian trypanosomes differ also in the shape of the posterior end of the body which may be sharply pointed (T. lewisi), bluntly pointed (T. brucei) or rounded (T. vivax and T. congolense). The position and size of the kinetoplast are also of diagnostic value. It may be far from the posterior end of the body (T. theileri), subterminal (T. brucei), marginal (T. congolense) or almost terminal (T. vivax).

1.1.3 Life history of trypanosomes

Members of the genus Trypanosoma are digenetic parasites, whose life cycle involves an alternation of two hosts. Normally a vertebrate animal is the final host, while diverse haematophagous invertebrate act as intermediate hosts or vectors, which transmit the infection to new vertebrate hosts. The course of development of trypanosomes in the mammalian host begins with the inoculation of metacyclic trypanosomes by the insect vector (in case of salivarian species). The trypanosomes find their way into the bloodstream where they change into trypomastigotes. In the bloodstream the trypomastigote forms multiply and the pattern of multiplication as well as the stages in which multiplication takes place vary considerably in different species of trypanosomes.

Development of trypanosomes in the insect vector is confined to the alimentary tract and involves migration to the proboscis or salivary glands depending on the species of trypanosome. The trypanosomes assume different stages in

different proportions at various periods of the invertebrate cycle. The most important and characteristic stage in the invertebrate cycle is the epimastigote form through which all the trypanosomes must pass before they are transformed into metatrypanosomes. The intermediate host is incapable of transmitting the infection to the mammalian host until the cycle from blood trypanosome through epimastigote stage to metatrypanosome is completed.

1.1.4 Geographical distribution of trypanosomes

The geographical distribution of most parasites generally coincides with that of their invertebrate hosts. This general rule applies to the global distribution of trypanosomes. The salivarian trypanosomes T. brucei brucei, T. brucei rhodesiense, T. brucei gambiense, T.(N) congolense and T. (N). simiae are found within latitudes 14°N and 30°S of Africa a region within which the tsetse flies are found. T.(T) evansi and T. equiperdum on the other hand have spread to areas outside Africa where they have become independent of Glossina and are transmitted mechanically by other biting flies (Hoare, 1972). T.(D) vivax occurs in tropical Africa where it is transmitted by Glossina but has also become established in some parts of South and Central America, where it is transmitted mechanically by other blood sucking flies.

The most important stercorarian trypanosome species, medically, T. cruzi, is transmitted by reduviid bugs and is restricted to Central and South America.

1.1.5 Control of trypanosomiasis

Programmes to control trypanosomiasis have been in operation for nearly 100 years. Initial attempts involved control of the tsetse vector either by destroying the wild animal population on which tsetse feed or clearing the vegetation the flies require as breeding and resting sites. More recently, most tsetse control programmes have been based on the application of insecticides.. Insecticide spraying is not entirely effective in areas of heavy tsetse infection where dense vegetation and high rainfall make spraying programmes very expensive. There is also a risk of environmental pollution as a result of repeated large scale insecticide spraying.

Regular treatment with trypanocidal drugs has been an important control method since the early 1920's. This approach has been successful in situations where competent livestock management has been achieved. Chemotherapy, though quite an effective control measure, must however be implemented indefinitely because domestic animals are exposed to an extensive reservoir of infection in the wildlife population.

Vaccination would be preferable to drug therapy since available drugs raise problems of resistance, toxicity and production costs. Attempts to develop an effective vaccine are in progress though the unique property of the trypanosome known as antigenic variation works to frustrate the efforts.

1.2. LITERATURE REVIEW

1.2.1 Clinical manifestations of African trypanosomiasis

How, when and which African trypanosomes cause disease and death of the host are influenced by factors relating to the tsetse fly the trypanosome, the definitive host and their environments. Most species of African trypanosomes are spread by tsetse flies (Glossina). Following infection through fly bite, there is an incubation period lasting for days or weeks before the first signs of illness are observed. The period corresponds roughly to the time it takes the organism to reach the bloodstream from the chancre in sufficient numbers to cause clinical disease. This period can be considerably reduced by experimental needle challenge. The onset of parasitaemia is characterized by fever and dullness. With time the fever becomes irregular and intermittent and there is progressive anaemia, poor hair coat, emaciation, enlargement of superficial lymph nodes, abortion, irregular vestrus and infertility. In addition, there may be subcutaneous oedema, haemorrages in mucous membranes, increased respiratory and heart rates, nasal and occular discharges, corneal opacity, and concurrent infections. With the Trypanozoon, there may be, in terminal stages marked nervous signs characterized by tremors, foamy mouth, incoordination and hyperexcitability (Apted, 1970; Ikede and Losos, 1975). Pigs infected with T. simiae die within hours or days after appearance of the trypanosomes in peripheral circulation (Isoun, 1968). However, the natural course of African trypanosomiasis lasts normally for weeks in acute cases or months and years in subacute and chronic cases. During this

period, there are several parasitaemic peaks with each peak progressively worsening the condition of the host. In ruminants, the peaks are best demonstrated with T. vivax infection, and the ability to suppress the level of parasitaemia will determine whether the animal will die or survive the infection. Suppression of parasitaemia has been postulated as one of the mechanisms of trypanotolerance. Such animals produce more efficient immunoglobulin M (IgM) which is involved in immune adherence and phagocytosis of the parasites (Nantulya, 1985).

Most animals that survive the acute phase of trypanosomiasis remain generally unthrifty and unproductive. Some may self-cure, others may relapse into the acute phase during stress from malnutrition, overwork or pregnancy, while others may die from superimposed viral, bacterial, mycotic, helminthic or other protozoan infections. Concurrent infections are believed to be the result of the immunosuppressive effects of trypanosomes. Immunosuppression also affects the response of infected hosts during vaccination programmes (Ilemobade et al; 1982).

1.2.2 Antigenic variation in trypanosomes

Antigenic variation is a powerful strategy adopted by certain species of parasitic protozoa to enable them to survive in the immunized host. It is exemplified by the African trypanosomes in which the phenomenon has been most studied. Trypanosomes reside extracellularly in plasma where they are directly exposed to the entire armoury of the host defense system. Their successful adaptation to that hostile environment is due to the phenomenon

of antigenic variation (Gray and Luckins, 1976; Vickerman, 1978; Turner, 1983).

Four of the seven subgenera of Trypanosoma have been shown to undergo antigenic variation. All the species of these subgenera are transmitted either cyclically by the tsetse fly or mechanically by other biting Diptera. The capacity to undergo antigenic variation is associated with the presence of a surface coat, which can be visualized in transmission electron microscopy as an electron dense layer 12 - 15 nm thick (Vickerman, 1969; Borst, 1982). This coat is made up of glycoproteins. It seems to be an adaptation to life in the bloodstream of the mammalian host since it is lost when trypanosomes are ingested into the tsetse fly midgut. In the fly's midgut, the trypanosomes develop into uncoated trypomastigotes and the surface layer is only re-expressed with the development of infective metacyclic trypomastigotes from uncoated epimastigote forms in the salivary glands. Each coat protects the organism from complement lysis (Vickerman and Tetley, 1979) and at the same time presents a different variety of antigens to keep ahead of specific antibody production by the host. The coat may also play a role in selective infectivity, tissue tropism and immunopathology (Cross, 1979).

Once injected into the bloodstream of the mammalian host, metacyclics differentiate into rapidly dividing bloodstream trypomastigotes. The population increases until antibody capable of bringing about parasite destruction appears in the bloodstream. Parasite numbers then fall rapidly, but before the infection can be resolved a new and antigenically distinct population of parasites

develops, which in turn provokes an antibody response, which controls the parasitaemia until a third variant population appears and so on (Barry and Hajduk, 1979). The cycle usually continues until death of the host in absence of any chemotherapy. Antigenic variation is therefore characterized by fluctuations in parasitaemia as the flagellates express an apparently inexhaustible series of variant antigen types (VAT's) against each of which the host mounts a new antibody response (Gray, 1965a; Vickerman, 1978). This process gives rise to consecutive antigenic types which are resistant to antibodies synthesized in response to preceding populations but not to subsequent ones. In many (but not all) immunocompetent hosts, the waves of parasitaemia crest at regular intervals of 3 - 7 days. This continuous antigenic variation enables trypanosomes to keep "one step ahead" of antibodies raised against them by the mammalian host. Goodwin (1979) in a vivid description had this to say about the surface coat of trypanosomes:

> "The glycoprotein garment is continually renewed like a creeping film, the spare bits being slashed off in redundant plasmanemes. The garment resembles a greasy field, almost all the glycoprotein blades belong to a single species, although there are a few shafts of related species here and there or an undergrowth of the shorter blades or cross-reacting determinants."

Antigenic variation is phenotypic, although the number of variants that can be generated from a single trypanosome is uncertain. Many serological types can be derived from a single strain of the T. brucei subgroup or even from a single trypanosome (Gray, 1965b). This suggests a limitless range of variants in nature, death of the host being the only limiting

factor. Serological analysis suggests 100 variants whereas gene counting using techniques of molecular genetics implies 1000 (Van der ploeg et al; 1982).

Expression of different variant antigen genes is non-random. Gray (1965b) working with T. brucei (subgenus Trypanozoon) and Wilson and Cunningham (1972) with T. congolense (subgenus Nannomonas) in cattle demonstrated that antigenic variants were formed in a definite pattern and that reversion to a parent type occurred. Such studies suggest that antigenic variation is not a random process and that the number of variants from a strain of trypanosomes may be large but not infinite. Only a comparatively small proportion, perhaps 0.1% of the gene pool (Crowe et al; 1983) is expressed within the metacyclic population, and these variants also appear in the first patent parasitaemia (Barry and Emery, 1984) within the bloodstream of the mammalian host. These are followed by 'predominant' antigenic types, so called because they always occur early in the host following either cyclical or syringe passage to non-immunized hosts (Gray, 1965a).

It was originally observed by Broom and Brown (1940) that if different antigenic types of the same stock of Trypanosoma brucei were cyclically transmitted to new hosts, the first detectable trypanosome populations in each case were similar. The antigenic composition of this first detectable trypanosome populations in each case were similar (Seed, 1964). The antigenic composition of this first population also appeared to be characteristic of each stock. Thus cyclical transmission of different stocks gave rise to the original population antigenic types. The effect of cyclical transmission on the process of antigenic variation in T.

congolense is similar to that in T. brucei infections (Sclappi and Jenni, 1977).

Antigenic variation occurs at a comparatively low frequency at any one time, about 1 in 10^4 or 1 in 10^5 of the population are switching (Seed and Gam, 1966; Doyle et al; 1980). There is no evidence that antibody acts in any way other than by selecting for new variants by destroying their predecessors. If there is an inductive event in antigenic variation it has yet to be identified.

The ability of trypanosomes to change the antigenic composition of their surface coat is the primary mechanism which prevents most domestic animals from developing an effective immune response against trypanosomiasis. The same mechanism enables the parasite to evade the effects of a conventional vaccine which works by priming an animals antibody responses against one or a small number of antigens.

The surface coat of trypanosomes is made up of a matrix of about 10^7 identical glycoprotein molecules, known as the variant surface glycoproteins (VSG's) (Cross, 1975). Expression of genes coding different VSG's is the basis for antigenic variation. Expression of VSG genes is controlled by complex gene rearrangements. Although much elegant work has been done on this subject (Borst and Cross, 1982; Turner, 1983), the exact nature of the control mechanism still remains to be elucidated.

For the variant molecule encoded by a particular VSG gene to reach the surface of the trypanosome, the gene must be located on a special 'expression site'. This site is called a telomeric site because of its location near the end of the chromosome. From this site the gene can be copied into a mRNA molecule which directs the

synthesis of the appropriate VSG protein chain (Cross et al; 1980). There are many telomeric expression sites in the trypanosome genome that carry VSG genes. Only one of these is actively copied into RNA at a time.

The mechanism of this exclusive expression is not yet understood. Neither is the process by which expression is switched to a different telomeric site leading to the appearance of a different VSG on the trypanosome surface. The telomeric VSG genes provide a pool of VSG's likely to be expressed early in an infection. Switching of VSG expression may also occur by replacement of the VSG gene in the active telomeric site by a duplicated copy of a non-telomeric VSG gene or of another telomeric gene. The understanding of the genetic basis of antigenic variation does not provide an explanation for the restricted size of the metacyclic antigen pool, and also for the phenomenon of predominance. It seems that gene positional effects are likely to be implicated.

The extraordinary diversity and flexibility of this system of antigenic variation seems certain to frustrate attempts to devise a vaccine. Current efforts are directed at analysis of the antigenic types expressed on metacyclic trypanosomes. Since these are the first variants encountered by the hosts immune system, and since they seem to be restricted in number, a cocktail vaccine covering this population could be effective. The feasibility of inducing immunity in this way has been demonstrated (Nantulya, Doyle and Jenni, 1980). Recent studies on T. congolense suggest that a cocktail of 12 variants in this species would be sufficient (Crowe et al; 1983), although another study suggests that in T. b. rhodesiense

the repertoire of metacyclic variants may be continously evolving (Barry, Crowe and Vickerman, 1983). This poses a serious difficulty to the development of a vaccine.

1.2.3 Trypanosome metabolism

The causative agents of African trypanosomiasis belonging to the subgenera Trypanozoon and Nannomonas spend a portion of their life cycle in an invertebrate host (Glossina) and part in a vertebrate host. The bloodstream forms possess a mitochondrion in the form of a single canal with few cristae and no functional citric acid cycle (Fulton and Spooner, 1959). The organisms apparently depend entirely on glycolysis for their energy requirements since they have no cytochromes (Ryley, 1956). Reoxidation of NADH under aerobic conditions is effected by a glycero-phosphate oxidase (GPO) system unique to the salivarian trypanosomes (Grant and Sargent, 1960). The GPo system consists of an α-glycerol-phosphate dehydrogenase and a terminal oxidase. The oxidase reduces molecular oxygen to water and is not coupled to ADP phosphorylation. This terminal oxidase is insensitive to inhibitors of the mammalian respiratory chain such as cyanide, azide and antimycin but is inhibited by hydroxamic acids (Hill, 1976). The inhibition of respiration by salicylhydroxamic acids (SHAM) can be bypassed by adding phenazine methosulphate which can accept electrons from the dehydrogenase. Under aerobic conditions the oxidation of 1 mole of glucose produces 2 moles of pyruvate and two net moles of ATP. However during anaerobiosis the amount of ATP produced is halved and pyruvate and glycerol are produced in equal

quantities.

Bloodstream trypanosomes can be divided into groups according to the end-products of aerobic glucose metabolism. One group produces mainly pyruvate and contains members of the subgenus Trypanozoon. The second group produces mainly carbon dioxide (CO_2) but also some acetate and succinate and contains South American trypanosomes. The African trypanosomes T. vivax and T. congolense of the subgenera Duttonella and Nannomonas respectively are intermediate, producing pyruvate, lactate, acetate, succinate and carbon dioxide (Gutteridge, 1976).

1.2.4 Electrophoresis of isoenzymes in trypanosomes

Classification within the genus Trypanosoma is problematical and this was noted. It started with Gruby in 1843. The history of the systematics of the mammalian trypanosomes has passed through several phases.

Previous means of subspecific or strain classification in trypanosomes have either been limited in application or too vague to be distinctive. They have included:
(i) biomensural data which overlap between groups, vary with the host and are laborious to obtain (Fairbairn, 1953), (ii) host specificity (Desowitz and Watson, 1953), (ii) Drug resistance (Williamson and Stephen, 1960), which though useful as a marker for particular strains, cannot distinguish between individual drug-sensitive organisms. (iv) pathogenicity sometimes linked with geographical location (Hoare, 1972) or biomensural data (Fairbairn, 1953). The common practice of labelling so-called

strains by geographical locality has, at the moment, little meaning since in each area there may be several strains any of which may occur elesewhere. Most species of trypanosomes can be distinguished by morphological criteria but a problem exists in subgenera comprising morphologically identical trypanosomes but with different biological characteristics.

Recent approaches to the intrinsic identification of parasitic protozoa (Lumsden, 1974) include direct biochemical methods (Newton, 1976) and indirect immunological methods (Gray and Luckins, 1976).

Isoenzyme electrophoresis is an effective method for identification and is being widely used in the characterization of parasitic protozoa such as Entamoeba (Reeves and Bischoff, 1968), Leishmania (Evans, 1978) and Plasmodium (Carter, 1973). The technique was first applied to soluble enzymes of trypanosomes (Kilgour and Godfrey, 1973; Bagster and Parr, 1973) and the potential usefulness of the technique as a means of distinguishing trypanosomes soon became apparent.

Enzyme profiles of protozoa have usually been determined with starch-gel electrophoresis, often using thin-layer gels. The main advantages of thin-layer starch gel electrophoresis are good resolution and reproducibility and the facility to compare a series of organism populations simultaneously under identical conditions.

In the subgenus Duttonella, Trypanosoma vivax has spread beyond the tsetse belt of Africa to tsetse free areas of Africa, as well as to distant countries. Although there is little morphological difference between T. vivax isolated from these different geographical areas, differences have been observed in fly infectivity (Hull et al.; 1971) and pathogenicity of the organism for bovines (Hoare, 1972).

Enzyme polymorphism amongst naturally occurring T. vivax was first shown by Kilgour et al; (1975). They demonstrated three zymodemes of alanine and aspartate aminotransferase among samples isolated in Nigeria from naturally infected cattle which came from different areas of the country. The criteria for division of T. vivax into the different enzyme groupings was that the same sample consistently showed a clear distinction in banding in alanine and aspartate aminotransferase. Murray (1982), using nine T. vivax stocks was able to detect differences in three out of the twelve enzymes studied.

T. cruzi organisms from man and other mammals of the western Hemisphere are morphologically indistinguishable (Hoare, 1972). Those organisms occurring in sylvatic animals are currently considered as a threat to man on the basis of morphology and infectivity to both rodents and triatomine bugs. Since it is difficult to prove otherwise it is prudent to treat all T. cruzi like organisms as the human pathogen. However, there is evidence of marked differences between T. cruzi of diverse origins, notably from behaviour and experimental hosts (Goble, 1970) and from the dispartate distribution of chagas disease syndrome in man (Rezende, 1975). There has been no reliable method for the intrinsic characterization of T. cruzi strains.

Recently biochemical methods have been used for the identification. Toye (1974) reports that multiple forms of two aminotransferases differed among T. cruzi isolates collected from widely separated geographical areas. Miles et al; (1977) showed two distinct combinations of electrophoretic patterns

for six enzymes. One combination was observed in stocks derived from human infections or domiciliated animals, the other occurred in stocks from sylvatic hosts.

The three varieties within the complex of Trypanosoma (Trypanozoon) brucei (Hoare, 1972) are morphologically identical. In the tsetse fly zones of Africa T. b. gambiense is associated with a chronic human disease in Western and some Eastern areas, T. b. rhodesiense causes an acute disease in Eastern areas, while T. b. brucei is widespread in animals but does not infect man. Each subspecies infects animals, and definitions based on infectivity present problems when attempting to identify T. brucei brucei. Experiments with human volunteers have been performed, but because it is impossible to test sufficient numbers, a negative result cannot be conclusive. T. b. brucei is also sometimes designated, possibly wrongly because it is isolated from regions where human trypanosomiasis is believed not to occur. Recently an isolate labelled as T. brucei brucei infected a laboratory worker (Robertson and Pickens, 1975). Even among human pathogens diagnosis can be difficult, since endemic areas as well as clinical conditions, which range from rapidly fatal infections to symptomless carrier states may overlap (Apted, 1970; Hoare, 1972). The need for accurate identification of Trypanozoon organisms is well recognized.

One method of identification is based on strain-specific electrophoretic mobilities of isoenzymes within the trypanosomes. Using enzyme electrophoresis Godfrey and Kilgour (1976) showed that T. b. gambiense could be identified by certain aminotransferase patterns. Out of 16 stocks reported to be T. b. gambiense 13 had

a slow alanine aminotransferase (ALAT) pattern (ALAT I), and the remaining 3 stocks had a slow moving pattern (ALAT II) found to be typical of T. brucei brucei and T. brucei rhodesiense. The isoenzymes of alanine aminotransferase (Kilgour and Godfrey, 1976) were also found in trypanosomes isolated from Liberian pigs (Gibson, et al; 1978) which reinforces the suggestion, based on the resistance of these trypanosomes to human plasma, that these animals may be the reservoirs of human Gambian trypanosomiasis. Tait et al; (1984) were able to establish, on the basis of enzyme electrophoresis, that T.b. gambiense (defined by isolation from man) could be distinguished from Nigerian and Ugandan T. brucei isolated from either tsetse or domestic animals. They demonstrated the presence of a variant peptidase which was exclusive to T.b. gambiense. Variants in the enzymes tyrosine, aspartate aminotransferase, phosphoglucomutase and malic enzyme were only found in T.b. gambiense and not in Ugandan or Nigerian stocks.

Otieno and Darji (1985) working with T. brucei rhodesiense from the South Nyanza district of Kenya showed the existence of two principal enzyme strains (zymodemes) of T. brucei isolated from G. pallidipes. One zymodeme was identical electrophoretically to T. brucei rhodesiense isolated from patients in the same locality. The second zymodeme did not have the ALAT I pattern characteristic of typical West African T. brucei gambiense.

Trypanosomes of the subgenus Nannomonas are the most important pathogenic parasites affecting domestic animals in Africa (Hoare,

1970). For classification of trypanosomes belonging to this subgenus, criteria such as morphometry, behaviour in the insect vector and host preference of the trypanosome have been used (Hoare, 1970). By the accepted criteria, there are only two recognized species of trypanosomes within the subgenus Nannomonas, namely Trypanosoma (N) simiae and Trypanosoma (N). congolense. The various kinds of T. congolense that differ in such attributes as pathogenicity and host preference are not easily defined, with the consequence that their distribution is unclear and the epidemiology of an important animal disease throughout sub Saharan Africa is poorly understood.

Distinct enzyme polymorphism has been amply demonstrated in T. congolense. Young and Godfrey (1983) using 78 stocks of T. congolense showed that three enzymes had the same electrophoretic mobility in every stock; these are probably typical of T. congolense and perhaps subgenus Nannomonas since these enzymes showed different mobilities in the few representatives of the subgenera Trypanozoon and Dutonella. Apart from this clear unity in the stocks, Young and Godfrey, (1983) also demonstrated a marked dichotomy between stocks from drier Savannah zones throughout the continent and stocks from humid coastal areas of West Africa.

A recent approach to the identification of T. congolense strains has been DNA hybridization tests which are used to investigate the genomic relationships among clones. Using this test, some clones designated as T.(N). congolense were shown to differ from other T.(N). congolense strains and stocks with which they were compared (Majiwa et al; 1985).

There is no consensus regarding the number of species, strains or forms of salivarian trypanosomes other than T.(N).

congolense and T.(N). simiae that belong to the subgenus Nannomonas (Godfrey, 1977). The two differ from each other in one important way, T.(N). simiae causes a rapidly fatal disease in pigs whereas T.(N). congolense causes a mild one (Hoare, 1970). There have previously been no other method of distinguishing T.(N). simiae from T.(N). congolense.

Recently a comparative analysis of repetitive DNA sequences and kinetoplast DNA has been carried out on T.(N) simiae and T.(N) congolense. Using this technique, Majiwa et al; (1985) have shown that T.(N). simiae is significantly different from its close relative T.(N). congolense.

In this study, isoenzymes have been used to characterize T. (N). simiae and also to distinguish it from T.(N). congolense. Differences in electrophoretic mobility of enzymes is a reflection of the genetic differences among organisms. The genetic basis of the differences among trypanosomes may be of epidemiological importance and may be relevant to the clinical differences caused by the various trypanosome strains.

1.2.5 Subgenus Nannomonas

The subgenus Nannomonas comprises relatively small trypanosomes measuring between 8 µm and 24 µm in total length. They have no free flagellum in any stage of development, whether in the mammalian or in the insect host. Trypanosomes of this subgenus are among the most important pathogenic parasites affecting livestock in Africa but there is still some uncertainty regarding the number of species in this group. This is because some morphological forms are common to all the species described and since

the proportion of such forms varies in different strains and populations it is not easy to identify the species in parasites taken randomly. The two parasites T. congolense and T. simiae are generally regarded as the only species belonging to Nannomonas. However, among various strains attributed to T. congolense, there are considerable morphological and biological differences on account of which some of them have been separated as independent species (Godfrey, 1977).

1.2.6 Trypanosoma (Nannomonas) simiae

A trypanosome with the characteristics of T. simiae was first seen in 1909 by Montgomery and Kinghorn, working in Kambole (Zambia), in a pig which suddenly developed severe symptoms of disease and died six hours later. These workers attributed the disease to T. nanum. Three years later Bruce and others (1912) isolated from monkeys on which wild tsetse flies had been fed, a trypanosome with characteristic similar to those described by Montgomery and Kinghorn. It took more than two decades for T. simiae to become generally accepted as the only trypanosome that is highly pathogenic to pigs. Some of the earliest observations on acute porcine trypanosomiasis were made by Lichtenheld (1912). He described outbreaks among pigs in Tanganyika (Tanzania) of a violent disease which rapidly killed the animals. He attributed the disease to nagana. However, the fact that bovines and equines kept in close proximity to the pigs escaped infection and the severity of disease in the pigs suggest that T. simiae was the cause of these outbreaks.

Trypanosoma simiae is a common parasite affecting pigs and other domestic mammals throughout the tropical zone of the continent. Hoare (1936) listed its occurrence in Tanzania, Congo,

Malawi, Zambia and Rhodesia. Stephen (1966) recorded its occurrence in Portuguese Guinea, Dahomey, Guinea, Sierra Leone, Mali, Ghana, Nigeria, Burundi, Uganda, Kenya, Mozambique and Somalia. T. simiae has also been reported in Rwanda, Burundi and Liberia.

T. simiae was described by Bruce et al; (1912) as a monomorphic species resembling T. congolense in general appearance. However, subsequent studies of T. simiae from infected pigs (Hoare, 1936; Culwick and Fairbairn, 1947; Stephen, (1966) established that it was polymorphic and could be differentiated on morphological grounds from T. congolense especially when parasitaemia is high. The general morphology of T. simiae conforms to that of T. congolense. Their common features are a medium sized kinetoplast (diameter 0.8 μm) occupying a marginal position near the posterior end of the body, and the absence of a free flagellum. Both T. simiae and T. congolense have a conspicous undulating membrane. However, in contrast to T. congolense which is relatively monomorphic, T. simiae is characterized by a high degree of pleomorphism (Hoare, 1936; Stephen, 1966). The overall lengths of T. simiae are similar to those of T. congolense.

T. simiae is primarily transmitted by several species of tsetse flies (Glossina) but there is evidence that mechanical transmission by other bloodsucking Diptera may play a role in its transmission during epizootics (Stephen, 1966). The complete cycle of development of T. simiae in the intermediate host (Glossina) was described by Bruce et al (1913). They showed that the cycle of development is similar to that of T. congolense.

It starts in the midgut where the flagellates are in the trypomastigote form with the kinetoplast far away from the posterior end of the body and near the nucleus. These flagellates migrate to the proboscis, assume the epimastigote form and attach themselves to the walls of the food canal, and finally invade the hypopharynx where the metatrypanosomes are produced. The metatrypanosomes resemble the blood forms of T. congolense (Culwick and Fairbairn, 1947). Like T. congolense, T. simiae is devoid of a free flagellum in all stages of its development in the vector. The entire developmental cycle in G. morsitans and G. brevipalpis takes about 20 days at 28.3°C (Bruce et al; 1912).

T.simiae (Bruce et al; 1912) is an important parasite of pigs. It causes an acute and fatal form of trypanosomiasis in pigs (Hoare, 1936; Unsworth, 1952). The animals die due to thromboses of medium sized blood vessels, large veins and arterioles and the plugging of capillaries with trypanosomes in the spleen, lungs, kidneys, adrenals, lymph nodes, cerebrum and cerebellum (Isoun, 1968). Trypanosoma simiae has been described as the "Lightning destroyer of the domestic pig" (Bruce, 1912). Although T. simiae causes acute outbreaks in pigs (Hoare, 1936) characterized by very rapid death, the possibility of chronic infections cannot be ignored. T. simiae strains of lower virulence have been noted. Bruce et al; (1913) observed a reduction in virulence of T. simiae after passage through a goat. Desowitz and Watson (1953) reported a change after passage through a rabbit. Mettam (1951) found a particularly mild strain of T. simiae in pigs at Agege in Western Nigeria and noted that experimentally infected

pigs survived for nine months.

Janssen and Wijers (1974) reported that the virulence of T. simiae in pigs depended on species of tsetse fly transmitting trypanosomes. Glossina brevipalpis transmitted virulent infections while G. pallidipes transmitted chronic ones. Roberts (1971) showed that G. tachnoides could transmit T. simiae to pigs though the infection rate in G. morsitans was higher than that in G. tachnoides. Similar observations were reported by Desowitz and Watson, 1953; Isoun, 1968; and Agu, 1984.

The classical form of acute porcine trypanosomiasis is characterized by a sudden onset of disease, where a pig which may have been healthy a day or several hours earlier suddenly falls ill, becomes progressively worse and usually dies within the next few days. The spread of disease within a herd is also sporadic. With atypical strain(s) of T. simiae the infection in pigs has a longer incubation period, the animals survive for more than a month and instead of a progressive rise of parasitaemia, there are periods when trypanosomes are numerous in the blood and periods when they disappear from the blood (Stephen, 1966).

T. simiae has also been reported in camels where the disease ranges from a hyperacute form, with animals dying in a few hours, to an acute form with animals dying within days, to the chronic form where the disease lasts for months. In many respects the disease in camels is similar to that in pigs.

In other mammals, the behaviour of T. simiae varies according to the strain of parasite, the donor host and the mode of inoculation. In general bovines appear to be refractory to this trypanosome.

However, atypical strains of T. simiae from Rwanda - Burundi (Chardome and Peel, 1967) were infective to cattle. T. simiae has been reported in sheep and goats but with variable effects. While some strains were pathogenic others produced a chronic infection or even a latent infection without clinical symptoms (Barnett, 1947). The susceptibility of Cercopithecus monkeys to infection with this parasite was also noted (Culwick and Fairbairn, 1947).

The natural reservoir for T. simiae is believed to be the warthog (Stephen, 1966), though other species of wild pigs may also harbour the parasite.

Relatively little is known about the biology of T. simiae compared to other species of trypanosomes. This may be mainly because of failure to maintain strains in suitable laboratory animals. The literature on T. simiae is meagre and a number of questions concerning its identity are yet to be answered. For instance, it is not at all clear whether T. simiae is a distinct species or only a strain of T. congolense. These two parasites are morphologically indistinguishable. They are infective to common hosts, sheep, goats, cattle and pigs with varying degrees of virulence. The only clear distinction is the rapid action of T. simiae on pigs.

T. congolense infections have been reported in pigs in Nsukka (Eastern Nigeria). There were few trypanosomes in thin blood film with little harm on the pigs. Mild strains of T. simiae have also been reported in pigs at Agege in Western Nigeria (Mettam, 1951). It was noted that experimentally infected pigs survived for nine months. There is therefore a danger of confusion between T. simiae infections in pigs of low virulence and low rates

of infection by T. congolense. Previously, a parasite found in the pig if infective to laboratory rodents was designated as T. congolense. Recently a laboratory adapted strain of T. simiae has been reported (Opiyo, 1985). The fact that a strain of trypanosome is infective to the pig does not automatically designate it as the porcine parasite simiae. There exists a need, therefore, to establish the identity of T. simiae. Many parasites can be identified using morphological features but characterization of behavioural, specific or subspecific differences by morphology has failed in the case of T. simiae and T. congolense. Biochemical and immunological characterization studies which are the subject of this study aim at throwing light on these two parasites and hopefully reveal the differences between the two parasites which are of great biological and economic importance in Africa. Such a study is not only of interest to taxonomists but is of great help to studies on cell biology, studies on epidemiology and comparative pathogenicity as all these require reliable and accurate labels on the parasites.

1.3. Aims and Objectives

Trypanosoma N. simiae is morphologically indistinguishable from its close relative Trypanosoma N. congolense. The only distinction between the two parasites is the rapid action of T. simiae on pigs. Studies on three characters of the biology of T. simiae were carried out with a view of seeing whether or not it differs from T. congolense.

The main objectives of this study were

(1) To study isoenzyme patterns of T. simiae isolates using starch-gel electrophoresis and compare them with isolates of T. congolense.

(2) To investigate the occurrence of antigenic variation in an isolate of T. simiae and to find out if T. simiae and and T. congolense share common antigens on their surface.

(3) To determine the end-products of the glycolytic pathway in T. simiae and group the parasite appropriately on the basis of its metabolism.

CHAPTER II

MATERIALS AND METHODS

2.1. PARASITES

2.1.1 Isolates

Trypanosoma simiae isolates EATRO 1786, 1806 and 1861 and Trypanosoma congolense EATRO 2386 and EATRO 2506 were obtained as stabilates from the Kenya Trypanosomiasis Research Institute (KETRI) Muguga. T. simiae TS 81 and TS 82 were obtained from the International Laboratories for Research on Animal disease (ILRAD) whereas T. congolense S 14 was obtained as a stabilate from ICIPE. Details of the brief history of stabilates are described in 2.3.1 . The parasites were maintained by serial passage through animals or as stabilates in 10% glycerol.

2.1.2. Maintenance of strains in host animals

All the T. congolense isolates and T. simiae EATRO 1786 were maintained by serial passage in male outbred Wistar rats of body weight 200 - 300 g. The rats were inoculated intraperitoneally with 1.5×10^6 trypanosomes suspended in Phosphate Saline Glucose (PSG) buffer. The course of parasitaemia in the rats was followed by examination of wet films of blood from the tail. When parasitaemia was high the parasites were harvested by cardiac puncture while the animal was under diethyl - ether anaethesia. First, the thorax was opened to expose the heart. Then the heart was sprayed with 5% (w/v) trisodium citrate in phosphate saline glucose (PSG) buffer as anticoagulant. It was then punctured with a needle fitted to a 10 ml syringe and blood drained from the heart to the syringe. The trypanosomes were counted in an improved Neubaeur haemocytometer(Lumsden, et al; 1973).

T. simiae isolates TS 81, TS 82, EATRO 1861 and EATRO 1806 were maintained in Piglets. Crosses of Large - White and Hampshire breeds of pigs were used. The piglets were intravenously inoculated with 1×10^5 trypanosomes suspended in Phosphate Saline Glucose buffer (PSG) via the ear-vein and parasitaemia followed. The parasites at peak parasitaemia were harvested via the jugular vein into a heparinized container.

2.1.3 Separation of trypanosomes from infected blood

Trypanosomes were separated from blood by passing infected blood through an anion exchange column of DEAE cellulose as described by Lanham and Godfrey (1970). The separation depends fundamentally on differences in surface charge; the DEAE cellulose adsorbs the more negatively charged blood components while the less negatively charged flagellates are eluted.

Although surface charge differs between species of salivarian trypanosomes it is always sufficiently different from that on the host blood cells and platelets for successful separation in standard conditions of pH and ionic strength.

The preswollen gel was suspended in phosphate buffered saline (PBS) whose pH was adjusted to 8.0. It was then freed from fine particles by repeated sedimentation, decantation of supernate and resuspension in PBS. The last resuspension was made in phosphate-buffered saline with glucose and heparin (PBSGH) and the gel was then sedimented over a filter paper support in a funnel to form the column. Air bubbles were excluded.

Parasites from infected blood were estimated by use of the haemocytometer and the infected blood was centrifuged for 1 min at 3,000 rpm. The pellet of trypanosomes and blood suspension was layered on top of the prepared column and run into the column under mild sunction. It formed a well defined layer in the cellulose. The blood cells were adsorbed tightly onto the cellulose. A flow of one drop/10 seconds was maintained for fast elution. The eluate was collected as successive fractions in tubes. The tubes were replaced in succession as they got filled up. The tubes containing trypanosomes appeared milky. The eluate was centrifuged at 3,000 rpm for 1 minute and the organisms were sedimented as a white pellet. These were resuspended in PSG buffer and final counts taken for total organisms eluted. They were kept at $4^{o}C$ until used.

2.2 ANIMALS

2.2.1 Rats

Male outbred Wistar rats of body weight 200 - 300 g were used in this study. The rats were bred in the Zoology Department animal house and kept in plastic rectangular cages with a wire mesh top. They were provided with a bedding of wood shavings and fed on a diet of mice pellets and water.

2.2.2 Pigs

Crosses of Large White and Hampshire breeds of pigs aged 2 - 3 months and of body weight 15 - 20 Kg. were used in this study. The piglets were purchased from the University of Nairobi

Animal Farm, Kabete. They were fed on a diet of weaner and sow meal and water three times daily.

2.2.3 Rabbits

Newzealand-White rabbits aged 9 - 12 months were used in the study. The rabbits were fed on a diet of rabbit pellets, green vegetables and water.

2.3. ELECTROPHORESIS

2.3.1 Trypanosome stocks

The trypanosome stocks used for electrophoresis and their histories are shown below:

Stock	Species	Location	Host	Year
EATRO 1786	T. simiae	Muhaha Forest	G. austeni	1970
EATRO 1806	T. simiae	" "	G. brevipalpis	1970
EATRO 1861	T. simiae	" "	G. brevipalpis	1970
TS 81	T. simiae	Ukunda		
TS 82	T. simiae	Ukunda		
S 14	T. congolense	Nkruman		
EATRO 2386	T. congolense	Serengeti		
EATRO 2506	T. congolense	Ukunda		

T. simiae and T. congolense parasites obtained from piglets and rats respectively at peak parasitaemia were cleaned by use of the anion exchange DE-52 column as in 2.1.3 . The clean trypano some preparations were divided into small aliquots and stored at -70°C. For electrophoresis the pellets were thawed at room

temperature, the trypanosomes resuspended in 2.5 volumes of lysis buffer (10 mM Tris - HCl pH 7.4, 1 mM dithiothreitol, 1 mM EDTA, 2.5% Triton x - 100) at 0°C and homogenized in a Tri - R - stir homogenizer. The homogenate was centrifuged at 40,000 g for 30 minutes and the resulting supernatant fluid was used for electrophoresis.

2.3.2 Electrophoresis

Electrophoresis was carried out on a thin-layer starch gel as described by Young and Godfrey (1983). Connaught Hydrolysed starch gel was prepared in the appropriate buffer and laid on a flat surface. The gel was allowed to cool for 1 hour at room temperature and then for 30 minutes at 4°C. Trypanosome lysates were inserted into slots made in the gel with a razor blade by adsorbing the extracts on cotton threads. Electrophoresis was carried out at 5°C in the refrigerator. This temperature was maintained by a water cooling device in the electrophoretic tank. The buffer conditions used for electrophoresis for the various enzymes are shown in Table I. After electrophoresis the top of the gel was sliced off, and the freshly cut gel surfaces overlayed with the appropriate specific enzyme stain. The enzyme stains are shown in Table II. After staining at 35°C in the dark for 30 minutes to 1 hour the gels were fixed in 7% acetic acid and photographed.

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TABLE I: ELECTROPHORETIC CONDITIONS USED FOR THE VARIOUS ENZYMES *

Enzyme	Tank buffer	Gel buffer	Volts cm^{-1} across gel	Time (Hours)
GPI	0.2M phosphate pH 7.0	3 in 40 dilution of TB	20	3
PGM	0.135M Tris 0.043 M citric acid pH 7.0	1 in 10 dilution	29	2
ALAT	0.15M Tris-0.007M citric acid PH 7.0	3 in 40 dilution of T B	40	1 hour 15 min
ASAT	0.15M glycine -NaOH pH 9.5	3 in 40 dilution of T B	40	1 hour 15 min.
GAPDH	0.1M KH_2 PO_4-KOH (PH 7.0) + 0.1% Mercaptoethanol	1 in 10 dilution KH_2 PO_4 KOH-buffer + paper overlay of T B	20	1 hour 30 min

TABLE I: Cont.

Enzyme	Tank buffer	Gel buffer	Volts cm^{-1}	Time
MDH	0.135M Tris - -0.043 M citric acid PH 7.0	1 in 15 dilution of T B	21	3
PEP I + PEP II	0.18M Tris - 0.02M $KH_2 PO_4$ (adjust to PH 9.0 with HcL).	1 in 10 dilution of T B	20	2
ME	0.2 M phosphate PH 9.0	3 in 40 dilution of T B	20	3

TABLE II SPECIFIC ENZYME STAINS FOR THE VARIOUS ENZYMES

Enzyme	Buffer	Substrate	Co-enzyme	Linking Enzyme	Ions	Dye	H_2O
GPI	5 ml 0.3M Tris/HCl PH 8.0	1 mg F_6P	5 mg NADP	500 G6PDH	1 ml 0.1M $MgCl_2$	10mg MTT 2mg PMS	7
PGM	8 ml 0.3M Tris/HCl	16 mg G 1P with 1% G16DP	10 mg NADP	500 G6PDH	1 ml 0.5M $MgCl_2$	10 mg MTT 2mg PMS	7
ALAT	6 ml 0.1M Phosphate PH 7.4	10 mg 2 - oxoglutaric acid 120 mg L-alanine	2 mg NADH	800 LDH	-	-	-
GAPDH	3 ml 0.3M Tris (HCl PH 7.4	120 mg F16DP 300 aldolase	6mg NAD	-	10 mg sodium arsenate	10 mg MTT 2 mg PMS	

TABLE II Cont.

Enzyme	Buffer	Substrate	Co-enzyme	Linking Enzyme	Ions	Dye	H_2O
MDH	6 ml 0.3 M Tris/HCl PH 7.4	1 ml 1.0 Malic acid PH 7.0 with NaOH	3 mg NADP	-	-	10 mg MTT 2 mg PMs	7
PEP I	2 ml 0.1 M Phosphate PH 7.4	7 mg L-leucyl glycl glycine	1 mg peroxidase 1 mg L-amino acid oxidase	-	0.2 ml 0.1 M $MnCl_2$	-	5
PEP II	2 ml 0.1 M Phosphate PH 7.4	7 mg L-Leucyl L-alanine	1 mg peroxidase 1 mg L-amino acid oxidase	-	0.2 ml 0.1M $MnCl_2$	-	5
ME	8.5 ml 0.2 M Tris/HCl PH 7.4	0.3 ml 0.1 M Malic acid PH 7.0 with NaOH	2 mg NADP	-	1 ml 0.5 M $MgCl_2$	10 mg MTT 2 mg PMS	6

* Young and Godfrey (1983)

2.4. ANTIGENIC VARIATION

Trypanosoma simiae EATRO 1786 and T. congolense S 14 were used for the antigenic variation studies.

2.4.1 Antigen and anti-trypanosome serum preparation

The antigens used in this study consisted of a suspension of trypanosomes in whole rat blood. A group of 20 male outbred Wistar rats were inoculated intraperitoneally with 1×10^6 trypanosomes suspended in PSG buffer. Parasitaemia was monitored in the rats until death. Counts of parasites were done by use of the improved Neubaeur haemocytometer. At each peak of parasitaemia 5 rats were bled by cardiac puncture while under diethyl-ether anaesthesia, 1 ml of blood was withdrawn from the heart of each rat. The parasites were cleaned by use of the DE-52 column and stored as stabilates in liquid nitrogen until needed for the serological tests. Several populations were generated and stored.

Antisera to the trypanosome populations were raised in albino rabbits. Cleaned parasites were inoculated intravenously into albino rabbits already bled for normal serum. The development of agglutinating antibodies to serotype antigens in the rabbit was monitored. The ideal day for bleeding for serum was the day when the 1st agglutinating antibodies appeared. Bleedings for antisera were made 7 days after infection as maximum agglutination titres had been reached by this time. The rabbits were bled via the marginal ear-vein. Serum was obtained by allowing blood to clot overnight, at $4^{\circ}C$. The clotted blood was freed from the walls of the container and the clot allowed to

retract. The expressed serum was collected by removing any loose blood cells by centrifugation. The serum was stored in small vials at -20°C.

2.4.2 Agglutination

This test is antigenic-type specific and is used for the identification of the antigenic type of trypanosome populations or for recognition of antigenic-type specific antibodies (Lumsden 1973). The test depends on the agglutination of living trypanosomes by antibody specific to their surface antigens binding the the organisms together.

The agglutination technique was carried out as described by Cunningham and Vickerman 1962. A slide agglutination test was used. Two-fold serial dilutions of antisera from 1/10 - 1/640 were made in microtiter plates. Phosphate Saline Glucose (PSG) buffer was used as diluent. Normal sera collected before infection of the rabbits used for antisera production was used as controls for the corresponding antisera.

1 drop of antigen ≅ 50 µl was added to each dilution of the test and control serum. The microtiter plates were incubated at 25°C for 1 hour in a moist atmosphere to prevent evaporation. Each dilution was examined by use of a haemocytometer under the microscope and the percentage (%) agglutination scored. Agglutinated trypanosomes were observed as groups of 3 or more trypanosomes. The % agglutination was estimated by use of the ruled area of a haemocytometer. A given area was selected and the % area occupied by agglutinated trypanosomes estimated.

Each population of antigens was tested for agglutination using the homologous and the heterologous antisera.

2.4.3 Lysis

The lysis-technique detects surface antigens and so, like the direct agglutination test is, antigenic-type specific. This technique involves the lysis of living trypanosomes using homologous immune serum in presence of complement. Lysed trypanosomes can be distinguished from unaffected ones by being non-motile, enlarged and non-refractile on phase-contrast microscopy. The difference cannot be seen by ordinary transmitted light microscopy.

The immune-lysis technique was carried out as described by Lumsden et al; 1973. Dilutions of antisera from 1/10 - 1/640 were made on a ceramic ring slide. Phosphate Saline Glucose (PSG) buffer was used as diluent. 1 drop of trypanosome suspension was added to each dilution of antisera. Normal rabbit serum was used for controls. Guinea pig serum at a concentration of 25% was used as complement. The mixture was incubated at 37°C for 1 hour. Microscopic examination with phase-contrast illumination was done for lysed trypanosomes. This was expressed as a percentage of the total number of trypanosomes.

2.4.4 Cross - reactions

Trypanosoma congolense organisms raised in male Wistar rats were tested for agglutination in anti-simiae serum raised in Newzealand-White rabbits.

2.5. METABOLISM

Trypanosoma simiae EATRO 1786 (Ukunda - 1970) was used in the respiration experiments.

2.5.1 Oxygen uptake by trypanosomes

The rate of oxygen utilization by trypanosomes was followed at 25°C using a Clark-type oxygen electrode connected to a Sargent Welch recorder. A closed system was used in which oxygen was not replenished, as it was depleted by the respiring material. The buffer used was Phosphate Saline (PS) buffer. The electrode was allowed to equilibrate with the buffer for 5 minutes at 25°C before the experiment was started. Glucose (1.0 M) was used as a substrate. To start the reaction 100 µ l of a trypanosome suspension containing about 10^8 trypanosomes was added to the cuvette. Rates of respiration were expressed as nanomoles of oxygen utilized per 10^8 cells/min.

2.5.2 Product assays

In order to determine the end-products of glycolysis, incubations were carried out in a Dubnoff shaking metabolic incubator in 25 ml Erlenmeyer flasks at 25°C for 1 hour. Glucose 10 mM was used. Metabolism was stopped by deprotenisation with ice-cold perchloric acid to a final concentration of 7% ($v/_v$). The deprotenized samples were neutralized with 6M KOH. Aliquots of the protein-free neutral extracts were used for metabolite determination. Most assays depended on enzyme reactions which could be followed spectrophotometrically by measuring the decreased

or increased absorbance of reduced pyridine nucleotides at 340 nm on a Unicam SP 800 spectrophotometer using cuvettes of 1 cm light path at 25°C. The spectrophotometer was coupled to a Pye - Unicam recorder.

An extinction coefficient of 6.22×10^{-6} or 3.34×10^{-6} per mole of NADH or NADPH used or generated was used in all the determinations. The concentration of the metabolites in each sample assayed was estimated using the following formula.

$$C = \frac{\Delta E}{6.22 \text{ or } 3.34} \times \frac{V}{v}$$

where C = Concentration of respective metabolite in µmoles/ml of sample.

ΔE = Change in absorbance during the assay due to volume v of sample in a cuvette containing a final volume V of assay mixture.

(i) Assay of Pyruvate

Pyruvate was assayed immediately after neutralization of the deprotenized extract as described by Bergmeyer and Bernt (1974). Pyruvate + NADH + H^+ $\rightleftharpoons^{LDH}$ Lactate + NAD^+

0.1 ml deprotenized sample was added to a reaction mixture containing 50 mM KCl, 10 mM $MgSO_4$, 0.2 mM EDTA and 0.15 mM. NADH. 5 units of LDH were used per 1ml cuvette to start the reaction. Absorbance was read at 340 nm.

(ii) Assay of Lactate

$$\text{Lactate} + NAD^+ + \underset{\text{LDH}}{\rightleftharpoons}, \text{Pyruvate} + NADH + H^+.$$

The formation of NADH was measured by the increase in extinction at 340 nm. The reaction mixture contained 0.4 M Hydrazine, 0.5M Glycine (PH 7.0) and 1.5 mM NAD^+. The reaction was started by the addition of 1.5 units of LDH. Absorbance was read at 340 nm.

(iii) Assay of succinate

$$\text{Succinate} + ATP + COA \underset{\text{thiokinase}}{\overset{\text{Succinate}}{\rightleftharpoons}} \text{succinyl CoA} + Pi + ADP.$$

$$ADP + PEP \xrightarrow[\text{kinase}]{\text{Pyruvate}} ATP + \text{Pyruvate}$$

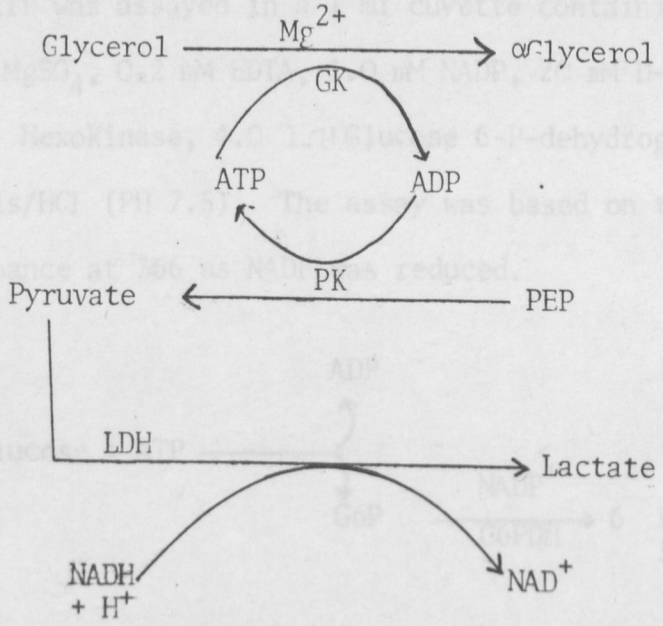
$$\text{Pyruvate} + NADH + H^+ \underset{\text{LDH}}{\rightleftharpoons} \text{Lactate} + NAD^+$$

The assay was carried out in a reaction mixture containing 50 mM Triethanolamine, 10 mM $MgCl_2$, 5mM EDTA, (PH 7.4), 5mM CoA, 10 mM ATP, 50 mM PEP, 2.4 mM NADH and 1.0 units of LDH and PK. The reaction was started by addition of 21 I.U of succinate thiokinase. The change in absorbance was read at 366 nm.

(iv) Assay of glycerol

The assay involves following ATP consumption by a coupled enzyme system consisting of pyruvate kinase (PK) and lactate dehydrogenase (LDH) with phosphoenol pyruvate (PEP). The adenosine diphosphate formed during the phosphorylation of glycerol is rephosphorylated by PEP to form pyruvate and ATP. Pyruvate is reduced by NADH to lactate.

The sequence of reactions is as follows:



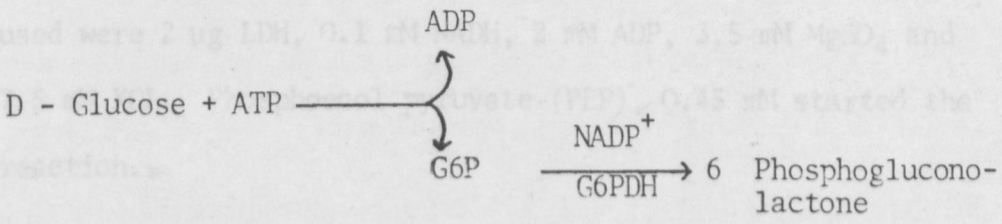
Deprotenised samples were added to a reaction mixture containing 50 mM KcL, 10 mM $MgSO_4$, 0.2 mM EDTA, 0.15 mM NADH, 4.0 mM ATP, 5.0 mM PEP and 5.5 units LDH, 4.0 units PK and 50 mM Tris - HCI (PH 7.5). The mixture was pre-incubated until a stable absorbance was obtained. 2.0 Units of GK were used to start the reaction. Absorbance was read at 340 nm.

(v) Assay of glycerol phosphate

The cuvette for the assay of α-glycerol phosphate (αGP) contained glycine - hydrazine buffer (pH 9.5) consisting of 0.19 M glycine, 0.69 M Hydrazine sulphate, 5.0 mM $MgSO_4$, 40 mM ATP, 2.5 mM NAD^+ and 4.0 I.U. αGPDH. During the assay for αGP, the reaction was started by addition of G6PDH. The change in absorbance was obtained after 10 - 15 minutes and was read at 366 nm.

(vi) Assay of ATP

ATP was assayed in a 1 ml cuvette containing 50 mM KCl, 10 mM $MgSO_4$, 0.2 mM EDTA, 1.0 mM NADP, 20 mM D- Glucose, 1.5 I.U. Hexokinase, 4.0 I.U Glucose 6-P-dehydrogenase and 50 mM Tris/HCl (PH 7.5). The assay was based on the increase in absorbance at 366 as NADP was reduced.



The reaction was started by addition of 20 µl of IM D-glucose. The ΔE at 366 nm was used to determine the amount of ATP in the sample assayed.

2.5.3. Enzyme assays

The methods described in "Methods of Enzymatic Analysis by Hans Urlich Bergmeyer Volume I - II (1974) were used. The 3 ml assays were buffered with either Tris - HCl 0.1M (PH 7.6), 80 mM or Triethanolamine 0.1M (PH 7.6) 50 mM.

(i) Hexokinase (HK) (EC 2.7.1.1.). This was assayed by measuring the rate of reduction of $NADP^+$ with glucose 6-P-dehydrogenase at 340 nm. The assay medium contained 75 mM Tris, 7.5 mM $MgCl_2$, 0.8 mM EDTA, 1.5 mM KCl, 4 mM mercaptoethanol, 0.4 mM NADP, 2.5 mM ATP. 1 mM D-Glucose initiated the reaction.

(ii) Glycerol kinase (GK) (EC 2.7. 1.30). The assay contained in final concentrations: 1.25 mM ATP, 2.5 mM $MgCl_2$, 3 µg G 6 PDH, 0.45 mM NAD^+ in 80 mM Glycine buffer (PH 10.0). The reaction was started by addition of 5 mM glycerol. *

(iii) Pyruvate kinase (PK) (EC 2.7 1.40). The method used was that described by Bucher and Pfleiderer (1955). Final concentrations used were 2 µg LDH, 0.1 mM NADH, 2 mM ADP, 3.5 mM $MgSO_4$ and 7.5 mM KCL. Phosphoenol pyruvate (PEP), 0.45 mM started the reaction. *

(iv) Lactate Dehydrogenase (LDH). The method used was that described by Bergmeyer and Bernt (1974). The assay system contained 0.1 M phosphate buffer, PH 7.5 and 0.15 mM NADH. The reaction was started by addition of 2 mM pyruvate at 340 nm.

(v) Glucose-6-phosphate dehydrogenase (G6PDH) (EC 1. 1. 49). The assay medium contained 2 mM $NADP^+$, 1.75 mM ATP, 2.5 mM $MgCl_2$. 5 mM glucose - 6 - phosphate was used to start the reaction. *

(vi) Glucose phosphate isomerase (GPI) (EC 5.3. 1. 9). Final concentrations were as for HK but fructose - 6- phosphate, 3.5 mM was substituted for glucose. *

(vii) Phosphofructokinase (PFK) (EC 27. 1. 11). Final concentrations were 1.25 mM ATP, 2.5 mM $MgCl_2$, 2 µg aldolase, 0.1 mM NADH, α - glycerol phosphate dehydrogenase 5 µg. The reaction was initiated with 2.5 mM fructose - 6 - phosphate. *

(viii) Malic enzyme - $NADP^+$ (EC 1.1.1. 40) and malic enzyme NAD^+ (EC 1.1.1.39). The method used was according to Hoek et al; (1976). The final concentrations used were 2.5 mM $MgCl_2$, 2 µg malate dehydrogenase, 0.5 mM NAD^+ or 0.25 mM $NADP^+$. The reaction was started with 5 mM malate.*

(ix) α- glycerolphosphate oxidase (GPO) (EC 1.1.1.8). The method used as as described by Hoek et al; (1976). Final concentrations in 0.1 M glycine - NaOH buffer (pH 10.0) and 0.5 mM NAD^+. The reaction was started with 5 mM α- GP.*

(x) Malate dehydrogenase MDH (EC 1.1.1.37). The method used is as described for GPDH, substituting α-GP with 5 mM L - malate.*

* 340nm

Protein determination: Protein was estimated by the Lowry-Folin Method described by Lowry et al; (1951). Samples were suitably diluted to give 200 µg protein/ml. Bovine serum albumin was used for protein standard curve plots.

CHAPTER III

RESULTS

3.1. ELECTROPHORESIS

Thin-layer starch gel electrophoresis of lysates obtained from T. simiae and T. congolense was carried out as described under Materials and Methods (2.3.2).

The results of isoenzyme electrophoresis of T. simiae and T. congolense isolates are presented in form of plates (Plates I - VIII). The mobilities of the isoenzymes of T. congolense and T. simiae can be best compared by use of their Rf values. The Rf value shows the distance each individual band has moved in relation to the entire gel distance. The Rf values are presented in Table III.

3.1.1. Phosphoglucomutase

Trypanosoma congolense S14 produced two bands which were quite distinct compared to Trypanosoma simiae isolates which showed only one band. The single band for the five T. simiae isolates migrated uniformly giving an Rf value of 64.7. This was a faster mobility than the two bands showed by T. congolense. The slower band in T. congolense had an Rf value of 42.25 whereas the faster band had an Rf value of 49.29.

3.1.2. Glucose phosphate isomerase

Plate II shows the isoenzyme patterns of Glucose phosphate isomerase (GPI). Three isolates of T. congolense from different localities were compared with five isolates of T. simiae. Although a multibanding pattern was observed in both T. congolense and T. simiae isolates, T. congolense S14, showed one band only.

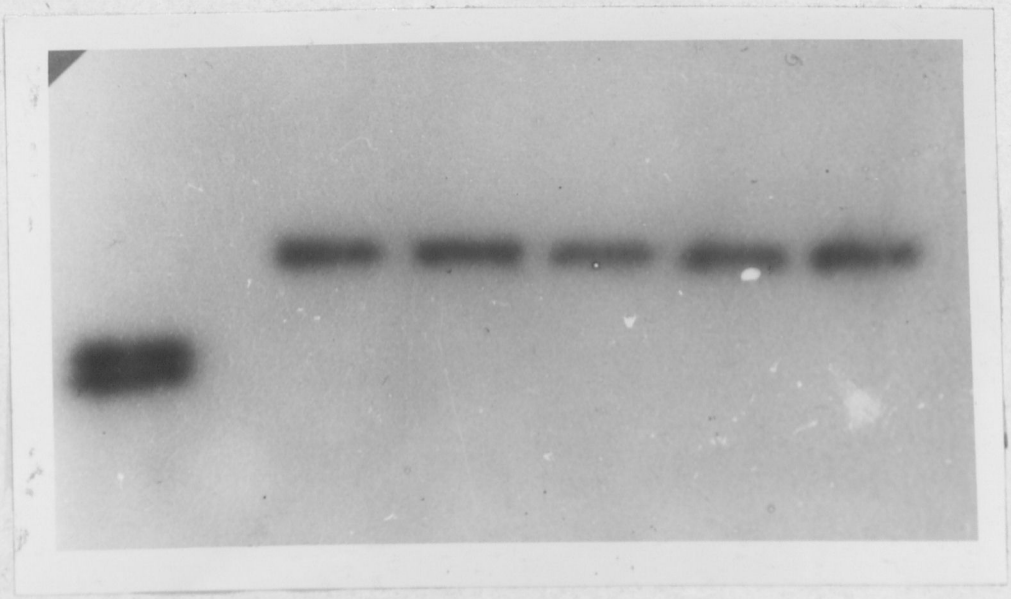


Plate I: Starch-gel electrophoresis of phosphoglucomutase (PGM) showing a comparison between one isolate of T. congolense and five isolates of T. simiae. From left to right 1 - Trypanosoma congolense S 14, 2-6 Trypanosoma simiae EATRO 1786, 1806, TS 81, TS 82 and EATRO 1861.

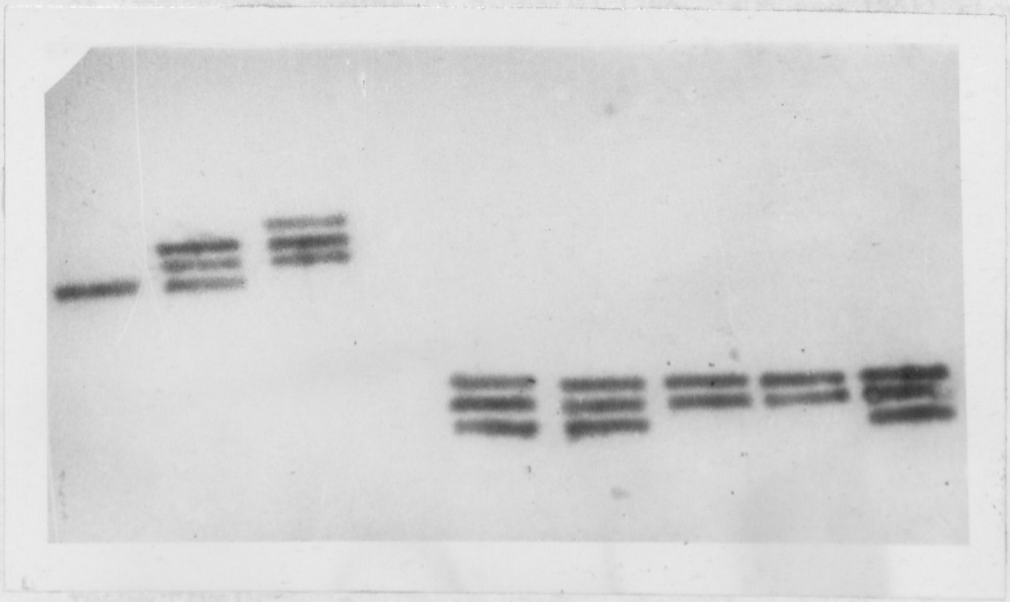


Plate II: Starch-gel electrophoresis of glucose phosphate isomerase (GPI) comparing three isolates of T. congolense with five isolates of T. simiae. From left to right 1-3 Trypanosoma congolense S 14, EATRO 2386 and EATRO 2506. 4-8 Trypanosoma simiae EATRO 1786, 1806, TS 81, TS 82 and EATRO 1861.

This band was similar to the slowest band obtained in T. congolense EATRO 2386. Two isolates of T. congolense EATRO 2386 and 2506 showed three bands each. The second slowest band of EATRO 2386 was similar to the slowest band in EATRO 2506. This indicates variations among isolates in the same species. Three isolates of Trypanosoma simiae (i.e. EATRO 1786, 1806 and 1861) showed three bands each, whereas two isolates (TS 81 and TS 82) showed two bands each. The slowest bands in TS 81 and in TS 82 were similar in electrophoretic mobility to the middle bands in EATRO 1786, 1806 and 1861. The bands of T. simiae isolates were slower than those of T. congolense isolates. The fastest moving band amongst the T. congolense isolates had an Rf value of 63.33 and the slowest had an Rf value of 55.54 compared to T. simiae Rf values of 50.0 and 27.96 for the fastest and slowest bands respectively.

3.1.3. Malate dehydrogenase

Plate III compares the isoenzymes of malate dehydrogenase (MDH) from one isolate of T. congolense with those from five isolates of T. simiae. T. congolense S14 showed a multibanding pattern of malate dehydrogenase of 3 bands. The three bands were distinct from one another. They were relatively slow moving with Rf values of 41.25, 46.35 and 48.25 for the slowest to the fastest moving band respectively. Trypanosoma simiae isolates on the other hand showed one band each. This band was similar and moved uniformly in all the five isolates studied. It had an Rf value of 64.5.

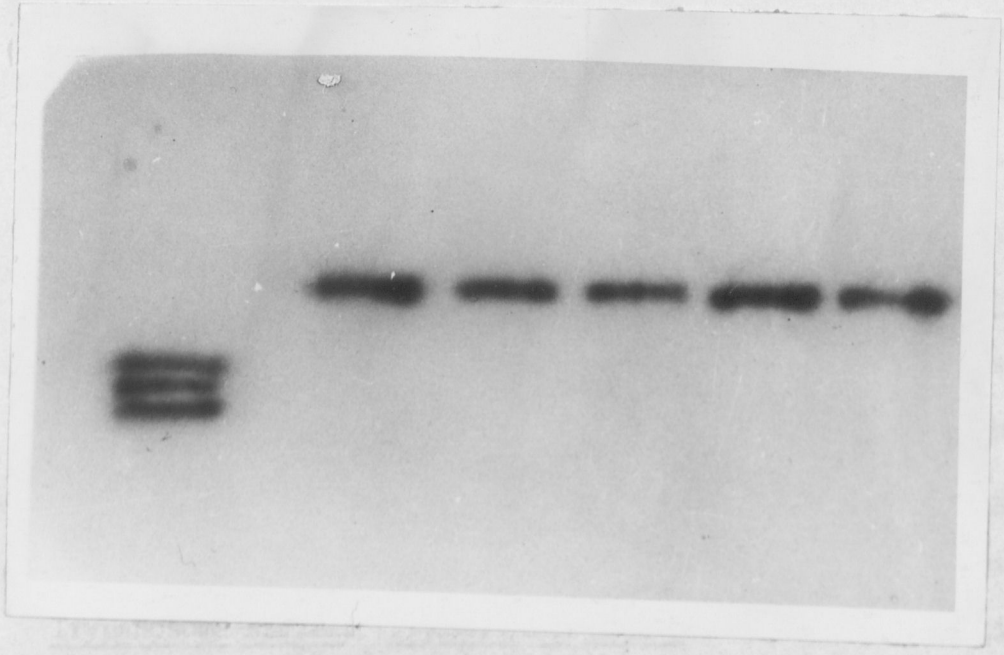


Plate III: Starch-gel electrophoresis of malate dehydrogenase (MDH) comparing 1 isolate of T. congolense and five isolates of T. simiae. From left to right 1 Trypanosoma congolense S 14, 2-6 Trypanosoma simiae EATRO 1786, EATRO 1806, TS 81, TS 82 and EATRO 1861.

3.1.4. Aspartate aminotransferase

Plate IV compares the isoenzymes of aspartate aminotransferase in three isolates of Trypanosoma congolense and five isolates of Trypanosoma simiae. Two of the isolates of Trypanosoma congolense showed one band each, while the third isolate showed three bands. These bands migrated at different rates although the band from T. congolense S14 was similar to the fastest band obtained from Trypanosoma congolense EATRO 2506. Both showed an Rf value of 63.79. Trypanosoma congolense EATRO 2386 showed one band. This band was similar to the slowest band in T. congolense EATRO 2506. These results confirm that intraspecific variations do exist among isolates of T. congolense. The bands of T. congolense were faster moving than those of Trypanosoma simiae. Trypanosoma simiae also showed a multi-banding pattern with three bands in three of the five isolates (i.e. EATRO 1786, 1806 and 1861) and two bands in each of the other two isolates (TS 81 and TS 82). The slowest band in TS 81 and in TS 82 migrated in a similar manner and were similar to the fastest band in EATRO 1786 and 1806 having an Rf value of 37.93. Two bands of EATRO 1861 and EATRO 1786 (slowest and the second slowest) were similar with Rf values of 32.06 and 36.89 respectively. Even with intraspecific variations amongst isolates, there were always shared bands among isolates in the same species.

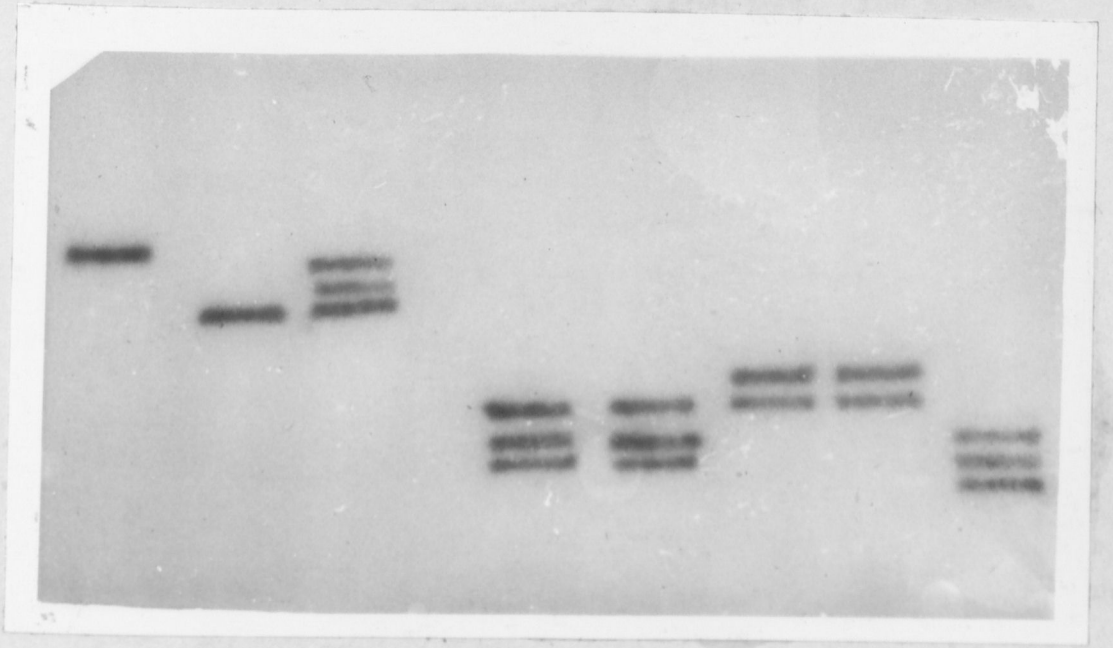


Plate IV: Starch-gel electrophoresis of aspartate aminotransferase (ASAT) showing the comparison of three isolates of T. congolense and five isolates of T. simiae. From left to right 1-3 Trypanosoma congolense S 14, EATRO 2386 and EATRO 2506. 4-8 Trypanosoma simiae EATRO 1786, EATRO 1806, TS 81, TS 82 and EATRO 1861.

3.1.5. Malic enzyme

Plate V compares the isoenzymes of malic enzyme (ME) in three isolates of Trypanosoma congolense and five isolates of Trypanosoma simiae. T. congolense S14 showed one band with an Rf value of 41.6. This was similar to the fastest band in EATRO 2386 and the slowest band in EATRO 2506. The slowest band in EATRO 2386 was slow moving compared to the other bands of the T. congolense isolates and it had an Rf value of 36.6. All the bands obtained in T. congolense isolates were faster moving than those in T. simiae isolates. Three Trypanosoma simiae isolates (EATRO 1786, 1806 and 1861) showed two bands each. The bands obtained in EATRO 1786 and 1806 were similar while those bands of EATRO 1861 were relatively faster moving. TS 81 and TS 82 showed one band each which seemed to be shared amongst all the isolates of T. simiae studied and had an Rf value of 21.6. The fastest band in T. simiae was showed by EATRO 1861 and had an Rf value of 25.0. This was a much slower mobility than that of the bands in T. congolense isolates.

3.1.6. Alanine aminotransferase

In plate VI the isoenzymes of alanine aminotransferase from one isolate of T. congolense and five isolates of Trypanosoma simiae are compared. There were distinct differences in the banding patterns of the two parasites. The one isolate of T. congolense S14 studied showed only one band which moved faster than the bands obtained in T. simiae. The band in T. congolense S14 had an Rf value of 51.47. All the five isolates

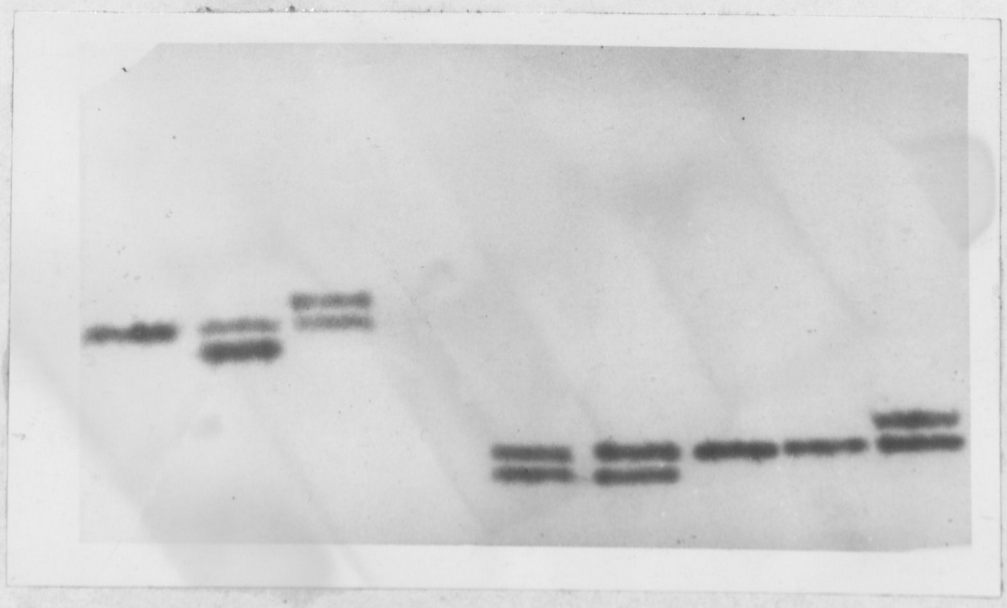


Plate V: Starch-gel electrophoresis of malic enzyme (ME) comparing three isolates of T. congolense and five isolates of T. simiae. From left to right, 1-3 Trypanosoma congolense S 14, EATRO 2386 and EATRO 2506. 4 - 8 Trypanosoma simiae EATRO 1786, EATRO 1806, TS81 TS 82 and EATRO 1861.

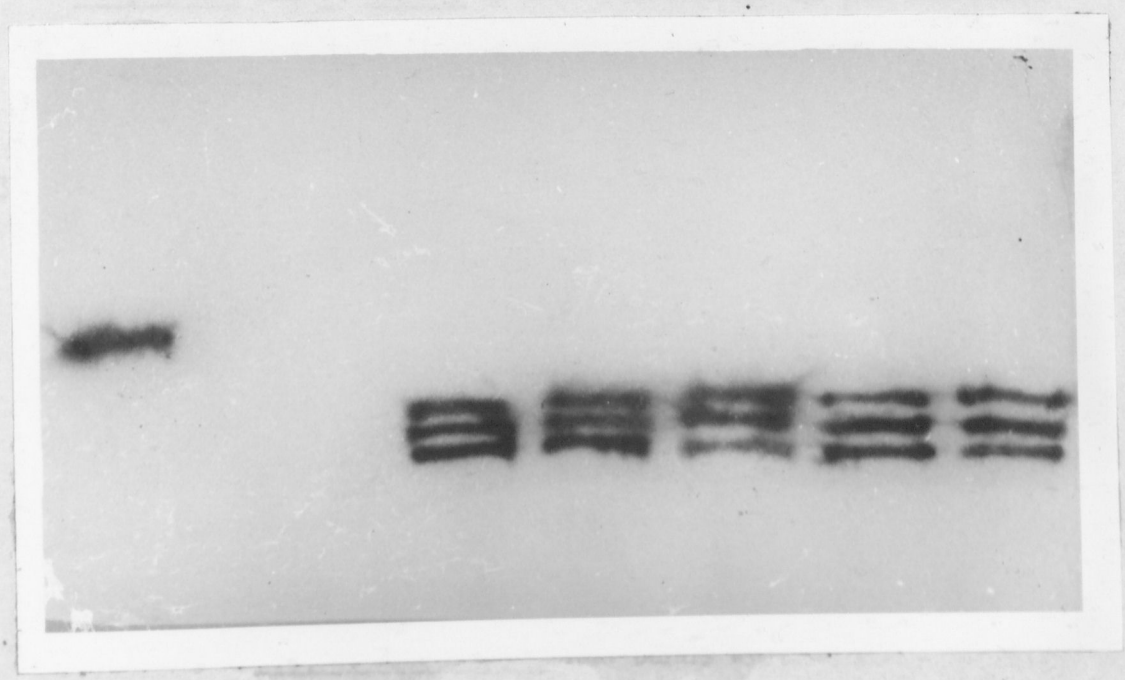


Plate VI: Starch-gel electrophoresis of alanine aminotransferase (ALAT) comparing one isolate of T. congolense with five isolates of T. simiae. From left to right 1 - Trypanosoma congolense S 14, 2-6 Trypanosoma simiae EATRO 1786, 1866, TS 81, TS 82 and EATRO 1861.

of Trypanosoma simiae showed three bands. These bands were distinct and moved in a similar manner in all the five isolates. They had Rf values of 32.64, 36.17 and 40.59 for the slowest to the fastest moving band respectively. The two parasites T. congolense and T. simiae showed differences in the number of isoenzymes and in their rates of movement.

3.1.7. Peptidase I.

Plate VII shows the isoenzymes of peptidase I. One isolate of T. congolense was compared with five isolates of T. simiae. There were two bands in each of the isolates studied. However, the bands in T. congolense S14 were faster moving than those in T. simiae isolates. The slower band in T. congolense had an Rf value of 53.12 whereas the faster band had an Rf value of 65.62. Trypanosoma simiae had bands which showed intraspecific variations. The faster moving band in T. simiae was shared among all the five isolates. It had an Rf value of 55.16.

3.1.8. Peptidase II

In plate VIII, one isolate of T. congolense was compared with five isolates of T. simiae using isoenzymes of peptidase II. In T. simiae peptidase II was characterized by having two bands in all the isolates studied. T. congolense S14 showed three bands which were quite distinct from one another and which were different from those of T. simiae isolates. The bands obtained in T. congolense had Rf values of 31.71, 37.80 and 46.34 for the slowest to the fastest band respectively. These

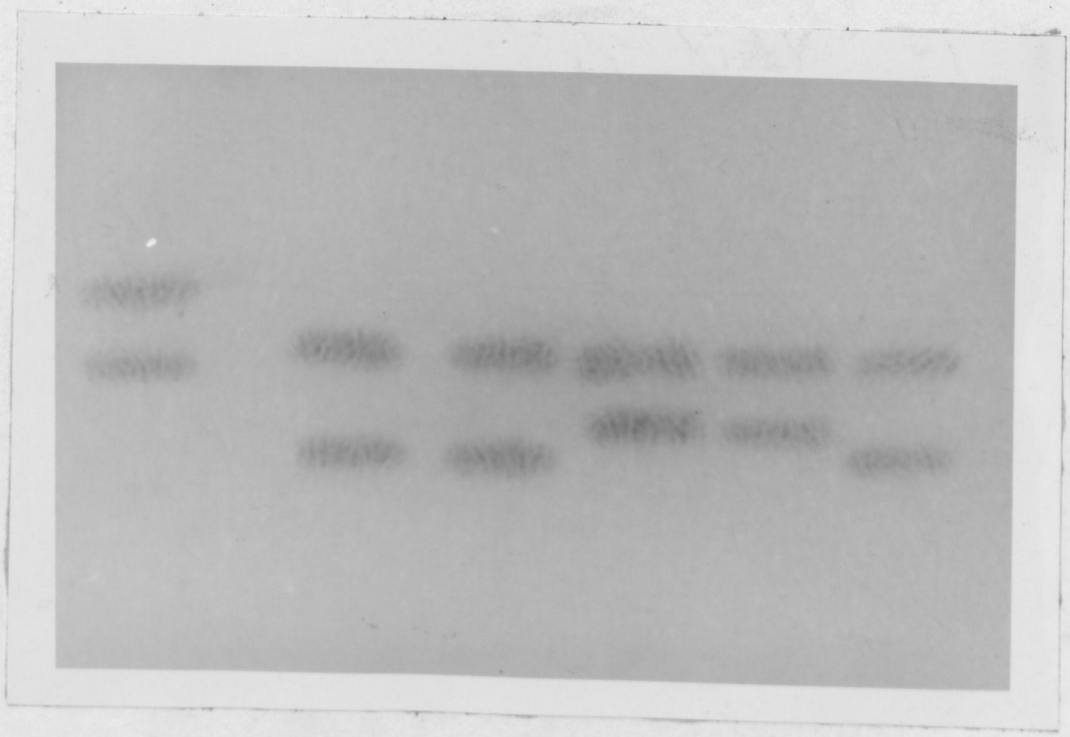


Plate VII: Starch-gel electrophoresis of peptidase I (PEP I) comparing one isolate of T. congolense with five isolates of T. simiae. From left to right 1- Trypanosoma congolense S 14, 2-6 Trypanosoma simiae EATRO 1786, 1806, TS 81, TS 82 and EATRO 1861.

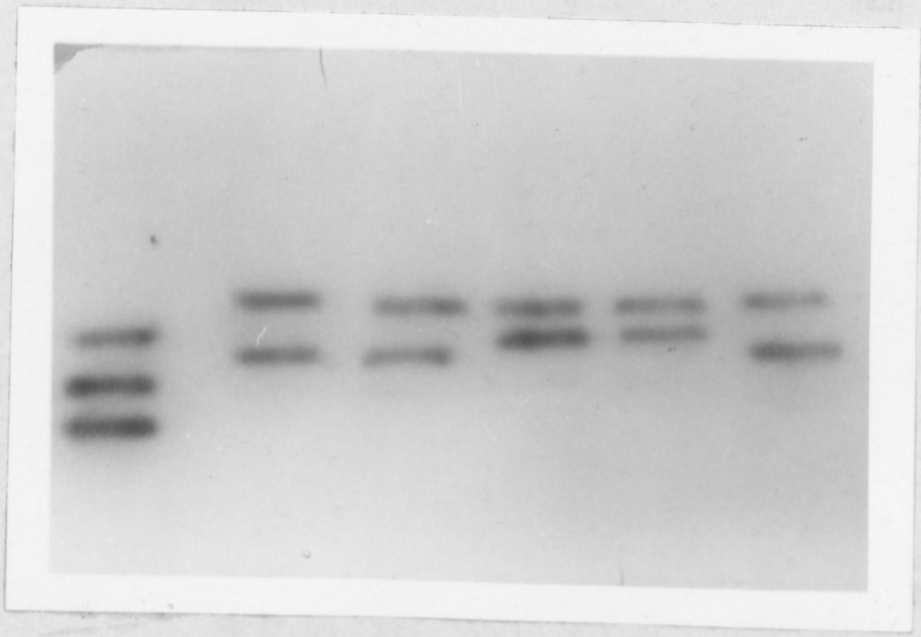


Plate VIII: Starch-gel electrophoresis of peptidase II (PEP II) comparing one isolate of T. congolense and five isolates of T.simiae. From left to right 1 - Trypanosoma congolense S 14, 2-6 Trypanosoma simiae EATRO 1786, EATRO 1806, TS 81, TS 82 and EATRO 1861.

Band	T.simiae	T.congolense	Enzyme
I	64.7	42.25	
II	-	49.29	PGM
III	-	-	
I	27.96	55.54	
II	31.32	59.99	GPI
III	50.0	63.33	
I	64.5	41.25	
II	-	46.25	MDH
III	-	48.75	
I	32.06	58.04	
II	36.89	58.6	ASAT
III	37.93	63.79	
I	18.66	43.89	
II	21.66	47.5	ME
III	-	-	
I	32.64	51.47	
II	36.17	-	ALAT
III	40.59	-	
I	39.69	53.12	
II	55.16	65.62	PEP I
III	-	-	
I	44.75	31.71	
II	52.19	37.80	PEP II
III	-	46.34	

Table III: The mean Rf values for the various enzymes.

bands were slower moving than the bands in T. simiae isolates. The faster band in T. simiae was shared amongst all the isolates and had an Rf value of 52.19. The slower band, however, showed variations amongst the T. simiae isolates suggesting intraspecific differences in the parasite.

3.2. ANTIGENIC VARIATION

Antigenic variation is believed to be the phenomenon responsible for the relapsing course of parasitaemia in organisms of the genus Trypanosoma. Experiments were carried out to study this phenomenon in Trypanosoma simiae. Initial experiments involved taking counts of parasites in infected host animals. The counts enabled us to establish a pattern of parasitaemia in rats infected with the rat-adapted strain of T. simiae. The results of these counts are shown in Figure 1. The results indicate that the parasites first appeared in the blood of the animal on day six after infection. Parasite numbers increased until a peak parasitaemia of 5×10^7 organism/ml of blood was reached. This was followed by a decrease, and then an increase in number of parasites. There were clear fluctuations in trypanosome numbers with well defined peaks. The waves of parasitaemia crested at regular intervals of two to three days. The fluctuations in parasite numbers were terminated by death of the host animal. The fluctuations in parasite counts were a clear indication of the occurrence of antigenic variation in the parasite.

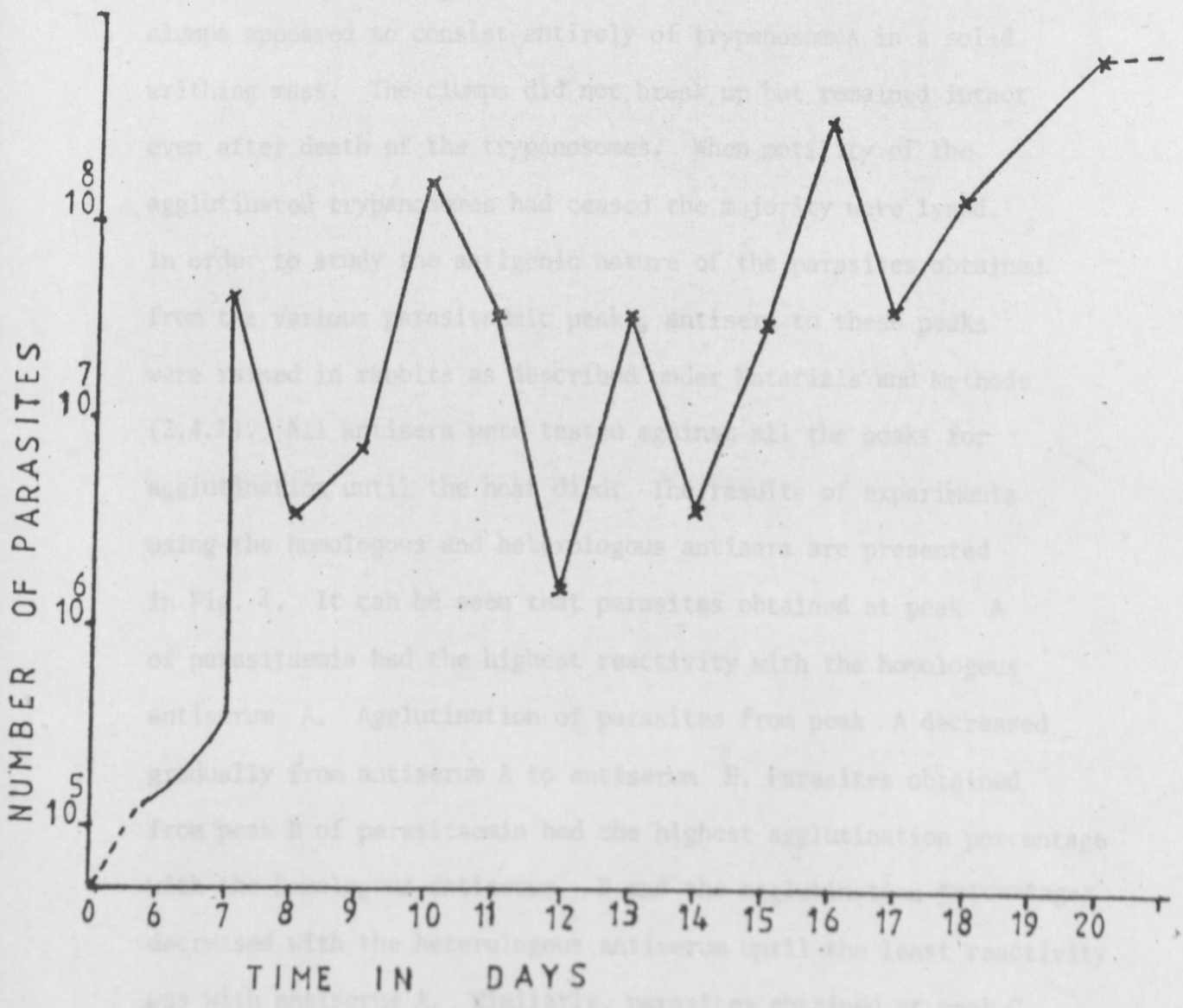


Fig 1. Pattern of parasitaemia in the Laboratory rat.

3.2.1. Agglutination

Serological tests including agglutination and lysis were carried out in order to compare the antigenic compositions of parasitaemic peaks. Agglutination was observed as clumps composed of densely packed organisms. Under high power, the clumps appeared to consist entirely of trypanosomes in a solid writhing mass. The clumps did not break up but remained intact even after death of the trypanosomes. When motility of the agglutinated trypanosomes had ceased the majority were lysed. In order to study the antigenic nature of the parasites obtained from the various parasitaemic peaks, antisera to these peaks were raised in rabbits as described under Materials and Methods (2.4.1). All antisera were tested against all the peaks for agglutination until the host died. The results of experiments using the homologous and heterologous antisera are presented in Fig. 2. It can be seen that parasites obtained at peak A of parasitaemia had the highest reactivity with the homologous antiserum A. Agglutination of parasites from peak A decreased gradually from antiserum A to antiserum E. Parasites obtained from peak B of parasitaemia had the highest agglutination percentage with the homologous antiserum - B and the agglutination percentages decreased with the heterologous antiserum until the least reactivity was with antiserum A. Similarly, parasites obtained at peak C, D and E of parasitaemia had the highest reactivities with the homologous antisera C, D and E respectively. These results show that agglutination occurred only when complementary antibody was present in the medium. This suggest that serologically different populations

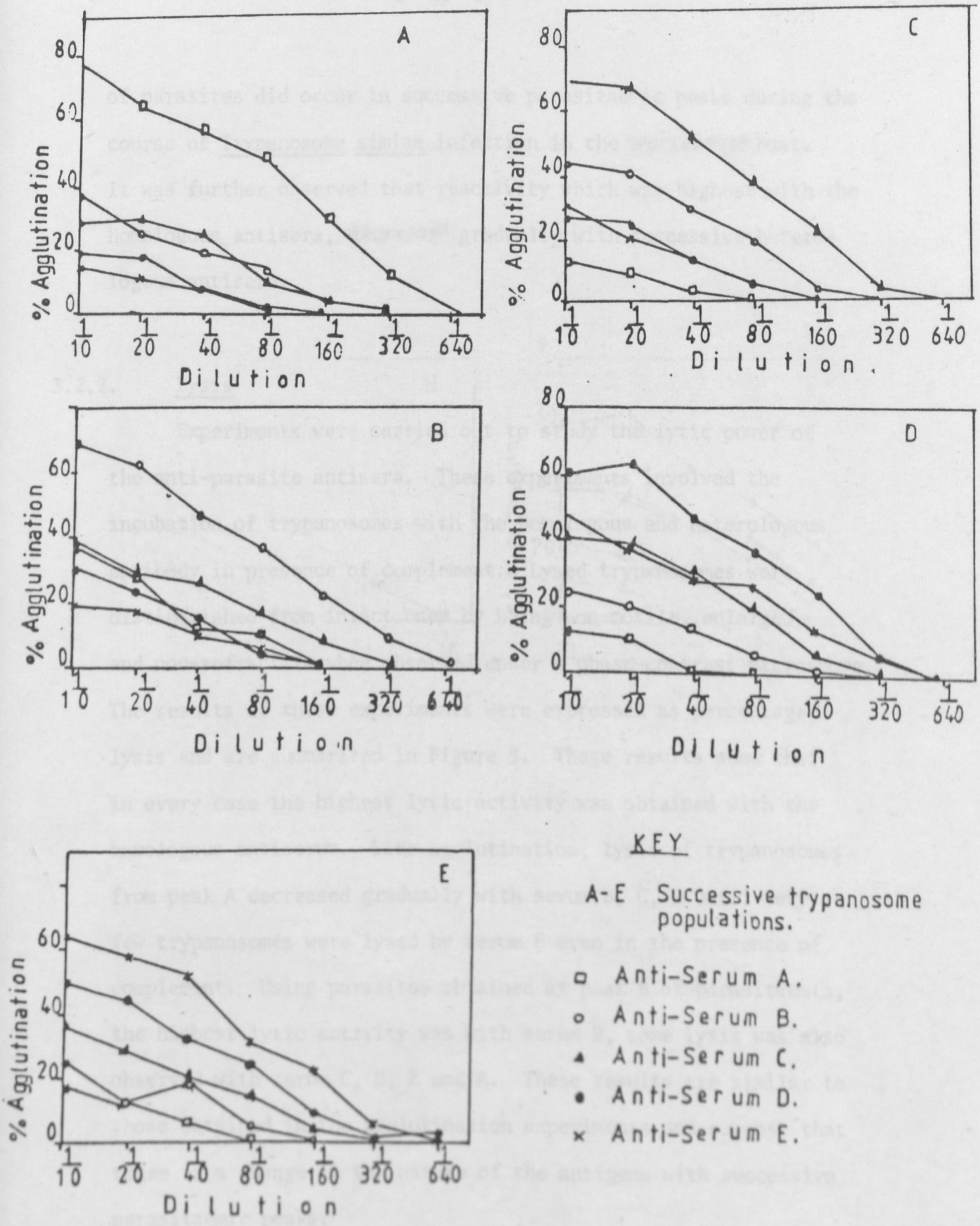


Fig 2. Agglutination assays of *T. simiae* populations(A-E) using the homologous and heterologous antisera.

of parasites did occur in successive parasitaemic peaks during the course of Trypanosoma simiae infection in the vertebrate host. It was further observed that reactivity which was highest with the homologous antisera, decreased gradually with successive heterologous antisera.

3.2.2. Lysis

Experiments were carried out to study the lytic power of the anti-parasite antisera. These experiments involved the incubation of trypanosomes with the homologous and heterologous antibody in presence of complement. Lysed trypanosomes were distinguished from intact ones by being non-motile, enlarged and non-refractile when observed under a phase-contrast microscope. The results of these experiments were expressed as percentage lysis and are summarized in Figure 3. These results show that in every case the highest lytic activity was obtained with the homologous antiserum. Like agglutination, lysis of trypanosomes from peak A decreased gradually with serum B, C, D until very few trypanosomes were lysed by serum E even in the presence of complement. Using parasites obtained at peak B of parasitaemia, the highest lytic activity was with serum B, some lysis was also observed with serum C, D, E and A. These results are similar to those obtained in the agglutination experiments and suggest that there is a change in the nature of the antigens with successive parasitaemic peaks.

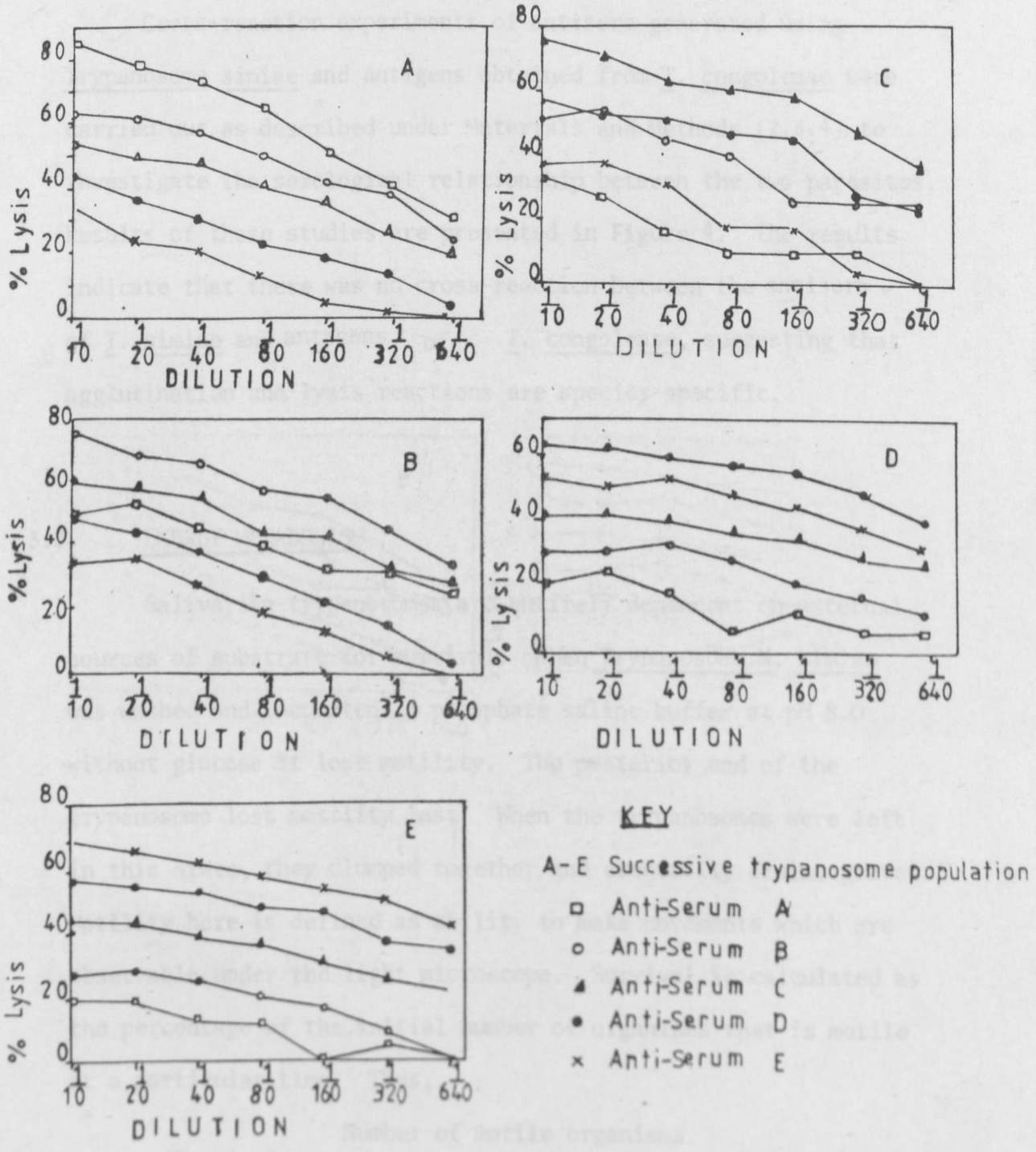


Fig 3. Lysis assays of T. simiae populations (A-E) using the homologous and heterologous antisera.

3.2.3. Cross-reaction experiments

Cross-reaction experiments of antisera generated using Trypanosoma simiae and antigens obtained from T. congolense were carried out as described under Materials and Methods (2.4.4) to investigate the serological relationship between the two parasites. Results of these studies are presented in Figure 4. The results indicate that there was no cross-reaction between the antisera of T. simiae and antigens of T. congolense, suggesting that agglutination and lysis reactions are species-specific.

3.3. ENERGY METABOLISM

Salivarian trypanosomes are entirely dependent on external sources of substrate for survival. When Trypanosoma N. simiae was washed and incubated in phosphate saline buffer at pH 8.0 without glucose it lost motility. The posterior end of the trypanosome lost motility last. When the trypanosomes were left in this state, they clumped together and eventually disintegrated. Motility here is defined as ability to make movements which are observable under the light microscope. Survival is calculated as the percentage of the initial number of organisms that is motile at a particular time. Thus,

$$\text{Survival} = \frac{\text{Number of motile organisms}}{\text{Initial number of organisms}} \times 100$$

The effects of glycerol, glucose and α-glycerol-phosphate upon survival of T. simiae were studied and the results of these experiments are shown in Table IV. The survival of T. simiae

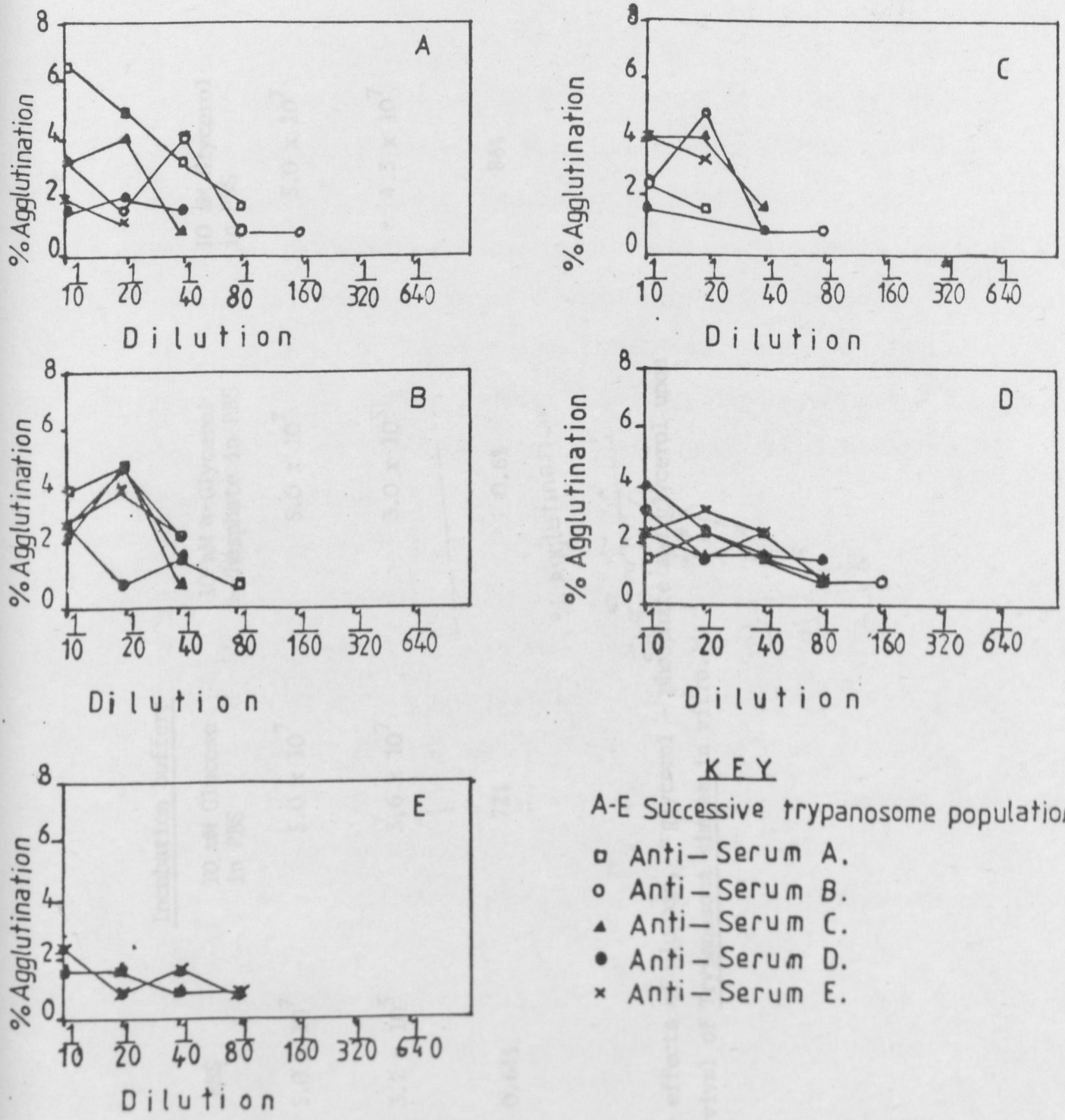


Fig 4. Cross-reaction assays of T. congolense organisms with T. simiae antisera.

	Incubation buffers			
	PBS	10 mM Glucose in PBS	10 mM α-Glycerol α-Phosphate in PBS	10 mM Glycerol in PBS
Initial counts of trypanosomes	5.0×10^7	5.0×10^7	5.0×10^7	5.0×10^7
Counts after 60 minutes	3.2×10^5	3.6×10^7	3.0×10^5	4.3×10^7
% survival	0.64%	72%	0.6%	86%

Table IV: The effects of glucose, α glycerol - phosphate and glycerol upon survival of Trypanosoma simiae in vitro.

after 1 hour's incubation in 10 mM glucose and in 10 mM glycerol was 72% and 86% respectively. When incubated for the same period in α-glycerol - phosphate or phosphate buffered saline only a few (0.6%) of the organisms survived. These results clearly indicate that both glucose and glycerol support the survival of simiae in vitro. Glycerol gave a better survival rate than glucose. It was further observed that if a suitable substrate was added to the culture when the organisms were almost immobile they became active.

In order to find out how substrates were utilized by the trypanosomes, incubations were carried out with 10 mM glucose in a closed system. The utilization and metabolic products of glucose under aerobic conditions were then investigated. In all the experiments the concentration of glucose was 10 mM at the start of incubation. T. simiae aerobically breaks down glucose. The rate of oxygen utilization is as shown in Figure 5. The rate of oxygen utilization was 40 nanoatoms O_2/min/10^8 trypanosomes. This rate is slightly lower than that reported for T. brucei (Subgenus Trypanozoon) by Hill, 1980. The oxygen uptake is a good indication of the fact that glucose is aerobically broken down.

The next step undertaken was to try and analyse the end-products of this glucose breakdown. The end-products were assayed after 1 hour's incubation of simiae freshly harvested in 10 mM D-glucose as described under materials and methods (2.5.2). The results of these experiments are summarized in Table V. The main organic end-products of simiae were acetate and pyruvate.

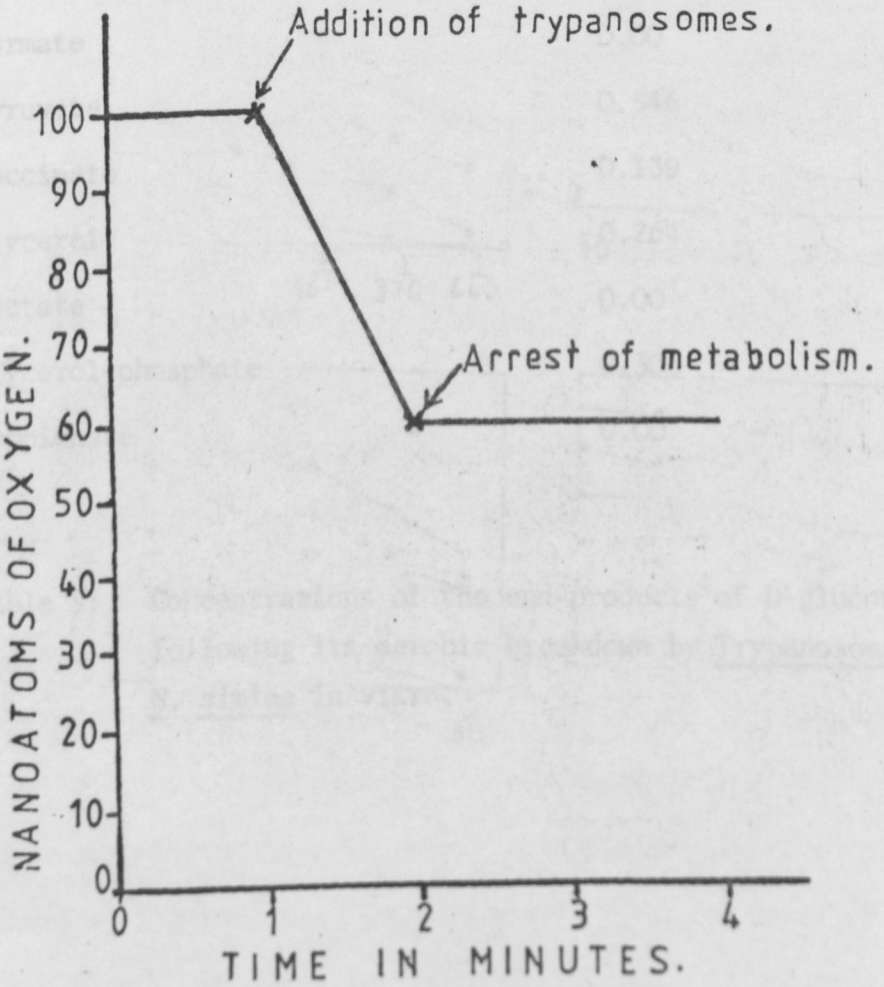


Fig 5. Oxygen uptake by bloodstream forms of *T. simiae*.

Metabolite	µmoles/ml sample
Acetate	1.04
Ethanol	0.00
Formate	0.00
Pyruvate	0.546
Succinate	0.139
Glycerol	0.264
lactate	0.00
Glycerol-phosphate	0.306
Propionate	0.00

Table V: Concentrations of the end-products of D-glucose following its aerobic breakdown by Trypanosoma N. simiae in vitro.

Succinate, glycerol and glycerol-phosphate were present in low amounts. There was no ethanol, lactate, formate or propionate. The absence of lactate conforms to the low activity exhibited by lactate dehydrogenase (LDH) reported below. The end-products could be a rough indication of the pathways followed in the aerobic degradation of glucose by the trypanosome under study.

A study of the enzyme activities was undertaken in an attempt to understand the relationship between the organisms' dependence on glycolysis for survival, the metabolic products and the high rate of oxygen utilization observed. Trypanosomes are unique in that NADH reoxidation is through the α-glycerol phosphate oxidase (αGPO) system. The activities of malic enzyme (ME) and malate dehydrogenase (MDH) were studied as possible pathways of NADH reoxidation in simiae. The activities of the enzymes may also be indicative of other possible catabolic pyruvate utilizing pathways or pathways by which alternative substrates may be utilized. A unit of enzyme activity was taken as that required to catalyse the conversion of 1 μmole of substrate/minute under the assay conditions. All the glycolytic enzyme activities were demonstrated in addition to other enzyme activities shown in Table VI.

Enzyme	µmoles/min/mg Protein	No. of determinations
Hexokinase	0.50 ± 0.12	3
Glucose-6-PDH	0.031 ± 0.019	3
Phosphoglucoisomerase	0.623 ± 0.11	3
Phosphofructokinase	0.56 ± 0.047	3
Lactate Dehydrogenase	0.022 ± 0.01	3
Glycerol Kinase	0.50 ± 0.05	3
Pyruvate Kinase	0.76 ± 0.16	3
Malate Dehydrogenase	0.073 ± 0.032	3
α-glycerol phosphate oxidase	0.035 ± 0.005	3
$NADP^+$ linked malic enzyme	0.058 ± 0.03	3

Table VI: Enzyme activities in µmoles/min/mg protein of homogenates of Trypanosoma N. simiae.

CHAPTER IV

DISCUSSION

DISCUSSION

Trypanosomes of the subgenus Nannomonas, T. congolense and T. simiae are morphologically indistinguishable (Hoare, 1972, Godfrey, 1977). Some authors regard T. simiae as a distinct species whereas others designate it as a sub-species of T. congolense. In the present work, three aspects of the biology of T. simiae, namely antigenic variation, electrophoresis of isoenzymes and metabolism were studied with a view to seeing whether or not it differs from T. congolense.

4.1. Antigenic Variation

Antigenic variation occurs in T. congolense (Wilson and Cunningham, 1972) and it was of interest to find out if it occurs in T. simiae. The initial experiments involved taking daily counts of parasites in the infected host animals until the animals died. Results of such counts clearly show a fluctuating pattern in the number of parasites in the host animal as the infection progressed. The pattern is a clear indication of antigenic variation in the parasite under study. To confirm this observation two serological tests were carried out. Immune sera against parasites from the various peaks of parasitaemia was raised in rabbits as described under materials and methods (2.4.1). Using these sera, both agglutination and lysis experiments were carried out. Agglutination results clearly showed that the highest reactivity occurred with the homologous antisera. There was then a gradual decrease in reactivity with successive heterologous antisera such that the lowest reactivity

was observed with the most heterologous antiserum. The lysis test also confirmed these observations. These results strongly suggest that the change in antigen constitution was gradual and continous and that several antigenic types co-existed together though at any one time there was a predominant type often called the "Variant antigen type" (VAT). This variant antigen type had the highest reactivity with the homologous antiserum. It is also clear from the results that each rabbit produced antibodies reacting with an increasing number of antigenic types. The results do not provide the order in which antigens developed in the host, but they certainly show antigenic change. These results do agree with the work by Wilson and Cunningham (1972) who using T. congolense demonstrated that antigenic variation is not a random process.

Results obtained in this study further suggest that at advanced stages of infection (Peak E), mixtures of antigenic types may be found in the bloodstream of the vertebrate host as parasites at peak E of parasitaemia were lysed and agglutinated to some extent by antibodies generated by the previous peaks. These results do agree with those of Van der ploeg et al (1982) who suggested that the number of antigenic variants that can be generated from a strain of trypanosomes is uncertain.

Antisera raised in rabbits infected with T. congolense failed to agglutinate T. simiae organisms. A cross-reaction would suggest that the organisms share common antigens on their surfaces. Lack of cross-reactivity implies that the antigens of T. simiae and those of T. congolense are serologically distinct

and that the two parasites are antigenically different. The low percentage agglutination observed in very low dilutions could be due to autoagglutination. Soltys (1957) using T. brucei and T. congolense organisms showed that agglutinins were species specific such that agglutinins produced by T. congolense agglutinated T. congolense organisms but not T. brucei organisms.

The results offered here are based on the interpretation that the different peaks of parasitaemia A, B, C, D and E pertain to different antigenic populations within the organism. The populations are in competition with one another and are subject to selection through the agency of antibodies and physiological responses of the host. This selection results in one population being dominant at a given time and this is the one detected most strongly by the homologous antibody in both the agglutination and the lysis reactions. The other populations are small.

In conclusion it can be stated that antigenic variation occurs in T. simiae. If the lysis and agglutination reactions are species-specific then the results show that T. simiae and T. congolense are distinct species since agglutinins which were produced by T. simiae agglutinated homologous and heterologous populations derived from T. simiae but failed to agglutinate populations produced by the closely related organism T. congolense.

The two serological tests may be of value in the diagnosis of low grade T. simiae infections in which it is not easy to demonstrate trypanosomes by parasitological methods.

4.2. Electrophoresis

In this study, starch-gel electrophoresis was carried out on 8 enzymes. The results (Plates I - Plate VIII) show differences between T. congolense and T. simiae. There were distinct differences in the patterns of migration of the isoenzymes and also in the rate of migration. These interspecific differences are due to structural differences in the proteins and have presumably, developed during the course of evolution.

With regard to the results for the enzymes glucose phosphate isomerase, aspartate aminotransfense, malic enzyme, peptidase I and peptidase II, it is evident that there are variations amongst the isolates of T. simiae. These are intraspecific differences which could be the outward manifestations of less profound modifications affecting some of the proteins. Although T. simiae patterns vary considerably with regard to the mentioned enzymes it was evident that the isolates shared common bands.

Results of this study indicate that T. simiae is different from T. congolense and therefore support the designation of simiae as a distinct species. They agree with those of Gashumba et al (1986) who using two isolates of T. simiae showed that it is distinct from T. congolense on the basis of isoenzymes. Enzymes being discrete proteins, enzyme variations may be considered an

expression of genetic variation and a valid taxonomic tool.

4.3. Metabolism

It has been demonstrated in this study that T. simiae depends largely on an external substrate for survival. In absence of an external substrate (glucose or glycerol) the organisms lyse and eventually disintegrate. The end products of aerobic glucose breakdown by T. simiae were found to include acetate, succinate, pyruvate, glycerol and glycerol phosphate. There was no lactate, ethanol or propionate. These results strongly suggest that glucose breakdown goes beyond the glycolytic sequence. The initial steps of carbohydrate catabolism however conform to those of classical glycolysis a fact supported by the high levels of activity for enzymes involved in the glycolytic sequence as reported later in this chapter. These results agree with those of Bowman and Flynn (1976), who reported that bloodstream trypanosomes of the subgenus Trypanozoon were entirely dependent on an external substrate for their energy requirements. Carbohydrate metabolism in trypanosomes is characterized by incomplete oxidation of the substrate (Grant and Fulton, 1957). This is most marked with the brucei - evansi group. Reiner et al (1936), found that T. equiperdum converted glucose into pyruvic acid, under aerobic conditions or into a mixture of pyruvic acid and glycerol under anaerobic conditions. Von Brand et al (1948) using T. gambiense found that pyruvic acid accounted for most of the glucose degraded under aerobic conditions. Similar observations were noted for T. brucei. Grant and Fulton (1957) using T. rhodesiense

noted that glucose catabolism results in the production of mainly pyruvate.

In contrast to the evansi-brucei group, T. cruzi, T. congolense and T. vivax degrade glucose beyond the pyruvic acid stage. Fulton and Spooner (1959) noted that the organisms produce appreciable quantities of succinic, acetic acids and carbon dioxide. Similar observations were made by Ryley (1956). Aerobic succinate formation is common to several trypanosomes (Ryley, 1956) but the pathway of biosynthesis of this metabolite is unknown. The system responsible for the formation of this metabolite may offer a possible target for chemotherapeutic attack.

A survey of enzyme activities in this study was carried out to try and explain possible pathways of glucose utilization. Hexokinase, the first enzyme in the glycolytic sequence showed one of the highest enzyme activities. Its activity compared with glycerol kinase the phosphorylating enzyme on the opposite end of the pathway. The two enzymes catalyse the initial steps in the breakdown of the two main substrates glucose and glycerol. The enzyme hexokinase also catalyses a rate limiting step in the glycolytic sequence. Phosphoglucoisomerase and phospho-fructokinase exhibited high activities (0.623 and 0.56 respectively). These are enzymes crucial in the glycolytic pathway. The enzyme lactate dehydrogenase was present but it had a very low activity. This low activity, the absence of lactate and the inability to accumulate pyruvate to oxidise NADH indicate that lactate dehydrogenase is not active in the organism. NADH reoxidation could be via the α-glycerol phosphate oxidase system

(Hill, 1976) as is the case in the other trypanosome species. There was very low activity exhibited by glucose-6-phosphate dehydrogenase whereas 6-phosphogluconate dehydrogenase activity was not demonstrated. The two observations rule out the presence of the pentose phosphate pathway in Trypanosoma simiae and suggests that glucose-6-phosphate dehydrogenase plays no active role in the organism.

The activities of the terminal oxidase system of trypanosomes were demonstrated. α-glycerol phosphate dehdrogenase had an activity of 0.525 comparable to that of hexokinase and glycerol kinase. Glycerol phosphate oxidase, on the other hand, exhibited very low activity (0.035) comparable to those of lactate dehydrogenase and glucose-6-phosphate dehydrogenase. Malic enzyme activities were effectively demonstrated by the ability of the homogenate at PH 7.6 to reduce $NADP^+$ and to a very low extent NAD^+ in presence of malate as substrate. The observed activity was then due to NAD^+ linked malic enzyme. Malate dehydrogenase activity observed at pH 10.0 was 0.073. This was at levels higher than that of NAD^+-linked malic enzyme.

In summary, glycolysis is fundamental for the survival of T. simiae in vitro as it is in other trypanosome species. The results suggest that a decarboxylase system exists for the catabolism of pyruvate to acetate probably via the formation and subsequent hydrolysis of acetyl Co A.

CONCLUSIONS

From the results of this study, it is possible to draw the following conclusions:

1. *Trypanosoma simiae* is antigenically distinct from its close relative *T. congolense*.

2. *T. simiae* is a distinct species from *T. congolense* on the basis of enzymic differences.

3. *T. simiae* is very similar to *T. congolense* in metabolism.

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