STUDIES ON THE RESISTANCE OF HOST RED BLOOD CELLS TO *TRYPANOSOMA VIVAX* PHOSPHOLIPASES

by

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DECLARATION

I declare that this work is wholly the author's and has not been part of any presentation for any other qualification.

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EMMANUEL O. OGBADOYI (Author)

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CERTIFICATION

This thesis tittled "Studies on the Resistance of Host Red Blood Cells to Trypanosoma Vivax Phospholipases" by Emmanuel Olofu Ogbadoyi meets the regulations governing the award of the degree of Master of Science, Ahmadu Bello University Zaria and is therefore approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to my cousin, Engr. B.O. Oche and God the Almighty Father who have made it possible for me to attain this level of education.

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ABSTRACT

Five goats were experimentally infected with Trypanesoma vivax and the packed cell volume (PCV), haemoglobin (Hb) levels and serum free fatty acid (FFA) levels were monitored. A cyolical fluctuation was observed in the development of parasitemia during the twenty-six-day period of infection with the highest peaks on days five and twelve. There was a general gradual decrease in both the PCV and the Hb levels of all the infected animals, an indication of progressive anaemia. The anaemia became very severe by day twenty-six post infection as shown by the very low values of the PCV and Hb levels. One of the infected animals died of acut infection on day twenty-six post infection. Analysis for serum FFA showed a general increase in the FFA levels of all the infected animals throughout the period of infection.

Investigation into the haemolytic activity of the isolated phospholipase A_2 showed that the enzyme does not cause any significant haemolysis of host (bovine) erythrocytes in the absence of serum albumin. However, in the presence of 3.5% albumin, the enzyme caused up to 70% haemolysis. In the presence of 20mM EDTA, a potent inhibitor of phospholipase A_2, only 8.0% haemolysis was observed. The development of drugs for the treatment of anaemia in trypanosomiasis using inhibitors of phospholipases is therefore suggested.

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CHAPTER ONE

INTRODUCTION

1.1 GENERAL INTRODUCTION

One major problem today on the whole African continent and most developing nations of the world is meeting the food needs of the rapidly growing population. Many of these countries therefore import food from the more agriculturally developed nations. Today, food importation which used to supplement the meagre local production is made difficult by the global economic recession. Farm animal protein is almost entirely out of reach for the poverty striken majority in many parts of Africa and some wild animals from which they used to derive most of their animal protein are now sold out to the privileged few, leading to widespread cases of childhood malnutrition, (particularly protein energy malnutrition) and adult malnutrition.

Thus the agricultural output derived from the predominantly peasant system is clearly incapable of meeting the domestic needs of the rapidly expanding population. While the problem of food scarcity may be largely attributed to the inadequacies of man, it is pertinent to appreciate the role of nature in this regard, vis-a-vis draught and desert enchroachment on the African continent. A concerted effort is therefore required of all concerned to tap all available potentials in the agricultural sector so that Africa can meet the food needs of her people and possibly obtain finances for the development of other sectors of the economy.

1.2 LIVESTOCK DEVELOPMENT

The potentials for appreciable increases in livestock production in many parts of Africa and Nigeria in particular cannot be over emphasized. This is however hampered by man power and animal diseases. Many diseases in Africa, such as Rinderpest, East Coast Fever (ECF) and tsetse-transmitted Trypanosomiasis affect animals. Infact trypanosomiasis affect both man and his domestic animals. Unlike rinderpest, ECF and trypanosomiasis still constitute a major constraint militating against the development and growth of the livestock industry in many parts of Africa thereby limiting total food production. These two diseases are believed to account for the death of 3 million cattle a year and one or both of these diseases occurs across more than one third of the continent (ILRAD 1987).

Trypanosomiasis is a disease caused by protozoan parasites of the genus Trypanosoma of which different species infect cattle, sheep, goats, pigs, horses, donkeys, camels, many types of wild animals and man. African trypanosomiasis is mainly transmitted by tsetse flies, Glossina species. In highly tsetse - infested areas, it is extremely difficult to keep livestock at all, and even human habitation may be threatened. The disease complex has a number of clinical forms which include preacute, acute, chronic and mild forms. The characteristic symptoms in both man and animals are intermittent fever and parasitemia, progressive anaemia,

emaciation, reduced production and sudden death. Affected animals show poor growth rates, low milk yields, reduced capacity for work, poor reproductive performance (ILRAD, 1986) and at slaughter, carcasses are of low weight and poor quality, all of which lead to large economic losses (Kyewalabye, Personal Communication). Trypanosoma vivax causes the most important form of trypanosomiasis in farm animals in West Africa (Soulsby, 1982) particularly in Northern Nigeria (Leeflang, 1978).

Control programmes for African trypanosomiasis have been on for many decades. These involve control of the tsetse vector by destroying the wild animal populations on which tsetse feed and/or clearing the bush vegetation required by tsetse as breeding and resting sites; application of insecticides in some instances in connection with tsetse traps and targets or screens (ILRAD, 1986); sterilization of the males (Dame, 1970); biological control using parasites and predators of Glossina (Nash, 1970) and Hand-catching and traps (Glasgow and Potts 1970). Considerable success has been achieved where control measures using insecticides have been properly implemented, notably in Nigeria, Botswana, Zambia and Zimbabwe (ILRAD, 1986). However, inspite of these control measures in many parts of Africa, the area infested by tsetse flies is expanding.

Regular treatment of infected animals with trypanocidal drugs has been in use since the first quarter of this century.

This approach has also been successful in areas where livestock management has been consistent overtime (ILRAD, 1986). There are however, some limitations. Domestic animals are exposed to tsetse flies that have fed on wild animals which act as an extensive reservoir of infection and an indefinite application of chemotherapy is therefore required to obtain any meaningful result. Secondly no new trypanocidal drug has been introduced since the past twenty-seven years and the possibility of drug resistance developing in local parasite populations is a serious threat (ILRAD, 1986). The phenomenon of antigenic variation in which variant antigenic types of the parasites are presented to the host further complicates the problem by making it difficult to develop an effective vaccine against African trypanosomiasis (ILRAD, 1986).

Research programmes aimed at developing improved control measures are on in many international organizations such as the International Laboratory for rResearch on Animal Diseases (ILRAD) and the International Livestock Centre for Africa (ILCA). For instance, ILRAD in conjuction with the Wildlife section of the Veterinary Research Laboratory in Kenya are currently studying the mechanisms of resistance to trypanosome with the aim of identifying resistance mechanism which could be induced in domestic livestock or genetic markers which could be used to select relatively resistant animals. Another major area of current research is understanding the pathogenesis of trapanosomiasis

with the aim of enhancing the host resistance.

It has been well documented that anaemia is one of the major pathological conditions of clinical significance in animal trypanosomiasis (Fiennes, 1954; Losos and Ikede, 1972; Mackenzie and Cruickshank, 1973; Woodruff, 1973; Mamo and Holmes, 1975; Kobayashi et al, 1976 Maxie et al, 1976). The mechanism of this anaemia is uncertain. However, hypoteheses such as hypoplasia of the bone marrow (Jennings et al, 1974), hydraemia (Fiennes, 1954), haemorrhagic syndrome (ILRAD, 1984), immunological reactions (Herbert and Inglis, 1973; Mackenzie and Cruickshank, 1973; Woo and Kobayashi, 1975; Kobayashi et al 1976, Ikede et al, 1977); and severe haemolysis (Landsteiner and Raubitscheck, 1907; Fiennes, 1950, 1954; Huan et al, 1975; Tizzard and Holmes 1976; Tizzard et al; 1977; Tizzard et al, 1978, ILRAD, 1984). The predominance of hemolytic anaemia in trypanosomiasis has been reported (Mamo and Holmes 1975; Valli et al, 1978). Haemolytic anaemia is believed to be caused by the action of trypanosome phospholipases on endogenous phospholipids during active trypanolysis in which the free fatty acids and lysophospholipids so generated exert their detergetnt like activity on the erythrocyte membranes leading to haemolysis (Tizzard et al, 1978). A clear understanding of this crucial role of these enzymes in the pathogenesis of the anaemia is possible only when the enzymes are isolated and their activities studied in vitro. Unfortunately, to the best of the author's knowledge, little or no work has been done in this direction with regard to Trypanosoma vivax

which incidentally is the major cause of bovine trypanosomiasis in Nigeria and West Africa.

Significant advances in the control of trypanosomiasis would lead to major improvements in agriculture, food production, economic growth and human well-being in nearly every country of subsaharan Africa.

It is against this background that the present study was undertaken to isolate trypanosoma vivax phospholipases and examine their effect on host erythrocytes in vitro.

CHAPTER TWO

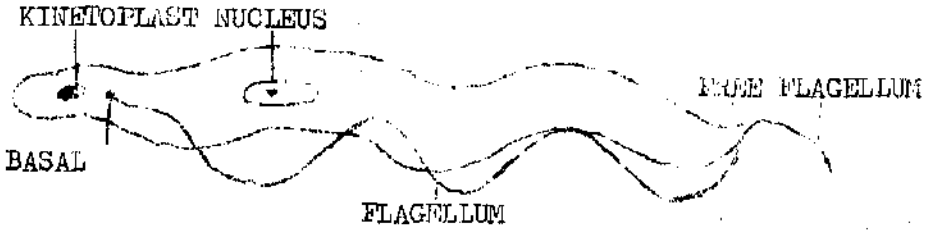
LITERATURE REVIEW

2.1 CLASSIFICATION OF TRYPANOSOMES

Trypanosomes are unicellular protozoan parasites and belong to the intestinal flagellates in the family trypanosomatidae. A systematic position of Trypanosoma among the protozoa and the revised classification of the mammalian trypanosomes are shown in table one.

2.2 MORPHOLOGY OF TRYPANOSOMES (UNDER LIGHT MICROSCOPE)

Typically, mammalian trypanosomes have a more or less spindle-shaped flattened body which is usually curved. However, the shape varies in different species. There is a single vesicular nucleus and a single flagellum which runs along the outer margin of a pellicle to form the undulating membrane. The flagellum may terminate or become free at the anterior end of the membrane. The flagellum originates in a structure called the 'basal body' which is sometimes referred to as blepharoplast. Close to the basal body is the kinetoplast. A number of volutin granules are also present in the cytoplasm. At the posterior end of the body is an ectoplasmic invagination called the reservior. A contractile vacuole close to the reservoir is present in some trypanosomes.



STRUCTURE OF A TRYPANOSOME UNDER LIGHT MICROSCOPE

TABLE 1 CLASSIFICATION OF TRYPANOSOMES

PHYLUM : PROTOZOA

↓

SUBPHYLUM : SARCOMASTIGOPHORA

↓

SUPERCLASS : MASTIGOPHORA

↓

CLASS : ZOOMASTIGOPHOREA

↓

ORDER : KINETOPLASTIDA

↓

SUBORDER : TRYPANOSOMATINA

↓

FAMILY : TRYPANOSOMATIDAE

↓

GENUS : TRYPANOSOMA

SUBGENUS SCHIZOTRYPANUM	SUBGENUS TRYPANOZOON	SUBGENUS HERPETOSOMA	SUBGENUS DUTTONELLA	SUBGENUS MEGATRYPANUM	SUBGENUS NANNOMONAS	SUBGENUS PYCNOMONAS
SPECIES	SPECIES	SPECIES	SPECIES	SPECIES	SPECIES	SPECIES
T. CRUZI	T. BRUCEI	T. LEWISI	T. VIVAX	T. THELERI	T. CONGOLENSE	T. SUIS
T. VESERTILIONS	T. RHODESIENSE	T. PERODICTICI	T. UNIFOME	T. HEYBERGI	T. SIMIAE	
	T. GAMBIENSE	T. PRIMATUM		T. HELOGALEI		
	T. EVANSI			T. ICHNEUMONI		
	T. EQUIPERDUM			T. IRANGIENSE		
				T. MEGADERMAE		

Different trypanosome species possess some morphological features not represented by the typical structure above. However, within the scope of this work, only those of T. vivax is considered. The subgenus Duttonella to which T. vivax belongs are generally monomorphic with a free flagellum and a typically rounded posterior end. The kinetoplast is large and terminal.

T. vivax has a somewhat club-shaped body which is typically swollen, rounded at the posterior end and tapering towards the anterior end. The undulating membrane is slightly developed and a distinct free flagellum is present. There is a large kinetoplast which is typically terminal but may be subterminal occupying a marginal position. T. vivax has a characteristic length which ranges from 18 to 31 (including the free flagellum, 3 - 6) with means from 21 to 25.4 . Fairbairn (1953) gave 2 main groups of the length distributions - The long forms, 26 - 30 (Means, 26 - 27) and the short forms 15 - 28 (Means, 21 - 24).

2.3 LIFE CYCLE

The mammalian trypanosomes fall into two main divisions on the basis of the course of development in their vector and mode of transmission (Wenyon, 1926; Hoare, 1949).

These divisions are:

A. Stercoraria: These are trypanosomes in which the entire invertebrate cycle of development is confined to the alimentary tract of the vector. Some examples are T. lewisi of rodents, T. theileri of ruminants and T. cruzi of man.

B. Salivaria: These are trypanosomes in which only the initial stages of development take place in the gut while the final stages

occur in the proboscis or salivary gland of the insect vector. In T. vivax, the entire development takes place in the proboscis. Some examples include T. brucei, T. congolense and T. vivax. The salivarian trypanosomes unlike the stercoraria (with the exception of T. cruzi) are generally pathogenic.

2.4 Transmission: The life cycle of trypanosomes involves an invertebrate host, the insect vector and a vertebrate host. The invertebrate host of the African trypanosome is tsetse fly (Glossina species). Trypanosomes are transmitted by about thirty species and subspecies of tsetse fly which infect about ten million km^2 (about 37%) of Africa (ILRAD 1986).

The trypanosomes are ingested by their invertebrate hosts when the tsetse flies feed on the vertebrate hosts. Depending on whether the trypanosomes are stercorarians or salivarians, they enter the alimentary tract or migrate to the proboscis/ salivary gland where they are transformed into trypomastigotes, through epimastigotes to metatrypanosomes. It is in this form that the trypanosomes are transmitted to the vertebrate hosts.

The metatrypanosomes are transmitted into the mammalian hosts passively by contamination of the mucous membranes or the skin, in the case of stercorarian species and actively by inoculation through the insect bite in the case of the salivarian species. The metatrypanosomes are then transformed into trypanosomes with the characteristic structure of the species to which they belong.

Although without a definite proof, biting flies other than tsetse flies probably transmit infection at the height of an epidemic as was suspected in the Mazwa epidemic (Swynnerton, 1923).

Congentital infection has been described on a number of occasions (Darre et al, 1987; David and Pape, 1942; Pinto, 1960). Experimental observations on the reason for its occurrence have been reported by Werner and Kunert (1958). It has also been claimed that infection can also be transmitted from mother to offspring through the breast milk (Lanfranchi, 1915). This has however been rejected by Werner (1954).

2.5 Reproduction: The blood forms of the mammalian trypanosomes have two main modes of reproduction - Unequal binary or multiple division in the amastigote or epimastigote stages in the invertebrate host and by equal binary division in the trypomastigote stage in the vertebrate host. The latter is characteristic of the tsetse - borne pathogenic trypanosomes and allied forms.

2.6 Energy Metabolism in Trypanosomes

Biochemical investigations are limted by the difficulty involved in getting sufficient quantity of materials directly related to natural infections. For the most part, biochemical

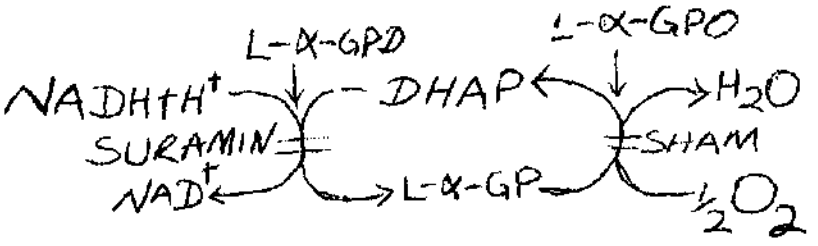
investigation is directed toward finding.

(1) differences from mammalian biochemistry to provide rational target for drug attack or prophylaxis (ii) means of identifying any different strains within a species responsible for the different disease symptoms (iii) the relationship of surface coat proteins and turnover with production of a vaccine.

The discussion which follows is for T. brucei but the information can be applied to other trypanosomes, including T. vivax.

Trypanosomes stop respiring and become immobile within one minute if there is no supply of exogenous carbohydrate because of their low endogenous energy reserves (Opperdoes et al; 1976). Energy is derived from the catabolism of glucose. However, in aging trypanosomes, when parasitemia begins to fall, 2 - oxoglutarate can replace glucose to some extent (Bowman and Flynn, 1977). Glucose catabolism occurs via a glycolytic pathway which terminates in pyruvate. The glycolytic enzymes are located in a microbody - like organelle called a glycosome (Opperdoes and Borst, 1977). There is no functional tricarboxylic acid cycle and no cytochromes, (Hill, 1976) and only two molecules of ATP are produced from each glucose molecule. Lactate dehydrogenase is absent (Dixon, 1966) and NADH is reoxidized by a unique - glycerophosphate oxidase system, consisting of glycerol - 3 - phosphate dehydrogenase and glycerol - 3 - phosphate oxidase with a shuttle between the glycosome and the undeveloped mitochondria. No ATP is produced by this oxidase

system and it is insensitive to cyanide but inhibited by hydroxamic acids notably salicylhydroxamic acid (SHAM) (Gutteridge and Coombs, 1977; Kilgour, 1980).



L -α- GPD: L -α-Glycerophosphate dehydrogenase

L -α- GPO: L -α- Glycerophosphate oxidase

DHAP: Dihydroxyacetone phosphate

L -α- GP: L -α- Glycerophosphate.

The figure above shows the - glycerophosphate system with areas of drug attack proposed by Gutteridge and Coombs (1977) and Gutteridge and Rogerson (1979).

2.7 Antigenic Variation: Ross and Thompson (1910) observed recurrent waves of parasitemia in the early stages of trypanosomiasis. These successive waves of parasitemia represent antigenically distinct populations (Levaditi and McIntosh, 1910). This seemingly endless succession of antigenic variants are associated with the changing protein coat present only during the trypanosome's infective stages in the vector and animal host (Gray and Luckins, 1976). Thus individual parasites present different antigenic guises to the host (Ritz, 1916). This is a barrier to finding an effective vaccine. It has been documented that there is a defined subset of antigenic types reproducibly found in the metacyclic trypanosomes. Thus there are twelve for T. congolense (Crowe et al., 1983) and at least 14 - 17 for T. brucei which are particular for a given strain (Barry et al.,

1979; Hajduk et al, 1981; LeRay et al, 1978).

Molecular Basis of Antigenic Variation

There is a presumption that the antigens are present on the surface of the intact parasite since antibodies against the variant-specific antigens could in the presence of complement lyse the corresponding trypanosomes in vitro. This presumption is strengthened by the report of Vickerman (1969) that blood stream trypanosomes are completely enveloped by a surface coat and that this likely contained the variable antigen. It has been demonstrated that this coat is composed of a single abundant species of glycoprotein which is characteristic for a given variant (Cross, 1975). This surface coat has been separated intact and to some extent characterized by Cross et al (1976), from homologous antigenic populations at successive peaks of parasitemia from cloned T. brucei. Immunization with these purified glycoproteins is variant - specific (Cross, 1975) and are therefore termed variant - specific surface glycoproteins (VSGs). Recent estimates put the number of VSG molecules at about 1.2×10^7 per trypanosome.

Significance of VSGs.

1. They enable the emergence of successive antigenically distinct populations in the course of an infection, allowing the organisms to avoid the consequences of the host response and persist within the blood stream. The cell surface glycoprotein when shed from the parasite is called an exoantigen. Exoantigen has a number of biological activities which include:

2. It is antigenic and may stimulate a protective immune response (Herbert and Inglis, 1973; Herbert and Lumsden, 1968).

3. It presumably combines with antibody to form immune complexes and may therefore conttribute to the immune-complex derived lesions observed in trypanosomiasis (Boreham and Kimber, 1970; Lambert and Houba, 1974).

4. Exoantigen can bind directly to erythrocytes (Mackenzie et al.1978; Woo and Kobayashi, 1975; Zuckerman, 1964), and may provoke their sensitization by trypanosome antibodies leading to the accelerated clearance of the erythrocytes (Tizzard et al., 1978).

5. Surface glycoproteins of trypanosomes (T. brucei) activate the classical complement pathway through C1, C4, and C2 (Muskoke and Barbet, 1977), since these glycoproteins can bind to erythrocytes, they may activate complement in the erythrocytes leading to significant erythrocyte destruction (Tizzard et al., 1978).

2.8 TRYPANOSOME ANESTHETICS: African trypanosomes catabolize aromatic amino acids producing compounds which may contribute to pathology characteristic of African trypanosomiasis (Stibbs and Seed, 1973, 1975, 1976). For instance, T. gambiense catabolizes tryptophan to indole - 3 - ethanol (Tryptophol), Indole - 3 - acetic acid and indole - 3 - lactic acid and 5 - hydroxytryptophan to 5 - hydroxytryptophol. (Stibbs and seed, 1976). These indoles produce a sleeplike or comatose state

(Tizzard et al., 1978), a characteristic feature of human trypanosomiasis, hence the name sleeping sickness. Tryptophol lowers body temperature (Seed and Sechelski, 1977) and suppresses the humoral immune response (Ackerman and Seed, 1976). Tryptophol also rapidly and irreversibly interacts with cell membranes leading to cell lysis (Tizzard et al., 1978). In the same manner, tryptophol alters the permeability of synaptic membranes leading to changes in the transmission of nerve impulses resulting in behavioural changes and systemic effects such as immunosuppression.

2.9 AFRICAN TRYPANOSOMIASIS

African trypanosomiasis is a spectrum of diseases caused by the salivarian trypanosomes in man, where it is referred to as sleeping sickness and in his domestic animals where it is called 'nagana'. There are variations in the symptoms and severity depending on a number of factors which may include; the causative species of trypanosome, virulence or invasiveness of the strain, level or size of challenge, duration of exposure to tsetse flies, species and breed of the animal host, the level of host resistance and age of the animals. In general, however pathological processes include erythrocyte haemolysis, damage to tissues including those of the nervous system and fever is almost often present.

Trypanosomiasis occurs in tropical Africa, central and south America, the Middle East and Asia.

Human African Trypanosomiasis

Trypanosomes were first discovered as the causative agents of human sleeping sickness as far back as 1900. In the late 1890s and early 1900s, about 200,000 people two-thirds of the population living around the north of Lake Victoria died from the disease (Boothroyd, 1985). Although accurate figures are difficult to obtain because of the association of trypanosomiases mainly with rural areas in developing countries, the general estimate is that thirty-five million people are at risk from sleeping sickness in Africa and over ten million are infected with chagas disease in Central and South America (WHO, 1976).

African human trypanosomiasis is caused by T. brucei rhodesiense, carrying the rhodesian sleeping sickness and T. brucei gambiense carrying the gambian sleeping sickness while the chagas disease of America is caused by T. cruzi. For the establishment of an infection, there is a minimum number of trypanosomes required known as the infective dose. The infective dose for man is 300 - 450 metacyclic trypanosomes (Fairbairn and Burtt, 1946).

Symptoms/Clinical Manifestation

The disease course is usually acute with T. rhodesiense and more chronic with T. gambiense. When an infected tsetse fly bites, trypanosomes are deposited in the subcutaneous tissues giving rise to a hard painful red nodule called the chancre which appears in the skin in 4 - 10 days after the tsetse bite.

The trypanosomes then invade the blood and the lymph vessels leading to swelling of the lymph nodes and the spleen and the stage of acute generalized trypanosomiasis continues with elevation in body temperature.

There is a gradual fall in red blood cell count, indicative of anaemia and the total white blood cell count also falls, there is elevation of serum globulin levels.

The heart is also affected, showing endocarditis, periarterial myocarditis, arteritis and various forms of electrocardiographic abnormalities. The effect on the heart is more in the rhodesian type of sleeping sickness than in the gambian type. There is early meningeal reaction which is manifested in severe headache, skin rash in white-skinned patients and deep hyperaesthesia.

The advanced form of the disease is characterised by further haematological, immunological, endocrine and central nervous system changes. There is increasing anemia, leucopenia; great wasting of the limb, abdominal and thoracic muscles; fibrosis of the lymph vessels; immunological failure; Inflammation of the brain (meningo-encephalitis) manifested by headaches, skin rash, etc; general increase in drowsiness during the day and insomania at night and mental deterioration, the symptoms which give rise to the common name of the disease complex - 'sleeping sickness'. The inflammation of the brain which leads to mental changes manifested by confusional behaviour, occasional hallucinations, marked apathy and lethargy is more commonly

associated with the gambian sleeping sickness than the rhodesian type. As the disease progresses, there is a gradual loss of muscle control, somnolence, coma, and death.

Endocrine dysfunction in women is shown by the occurrence of sterility, menstrual disorders and a high abortion rate. Perinatal deaths are common features of trypanosomiasis in women.

In men there may be impotence and in the later stages of the disease, some develop gynaecomastia.

Epidemiology of Human Trypanosomiasis

The disease pattern is influenced by a number of factors which may include existence of reservoir of infection in the form of human asymptomatic carriers; variation in strain and in its virulence; susceptibility of the population; development of a degree of resistance by the host or reduced virulence of the parasite arising from continued exposure of communities to the infection; possible existence of epidemic strains, the level of parasite challenge; susceptibility of the tsetse fly vector.

In terms of distribution, the gambian sleeping sickness is found mainly in the riverine areas particularly in West Africa while the rhodesian type is concentrated in the East and South African states. Both the gambian and rhodesian sleeping sickness are occupational hazards, being more prevalent in the males than in the females and even in the males, it is more commonly found in the working class.

AFRICAN ANIMAL TRYPANOSOMIASIS

Three major trypanosome species, Trypanosoma brucei, Trypanosoma congolense and Trypanosoma vivax are of great concern in veterinary pathology because they cause trypanosomiasis in domestic animals. T. vivax is of most economic importance in Nigeria out of these three trypanosome species because it is the major cause of bovine trypanosomiasis. T. evansi has some local importance in the sudan and sahel vegetation zones where it is pathogenic for camels, horses and donkeys.

The symptoms of the disease differ depending on the nature and stage of the infection being observed. The disease courses may also differ even for the same species. In some cases the disease may run an acute course, causing death of the host in about ten days. Alternatively, the disease may run a chronic coursefrom which the animal may ultimately make a complete recovery. The infection may however run a course between these two extremes. These striking variations are determined by factors similar to those of man. In general, T. congolense causes a more acute form of the disease than does T. vivax (Fiennes, 1970). Animal trypanosomiasis has a characteristic tendency to relapse after chemotherapy. Relapsing T. vivax infections in ruminants following homidium chloride treatment has been reported (Leeflang, 1978).

CLINICAL MANIFESTATIONS

There are essentially no apparent differences in the disease processes of the infection caused by T. congolense and

T. vivax and infact the pathological processes in all forms of animal trypanosomiasis are probably similar (Fiennes, 1970).

The disease processes of animal trypanosomiasis are complex but are generally characterized by intermittent fever and parasitemia, progressive anaemia, emaciation and sometimes sudden death of the infected animal. There are two clearly defined stages of infection. These are the acute stage with precritical and critical phases and the chronic stage which is post critical. Anaemia of the macrocytic type is present in the acutestage while microcytic anaemia occurs in the chronic stage. All these changes operate in different proportions and determine four different courses of the disease.

(a) The Hyperacute course. This form of the disase is characterized by severe haemolysis accompanied by hydraemia and it is rapidly fatal. A similar rapidly fatal course in West African cattle has been reported by Stephen (1970).

(b) The Acute Course. In this form the haemolysis is less severe in the early stages. There is initial hydraemia which is followed by dehydration. Death occurs at the time of haemolytic crisis.

(c) The Chronic Course. The early stages of the chronic course are similar to those of the acute course but hydraemia persists without dehydration. The haemolytic crisis if present does not prove fatal. The animal enters a chronic wasting disease which may persist for months and may eventually prove fatal.

(d) The Recovery Course. Recovery without treatment occurs exceptionally after several months infection. The hydraemic factor is either absent or of rare occurrence in this form of the disease.

Other important features include: elevation of body temperature; the animal becomes dejected with the head frequently hanging down at pasture; there is reduction in appetite and respirations are rapid and laboured; there is a significant loss of body weight and parasitemia is often massive in goats and cattle. The parasitemia is sustained in cattle (Stephen, 1970). In the less severe cases in cattle, the haircoat becomes stary because of loss of its lustre and the body loses its rounded contours and appears bony. The superficial lymph nodes, particularly the prescapulars and prefemorals become swollen and prominent and the animal develops the characteristic 'fly-struck' appearance. The animal is unable to rise and dies in a state of exhaustion in the terminal stages. Generally the only clinical features of any significance in infected sheep and goats are anaemia and loss of bodily condition.

A salient pathological change usually in chronic trypanosomiasis is the extensive involvement of lymphoid and reticular tissues, especially those commonly referred to as the reticulo-endothelial or lymphoid-macrophage system. The lining endothelium of the blood vessels may be hypertrophied and severely denuded (Fiennes, 1946). It is suggested (Fiennes, 1970) that this is due to intense mobilization of macrophage reserves. Thrombosis

may occur where the endothelial lining of the blood vessels is deficient There is widespread infiltrations of round cells, almost entirely of the lymphoid type in many organs just as those which occur in protein deficiency states like kwashiorkor.

ANEMIA IN TRYPANOSOMASIS. Aneamia is a recognised feature of clinical trypanosomiasis in man and his domestic animals (Fiennes 1953, Mackenzie and Cruickshank 1973, Mamo and Holmes 1975, Kobayashi et al. 1976). It is also regarded as the main pathological feature in livestock infected with T. congolense and T. vivax (Fiennes, 1970, Losos and Ikede, 1972). In some forms of trypanosomiasis, death may occur as a direct result of a servere haemolytic crisis (Losos and Ikede, 1972). The pathogenesis of the anaemia is unclear but a number of hypotheses have been put forward to explain the mechanism. These are hypoplasia of the bone marrow and hydraemia (Fiennes, 1954, 1970), hemorrhagic syndrome (ILRAD),1984), immunological reactions (Herbert and Inglis 1973, Woo and Kobayashi 1975, Kobayashi et al. 1976), erythrophagocytosis (Mackenzie and Cruickshank, 1973, Esievo et al. 1982), and severe haemolysis (Fiennes, 1950, 1954; Huan et al. 1975, (ILRAD1984). These hypotheses which have failed to find common ground of acceptable specificity are each discussed below.

(a) Immunological Mechanism and Erythrophagocytosis:

Erythrocyte destruction may be mediated by immunological mechanisms (Woo and Kobayashi, 1975; Herbert and Inglis, 1973; Kobayashi et al., 1976). In cattle suffering from naturally acquired trypanosomiasis, erythrophagocytosis has been found in the spleen (Ikede et al., 1977) and in amimals experimentally infected with T. congolense erytrophagocytosis has also been observed in the spleen as well as haemolymph nodes and lymph nodes (Fiennes, 1954). It has also been demonstrated in rabbits infected with T. brucei following the development of anaemia and was considered to be a major factor in its development (Boycott and Price Jones, 1913). The Onset of anaemia has also been related to the decline of the first wave of parasitemia and the rise of serum antibody, (Balber, 1974; Kobayashi et al., 1976; Mackenzie et al., 1978). Treatment with immunosuppressive drugs was found to increase T. brucei parasitemia in mice but delayed the onset and reduced the degree of anaemia (Balber, 1974). This indicates a relationship to the antibody response. Yorke (1911) reported the potential involvement of an autoimmune mechanism because of the detection of autoantibodies (Cold agglutinins) to red blood cells in human trypanosomiasis.

It has been suggested that the anaemia is due to sensitization of erythrocytes with immune complexes resulting in erythrophagocytosis; (Woo and Kobayashi, 1975; Kobayashi, et al., 1976). This is supported by positive Coombs tests with

antisera against euglobulins (Zoutendyk and Gear, 1952; Kobayashi *et al.*, 1976), antiserum against IgG (Maxie, et al., 1976), and antisera against C3 (Woodruff, 1973; Kobayashi *et al.*, 1976). However, negative results with direct Coombs tests have also been reported in human and rabbit trypanosomiasis (Mackenzie and Boreham, 1974a) and in trypanosomiasis of cattle (Mackenzie and Boreham, 1974a; Maxie etal., 1976) and also in mice (Ikede *et al.*, 1977).

Mackenzie et al, (1978) using the indirect fluorescent antibody test with hyperimmune antisera applied to whole blood demonstrated the presence of trypanosomal antigen on erythrocytes of sheep infected with *T. congolense*. Kobayashi, *et al.*, (1976) reported the elution of IgG from erythrocytes of cattle infected with *T. congolense* and the IgG had antibody activity against *T. congolense*. It has also been shown that erythrocytes can be coated with trypanosomal material *in vitro* (Dodin and Fromentin, 1963; Herbert and Inglis 1973; Woo and Soltys 1972; Woo and Kobayashi, 1975). Tabel *et al.*, (1978) have also suggested that coating of erythrocytes by cellular material of disintegrated trypanomes *in vivo* alters the erythrocytes surface and cause adherence to macrophages and subsequent erythrophagocytosis. Woo and Kobayashi (1975) observed adsorption of *T. brucei* antigen onto normal rabbit red cells after incubating both *in vitro* at 37°c and they then postulated that trypanosome antigen-antibody complexes were coated on the red cell surfaces and these cells under certain conditions were lysed by complement

or agglutinated and removed by the spleen. Autoagglutination resulting from alteration of red cells by material of disintegrated T. congolense has also been reported (Tabel et al., 1978).

It has been shown that T. vivax hydrolyses neuraminic acid in vitro and the neuraminidase activity is proportional to the concentration of trypanosomes (Esievo 1979, 1980). If this is operative in vivo, sialic acids might be cleaved off erythrocytes with subsequent clearance from circulation which might possibly lead to clinical anaemia (Esievo et al, 1982). A reduced erythrocyte sialic acid concentration occurred concurrently with an increased number of trypanosomes in the circulation, accompanied by a significant development of anaemia thus providing an indirect evidence that the trypanosomes might be producing neuraminidase in vivo and that these factors might be functionally and casually related. Esievo et al. (1982) therefore postulated that the early anaemia observed in infected animals may be attributable to the activities of the circulating trypanosomes which produce neuraminidase, which in turn cleaves off erythrocyte surface sialic acid thus rendering the erythrocytes more prone to erythrophagocytosis. The reported observations suggest that immunologic mechanisms play a role in the development of the anaemia, but the exact nature and the extent of this involvement remain unclear.

Haemodilution or Hydraemia

Hydraemia is a distinguishing feature of chronic trypanosomiasis (Fiennes, 1954). Naylor (1971) suggested haemodilution as a cause of anaemia after observing increases of up to 30% in plasma volumes of cattle infected with . congolense. Reduction in red cell count exists in hydraemia because of the increased plasma volume but there is often an increase in the actual numbers of circulating red cells (Fiennes, 1954). This factor is however considered today not to be very important in the pathogenesis of the anaemia in early infections (Dargie et al 1979a, b).

Hypoplasia of the Bone Marrow: There is degeneration of the red bone marrow in the presence of trypanosomes leading to impairement of the bone marrow activity (Fiennes, 1954). Thus there is inadequate bone marrow response and destroyed red blood cells cannot be adequately replaced.

Haemolysis: Fiennes (1950) observed the haemolysis of red cells clustered round agglutination rosettes of T. congolense. Haemolysis occurs simultaneously with active trypanolysis in which a haemolytic factor is released (Fiennes, 1954). During trypanolysis, some hydrolytic enzymes, phospholipases are released into the circulation and these enzymes directly or indirectly via the products of their hydrolytic activity cause erythrocyte haemolysis.

Role of Phospholipases: Landsteiner and Raubitschek (1907) demonstrated that T. equiperdum could release a haemolysin

after overnight incubation in the cold. They also showed that this haemolysin was a lipid and suggested that it might be fatty acid in nature. Huan et al (1975) demonstrated that autolysed T. brucei was also haemolytic although they indicated that the haemolytic factor was probably a protein. Tizzard and Holmes (1976) demostrated that T. congolense also produced a haemolysin which was lipid in nature. Tizzard et al. (1977) went further to show that the haemolytic material from T. congolense was composed of a mixture of free fatty acids and possibly some lysolecithin generated by the action of trypanosome Phospholipase A on endogenous phosphatidylcholine. They also reported that the lysolecithin caused about 12% haemolysis while the remaining haemolytic activity was due to free fatty acid.

The haemolytic activity of these lipids is due to their detergent-like properties which make them to be powerful surface-active agents and are thus membranolytic (Tizzard et al., 1978). It has also been suggested that the lytic activity of free fatty acids may be due to the activities of their oxidized products (Tizzard et al., 1978). Another observation is the stimulation of phospholipase activity by free fatty acids particularly, oleic, myristic and linoleic acids probably because they disrupt membrane and thus reveal more substrate to the enzyme (Waite et al., 1969).

Cobra venom phospholipase A_2 hydrolyses phospholipids in washed human erythrocytes without causing significant

haemolysis in an insotonic medium (Gul and Smith, 1972) Condrea et al. (1970) also reported that appreciable amounts of fatty acids are cleaved as a result of action of cobra phospholipase A_2 on intact erythrocytes in isotonic medium. The apparent absence of haemolysis in these conditions is attributed to the cells becoming crenated after washing with isotonic saline probably resulting to some structural rearrangement so that inspite of membrane damage as evidenced by phospholipid splitting, the membrane is capable of retaining haemoglobin (Gul and Smith, 1972). Addition of 2% albumin to the incubation medium either prior to or subsequent to the enzyme action caused haemolysis without any further increase in phospholipid splitting (Gul and Smith, 1972). This they explained was due to the albumin binding the free fatty acids from the membranes which thus can nolonger retain haemoglobin and haemolysis becomes evident.

It has been reported that purified phospholipase A_2 does not haemolyse washed red blood cells unless the medium contains an external source of phospholipids (Bowman and Kaletta, 1957) or contains sublytic concentrations of detergents which are themselves potentially lytic at higher concentrations (Heemskerk and Deenen, 1964; Roelofsen et al., 1971) or is hypotonic (Lankisch and Vogt, 1972).

Failure of purified phospholipase A_2 to haemolyse washed erythrocytes in isotonic medium has been attributed to the inaccessibility of the membrane phospholipids to the enzyme.

Lankisch and Vogt (1972) have proposed that in a hypotonic medium, the membrane configuration changes due to swelling of the cells and thereby exposing the phospholipids to the action of the phospholipase leading to haemolysis. Direct lytic factors and other agents exert their action by swelling and strectching the cell membrane (Gul and Smith, 1972).

Condrea, et al. (1964) have reported that phospholipase A_1 is in general poorly lytic by itself but in conjuction with detergent - like molecules, such as free fatty acids, it is capable of causing significant cell membrane disruption. An in vivo synergy of cardiotoxin and phospholipase A_2 in erythrocyte haemolysis has also been reported (Bougis et al., 1987). In this synergy, trace amounts of phospholipase A_2 is sufficient to effect haemolysis in vitro which is highly dependent on the assay conditions such as source and preparation of the erythrocytes, composition and ionic strength of the medium (Bougis et al., 1983).

Indirect Effect of Phospholipases:

As has earlier been mentioned, phospholipases indirectly act on erythrocytes via the products of their esterase activity, free fatty acids and lysoderivatives. The involvement of the hydrolysis products of phospholipase activity has also been suggested (Bougis et al., 1987). Free fatty acids do not usually exist free in the blood stream but are rapidly bound to serum albumin (Goodman, 1958; Spector et al, 1969). Therefore it is anticipated that the

presence of serum albumin or other proteins effectively blocks the haemolytic activity of fatty acids both *in vitro* and *in vivo*. If fatty acids are to exert their cytolytic properties *in vivo*, they must accumulate in sufficient quantities within the blood stream in order to saturate the binding sites of serum albumin. In trypanosomiasis due to *T. congolense*, the organism accumulates in large numbers in the microcirculation and the local destruction of these packed organisms especially if associated with a local reduction of blood flow might permit fatty acids to accumulate sufficiently to permit erythrocyte modification and microvascular damage to occur (Losos and Ikede, 1972).

Serum albumin concentrations in trypanosome infected calves drop to about 2.2g/100 ml (Tizzard *et al.*, 1978) and this amount can fully adsorb between 0.4 and 0.5mg free fatty acid per ml (Spector *et al.*, 1969) before it becomes saturated. Tizzard *et al* (1978) in their experiment with calves and rats infected with *T. congolense* found that only 0.002mg free fatty acid per ml was generated in the rats. They therefore inferred that *T. congolense* haemolysin was unable to influence erythrocyte survival in cattle while the haemolysin (free fatty acid) in infected rats, especially under conditions of reduced capillary blood flow could fully saturate rat serum albumin and cause cell damage. However, these results do not rule out any role for fatty acids in bovine trypanosomiasis since albumin does not block their mitogenic effects (Arai *et al.*, 1977).

The existence of a haemolytic factor in trypanosomiasis is beyond doubt but it is present only intermittently in the course of the disease and in highest quantity at times of trypanolysis (Fiennes, 1954). Therefore, while haemolysis may be responsible for an acute course of the disease or for a fatalterminat:on at the time of crisis, it is not the underlying cause of the progressive anaemia (Fiennes, 1954).

EPIZOOTIOLOGY OF ANIMAL TRYPANOSOMIASIS

The Insect Vector:

Variations in species, density, infection rate and distribution of Glossina greatly affect the pattern of the disease incidence in livestock. The savanna species, G. morsitans and G. longipalpis and the forest species of the fusca group exhibit higher infection rates than the riverine species such as G. Palpalis and G. tachinoides.

The density of the insect vector also influences the severity of the disease. Thus an efficient vector with high density may result in frequent transmission of fresh strains of the parasite which may overwhelm the hosts' defensive mechanism which may lead to severe infection. On the other hand, close animal-fly contact may even at low fly density lead to serious disease incidence.

The distribution and changes in distribution of Glossina significantly affect the disease pattern. Human activities such as cultivation and hunting are important factors in determining tsetse distribution.

There could be large scale advances of Glossina in some areas and regression in others. When a livestock population is overtaken by an advance of tsetse flies, a high incidence of trypanosomiasis may result and is usually apparent before the presence of tsetse is detected. Influxes of human population have caused large shrinkages in the areas infested by savanna tsetse but usually have less effect on the riverine species. In such localities, the incidence of trypanosomiasis may be sufficiently low to permit seasonal or all-season use for rearing of livestock. However, the intensive cultivation of trees such as mango, guava, banana and tall crops such as sugar-cane can provide suitable habitats for tsetse, thereby causing a serious incidence of trypanosomiasis in susceptible livestock.

Tsetse Fly Host Preferences: Host preferences of Glossina is an important factor which influences the pattern of disease in domestic stock. The degree of risk to which animals are exposed is greatly influenced by the frequency with which tsetse feed on them. This probably explains the apparent good health of certain classes of suceptible mammalian livestock in some areas of risk. However, where tsetse population is very high, even stock which are not ordinarily used as sources of food may be fed upon sufficiently often to cause severe trypanosomiasis.

Effect of Seasonal Fluctuations on Livestock Distribution

Seasonal variations in climatic conditions strongly affect the distribution of livestock and tsetse flies and therefore the pattern of trypanosomiasis. For instance during the dry season, streams dry out or shrink and there is partial destruction of the vegetation which provides suitable microenvironment for tsetse to thrive in times of adverse ambient conditions. Thus in West Africa, the savanna species become more restricted during the hot dry season, though this tendency is less marked in the southern zones where there is high humidity. There is also an obligatory movement of livestock from the dry areas in search of water and green pasture for grazing. This may introduce livestock from relatively tsetse free areas into tsetse - infected areas In general, the exposure of livestock to tsetse bites becomes progressively less general as the hot season advances.

The Parasites: There are as many trypanosomiases as there are specific trypanosomes (Stephen, 1970). This arises as a result of variations in the pathogenicity of different trypanosome species and strains of t different animals. For instance, cattle previously exposed to some risk but nevertheless thriving may be taken into a new area where a particularly virulent strain of T. vivax may cause a severe disease with such rapid fatalities that considerable losses may be sustained before treatment can be made available (Maclennan, 1970). Fairbairn (1953) reported that the longer strains of T. vivax give rise to a less acute type of disease than the shorter strains do.

Long term Chemotherapy in a given Region

The incidence of the species of trypanosomes causing disease may be influenced by long term curative and prophylactic therapy. There is some evidence (Folkers and Jones - Davies, 1966) to suggest that the widespread use of homidium over a period of years leads to a decrease in the incidence of T. vivax infections as compared to those caused by T. congolense in cattle in Northern Nigeria. The extent to which this influence affects the type of infection depends on the extent to which infections in tsetse are themselves derived from feral or domestic animals, and on the relative effectiveness of the treatment for the two species of trypanosomes.

Species and Breed of Host: Susceptibility to trypanosome infection and the ability to tolerate the effects of infection are the most salient features of the host that influence the incidence of trypanosomiasis. Livestock vary greatly in their reaction to trypanosome infection, depending upon breed, strain or even the individual animal. A lot also depends on the conditions under which livestock have been raised and maintained. Animals may be able to survive and even thrive in tsetse - infested localities when they acquire a degree of tolerance or immunity resulting from earlier exposure to infection, and this is in turn influenced by the degree of risk to which they were previously exposed. In general, the Ndama, Kateku and Muturu breeds are more trypanotolerant than the other breeds of cattle.

The exotic breeds of cattle are highly susceptible to trypanosome infection while the various types of Zebu occupy an intermediate position. The various types of Zebu show distinct differences in their susceptibility to trypanosomiasis, varying from the 'white Fulani' type which withstands exposure to continuous slight or periodic risk to the larger 'Bororo' or 'Red Fulani' cattle which are more vulnerable. In thesame manner, the dwarf types of goat and sheep are generally more resistant than the larger animals.

Livestock Husbandry. The methods of animal husbandry adapted greatly influence the epizootiology of trypanosomiasis in livestock. Under intensive systems of husbandry it may be possible to avoid infection by maintaining stock in fly-proof accommodation, but range and migrant stock are often exposed seasonally or continuously to varying degrees of risk.

In practice, the extent to which the effects of trypanosomiasis in livestock can be ameliorated by better methods of husbandry is often determined by economic factors, but there is a critical degree of risk beyond which animals will not respond to improved methods of husbandry. A herd of Zebu (white Fulani) cattle maintained at the university of Ibadan in Nigeria offers a good example of the effect of husbandry on epizootiology. These cattle are more or less continuously exposed to a relatively light risk from G. palpalis but the standard of husbandry is so good that the herd thrives,

and clinical symptoms of trypanosomiasis are only occassionally seen, whereas similar cattle maintained in a similar locality on lower standards of husbandry would suffer more severely. However, these animals experience trypanosomiasis breakdowns when stressed, or at parturition, and may then require chemotherapeutic support (Hill, 1956).

Other factors that may affect the disease pattern are the state of nutrition, the occurence of intercurrent bacterial, viral, helminth and other parasitic diseases and stress due to vaccination regimes, trekking, thirst and temperature changes.

2.8 DIAGNOSIS OF TRYPANOSOMIASIS

Most of the symptoms of trypanosomiasis are also common features of other diseases. This makes diagnosis based on physical examination difficult. However, technical methods for the detection of trypanosomes are available and serological and other methods are also in use.

Apted (1970) grouped the diagnostic methods used in laboratory and field practice into two categories. Those by means of which a positive diagnosis can be made by demonstrating the presence of the trypanosome itself and the others are those that may give valuable suggestive or supportive evidence of trypanosome infection but which by themselves do not provide unequivocal proof of infection.

Detection of Trypanosomes

Trypanosomes may be found in the initial chancre, in the blood, the lymph glands, the cerebrospinal fluid or the bone marrow. They may also be isolated by cultural methods or after

animal inoculation.

The Chancre: This is more easily seen in man than in animals. If a chancre is present, it provides the earliest means of making a definite diagnosis since trypanosomes may be detected in the lesion up to two days before they can be found in the blood. The technique involves puncturing the chancre and then examining a drop of the exuding fluid under a cover-slip as a wet preparation.

The Blood: This is the most applicable specimen both in man and animals. Infection may be detected by examination of a fresh drop of blood in the form of wet smear preparations, stained thin blood films, lysed and stained thick films or smears of centrifuged deposits. Other methods for the detection of trypanosomes in blood are the animal subinoculation method and the haematocrit centrifuge technique ( ). The animal subinoculation method which is a good technique in detecting all trypanosomes except T. vivax involves taking blood from the patient and inoculating it into a susceptible host. The establishment or development of an infection in the recipient host is a confirmatory diagnosis of the disease in the patient whose blood was taken for inoculation. This procedure can be a very valuable diagnostic aid especially in suspected cases in which ordinary methods have failed to reveal the presence of trypanosomes. The HCT has the advantage of being suitable for use in the field and the estimation of the packed cell volume (PCV) which it affords is an additional valuable information.

The Lymph Glands: This is more applicable in humans and animals infected with T. brucei and T. congolense but less so with T. vivax. Fluid is taken from one of the enlarged cervical glands and a wet preparation of this fluid is examined for trypanosomes which if present are detected by their movements among the mass of cells.

The cerobrospinal Fluid: This is more often applicable to human trypanosomiasis and not animal trypanosomiasis. Although very unusual, trypanosomes may be present in the cerobrospinal fluid in the early stages of the infection. Ordinarily, they are not seen until a rise in the cell and protein content of the fluid provides definite evidence of nervous system involvement. Trypanosomes may be detected by direct examination of a drop of the fluid, but they are more likely to be found if a centrifuged deposit is searched (Apted, 1970). The examination of this fluid is usually undertaken to determine the stage of the disease, or its progress under treatment, and not primarily as a diagnostic measure except in suspected infections in which trypanosomes are not demonstrable in blood or glands, as is usually the case in the later stages of the disease.

The Bone Marrow: This method is rarely used because of its being unsuitable in the field and largely due to the dangers inherent in the method. Where it is used at all, it is applicable only to man. Trypanosomes may be found in bone marrow taken by sternal puncture, in situations where they cannot be demonstrated in blood, glands or cereborospinal fluid.

Cultural Methods: This method which has only recently been established at the laboratory level for the African trypanosomiases is mainly used for American trypanosomiasis. The superiority of cultural methods over other laboratory techniques for the diagnosis of sleeping sickness has been reported (Weinman, 1963). The method essentially involves the isolation of trypanosomes from body fluids such as blood, cerebrospinal fluid etc using suitable culture media. Results are obtained within five to thirty days.

Other indirect laboratory tests that may aid diagnosis are:-

(a) Examination of the cerebrospinal fluid for increase in number of cells and elevation of the protein content.

(b) Test for increase in erythrocyte sedimentation rate.

(c) Complement fixation test.

(d) Fluorescent antibody test

(e) The formol-Gel or serum formalin reaction.

(f) The immunoglobulin reaction.

CONTROL OF TRYPANOSOMIASIS

There are two major ways used in the control of trypanosomiasis. These are

i. Elimination or limitation of tsetse fly population.

ii. Chemotherapy and Chemoprophylaxis.

The elimination or limitation of tsetse fly population can be achieved in a number of ways which include Hand-catching and use of traps and chemical attractants, (Glasgow and Potts, 1970), clearing of vegetation (Ford et al. 1970), Destruction of Game animals (Ford, 1970), use of inseticides

(Burnett 1970), Biological control using parasites and predators of Glossina (Nash 1970) and Sterile male technique (Dame 1970).

Extermination of tesetse flies may be directed against all species of tsetse present in an area or may be restricted to the species responsible for the transmission of trypanosmiasis (Du Toit 1954, Maclennan 1967).

Hand-catching and use of Traps and Chemical Attractants.

Hand-catching operations involve the use of fly catchers to catch tsetse flies. Although, the most ancient method of insect control, hand-catching has two important advantages over all other methods of control.- Its absolute specificity and enabling the maintenance of an exact record of the number of insects destroyed (Glasgow and Potts 1970).

Use of Traps and Attractants: From the very early days of tsetse investigation, screens covered with bird-lime have been used for catching tsetse flies. Tsetse traps consisting of a cage and an attractant part are in use for catching tsetse flies. The attractant entices the tsetse flies into the cage from which they cannot escape. The first of these devices were the famous traps of Harris (1930) used in catching very large numbers of G. pallidipes in Zululand. This mehod also has the advantages mentioned in the preceeding section.

Clearing of Vegetation and Destruction of Larger Fauna: The vegetation which provides habitat for the tsetse flies when completely or partially cleared leads to a more or less permanent elimination of the tsetse flies. Similarly, the destruction of game animals on which some species of

tsetse flies depend for their food limits tsetse population.

The use of insecticides. The application of insecticides by aerial spray or otherwise greatly limits tsetse infestation. The insecticides commonly used are dichlorodiphenyltrichloroethane (DDT), benzene hexachloride (BHC) and the group of organochlorines known as the cyclopentadiennes. Others include endosulfan (thiodan), fenthion, bromocyden (bromodan), aprocarb and pyrethrum.

Biological Control. Tsetse fly population can be controlled using insect parasites and predators of tsetse flies and some organisms which are pathogenic in adult tsetse flies. A fungus belonging to the phycomycete is believed to be pathogenic to G. morsitans (Nash, 1970). An unidentified fungus has also been reported to be infective in G. morsitans and G. tachinoides (Lester, 1934).

Some species of Hymenoptera and Diptera are insect parasites of tsetse pupae (Jenkins, 1964). A number of organisms are predators of puparia tsetse and adult tsetse flies and they play significant role in regulating the size of tsetse population. Some examples of predators of puparia tsetse are the elephant shrew (Petrodromys tetradactylus), guinea-fowl (Numida, etc), bush-fowl (Francolinus, etc), and ants of the genus Pheidole while those of adult tsetse fly include hunting wasps (Bembix), robber-flies (Asilidae), the dragonfly Orthetrum chrysostigma, spiders of the genus Nephila.

Sterile Male Technique: Sterilization of male Glossina greatly reduces the population density of tsetse flies. Sterilization can be accomplished by use of high energy ionizing radiation-radiosterilization (Potts 1958, Dean and Wortham 1969, Dame 1970), or by the use of mutagenic chemicals-chemosterilization (Chadwick 1964, Dame 1970).

Chemotherapy and Chemoprophylaxis. A number of drugs are available for both prophylatic and curative treatment of human and animal trypanosomiasis. The drugs commonly used are pentamidine, tartar emetic, antimonysodium tartrate, tryparsamide, melarsoprol, melarsonyl potassium, melarsan, Nitrofurazone and furaltadone (for humans). Others include antrycide, ethidium bromide, prothidium, samorin, homidium chloride and suramin - antrycide complex (for animals). Suramin and berenil are both used for both human and animal trypanosomiases.

Other control measures which might eliminate or reduce the incidence of trypanosomiasis may include:-

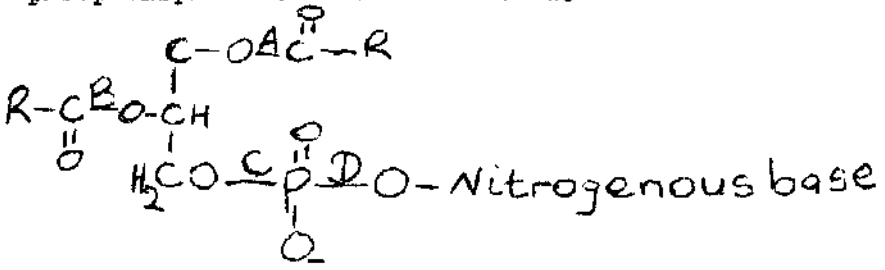
Selection of healthy areas: The selection of localities free from tsetse flies for livestock production or human habitation reduces the incidence of tsetse-borne trypanosomiasis.

The selection of suitable livestock: By selecting trypanotolerant breeds of animals for livestock development, the chances of contracting infection will be greatly reduced.

Erection of fences: The erection of fences around animals prevents their movement into areas in which they would be exposed to greater risk of contracting trypanosomiasis and from which they would be liable to bring back trypanosomes and tsetse flies into clean areas.

2.10 PHOSPHOLIPASES

Phospholipases are a group of hydrolytic enzymes involved in the degreadation of phospholipids which form about 50% of the total lipids in biological membranes. They are of widespread occurrence in nature. There are four well documented types which can attack a typical phosphoglyceride. These are phospholipases A, B, C. and D each of which is specific for the hydrolytic cleavage of one of the susceptible ester bonds labelled A to D in the general structure of a phospholipid molecule shown below.

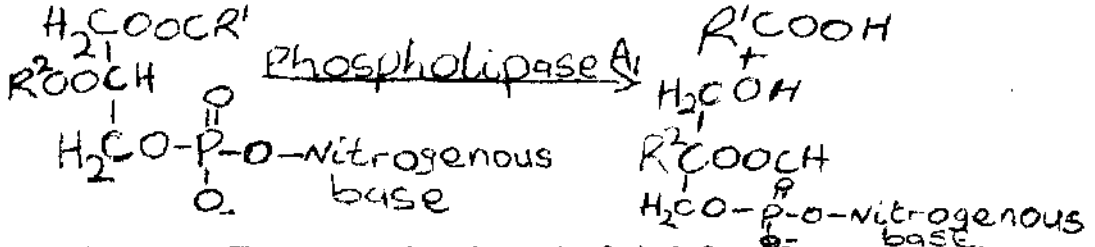


The nomenclature adapted is that used by Hanahan (1971) in which phsopholipases A_1 and A_2 hydrolyze the acyl groups in the 1 - and 2 positions of an sn - 3 - phosphoghlceride (or the 2 - position of an sn - 3 - Plasmalogen) respectively. Phospholipases hydrolyzes the phosphoglyceride to the corresponding diglyceride and phospholipase D cleaves it to the corresponding phosphatidic acid and free nitrogenous base. Thus phospholipase A_1 cleaves bond A above, phspholipase A_2 cleaves bond B while phospholipases C and D cleave bonds C and D respectively. Phospholipase B cleaves both bonds A and B.

Phospholipases A_2 and C have been the most frequently studied and widely used enzymes in this class of hydrolases. The specific attack of phospholipase A_2 on the 2 - position of

phosphoglycerides such as phospatidylcholine and phsphatidylethanolamine has provided a lot of information on the specific positioning of fatty acids and the relative metabolic behaviour of these acids. Phospolipase C which in many instances releases diglyceride and phosphorylcholine from phosphatidylcholine has allowed further examination of the structure of the phosphoglycerides and a convenient route to preparation of diglycerides. These phospholipases are also being used to establish a role for phosphoglycerides in the structure and behaviour of membranes and for studying lipid-protein interactions.

Phospholipase A_1 (EC 3. 1. 1. 32 phosphatidate 1 - acylhydrolase). Phospholipases A_1 are a group of hydrolases that catalyze the removal of the acyl group from position 1 of phosphoglycerides.



SOURCE: The enzyme has been isolated from lysosomes and plasma membranes of rat liver (Waite et al, 1981) and also from T. brucei (Hambrey et al, 1984).

PROPERTIES: The properties of phosphlipases A_1 vary depending on the source of the enzyme. For example the lysosomal enzyme has P^I range between 2.8 to 4.8 (depending on the treatment of the lysosomes) while the plasmalemma enzyme has P^I range between 3.8 and 5.6.

Molecular Weight: The molecular weight of the lysosomal enzyme as determined by SDS - gel electrophoresis is 32,000, that of the plasmalemma enzyme is about 75,000 while that of the trypanosomal enzyme is 26,000.

PH Optimum: The optimum PH of the lysosomal enzyme is 4.0, that of plasmalemma enzyme is between 8.0 and 9.0 while that of the trypanosomal enzyme is 6.0 to 8.5.

Activators and Inhibitors: The lysosomal enzyme is inhibited by metal ions, lysophospholipid, free fatty acid and some proteins such as bovine serum albumin and cytochrome C. The trypanosomal enzyme is inhibited by thiolreactive reagents such as P-chloromercuricbenzoic acid and N-ethylmaleimide (Sage et al, 1981) and is strongly activated by Triton X - 100.

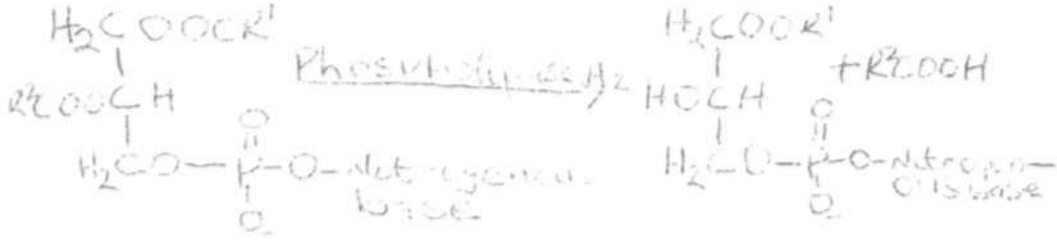
Thermal Stability: Phospholipases A_1 are generally thermally unstable. For instance, the trypanosoma enzyme is inactivated at PH 8.0 by heating at 60° C for 5 minutes (Hambrey et al, 1984) and thermolability of phospholipase A_1 from E. Coli K - 12 has been reported (Nishijima et al, 1977). The E Coli enzyme becomes inactivated at 37° C.

Storage Stability: Purified phospholipases A_1 rapidly lose activity on storage. Despite the presence of detergents and glycerol, the purified enzyme from Trypanosoma brucei lost activity on storage even at - 20°C (Hambrey et al, 1984). The purified rat liver enzyme lost activity in a matter of days.

Phospholipase A_2 (EC 3.1.1.4 Phosphatide 2 - acylhydrolase).

Phospholipases A_2 catalyze the specific

removal of the acylgroup from position 2 of an sn-3-phosphoglyceride. Phospholipase A_2 is the most widely studied of all phospholipases.



Source: The enzyme is present in high concentrations in the venom of many domestic and foreign snakes, in bee and scorpion venoms and in many different mammalian tissues with particularly significant concentrations in pancreas and small intestine. Homogenous preparations have been obtained from Crotalus adamanteus (Wells and Hanahan, 1969), C. atrox (Wu and Tinker, 1969), Naja naja (Salach et al, 1971; Deems and Dennis 1981), Vipera russelli (Salach et al, 1971) Apis mellifica (Shipolini et al, 1971). The enzyme has also been purified from red cell membranes of sheep (Zahler and Kramer 1981) from bee venom (Cottrel, 1981) and from the venom of Agkistrodan Halys pallas (Chen et al, 1987). Jiang et al. 1987). With few exceptions, most of the preparations of the enzyme from snake venom contain from two to eleven isoenzymes with differing specificities (Salach et al. 1971).

Properties.

Molecular weight. The molecular weight of enzyme from erythrocyte membrane of sheep is approximately 12,000 using sephadex G - 75 chromatography and 18,500 using dodecylsulfate

electrophoresis. The enzyme from Naja naja has a molecular weight of 13,000 ± 2000 using various techniques. This is however for the monomer.

PH Optimum. The optimum PH for the erythrocyte membrane enzyme and that of the bee venom enzyme is 8.0.

Activitors and inhibitors: All phospholipases A_2 so far studied have a strong requirement for calcium as cofactor. Some divalent cations such as Mg^{2+} Sr^{2+} and Ba^{2+} (Cottrel, 1981) can replace Ca^{2+} but with reduced efficiency. However, Deems and Dennis (1981) have reported that Sr^{2+} and Ba^{2+} are competitive inhibitors. Maleic anhydride, EDTA, P-Bromophenacylbromide and its analogs are inhibitory. Fatty acids under various conditions can act as activators or inhibitors (Jarvis et al., unpublished experiments).

Thermal Stability. Most phospholipases A_2 are extremely stable to heat at acidic PH. for example, the enzyme from Naja naja loses only 5% activity when heated at 100°C for 10 minutes at PH 3 to 4 (Deems and Dennis, 1981).

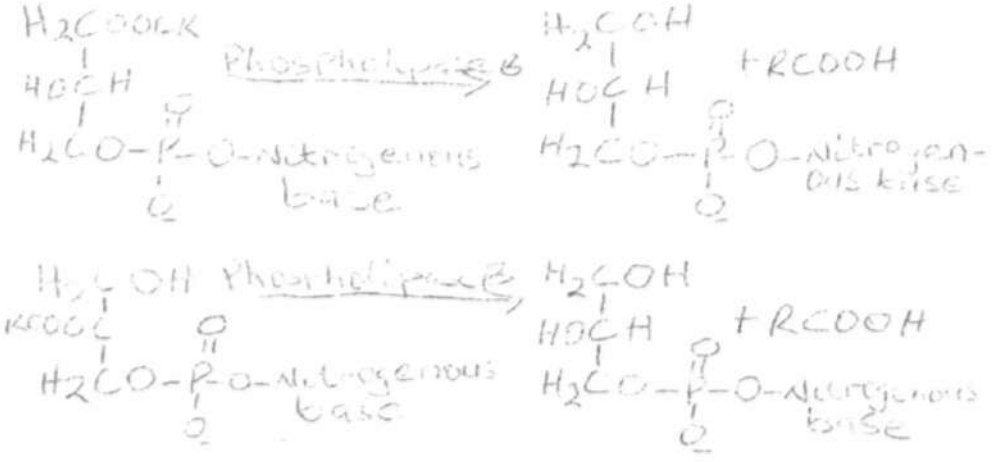
Storage Stability: Phospholipases A_2 do not easily lose activity on storage. For instance, the bee venom enzyme is extremely stable in the lyophilized form at room temperature.

Biological significance: Phospholipases A_2 exhibit pronounced differences relative to function. For instance, the venom of the Chinese water moccasin, Agkistrodan halys pallas contains three phospholipases A_2 (Wu and Chen 1981, Chen et al., 1981 Wu et al. 1984), an acidic enzyme with $P^I = 4.5$ and neutral

and basic enzymes with P^I = 6.9 and 9.3 respectively. These three enzymes differ greatly in pharmacologic and cytotoxic properties. Thus the acidic enzyme is a potent inhibitor of platelet aggregation (Ouyang et al. 1983, Chen 1987). This enzyme also hydrolyzes the phospholipids of the erythrocyte membrane without causing haemolysis (Chen et al 1987). On the other hand, most basic phospholipases A_2 haemolyze erythrocytes. This may be due to a specificity governed by electrostatic interactions between the electric charge of the enzyme and the charge at the surface of the erythrocyte membrane. The neutral phosppolipase A_2 exhibits a high lethal potency toward mice (snake Venom Research Group, Shanghai Institute of Physiology, 1976). The localization of the site of action of this enzyme to the neuromuscular junctions (Yang and Hsu 1977) led to the conclusion (Chen et al. 1987) that this enzyme is a neurotoxin. The basic phospholipase A_2 from Agkistrodon h. pallas and A. h. blomhoffi differ from other phospholipases A_2 because they hydrolyze phospholipids of E. coli membranes in the presence of a bactericidal permeability - increasing (BPI) protein from neutrophil sources. Phospholipase A_2 from Agkistrodan h. pallas is neurotoxic by disrupting the presynaptic membrane, thus interferring with the transmitter release processes and depleting the stores of transmitter (Jiang 1987).

Phospholipase B (EC 3.1.1.5 lysophosphatide acylhydrolase). It is a matter of dispute whether phospholipase B is an independent enzyme or a part of phospholipase A activity. However, various studies carried out have provided

considerable evidence for the existence of a specific enzyme which attacks lysophosphatides but not phosphatides. The pure enzyme is completely inactive toward phosphatides. Phospholipase B catalyzes the removal of the acyl group from position 1 or 2 of the lysophospholipid.



Source: The enzyme has been obtained in relatively pure form from various sources such as wasp venoms and castor beans (Hayaishi, 1955),), Molds (Fairbairn 1948), bacteria (Hayaishi and Kornberg 1954), Vipera Palestinae venom and very recently from the venom of the Australian elapid snake, Pseudechis colletti (Bernheimer et al. 1987).

Properties.

Molecular weight. The molecular weight of the Pseudechis colletti enzyme as determined by gel Filtration is approximately 35,000 while that determined by sodium dodecylsulfate - polyacrylamide gel electrophoresis is 16,500. These are presumably dimeric and monomeric forms of the enzyme.

PH optimum. The optimum PH for the bacteria enzyme is 6.0 while that for the wasp venom and mold enzymes is 3.8 to 5.5.

Activators and inhibitors: Cyanide inhibits and less readily heavy metals inactivate the enzyme. Phospholipases B from Vipera Palestinae and Pseudechis colletti both require Ca^{2+}. Activation of phospholipase B by Ca^{2+} has earlier been reported by Contardi and Ercoli (1933). However, fairbairn (1948) reported that the enzyme had no requirement for Ca^{2+}.

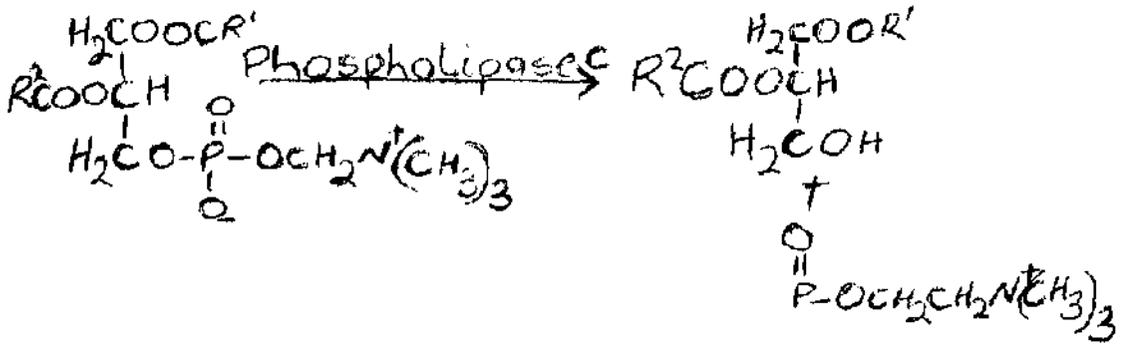
Stability: Phospholipase B of animal origin is rather heat labile compared to the accompanying phospholi;ase A (Noguchi 1944). Phospholipase B from Penicillium notatum is most stable at PH 4.5 and is rapidly inactivated at PH higher than 7.0 (Zamecnick et al. 1947) while the serratia enzyme on the other hand appears to be more heat-resistant than phospholipase. A from thesame origin or atleast is as stable as the later.

Biological Significance: Phospholipase B is strongly haemolytic for rabit and human erythrocytes in vitro but not for bovine or ovine erythrocytes, cytotoxic for cultured rhabdomyosarcoma cells and lethal for mice in which death was preceded by massive myoglobinuria (Bernheimer et al. 1987).

Phospholipase C (EC 3.1.4.3 Phosphatidylcholine Choline Phosphohydrolase). This enzyme which is also referred to as

- toxin catalyzes the hydrolytic cleavage of diacylsn-glycero - 3 - phosphorylcholine, for instance to a 1, 2 - diacylglycerol and phosphorylcholine.

The enzyme also attacks sphingomyelin.



Source: The enzyme has been isolated from Bacillus cereus (Kleiman and Lands 1969, Little, 1981), Clostridium Perfringens (Pastan et al. 1968, Takahashi et al. 1981) and the marine planktonic chrysomonad Monochrysis lutheri (Bilinski et al., 1968). The enzyme has also been isolated from Pseudomonas aeruginosa (Kurioka and Liu, 1967) and some strains of staphylococus aureus (Doery et al., 1963; O'Reilly et al., 1986).

Properties: Phospholipase C from Clostridium perfringens has multiple molecular forms designated α_0 -, α_1 -α_2 - and α_3-toxins with their PIs being 5.2, 5.3, 5.5 and 5.6 respectively (Takahashi et al., 1981). α- toxin with PI 8.5 has also been reported (Thelestam and Blomquist, 1988).

Molecular Weight: Molecular weight values range from 30,000 to 106,000 depending on preparation and method of estimation (Takahashi et al., 1981).

PH Optimum: The PH optimum for the clostridium enzyme lies between 7.0 and 8.0 while the Bacillus enzyme has double PH optima of 6.6 and 8.0.

Activators and Inhibitors: Detergents and calcium are required for maximal activity of the clostridium enzyme. Sodium deoxycholate also greatly enhances the activity of the enzyme and to a lesser extent sodium cholate and soidum dehydrocholate activate the enzyme. Magnesium ion and to a lesser extent Co^{2+}, Mn^{2+}, and Zn^{2+} activate the enzyme. The Bacillus enzyme is stabilised by presence of free zinc ions. The clostridium enzyme is inhibited by Cd^{2+}, Pb^{2+} and Hg^{2+} which act by strongly suppressing the stimulating effect of Ca^{2+}. The enzyme is also inhibited by metal chelating agents such as EDTA and O-Phenanthroline. Citrate, Phosphate, fluoride, ferricyanite, sodium dodecyl-sulfate and reducing agents such as glutathione, cysteine and thioglycolic acid are inhibitory. Polyamines such as spermine, spermidine and putrescine also inhibit the enzyme.

Stability: The clostridium enzyme is extensively inactivated at 60 - 70°C but is reactivated at higher temperatures with almost complete restoration of activity at 100°C. It is easily inactivated by surface denaturation when diluted. The Bacillus enzyme is extremely stable towards thermal denaturation and chaotropic agents.

Biological Significance:

Phospholipase C is strongly lethal, necrotizing and hemolytic (Macfarlane and knight, 1941). The lethal effect of phospholipace C which is rapid and most dramatic is the least understood of the activities of phospholipase C (Thelestan and Blomqvist, 1988). Circulatory disturbances and

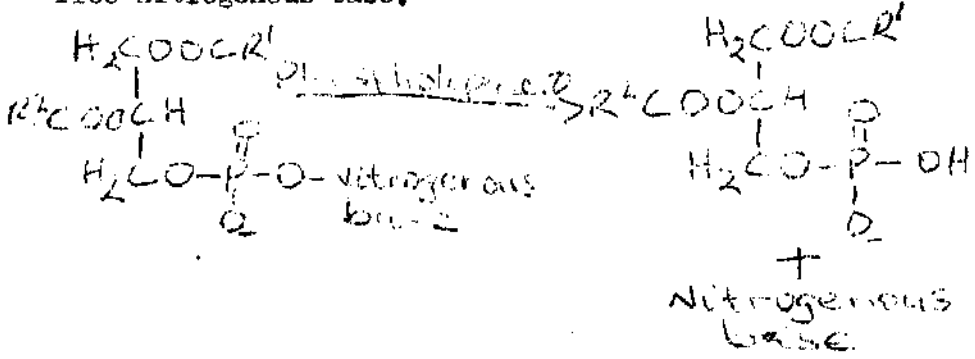
neurotoxicity have been suggested to be responsible for the lethal action of the enzyme. That phospholipase C is a neurotoxin has been reported by many workers (Harshman, 1979; Harshman et al, 1985). However, recent results do not support the conception of the enzyme being a neurotoxin. Rather, it appears disturbances are primary to the changes in the brain bioelectric activity (Thelestan and Blomqvist, 1988).

Phospholipase C is extensively used as a tool in cell biology. Controlled permeabilization of plasma membrane if attained would facilitate the study of some intracellular processes in situ (Heppel and Makan, 1977). Various methods such as hypertonic (Castellot et al., 1978) or high voltage treatment (Knight and Baker, 1982) or exposure to detergents such as saponin or digitonin (Burgess et al., 1983 ; Cook et al, 1983) which are being used have several disadvantages. For instance, they may induce large membrane lesions, so that the treated cell becomes more injured than desired. Detergents may possibly traverse the membrane and cause damage also to intracellular membranes.

Phospholipase C appears a highly suitable alternative tool for controlled and selective permeabilization of plasma membrane since it induces small lesions which do not seem to permit passage of the enzyme itself. Thus the plasma membrane is permeabilized, but internal membranes appear to remain intact (Ahner-Hilger et al., 1985a, b). Therefore, Plasma membrane permeabilization with phospholipase C should enable the study of how various intracellular processes are

regulated by ions, nucleotides, sugars and drugs. MCEween and Arion (1985) have reported the permeabilization of hepatocytes to glucose - 6 - phosphate, apparently with successful retention of the glucose - 6 - phosphatase system intact. Calcium -triggered dopamine released from phospholipace C permeabilized cells was further characterized recently (Ahnert Hilger and Gratzl, 1987). In this manner, phospholipase C can probably be applied for forcing substances such as drugs into cells which are otherwise impermeable to these substances (Thelestan and Blomqvist, 1988).

Phospholipase D (EC 3.1.4.4 phosphatidylcholinephosphatidohydrolase). Phospholipase D catalyzes the hydrolytic cleavage of a phospholipid molecule into phosphatidic acid and a free nitrogenous base.



Source: Phospholipase D has been purified from rat brain (Taki and Kanfer 1981) and from a variety of plant tissues (Kates and Sastry, 1969).

Properties

PH Optimum. The optimum PH for the rat brain enzyme is 6.0 while that for the plant tissue enzyme varies with the source. Thus for spinach and sugar beet chloroplasts, the PH optimum is 4.7 (Kates, 1954), 5.5 to 5.8 for cabbage chloroplasts and carrot root chromoplasts (Kates, 1954).

Activators and inhibitors: Calcium chloride and ferrous chloride cause 65 - 80% stimulation of the rat brain enzyme. The plant tissue enzyme also has an essential requirement for calcium ion with an optimal concentration of 40 - 100mm (Davidson and Long, 1958; Dawson and Hemington 1967). Ethylether and some linear aliphatic ketones and esters such as n-propylketone or ethylbutyrate (Kates 1957) are potent stimulators. The enzyme from the rat brain is inhibited by choline, ethanolamine, fluoride and EDTA.

Stability. The purified enzyme is generally unstable.

ASSAY OF PHOSPHOLIPASES

A number of methods are available for the assay of phospholipases. In most cases, phosphatidylcholine or phosphatidyethanolamine is used as substrate. Generally, phospholipases A_2 are more active toward phosphatidylcholine than other phospholipids, while phospholipases A_1 are more active towards phosphatidylethanolamine. Phospholipase C acts mainly on phosphatidylcholine while D acts on both phosphatidylcholine and phosphatidylethanolamine.

The available assay methods are Bioassays, physical methods, Acidimetric (Titrimetric) methods and methods based on chromatographic separation of products. Colorimetric and Radiochemical methods are also in use.

BIOASSAYS: Bioassays are used in the determination of the activity of phospholipases A and C. Erythrocyte haemolysis by the lysophosphatides and free fatty acids

liberated by the action of phospholipases A forms the basis for this determination in the case of phospholipases A. The activity is estimated by the extent of lysis. In the case of phospholipases C, three different biological assays are in use. These are determination of lethality (Macfarlane and Knight, 1941), Measurement of hemolytic activity (Izekawa, 1963; De Boer and Loyter, 1971; Sabban et al., 1972) and measurement of the delay in clotting time of plasma treated with various dilutions of tissue thromboplastin (Otnaes et al, 1972; Ottolenghi, 1969). The chemical bases of these activities have not been satisfactorily elucidated and the assays are not known to be specific for phospholipases C.

PHYSICAL METHODS: Physical methods are employed in the assay of phospholipases A, C. and D. The physical assay method is based on measurement of turbidity (phospholipases A and C), measurement of the fall in surface potential of monolayers of lecithin. Other physical methods include conductrimetric measurement and the coagulation test of Habermaun and Neumann (1954).

Dawson and Quarles (1969) devised a physical method for assaying the activity of phospholipase D. The method involves using a radioactively labelled substrate in a monolayer and following the course of the enzymatic reaction by measuring surface pressure, phase boundary potential and surface radioactivity.

ACIDIMETRIC METHODS: These are used for the assay of phospholipases A and C. It involves the titration of the liberated fatty acids by the action of phospholipase A or measurement of the liberation of H^{+} during hydrolysis of the phosphate ester bond by phspholipase C. Three alternative titrimetric methods are in use. These are: (a) stopping the reaction after a specified period, extracting the fatty acids liberated and titrating them; (b) continuous titration during the course of the enzyme action with a PH - stat; and (c) recording the PH change during the course of enzyme action in weakly buffered solutions. The fatty acids liberated can infact be titrated directly in the reaction mixture without having to extract them.

COLORIMETRIC METHODS: Three colorimetric methods differing in principle are available for the assay of phospholipase A. The first which is a variant of the acidimetric method involves the use of an internal PH indicator in the assay mixture. The two other ones are a two-stage assay involving incubation of the enzyme with the substrate, stopping the reaction and spectrophotometric determination of the copper soap of the liberated fatty acid or of the residual ester bonds by the hydroxamate procedure. The colorimetric method for the assay of phospholipase D involve spectrophotometric determination of choline as choline reineckate or periodide or determination of ethanolamine as the periodide or by using the ninhydrin method.

Radiochemical Methods: These methods which are used in the assay of phospholipases A C and D activity involve incubation of the enzyme with a labelled phosphatide stopping the reaction, extraction and chromatographic separation of the products and then counting. This method of assay has the advantage of great sensitivity and specificity.

Methods Based on Chromatographic Separation of Products:

This assay method which has only been described for phospholipase A involves the separation of the products of phospholipase A action by paper or thin-layer chromatography and then quantitatively estimating them.

Chemical Methods: This method which was first introduced by Macfarlane and Knight (1941) is used for the assay of phospholipase C. The method involves the measurment of liberated acid-soluble phosphorus. A similar method of following the release of phosphorus is used in the assay of phospholipase B (Bernheimer et al., 1987).

SIMULTANEOUS ASSAY OF PHOSPHOLIPASES A, C AND D.

The radiochemical procedure based on the use of uniformly labelled ^{14}C-lecithin has been used in the assay of phspholipases A, C and D concurrently in mixtures of the three enzymes (Grossman et al, 1974). The conditions of incubation necessarily represent a compromise in regard to PH and calcium ion concentration, since the PH optima of phospholipases A and C are on the alkaline side of neutrality, whereas the optimum value of phospholipase D is 5.6 (Kates

and Sastry, 1969). Moreover, the high calcium ion concentration used in phospholipase D assay inhibits phospholipase C (Ottolenghi, 1969). Consequently, the activities measured will not be maximal.

CHAPTER THREE

MATERIALS AND METHODS

3.1 ANIMALS

Goats and cattle were purchased from local markets in Zaria. The goats were kept in fly-proof pens in the faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. It was ensured that all the goats were in good condition and apparently free from trypanosomiasis and any other forms of parasitic disease before the experimental infection.

3.2 TRYPANOSOMES.

Trypanosoma vivax stabilate, stock Kabam/84/NITR/7.4 was obtained from the Nigeria Institute of Trypanosomiasis Research, Vom. This was inoculated in a sheep's blood and kept under liquid nitrogen at - 196°C before being used in goats.

3.3 CHEMICALS.

Phosphatidylcholine was supplied by Sigma Chemical Company, U.S.A. DEAE-Cellulose (DE - 52) was supplied by Whatman, Dithiolthreitol was supplied by Bio-Rad Laboratories, Richmond, Calif. Bovine Serum albumim (BSA), Heparin, D-Glucose, Triton X - 100, monosodium dihydrogen orthophosphate, Disodiumhydrogen orthophosphate, Ethanol, Chloroform and Methanol were supplied by BDH chemicals, Poole, England while Potassium dihydrogen orthophosphate and Dipotassiumhydrogen orthophosphate were supplied by Hopkin and Williams, England. Other chemicals used were available in the department and were either obtained from BDH chemicals or Hopkin and Williams, both in England.

3.4 EQUIPMENT

Sonicator - MSE

Micro-Haematocrit Centrifuge, Hawkley, England

Microscope. Olympus Monocular

Coulter Haemoglobinometer

Refrigerated Centrifuge

Benchtop centrifuge, MSE

Spectrophotometer, Model SP-200 PYE UNICAM.

Vortex mixer.

3.5 MONITORING THE CLINICAL COURSE OF INFECTION

The clinical course of infection was monitored by following the degree of parasitemia and anaemia. The latter was estimated by measuring the packed cell volume (PCV) and the haemoglobin (Hb) levels of the infected animals.

Procedure. Fresh blood samples were collected by vein puncture from both infected and uninfected animals using heparin (IOIU/ml) as anticoagulant and kept on ice. Estimation of the degree of parasitemia and measurement of PCV was done according to Woo (1970). The blood samples were taken into capillary tubes and the tubes were flame sealed at one end using a bunsenflame. The sealed tubes were then centrifuged at 12,000 rpm for three minutes. The PCV was measured by using the micro-haematocrit centrifuge reader. The tubes were placed on microscope slides on which a small quantity of oil immersion had been applied and examined under the microscope for detection of parasites.

The Hb levels were estimated using the coulter Haemoglobinometer.

3.6 ESTIMATION OF SERUM FREE FATTY ACID (FFA) LEVEL

The serum FFA was extracted as described by Falholt et al. (1973) and estimated by titration as described by Wells and Hanahan (1969) with a slight modification in which the indicator solution was prepared according to Kelley (1965). The extraction medium (CHM) was prepared by mixing chloroform and heptane 1:1 v/v and then adding 2% of methanol. The titrant was 0.02 M ethanolic NaOH prepared by diluting 0.2 M eq. NaOH 1:10 with 95% ethanol. The indicator solution was a dilute solution of phenolred prepared by diluting 1.0ml of a 1% aq. phenolred solution with 99ml of absolute ethanol and 200ml of heptane. Procedure. 50Ul of serum and 50Ul of distilled water (as blank) were added to test tubes with polythene caps. To these, aq. 6.0 ml of the extraction medium was added and the tubes were shaken vigorously for 90 seconds on a vortex mixer. The tubes were then allowed to stand for 15 minutes and were centrifuged at 4000rpm for 10 minutes. 5.0ml of the CHM phase was transferred to 25.0ml conical flasks and titrated with 0.02M ethanolic NaOH. The amount of FFA was estimated by the amount of NaOH consumed.

3.7 ISOLATION OF TRYPANOSOMES

Trypanosomes were harvested by the method of Lanham and Godfrey (1970).

Buffer Solutions: The stock buffer solution was sodium phosphate buffer (PH 8.0) in a final concentration of 0.09M in disodiumhydrogen orthophosphate, 0.005M in monosodiumdihydrogen orthophosphate (dihydrate) and 0.43% in sodium chloride. This is stock phosphate - buffered saline (PS). Phosphate-buffered saline Glucose (PSG) was prepared by mixing the stock PS and glucose solution in the ratio of 4:6 such that the final glucose concentration was 1% (W/V).

Equilibration of DEAE - cellulose. Preswollen DE-52 was suspended in PSG (250 g/litre) and the PH of the suspension lowered to 8.0 with orthophosphoric acid diluted 1:20. The suspension was washed four times with the PSG and the PH of the washed DE - 52 was found to be the same as that of the PSG. The equiliberated DE - 52 was stored in the refrigerator until required.

Procedure

Column preparation: Slit-sieve glass Buchner funnels (8cm x 12cm) were fitted with rubber tubings and screw-clips were fixed to the tubes to control the flow of eluate. A Whatman number 41 fitter paper was placed on slit-sieve disc and the column was carefully packed with successive volumes of the DEAE-cellulose slurry to within 1-2cm of the top. The slurry was allowed to settle and a Whatman number 41 filter paper was placed on top.

Blood samples and column loading: Blood samples were taken from the jugular vein of the infected goats using heparin as anticoagulant and kept on ice. The blood samples were immediately diluted 1:4 with PSG. About 5 - 8 ml of the diluted blood samples were loaded onto the column. The column outlet was then opened to allow the blood sink into the adsorbent followed by elution with PSG, the eluate being collected on ice packs. The eluate was frequently examined under the microscope for detection of trypanosomes. When trypanosomes stopped coming out of the column, the collected eluate was centrifuged at 2000*g* for 15 minutes and the supernatant discarded. The pellet was washed once to free the trypanosomes of host enzymes and suspended in 5 mM phosphate buffer, PH 7.4. The harvested trypanosomes were then stored at - 20°C until required.

3.8 ENZYME ISOLATION

The enzyme was isolated by the method of Hambrey et al. (1984) with the following modifications. Centrifugation was done at 25,000rpm for two hours instead of 54,000rpm for one hour and 5mM phosphate buffer, PH 7.4 was used instead of 10mM Tris buffer, 8.0.

Procedure: The harvested trypanosomes were sonicated at maximum speed until all the trypanosomes were disrupted. The sonicate was diluted 1:1 with water containing 2mM dithiolthreitol (DTT) and 2mM EDTA. The diluted sonicate was centrifuged at 25,000rpm for 2 hours. The supernatant was collected and the pellet resuspended to thesame volume

as dilute sonicate in 5mM phosphate buffer PH 7.4 containing 1mM DTT, 1mM EDTA and 0.1% v/v Triton X-100. This was again centrifuged for 2 hours and the supernatant collected. The two supernatants were pooled together and stored in the refrigerator.

3.9 PROTEIN ESTIMATION

Protein was estimated by the Biuret method.

3.10 ASSAY OF ENZYME ACTIVITY

The enzyme was assayed according to Wells and Hanahan (1969).

Buffer: The buffer solution was 5mM phosphate buffer (PH 7.4) containing 20mM $Cacl_2$, 1mM EDTA and 220mM Nacl.

Substrate solution: This was prepared as described by Wu and Tinker (1969), a slight modification of that of Wells and Hanahan (1969). Phosphatidylcholine was dissolved in chloroform containing 2% methanol (14 mg/ml).

Working alkali: 0.02M NaoH in 90% ethanol, prepared by diluting 0.2maq. NaOH 1:10 with 95% ethanol.

Enzyme solution: The crude enzyme preparation was diluted 1:1 with the buffer solution.

Procedure: 2.0ml of the substrate solution was taken in 5.0ml volumetric flask and 0.1ml of the enzyme solution was added. The mixture was shaken vigorously for 30 seconds and allowed to stand at room temperature for 15 minutes, after which 5.0ml of 95% ethanol was added. The mixture was titrated with 0.02M ethanolic NaOH using a 0.1% phenolred in water as indicator. Blank values were run with each assay.

3.11 ESTIMATION OF HEMOLYTIC ACTIVITY

The hemolytic activity of the enzyme was estimated as described by Gul and Smith (1972).

Enzyme solution: Crude extract was diluted 1:1 with 100mM $CaCl_2$ to obtain the enzyme preparation.

Procedure: Fresh host blood was collected in 3.8% sodiumcitrate (1ml/10ml) and centrifuged at 800g for 15 minutes. The supernatant plasma and buffy coat were removed and the cells were washed three times with 10 volumes of 0.95% saline, centrifuging each time at 1000g for 5 minutes. A fourth wash was done with equal volume of phosphate-bufferred saline. 0.1ml samples of the packed red cells were pipetted into glass stoppered tubes containing 0.8ml of phosphate-buffered saline. The tubes were incubated for 10 minutes at 37°C in a shaking water bath and the reaction was initiated by adding 0.1ml of enzyme solution. The incubation was then continued for 15, 30, 60, 120, 180, 240, and 300 minutes after which the reaction was stopped by adding 5.0ml ice-cold saline containing 1% 200mM EDTA. Bovine serum albumin was added to a final concentration of 3.5% and the tubes were left on ice packs until centrifugation. The supernatants were collected and the degree of haemolysis was estimated spectrophotometrically by measuring absorbance at 540nm. Absorbance of 1.0 was taken as 100% haemolysis.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Parasitemia

Samples were taken for detection of trypanosomes on a daily basis. Trypanosomes were first detected on day two post infection and by day five, parasitemia had shot up to about four parasites per field. On day eight parasitemia had dropped to as low as one parasite per field. Thus, as figure 4.1 clearly shows, there was a cyclical fluctuation in the development of parasitemia with the highest peaks on days five and twelve.

The observed cyclical fluctuation in parasitemia is in accordance with the successive wavesof parasitemia in the early stages of trypanosome infection reported by Ross and Thompson (1910). This is due to antigenic variation in which individual parasites have the capacity to present different antigenic guises to the host (Ritz, 1916) so that as one parasite population is cleared by the host's immune system, another set springs up. The successive waves of parasitemia therefore represent antigenically distinct populations (Levaditi and McIntosh, 1910).

4.2 Packed Cell Volume (PCV) and Haemoglobin (Hb) Levels.

There was a general decrease in both the PCV and the Hb levels of all the infected animals in the course of the infection, as can be seen from tables 4.2a and 4.2b (see Appendix) and figures 4.2a and 4.2b.

The progressive decrease in the PCV and haemoglobin levels observed through out the course of infection offers a supportive evidence for an earlier observation that anaemia

FIGURE 4.1

Figure 4.1 is a graph showing the development of parasitemia in five goats infected with Trypanosoma vivax over a twenty-six day period of infection.

Each point on the graph represents the mean of five experiments ± the standard error of mean (SEM)

FIGURE 4.1

DEVELOPMENT OF PARASITEMIA IN GOATS INFECTED WITH T. VIVAX

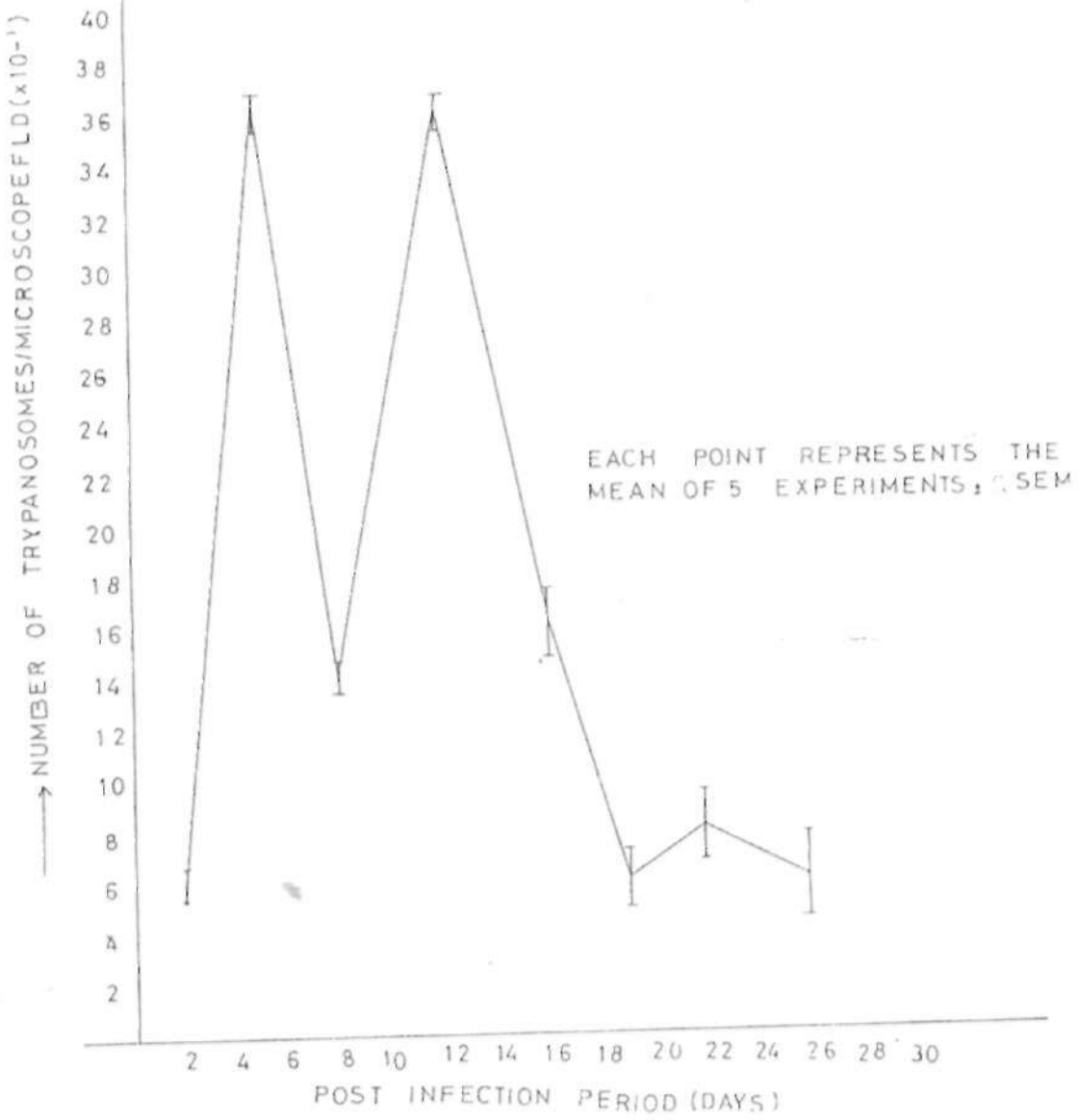


FIGURE 4.2a

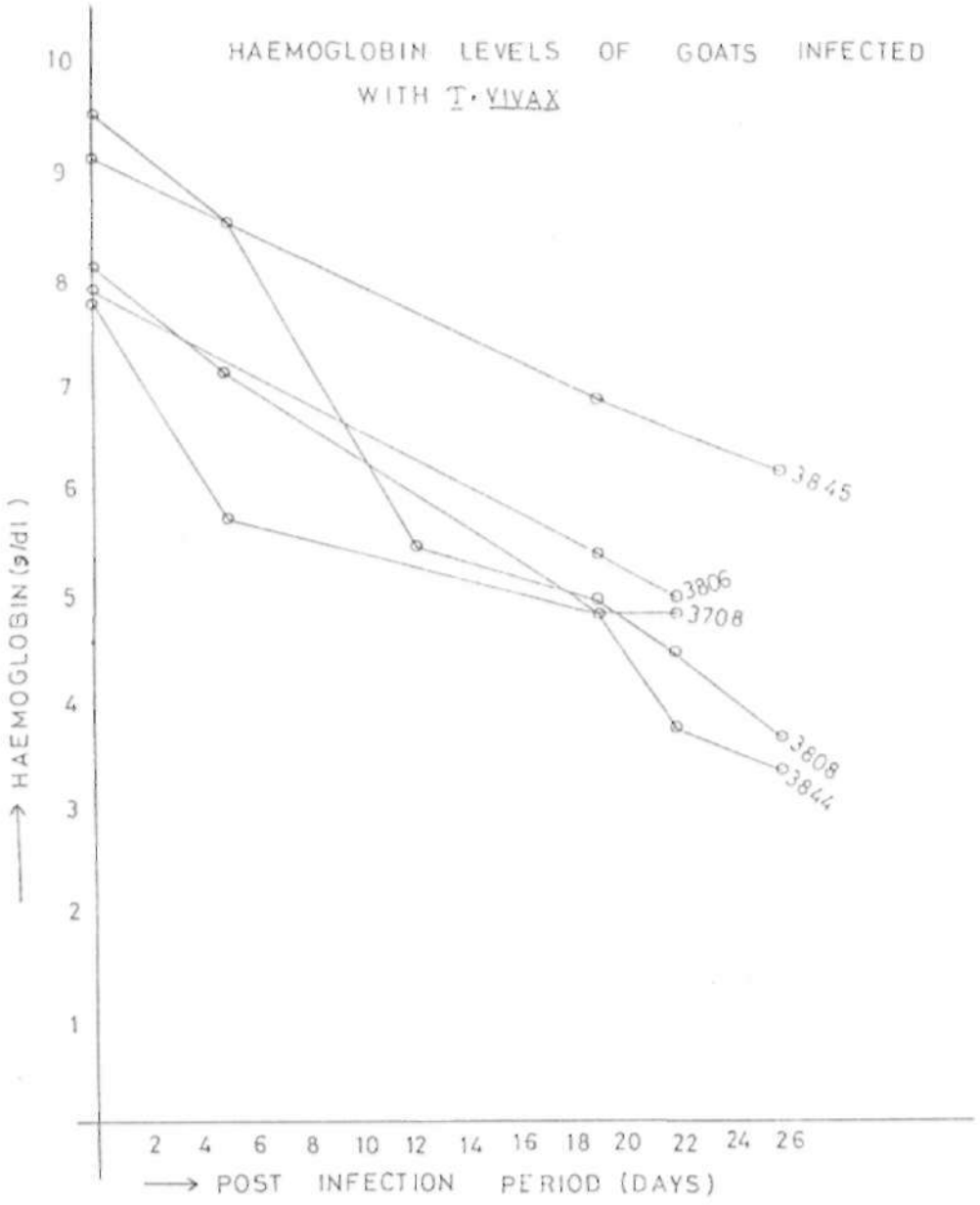

HAEMOGLOBIN LEVELS OF GOATS INFECTED WITH T. VIVAX
10
9
8
7
6
5
4
3
2
1
HAEMOGLOBIN (g/dl)
3845
3806
3708
3608
3844
2 4 6 8 10 12 14 16 18 20 22 24 26
POST INFECTION PERIOD (DAYS)

FIGURE 4.2a

Figure 4.2a are graphs showing individually, the haemoglobin levels of five goats infected with Trypanosoma vivax over a twnty-six day period of infection.

The numbers written at the end of each line on the graph (eg 3808, 3844, etc) are identification numbers of the experimental animals.

FIGURE 4.26

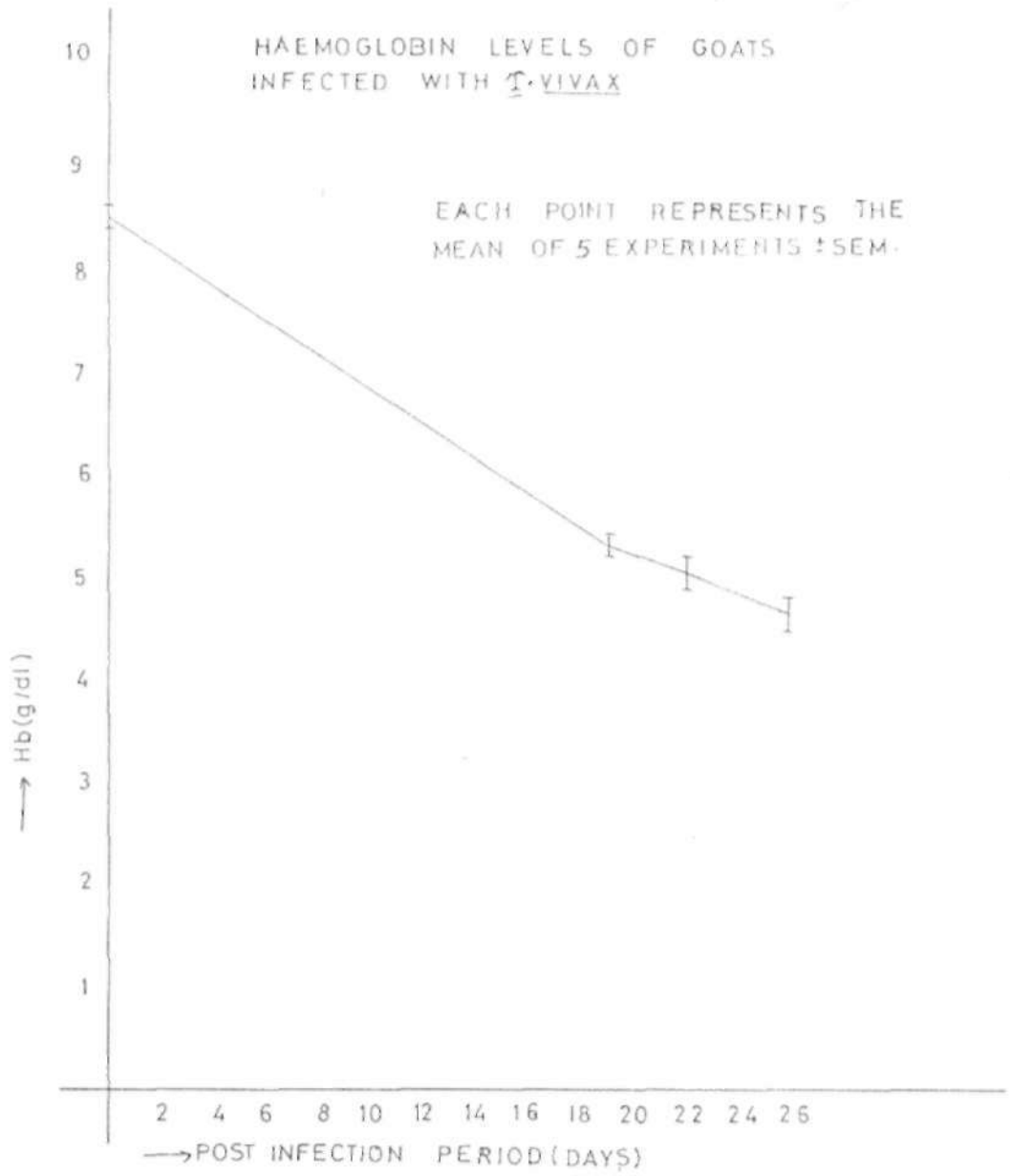
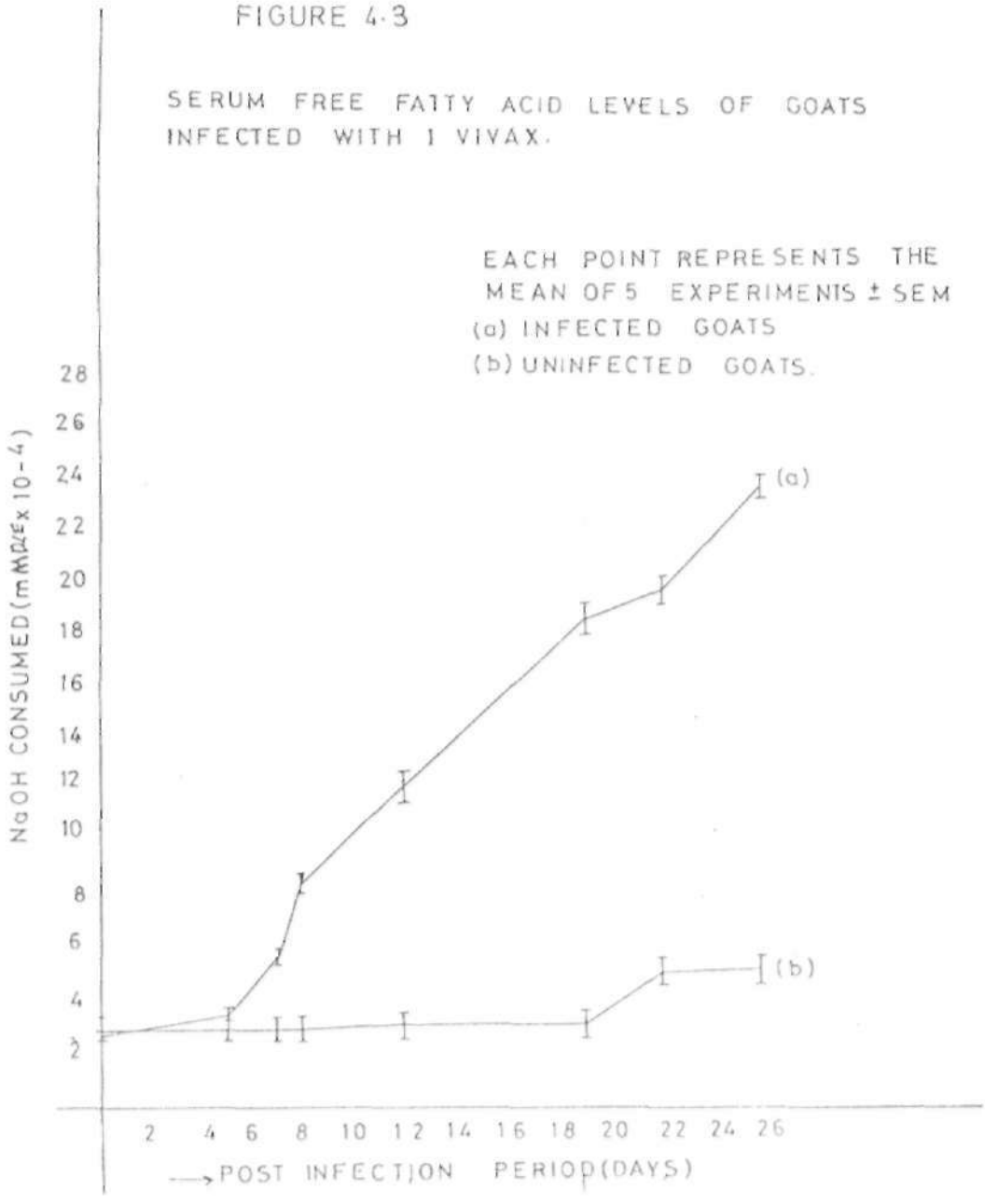

HAEMOGLOBIN LEVELS OF GOATS INFECTED WITH T. VIVAX
EACH POINT REPRESENTS THE MEAN OF 5 EXPERIMENTS ± SEM.
10
9
8
7
6
5
4
3
2
1
Hb(g/dl)
2 4 6 8 10 12 14 16 18 20 22 24 26
POST INFECTION PERIOD (DAYS)

FIGURE 4.2b

Figure 4.2b is a graph showing the haemoglobin level of five goats infected with Trypanosoma vivax. Each point on the graph represents the mean of five experiments ± the standard error of mean (SEM)

FIGURE 4·3

SERUM FREE FATTY ACID LEVELS OF GOATS INFECTED WITH I VIVAX.



is one of the major pathological features of trypanosomiasis in man and his domestic animals (Fiennes, 1954; Mackenzie and Cruickshank, 1973; Mamo and Holmes, 1975; Kobayashi et al;, 1976; Ikede et al., 1977). The rate of decrease in these two parameters vary in all the infected animals which might be due to differences in their extent of susceptibility to the infection. Thus there were rapid decreases in the PCV and Hb levels of goats number 3808 and 3844. Infact goat number 3844 died twenty-six days after being infected. The PCV of this animal by this day had dropped to slightly less than half of the preinfection value. Therefore, among other factors, severe anaemia might have been a contributing factor to the sudden death resulting from acute infection.

4.3 Free Fatty Acid Levels

Sera from both infected and uninfected animals were taken twice a week for analysis for free fatty acids (FFA). All the infected animals showed increases in their FFA levels while the values for the control animals only increased slightly toward the end of the period of analysis. These are clearly shown in tables 4.3a and 4.3b and figure 4.3.

Table 4.3a and figure 4.3 show a striking feature in the results of free fatty acid analysis in which a considerable rise in the FFA levels of all the infected animals between days twelve and nineteen post infection was observed. This period incidentally coincides with the period of active trypanolysis as shown by the marked drop in parasitemia

FIGURE 4.3

Figure 4.3 are graphs showing the serum free fatty acid (FFA) levels of infected (a) and uninfected (control) (b) goats over a twenty-six day period.

Each point on the graph represents the mean of five experiments ± the standard error of mean (SEM)

clearly shown in figure 4.1. It has been reported that as trypanosomiasis progresses in an infected animal, the trypanosomes begin to die, releasing a number of biologically active products among which are phospholipases (Tizzard et al. 1978a, 1978b; Akanji, 1985). The released phospholipases act on endogenous phospholipids, releasing FFA and lysophospholipids. The observed considerable increase in FFA in the sera of the infected animals during the period of active trypanolysis by this author is in agreement with this report. This possibly explains the observed insignificant increase in FFA levels during the period of growing parasitemia (day 1 - 5 post infection).

Another important observation in the course of this investigation was the coincidence of severe haemolytic crisis with the highest peak of FFA level as evidenced by the death of one of the infected animals on day twenty-six post infection, resulting from among other pathological processes, the severe haemolytic crises. That there was a haemolytic crisis is indicated by the marked drop in the Hb level and the packed cell volume. The FFA level of this animal by day twenty-six was highest of all the infected animals and the Hb level by this day had dropped from 7.8 on the day of infection to 3.3 while the PCV dropped from 25 to 12. Condrea et al (1964) had earlier reported a similar correlation between the release of FFA and erythrocyte haemolysis. Logos and Ikede (1972) have reported that in some forms of trypanosomiasis, it is

possible that death occurs as a direct result of a severe haemolytic crisis. It is therefore possible that the death of this animal is not unconnected with the severe haemolytic crisis.

The significance of free fatty acids in the pathogenesis of trypanosomiases has been highlighted in a review by Tizzard et al. (1978). These include: Haemolytic and cytotoxic due to their detergent-like properties. This they do by dissolving in the lipid component of cell membranes. At low concentrations, this may result in membrane stabilization whereas at higher concentrations, they destabilize the membrane and so cause lysis (Bachmann and Zbinden, 1973). Other toxic effects of FFA are induction of thrombosis (Connor et al., 1963, Hoak et al., 1964), lipid accumulation in cultured cells (Moskowitz 1967), myocardial lesions (Willebrands et al., 1973) and thrombocytopenia (Shore and Alpen 1963, Zbinden 1964). Free fatty acids also actively stimulate insulin secretion (Crespin et al. 1969) and as a result may induce hypoglycemia. This, too has been reported to contribute to deaths of trypanosome infected animals (Herbert et al 1975) .

44 Protein Estimation and Assay of Enzyme Activity

The total volume of crude enzyme was 12.0ml. The total quantity of protein was 67.2mg. The total activity of the enzyme was 16U molmin $^{-1}$ The specific activity was 0.24U molmin $^{-1}$ mg $^{-1}$.

4.5 Haemolytic Activity:

Washed host erythrocytes were incubated with crude phospholipase A_2 preparation for various time intervals ranging from 15 - 300 minutes after which the samples were examined for haemolysis. There was no significant haemolysis when this was done in the absence of albumin. However, when 3.5% bovine serum albumin (BSA) was added subsequent to the enzyme action, considerable haemolysis (up to 70%) was detected. Figure 4.4 shows the percentage haemolysis caused in the presence of phospholipase A_2 and 3.5% BSA, in the absence of phospholipase A_2 and 3.5% BSA, and in the presence of phospholipase A_2 without BSA.

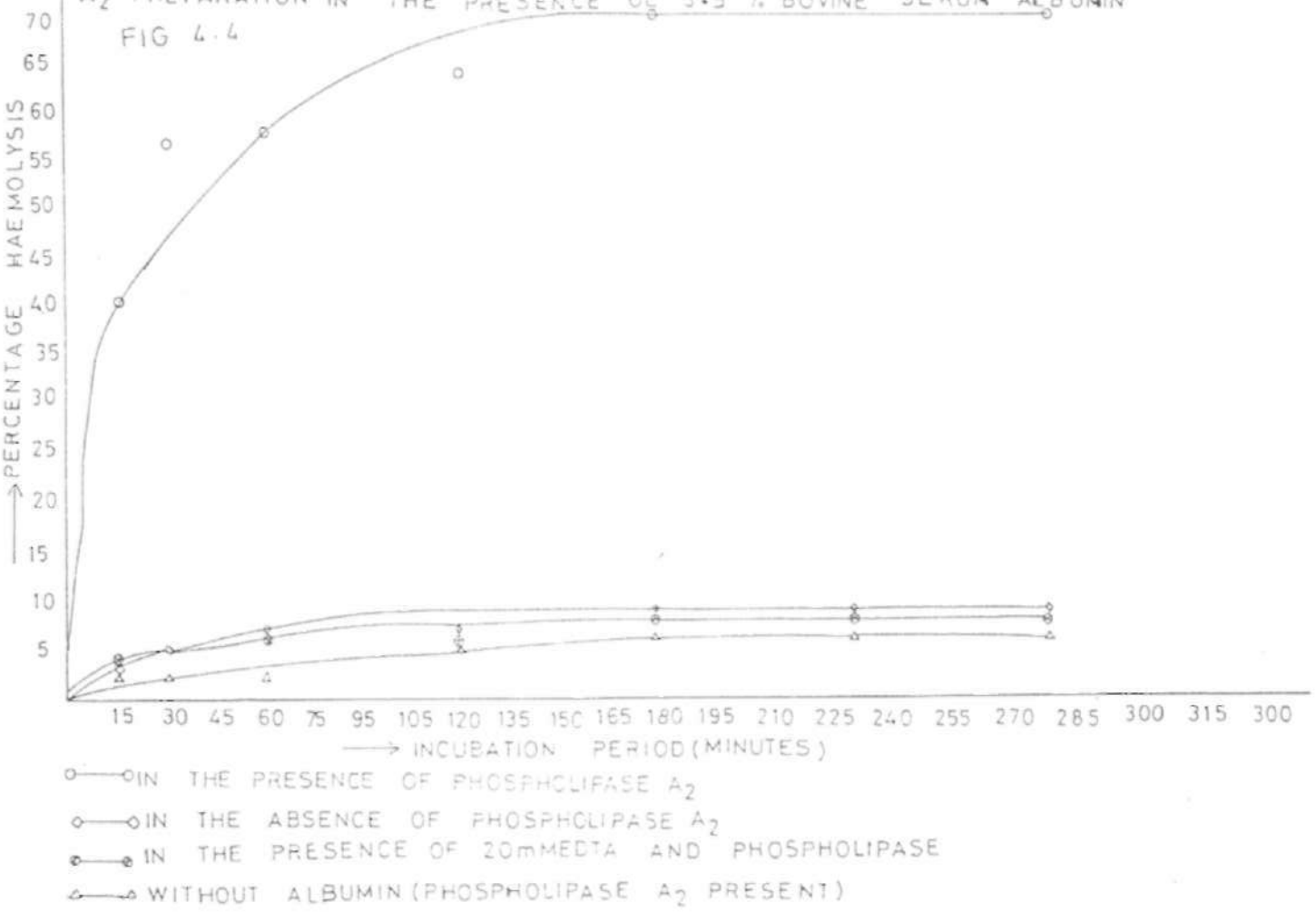
There has been reports (Ibrahim and Thompson 1965, Opden Kamp et al. 1972, Gul and Smith 1972, Colley et al. 1987) that purified phospholipases degrade erythrocyte membrane phospholipids without causing haemolysis. It has also been reported that purified phospholipase A_2 does not haemolyse washed erythrocyte unless the medium contains an external source of phospholipids (Bowman and Kaletta 1957) or contains sublytic concentrations of detergents which are themselves potentially lytic at higher concentrations (Heemskerk and Deenen 1964, Roelofsen et al 1971) or is hypotonic (Lankisch and Vogt 1972) or contains serum albumin (Gul and Smith 1972). The absence of haemolysis without albumin and the observed haemolysis in the presence of albumin is in accordance with these earlier observations. The enhancement of haemolysis by serum albumin has been attributed to

FIGURE 4.4

Figure 4.4 is a graph showing the percentage haemolysis of host (bovine) erythrocytes caused by crude phospholipase A_2 preparation and 3.5% bovine serum albumin.

HAEMOLYSIS OF WASHED HOST ERYTHROCYTES BY CRUDE PHOSPHOLIPASE A_2 PREPARATION IN THE PRESENCE OF 3·5 % BOVINE SERUM ALBUMIN

FIG 4.4



the removal of the split fatty acids and lysocompounds by the albumin (Gul and Smith 1972). That albumin is not involved in phospholipid splitting is supported by the reported observation of Gul and Smith (1972) in which no difference in the extent of Phospholipid breakdown was detected in the presence or absence of albumin. There is a report (Tizzard et al. 1978) that serum albumin blocked haemolysis of red cells both in vitro and in vivo. Their observation would be true from the point of view that the phospholipases do not directly hydolyse phospholipids of intact erythrocyte membrane and will only cause haemolysis indirectly through free fatty acids released by their hydrolytic action on released trypanosome phospholipids when the Parasites lyse. Therefore, the question of whether serum albumin blocks haemolysis or promotes it depends on which effect (direct or indirect) of the phospholipases is being considered.

Albumin - bound fatty acids though not cytolytic, they are capable of modifying other cellular functions such as the suppression of immune responses by certain free fatty acids which might include margaric (C17:0), Linoleic ($C_{18:2}$), linolenic ($C_{18:3}$) and arachidonic ($C_{20:4}$) acids (Arai et al. 1977.

From the present study, it cannot be conclusively said that the observed haemolysis was entirely due to the isolated phospholipase A_2 since the enzyme preparation was a crude one. Therefore, the possibility of the involvement of other factors which might have acted synergistically

with the enzyme cannot be ruled out. The reports of Condrea et al (1964) and Lankisch and Vogt (1972) of the synergy of a direct lytic factor and phospholipase A in the haemolysis of erythrocytes give credence to this view. Similar reports involving phospholipase A_2 and C (Cobley et al 1973) and phospholipase A_2 and cardiotoxin (Baugis et al. 1987) have also been made. In addition to other lytic factors which might potentiate erythrocyte haemolysis by the phospholipase A_2 preparation, it is also possible that the observed haemolysis was due to a combined action of phospholipase A_2 and A_1. This is because activity of phospholipase A_1 was also detected in the crude extract although this was relatively very low compared to that of phospholipase A_2. The observed low phospholipase A_1 activity might be due to the fact that phosphatidylcholine was used as substrate in the assay as against phosphatidylethanolamine which is the preferred substrate for most phospholipases A_1. In the erythrocyte membrane however, the availability of the preferred substrate makesthe activity of phospholipase A_1 to become significant. Infact Ibrahim and Thompson (1965) reported that the direct lytic factor of Condrea et al. (1964) might be a phospholipase A which is inactive against phosphatidylcholine but active against phosphatidylethanolamine or phosphatidylserine or both. Nevertheless, the dramatic drop in haemolysis observed in the presence of EDTA, a potent inhibitor of phospholipase A_2 strongly suggests the involvement of this

enzyme in the observed haemolysis. Therefore, it can be tentatively concluded that phospholipase A_2 of T. vivax can cause in vitro haemolysis of host (bovine) erythrocytes. A safe conclusion can however be made only when the enzyme is obtained in a relatively pure form and its effects on the host erythrocytes studied. An attempt in this direction is being contemplated by this author. From this study, the utilization of potent inhibitors of phospholipases A_2 in the synthesis of drugs for the treatment of anaemia in trypanosomiasis is suggested.

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APPENDIX - TABLES OF RESULTS

TABLE 4.1: DEVELOPMENT OF PARASITEMIA

Post Infection Period (Days)	No. of Parasites (Mean)	SEM
2	0.6	0.06
5	3.6	0.06
8	1.4	0.06
12	3.6	0.06
16	1.6	0.13
19	0.6	0.11
22	0.8	0.13
26	0.6	0.16

TABLE 4.2a: HAEMOGLOBIN LEVELS OF GOATS INFECTED WITH T. VIVAX

Post Infection Period (Days)	Means Hb Level	SEM
0	8.48	0.09
5	-	-
12	-	-
19	5.32	0.10
22	5.02	0.16
26	4.62	0.14

TABLE 4.2b: PACKED CELL VOLUME (PCV)% AFTER DAYS OF INFECTION

GOAT NUMBER	DAY 0	DAY 2	DAY 5	DAY 8	DAY 12	DAY 16	DAY 19	DAY 22	DAY 26
3808	29	29	25	25	20	16	16	15	12
3845	28	21	20	20	114	23	22	20	20
3708	27	25	22	22	18	16	16	16	15
3806	22.5	19	10	18.5	17	16	14	17	16
3844	25.0	20	18	18	18	14	15	21	12

TABLE 4.3a: FREE FATTY ACID LEVELS OF GOATS INFECTED WITH T. VIVAX

Post Infection Period (Days)	Free Fatty Acid Level (Mean)	SEM X 10^{-5}
0	2.7×10^{-4}	1.21
5	3.42×10^{-4}	1.84
7	5.70×10^{-4}	2.70
8	8.36×10^{-4}	3.68
12	1.20×10^{-3}	5.43
19	1.84×10^{-3}	6.21
22	1.96×10^{-3}	3.81
26	2.36×10^{-3}	4.14

TABLE 4.3b: FREE FATTY ACID LEVELSOF UNINFECTED GOATS

Days	Mean Free Fatty Acid Level x 10^{-4}	SEM X 10^{-5}
0	2.85	4.78
5	2.85	4.78
7	2.85	4.78
8	2.85	5.04
12	3.00	5.04
19	3.00	5.04
22	5.00	5.04
26	5.00	5.04

TABLE 4.4: PERCENTAGE HAEMOLYSIS CAUSED BY PHOSPHOLIPASE A_2

Incubation Time (Minutes)	In the Presence of Enzyme and 3.5% BSA	In the Presence of Enzyme and 20m MEDTA	In the Absence of BSA
15	40	4	2
30	57	5	2
60	58	6	2
120	64	6	5
180	70	8	6
240	70	8	6
300	70	8	6