# THE SUSCEPTIBILITY OF ERYTHROCYTES TO IN VITRO PEROXIDATION IN ANIMAL TRYPANOSOMIASIS

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# DECLARATION

I herby declare the originality of this work carried out by me in the Department of Veterinary Pathology and Microbiology under the supervision of Prof. K.A.N. Esievo, Prof. D.I. Saror and Dr. (Mrs.) O.K. Obagaiye. The works of other investigators are duly acknowledged and referred to. No part of this thesis has previously been submitted for a degree or diploma.

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CERTIFICATION

This thesis by IKECHUKWU ONYEBUCHI IGBOKWE meets the regulation governing the degree of DOCTOR OF PHILOSOPHY of Ahmadu Bello University and is approved for its contribution to scientific knowledge and literary presentation

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DEDICATION

To my FRIENDS,

Who inspired me,

Whose love drove me

To search and know

To my beloved FAMILY

For their care and support

To EZEKIEL and JONATHAN

Who passed on years ago

But now see with distant eyes

To the sun

In the night

That gave light

To the scrub

Of earth's womb

For the scoop of scum

And show of sheen

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## ABSTRACT

In order to assess the role of peroxidative injury to erythrocytes in animal trypanosomiasis, the effect of acute trypanosome infections on the susceptibility of erythrocytes to in vitro peroxidation was studied in two experimental models involving Trypanosoma brucei infection of mice and Trypanosoma yivax infection of calves. In the in vitro erythrocyte peroxidation, washed erythrocytes from the infected and control animals were incubated with 1.5% hydrogen peroxide (v/v) and the by-products of lipid peroxidation produced were measured spectrophotometrically as thiobarbituric acid reactive substances (TBARS). Glutathione (GSH), the primary antioxidant involved in peroxide neutralization in erythrocytes, was also measured in the trypanosome-infected calves.

Trypanosoma brucei produced an acute infection in 24 mice with parasitemia occurring 3-5 days post-infection (pi). Ten mice served as uninfected controls. Nine infected mice died before day 5 pi and another 4 died before day 8 pi, bringing mortality rate to 54.2%. The infection caused a significant ($P < 0.025$) decrease in the packed cell volume (PCV) of the tail blood of 11 survivors on days 5. and 8 pi, when compared with the control. The heart blood PCV of the control and infected animals were not significantly ($P < 0.05$) different. The peroxidized erythrocytes of the heart blood of the infected mice produced significantly (P 0.05) greater amounts of TBARS than those of the controls,

which suggested that the infected mice seemed to have reduced ability to prevent free radical-mediated lipid peroxidation in the erythrocytes.

Trypanosoma vivax infection of 6 calves caused an acute disease. Three calves served as uninfected controls. Parasitaemia occurred from day 2 pi in the infected animals. The infection was moderately severe with one of the infected animals moribund on day 13 pi. The mean PCV and red blood cell count of the infected animals decreased significantly ($P<0.05$) between days 6 and 13 pi, whereas the mean corpuscular volume was not significantly ($P>0.05$) different when compared with the control values. The amount of TBARS produced by the peroxidized erythrocytes of the infected and control calves did not differ significantly ($P>0.05$). It was deduced that the ability of the bovine erythrocytes to prevent free radical-mediated lipid peroxidation was not affected by the infection.

Erythrocytes of healthy mice produced significantly ($p<0.05$) greater amounts of TBARS than those of healthy calves, suggesting that bovine erythrocytes had superior ability to protect themselves against peroxidation when compared with the mouse erythrocytes.

Erythrocyte GSH concentrations of 62 apparently healthy cattle in Zaria and Maiduguri ranged from 21.7 to 135.1mg/100ml RBC. The GSH concentrations and PCV were significantly ($P<0.05$) and positively correlated ($r=0.58, 0.59$), but GSH and plasma ascorbic acid (0.35-1.00mg/dl) concentrations were not significantly correlated ($r=-0.01$).

In the T. vivax-infected calves, erythrocyte GSH decreased marginally at the peak of the first wave of parasitaemia occurring on days 4-5 pi. The GSH values recovered on day 6 and improved steadily until day 12 pi, which coincided with the second peak of parasitaemia, when another slight GSH decline occurred. On day 13 pi, the GSH value was significantly ($P<0.05$) higher than the control values. Therefore, the GSH values in the infected animals seemed to improve despite the decrease in PCV, a situation that differed from the positive correlation of both parameters in healthy animals. Hence, the erythrocytes of the infected animals might have enhanced GSH regeneration which would favour the maintenance of the antioxidant capacity of the erythrocytes, thereby reducing the risk of peroxidative damage.

In conclusion, while the possibility of peroxidative injury to erythrocytes seemed to exist in acute T. brucei infection of mice, it was unlikely in acute T. vivax infection of calves, suggesting that the pathogenesis of the anaemia in trypanosomiasis, in this particular respect, might be different in the two parasite-host models studied.

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# LIST OF ABBREVIATIONS

| | | | |
|---|---|---|---|
| PVC | Packed cell volume | ROOH | Hydroperoxide |
| EDTA | Ethylene diaminotetra-acetate | $H_2O$ | Water |
| | | $O_2$ | Oxygen |
| TBARS | thiobarbituric acid reactive substance | $O_2$ | Superoxide anion |
| | | HO | Hydroxyl free radical |
| CTB | Control tail blood | RH | Fatty acid |
| ITB | Infected tail blood | ROO | Peroxyl radical |
| CHB | Control heart blood | RO | Alkoxyl free radical |
| IHB | Infected heart blood | R | Alkyl free radical |
| GSH | Glutathione | H | Hydrogen free radical |
| DTNB | 5,5'-dithiobis-(2-nitrobenzoic acid) | ROH | Alkyl alcohol |
| | | Fe | Iron |
| DNPH | Dinitrophenyl hyrazine | Cu | Copper |
| RBC | Red blood cell | OH | Hydroxyl ion |
| pi | Post-infection | | |
| NADP | Nicotinamide adenine dinucleotide phosphate | | |
| NAD | Nicotinamide adenine dinucleotide | | |
| $H_2O_2$ | Hydrogen peroxide | | |
| GSSG | Glutathione disulfide | | |

## LIST OF PAPERS FROM THESIS

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# CHAPTER ONE

## INTRODUCTION

Trypanosomiasis (or trypanosomosis) is a haemoprotozoan disease of animals and man caused by various species of trypanosomes. It is a major constraint to livestock production in most parts of Africa. In Nigeria, recent surveys have reported outbreaks and prevalence of trypanosomiasis among livestock (Opasina and Ekwuruke, 1988; Nawathe et al., 1988; Joshua and Shanthikutmar, 1989; Ilemobade et al., 1991; Onah, 1991; Anene et al., 1991a,b; Kalu, 1991; Kalu et al., 1991; Ahmed, 1992; Ahmed et al., 1994; Ahmed and Agbede, 1993; Anosa et al., 1994). Some features of the disease in animals which cause enormous economic losses to livestock farmers include loss of body weight , stunting, muscular wasting, drop in milk yield, infertility, abortion stillbirth and deaths. (Esuruoso, 1973; Ilemobade and Balogun, 1981; Stephen, 1986; Ikede et al., 1988; Agyemang et al., 1990; Paling et al., 1991; Sekoni, 1994).

The development of anaemia has long been recognized as a consistent consequence of trypanosome infections in animals (Hornby, 1921). The anaemia is caused mainly by extravascular haemolysis in the expanded mononuclear phagocytic system (MPS) of the trypanosome-infected host (Anosa, 1988; Murray and Dexter, 1988; Suliman and Feldman, 1989), but intravascular haemolysis may occur during the early acute phase when the MPS has not been fully mobilized (Mamo et al., 1977; Esievo et al., 1984). Several Mechanisms have been reported to predispose the red blood cell to phagocytosis in the MPS (Anosa, 1988; Murray and Dexter, 1988). The immunological mechanisms involve the proliferation and activation of macrophages and the

immunochemical alteration of red cell membranes associated with the onset of parasitaemia and the rise of serum antibodies (Balber, 1974; MacKenzie et al., 1978; Esievo and Saror, 1991).

In Trypanosoma brucei infection of mice, anaemia was observed earlier than 7 days after infection when immune response was not expected to have commenced (Jennings et al., 1974). Anaemia occurred in rats subjected to total body irradiation and infected with T. brucei, indicating that immunological competence may not be essential for the development of the anaemia (Murray and Dexter, 1988). Within 2 to 3 days of infection of mice with T. congolense, T. vivax or T. brucei (Murray et al., 1979) and rats with T. brucei (Murray, 1979), the presence of haemolysin was detected in the plasma of the hosts. The haemolysin may include biologically active substances produced by the trypanosomes such as proteases (Londsdale-Eccles and Grab, 1986), phospholipases (Mellor, 1985), neuraminidases (Esievo, 1979, 1983; Engstler and Schauer, 1993) and a heat stable trypsin sensitive substance (Huan et al., 1975).

Because bloodstream forms of T. brucei lack catalase (Fulton and Spooner, 1956) and glutathione peroxidase (Meshnick et al., 1978), enormous amounts of hydrogen peroxide accumulate in the trypanosomes (Meshnick et al., 1977) and may be released into the extracellular fluid before and during trypanolysis. In the cultivation of T. brucei, T. equiperdum, T. evansi, T. rhodesiense and T. gambiense, it was reported that there was need to eliminate activated oxygen produced by the trypanosomes (Baltz et al., 1985). During trypanosome infections, activated macrophages may be producing activated oxygen (Klebanoff, 1982; Schwacha and Loegering, 1992) which may diffuse into the extracellular fluids. Ameh (1984) showed that blood and hepatic glutathione

levels decreased and the susceptibility of erythrocytes to oxidative haemolysis increased during the early acute phase of T. brucei gambiense infection of rats, which suggested that the infection might have imposed an oxidative stress on the erythrocytes of the infected rats.

In cattle, T. vivax and T. congolense could cause acute anaemia (Murray and Dexter, 1988), but T. brucei often caused mild chronic anaemia (Ikede and Losos, 1972). However, T. brucei caused a severe acute infection in mice (Jennings et al., 1974). It is well recognized that the pathogenesis of anaemia in trypanosomiasis varies with the species of trypanosomes, the species of the host and the duration of the infection (Ikede et al., 1977). Because of the complexity of the mechanisms of erythrocyte damage in trypanosomiasis (Anosa, 1983a), there was need to investigate erythrocyte peroxidation during the acute disease in animals.

The purpose of the present study was therefore to investigate the susceptibility of erythrocytes to in vitro peroxidation during acute T. brucei and T. vivax infections of mice and cattle respectively, in order to provide some evidence of the possibility of peroxidase damage of erythrocytes during acute animal trypanosomiasis.

The three phases of the study were:

a) Effect of acute T. brucei infection of mice on the susceptibility of erythrocytes to in vitro peroxidation

b) Erythrocyte glutathione and plasma ascorbic acid concentrations in apparently healthy cattle, to provide baseline data on these antioxidants.

c). Effect of acute T. vivax infection of cattle on erythrocyte glutathione and susceptibility to in vitro peroxidation.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 PATHOGENIC TRYPANOSOMES AND THEIR TRANSMISSION

The pathogenic trypanosomes of domestic animals and man and their preferred hosts are as follows (Soulsby, 1982):

Trypanosoma vivax: Ruminants, horses, camels;

T. congolense: Ruminants, horses, dogs, cats;

T. brucei: Ruminants, horses, dogs, cats, pigs;

T. evansi (T. hippicum): Camels, horses, pigs, dogs, cats;

T. equiperdum: Horses, donkeys;

T. equinum: Equines;

T. simiae: Pigs, camels,

T. suis: Pigs;

T. uniforme: Ruminants

T. gambiense, T. rhodesiense, T. cruzi: man.

Based on the nature of their life cycle and transmission, trypanosomes may be classified as stercorarian or salivarian (Siegmund, 1979). The stercorarian trypanosomes (T. cruzi) follow a cycle of development in an insect vector (Kissing bug, Triatoma sp.) with the infective form being transmitted to the mammalian host in the faeces oarf the vector; whereas the salivarian trypanosomes (T. vivax, T. congolense, T. brucei, T. simiae, T. rhodesiense, T. gambiense) follow a cycle of development in tsetse flies

(Glossina sp.) and the infective (metacyclic) forms are transmitted to the mammalian hosts through the vectors sali5va during the bites of the flies (Siegmund, 1979).

T. equiperdum is transmitted mechanically by coitus. T. vivax and T. evansi may be transmitted mechanically by horse flies- Tabanidae and Stomoxys (Hoare, 1947; Mwongela et al., 1981). Transplacental transmission of T. vivax in ruminants (Ikede and Losos, 1972a; Ogwu et al., 1986) and transmission of T. brucei to cats and dogs by feeding infected goats (Moloo et al., 1973) have been reported. All the trypanosomes can be transmitted mechanically by syringe inoculation. For experimental purposes, laboratory rodents easily develop and maintain infections with all the trypanosome species after syringe inoculation except T. vivax, T. simiae and T. suis (Soulsby, 1982). Rat strains of T. vivax was established by supplementing rats with sheep serum (Desowitz, 1963).

## 2.2 COURSE OF TRYPANOSOME INFECTIONS IN ANIMALS

Trypanosomiasis has been divided into acute and chronic phases (Anosa, 1988; Murray and Dexter, 1988). After deposition of the infective forms of trypanosomes in the subcutaneous tissues of the host by the vector, the trypanosomes multiply at the site of infection to produce a chancre and invade the blood stream either directly or through the lymphatics (Losos, 1978). The acute phase begins with the rise in parasitaemia and decline in packed cell volume. The peaks of the parasitaemic waves are usually high within this acute period and the degree of anaemia correlates closely with the intensity and duration of the parasitaemia (Dargie et al., 1979a,b; Esievo et al., 1982; Murray and Dexter, 1988). During this phase, T. vivax and T. congolense cause much higher parasitaemia and more severe anaemia than T. brucei in ruminants (Murray and Dexter,

1988). The acute infection causes death within one week in rodents whereas, in ruminants, the animal may die within a few weeks to several months (Anosa, 1988).

The animals which progress into chronic phase usually have lower parasitaemia and less severe anaemia than those that die in the acute phase (Anosa, 1988). The chronic phase is characterized by low transient parasitaemia or aparasitaemia and the animals involved maintain low packed cell volume (Murray and Dexter, 1988). Death may occur during this late stage or the animal recovers through self-cure (Uzoigwe, 1986).

## 2.3 *MECHANISMS OF CELLULAR INJURY IN TRYPANOSOMIASIS*

Trypanosome infections initiate pathological mechanisms which cause cellular injury resulting in anaemia, leucopenia, thrombocytopenia and tissue inflammation and damage. There are reviews on the pathogenesis of the haematological changes (Anosa, 1988; Murray and Dexter, 1988; Suliman and Feldman, 1989; Igbokwe, 1989; Esievo and Saror, 1991) and some aspects of the pathogenesis of the tissue lesions in trypanosomiasis (Clarkson, 1976; Goodwin, 1976; Nielson et al., 1978c; Zwart and Veenendaal, 1978; Boreham, 1978; Morrison et al., 1978).

Many factors and mechanisms act in concert to cause cellular injury in trypanosomiasis. It is difficult to identify the primary and secondary mechanisms and the sequence in which they play their roles in the pathogenesis of the disease. This section presents an overview of the concepts on the mechanisms of cellular injury during trypanosome infections.

### 2.3.1 Immunological mechanisms

In trypanosomiasis, there is an extensive proliferation of macrophages (Ikede and Losos, 1972; MacKenzie and Cruickshank, 1973; MacKenzie et al., 1978; Anosa and Isoun, 1983) which have been shown to be considerably activated (Anosa and Kaneko, 1983c). The macrophage proliferation may be due to the massive intravascular presence of living, dying and dead trypanosomes and immune complexes (Murray and Dexter, 1988). The activation of macrophages may be mediated by lymphokines, antigen-antibody complexes and complement fragment C3b (Allison, 1978). Phagocytosis of erythrocytes, leucocytes, platelets and some haematopoietic cells in trypanosome-infected animals has been documented (MacKenzie and Cruickshank, 1973; Anosa et al., 1992). The cells become susceptible to phagocytosis after alteration of their cell membrane partly by immunological mechanisms. Immunoglobulin M, IgG, immune complexes and complement fragment C3 were demonstrated on erythrocytes of infected animals (Kobayashi et al., 1976; Facer et al., 1982; Amole et al., 1982). The IgM and IgG had antibody activity against trypanosome antigens. Trypanosomal antigens were demonstrated on the surface of erythrocytes of trypanosome-infected sheep (MacKenzie et al., 1978). It was, therefore, suggested that soluble trypanosome antigens might adsorbed on erythrocytes with subsequent opsonization of antibody and complement (Murray and Dexter 1988). Autoantibodies against erythrocytes of infected animals have been reported (MacKenzie and Boreham, 1974; Kobayakawa et al., 1979). Coating of leucocytes, platelets and other cells with trypanosome antigens, antibody, immune complexes and autoantibodies may cause their damage and predispose them to phagocytosis (MacKenzie et al., 1978; Kaaya et al., 1979; Assoku and Gardiner, 1985).

Also, immune complex formation in trypanosomiasis may lead to the release of phamacologically active substances (MacKenzie and Boreham, 1974). It seems that the immune complexes absorb and activate Hageman factor (Factor XII) which then activates prekallikrein to Kallikrein (Boreham and Wright, 1976; Boreham, 1978). Kallikreins are chemotactic and act on kininogen to form kinins (bradykinin and kallidin) which probably combine with acidic groups on mucopolysaccharides and glycoprotein component of blood vessel walls to increase permeability (Boreham, 1978). Immune complexes also cause aggregation and degranulation of platelets leading to release of serotonin which is a vasodilator (Slot et al., 1977; Zwart and Veenendaal, 1978).

In trypanosomiasis, complement is activated by the classical or alternate pathway or both (Nagle et al., 1974; Greenwood and Whittle, 1976; Nielson et al., 1978c). Complement activating materials have been derived from trypanosomes (Musoke and Barbet, 1977; Nielson and Sheppard, 1977; Nielson et al., 1977, 1978a,b). Immune complexes also contribute in causing complement activation (Lambert and Houba, 1974; Nagle et al., 1974). Activation of complement leads to the formation of chemotactic fragments (C3a, C5a, C5b67 complex), anaphylactic fragments (C3a and C5a) which cause mast cell degranulation and C3b which enhances phagocytosis (Boreham, 1978).

Infections with T. brucei group in man (T. gambiense, T. rhodesiense) and animals cause tremendous inflammatory reactions, and large numbers of mononuclear cells and few polymorphonuclear leucocytes infiltrate the inflammatory sites (Anosa, 1983a,b). Complement fixation is one of the mechanisms by which the polymorphonuclear leucocytes are attracted to the site as in Arthus reaction (Morrison et al., 1978). During phagocytosis of immune complexes by the polymorphonuclear

leucocytes, enzymes that leak from their granules as the cells die may be cytolytic to the surrounding tissues (Morrison et al., 1978). This mechanism of tissue injury may not feature in T. vivax infections of sheep and goats since neutrophils were not present in the inflammatory reactions in various organs (Anosa and Isoun, 1980, 1983).

Deposits of immunoglobulin and trypanosome antigens occur between the muscles fibres of the heart and skeletal muscles of T. brucei-infected mice (Galvo-Castro et al., 1978). Rhesus monkeys infected with T. rhodesiense developed glomerulonephritis associated with deposits of C3, properdin and IgM in the glomeruli (Nagle et al., 1974). Further studies have associated development of glomerulonephritis in T. rhodesiense and T. gambiense infections with deposition of immunoglobulins and complement along the glomerular capillary wall (Lindsey et al., 1978; Van Marck et al., 1983; Nagle et al., 1980; Oemar et al., 1983). Immune complex deposition occurred in the renal glomeruli of trypanosome-infected animals (Nagle et al. 1974; Lambert and Houba, 1974; Murray et al., 1975) and it was suggested that preformed immune complexes may not be derived from circulation (Morrison et al., 1978), but may be formed by a local interaction of antibody with trypanosome antigen. Also, a delayed hypersensitivity reaction has been reported in T. brucei infection of rabbits after injection of the trypanosome products into the skin (Tizard and Soltys, 1971).

Various autoantibodies have been detected in the sera of trypanosome-infected human and animals (Houba and Allison, 1966; Houba et al., 1969; Mansfield and Kreier, 1972; MacKenzie and Boreham, 1974; Lindsley et al., 1974; Bruijin et al., 1987) and are thought to be involved in the development of tissue lesions. Autoantibodies directed against laminin and type IV collagen of the glomerular basement membrane have been

shown to cause both linear and granular immune deposits with slight inflammatory reaction (Bruijin et al., 1987).

Persistent antigen stimulation of the lymphoid system in trypanosome-infected animals is associated with structural and functional changes in the lymphoid organs. There is marked hypoplasia of the lymphoid organs with loss of germinal centres and thinning of the cortex (Losos et al., 1973; Brown and Losos, 1977; Masake, 1980; Masake and Morrison, 1981; Morrison et al., 1982; Anosa and Isoun, 1983). Polyclonal stimulation of B-cells by trypanosomes and their antigens (Esuruoso, 1976; Mansfield et al., 1976; Campbell and Phillips, 1976) and some impairment in suppressor T-cell function (Mansfield, 1978) may lead to loss of B-cell clones (Greenwood, 1974a,b; Hudson et al., 1976; Assoku et al., 1977; Askonas et al., 1979), and the development of abnormal plasma cells (Mott cells) with ballooned rough endoplasmic reticulum (Anosa, 1983a).

A serum factor, the same as the cytokine responsible for tumor necrosis (Tumor necrosis factor, TNF), was found in trypanosome-infected rabbits to cause wasting and was called cachectin (ILRAD, 1993). Production of TNF by peripheral blood mononuclear cells from cattle with acute T. vivax infection rose to a peak 17 days after infection and decayed thereafter (ILRAD, 1993; Sileghem et al., 1994). The results suggested a correlation between TNF production and the severity of anaemia (ILRAD, 1993). No TNF production was detected from monocytes taken from cattle infected with a less acute stock of T. congolense (ILRAD, 1993; Sileghem et al., 1994). At 2-3 weeks after infection, only a low activity of TNF was detected in the plasma of the T. vivax-infected cattle (Sileghem et al., 1994). Using polymerase chain reaction assay, complementary DNA sequences for bovine TNF were detected in the monocytes from

cattle infected with both T. vivax and T. congolense and the level indicated that the earlier failure to detect TNF during the T. congolense infection was due to insufficient sensitivity of immunological method employed (ILRAD, 1993). In human African trypanosomiasis, plasma concentrations of TNF and interleukin-6 were elevated in patients and appeared to be associated with impairment of adrenocortical function (Reincke et al., 1994).

### 2.3.2 Trypanosome potent enzymes (toxins) and metabolites

Living and dead trypanosomes produce a number of biologically active substances which are involved in the causation of cellular damage. These trypanosome components and metabolites associated with the alteration of cellular structure and function are:

(a) Proteases

Proteolytic lysosomal enzymes, both thiol-dependent and thiol-independent, are found in trypanosomes (Rautenberg et al., 1982; Londsdale-Eccles and Grab, 1986; Londsdale-Eccles and Mpimbaza, 1986) and may be released into the circulation and tissue fluid from probably the flagellar pockets of living trypanosomes as earlier suggested by Vickerman and Tetley (1978) or from damaged or dead trypanosomes. A peptidase of parasite origin has been identified in the plasma of cattle infected with T. congolense and mice infected with T. brucei (ILRAD, 1986; Knowles et al., 1987). The proteolytic enzymes in circulation may damage the vascular endothelial cells and blood cellular components especially the erythrocytes by removing fractions containing sialic acid in the form of glycopeptides from the cell membrane (Cook et al., 1960). The proteases in tissue fluid may cause tissue damage (Seed and Hall, 1985). Specific antibodies directed

against the T. congolense protease were considered to partly limit the pathogenic effects of the trypanosomes in trypanotolerant Ndama and Buffalo (Authié et al., 1993).

(b) Neuraminidases

Neuraminidase activity was generated in vitro by T. vivax (Esievo, 1979, 1983), T. cruzi (Pereira, 1983) and insect-stage of T. brucei (Engstler et al., 1991; Engstler and Schauer, 1993). Esievo et al. (1982) suggested that the trypanosomes may be producing neuraminidase in vivo during infection which may cleave off erythrocyte surface sialic acid rendering the erythrocytes more prone to phagocytosis. It was also suggested that the neuraminidase might damage leucocytes in a similar way (Esievo and Saror, 1983).

(c) Phospholipases and free fatty acids

Trypanosomes generate relatively high levels of phospholipases after death and autolysis (Tizard et al., 1978c). Phospholipases can act on cell membrane phospholipids to cause cell destruction or haemolysis (Colley et al., 1973). However, purified phospholipase A1 was unable to penetrate the membrane and reach the membrane phospholipids except in combination with free fatty acids which act on the cell membrane (Condrea et al., 1964). Phospholipase A1 was found in large amounts in the tissue fluids and in considerably lower amounts in the plasma of rabbits infected with T. brucei, but an inhibitory factor, which might be an antibody, was detected later in infection (Hambrey et al., 1980). Cutaneous injection of trypanosome autolysate containing phospholipases into rabbits increased vascular permeability and caused local inflammatory reaction (Seed, 1969; Tizard and Holmes, 1976). Also, trypanosomes in intraperitoneal diffusion

chambers released soluble vasoactive materials (Tizard and Holmes, 1977). Although the vasoactive substances might have been complement fragments produced after complement activation by trypanosome components (Nielson et al., 1978b), they might also be products of mast cell degranulation caused by phospholipase A in the trypanosome autolysate (Orr and Cox, 1969).

The trypanosome phospholipases act on endogenous phosphatidylcholine to release about 15 free fatty acids (FFA), the predominant ones being palmitic, stearic and linoleic acids (Tizard et al., 1977, 1978a,c). Linoleic acid was shown to have the greatest capacity to cause haemolysis and cytotoxicity in vitro as a result of its detergent action on cell membrane. In vivo, the FFA are not likely to cause cellular injury by their detergent action since they are bound to plasma albumin (Tizard et al., 1978b) except in situations such as in T. congolense infection where there is heavy parasitaemia in capillaries. With the death and autolysis of trypanosomes, and massive release of phospholipase, large amounts of FFA will be released to exceed the binding capacity of the plasma albumin (Tizard et al., 1978a) and lead to local haemolysis. It was considered that the bound FFA might also be cytotoxic due to the activities of its oxidized products. The bound FFA may modify lymphocyte function by being immunosuppressive or immunostimulatory (Assoku et al., 1977; Assoku and Tizard, 1978; Hazlett and Tizard, 1978). In support of the association between pathogenicity and the possession of activable phospholipase by pathogenic trypanosomes is the absence of activable phospholipase in, and hence inability to generate FFA by the non-pathogenic T. theileri (Tizard et al., 1978b).

(d) Pyruvate

Pyruvate , which is an end-product of glucose metabolism by trypanosomes, has been shown to accumulate in the blood of infected animals in amounts directly proportional to the number of parasites in the blood (Grant and Fulton, 1957; Coleman and Von Brand, 1957). In T. brucei infection of rabbits, pyruvate levels in blood and tissue fluids increased by five folds (Goodwin and Guy, 1973). Although it has been suggested that the pyruvate may not reach toxic levels in the blood (Coleman and Von Brand, 1957), it has also been suggested that pyruvate in blood may lead to acidosis and a lowered affinity of haemoglobin for oxygen. In the tissues, the pyruvate may be responsible for, or at least contribute to, the degenerative changes observed in the connective tissues of the infected animals (Newton, 1978).

During the metabolism of glucose by T. gambiense, a dimer of pyruvate was reported to be formed which may inhibit the tricarboxylic acid cycle in host cell mitochondria (Seed and Hall, 1985), thereby causing energy deficiency in the cell. This is explained further in section 2.5.3.

(e) Aromatic by-products

Trypanosomes are capable of metabolizing aromatic amino acids to form toxic by-products whose pathophysiological effects have been reviewed by Seed and Hall (1985). Phenylalanine may be catabolized to phenylpyruvate which has proteolytic activity and may inhibit gluconeogenesis and mitochondrial function. One of the by-products of

tryptophan catabolism is indole-ethanol (tryptophol) which may act on the cell membrane to cause osmotic fragility and lysis.

### 2.3.3 Nutritional, electrolyte and hormonal imbalances

In T. congolense infection of goats, there was hypercortisolism (Mutayoba and Gombe, 1989) which might cause an anti-insulin effect and reduce glucose supply to the tissues (Zilva and Pannall, 1984). Moreover, $Na^+K^+$-ATPase activity has been reported to decrease in the brain and kidney of T. congolense-infected rats (Nok et al., 1992a,b) which implies that energy-requiring transport of metabolites such as glucose and amino acids into tissues and the differential permeability of the cell membrane to $Na^+$ and $K^+$ may be impaired. Inadequate glucose supply to the brain could be highly injurious. It was suggested that inadequate energy supply in erythrocytes may alter the erythrocyte membrane surface during infection (Jennings, 1976).

There was accumulation of cholesterol in the brain of T. congolense-infected rats (Nok et al., 1992a). The major carrier of cholesterol from the blood stream to the tissues is the low density lipoprotein (LDL) (Brown and Goldstein, 1986) and LDL contains large amounts of unsaturated fatty acids (Heinecke, 1987). It is probable that the cholesterol will accumulate in the brain along with greater amounts of unsaturated fatty acids so that the possibility of peroxidative tissue injury may be enhanced. Decreased membrane fluidity was indicated in the brain of the infected rats (Nok et al., 1992a) which might be due to cholesterol infiltration or lipid peroxidation or both.

The loss of differential permeability of plasma membrane of the cell suggested by $Na^+K^+$-ATPase activity in the brain and kidney (Nok et al., 1992a,b) and $Ca^{2+}Mg^{2+}$-ATPase activity in the testis and kidney (Nok et al., 1992c) of *T. congolense*-infected rats might have serious consequences on the viability of the cell. Since a large cellular concentration of $K^+$ is required for the maintenance of pyruvic kinase activity in glycolysis and for optimal protein synthesis (Conn and Stumpf, 1976), loss of cellular $K^+$ as was reported in rat trypanosomiasis (Zwemer and Culbertson, 1939; Ikejiani, 1946), may be injurious. Decreased activity of the $Ca^{2+}$-pump is expected to lead to influx of $Ca^{2+}$ into the cell resulting in damage of the cell (Nok et al., 1992c).

Trypanosomiasis was associated with pituitary lesions (Ikede and Losos, 1975; Mutayoba et al., 1988) and it was suggested that gonadal, thyroid and adrenal lesions in the disease (Fiennes, 1970; Ikede and Losos, 1975; Mutayoba et al., 1988; Ogwu et al., 1992) might have occurred due to loss of trophic activity on target organs (Mutayoba et al., 1988). Hypercortisolism probably caused by stress during the early phase of trypanosomiasis (Mutayoba and Gombe, 1989) may contribute in causing tissue injury due to its catabolic effect on tissue proteins.

### 2.3.4 Fever

The undulating fever associated with trypanosomiasis may contribute in causing erythrocyte destruction (Anosa, 1988; Murray and Dexter, 1988; Suliman and Feldman, 1989). This view was supported by the observation that exposure of erythrocytes to temperatures above the normal body temperature increased their osmotic fragility,

membrane permeability and decreased their plasticity such that their life span was decreased in vivo (Karle, 1974).

### 2.3.5 Microangiopathy

Platelet aggregation may be caused by whole trypanosomes or trypanosome components (Davis et al., 1974) or by immunological reactions involving immune complexes (Slot et al., 1977) or platelet autoantibodies and it may result in the release of procoagulants. Microthrombosis due to disseminated intravascular coagulation has been reported in T. vivax infection of cattle (Isoun and Esuruoso, 1972; Van den Ingh et al., 1976a), sheep and goats (Van den Ingh et al., 1976b; Anosa and Isoun, 1983). Erythrocytes with weak membranes become fragmented as they pass through the fibrin deposits of microthrombi and such fragmented erythrocytes have been reported in T. brucei-infected mice (Anosa and Kaneko, 1983b) and T. congolense-infected cattle (Murray and Dexter, 1988). The microthrombi may lead to vascular obstruction and there may be tissue damage due to tissue hypoxia or anoxia which may be exacerbated by anaemic hypoxia (Ndung'u, 1991).

### 2.3.6 Serum factors of unknown origin

Sera from cattle acutely infected with T. vivax depressed granulocyte/macrophage colony formation in vitro (Kaaya et al., 1979). The inhibitor was found not to be a trypanosome material, but was a heat stable protein (Kaaya et al., 1980). Plasma from T. vivax infected sheep with moderate leucopenia was intraperitoneally injected into mice and the plasma did not appear to depress peripheral leucocyte count in the mice,

suggesting that any leucopoiesis inhibitory factor in the plasma may be small in amount and/or did not act for long enough time to produce an observable effect (Igbokwe and Anosa, 1989b).

### 2.3.7 Trauma

Trypanosomes may cause direct mechanical injury to erythrocytes and other cells by lashing action of their powerful locomotory flagella and microtubule-reinforced bodies (Vickerman and Tetley, 1978). Erythrocytes, reticulocytes and platelets have been observed to adhere firmly to trypanosomes through sialic acid receptors leading to cellular injury (Bungener and Muller, 1976; Banks, 1979, 1980; Anosa and Kaneko, 1983a; Anosa, 1983a). *Several tiny foci of discontinuity were present on the erythrocyte* membrane at the points of contact between trypanosome and erythrocyte (Anosa and Kaneko, 1983b). The trypanosomes may also inflict mechanical injury on the vascular endothelial cells as well as cells of solid tissue as the trypanosomes penetrate the tissues through the interstices.

## 2.4 PATHOGENESIS OF ANAEMIA IN TRYPANOSOMIASIS

Anaemia is a consistent feature of trypanosomiasis and haemolysis, bone marrow dyserythropoiesis, haemodilution and haemorrhage have been implicated in the pathogenesis of the anaemia according to reviews by Anosa (1988), Murray and Dexter (1988), Suliman and Feldman (1989), Igbokwe (1989) and Esievo and Saror (1991).

### 2.4.1 Haemolysis

Haemolysis is the most important mechanism of the anaemia caused by trypanosomiasis. This is supported by radioisotope studies which showed that red cell life span was reduced in T. brucei infection of mice (Jennings et al., 1974; Anosa et al., 1977), rabbits (Jenkins et al., 1980) and rats (Jennings et al., 1974), T. vivax infection of sheep and goats (Anosa and Isoun, 1976) and T. congolense infection of cattle (Mamo and Holmes, 1975; Holmes, 1976; Valli et al., 1978; Preston et al., 1979; Dargie et al., 1979a,b). The evidence of extravascular haemolysis was provided by the existence of erythrophagocytosis in the expanded and active mononuclear phagocytic system in T. brucei infection of mice (Jennings et al., 1974; Murray et al., 1974; Anosa et al., 1977; Ikede et al., 1977; Amole et al., 1982; Anosa and Kaneko, 1983b, 1984), T. congolense infection of mice (Ikede et al., 1977), sheep (MacKenzie and Cruickshank, 1973; MacKenzie et al., 1978) and cattle (Losos et al., 1973; Wellde et al., 1974; Valli et al., 1979), T. vivax infection of sheep and goats (Saror, 1980; Anosa and Isoun, 1983) and cattle (Mwongela et al., 1981) and T. rhodesiense infection of man (Woodruff, 1973; Woodruff et al., 1973). The erythrophagocytosis occurred mainly in the spleen and liver and, to a lesser extent, in the other parts of the body such as the lungs, haemal nodes, bone marrow and circulation (Murray et al., 1980; Saror, 1980; Anosa and Kaneko, 1983c; Murray and Dexter, 1988) The extensive haemosiderosis in the spleen, liver, (Fiennes, 1953; Losos et al., 1973; Anosa et al., 1977; Anosa and Isoun, 1983; Valli and Forsberg, 1979) give further evidence of extravascular red cell destruction. Some degree of intravascular haemolysis, however, may occur as suggested by a report of decreased plasma haptoglobin levels in acute T. vivax infection of cattle (Esievo et al., 1984).

The factors and mechanisms which predispose the erythrocytes to haemolysis have already been broadly reviewed in section 2.3 and includes: (1) immunological mechanisms associated with the expansion of the mononuclear phagocytic system (2) trypanosome proteases, neuraminidases, phospholipases and other haemolytic factors like free fatty acids; (3) fever; (4) microangiopathy and erythrocyte fragmentation and (5) trauma to red cell membrane by trypanosomes.

### 2.4.2 Dyserythropoiesis

In the acute phase of trypanosomiasis, there was expansion of the bone marrow with an erythroid hyperplasia, a drop in myeloid-erythroid ratio and an increase in iron uptake (Valli et al., 1978; Maxie and Valli, 1979; Valli and Forsberg, 1979; Anosa and Isoun, 1980; Valli and Mills, 1980; Anosa et al., 1992). However, reticulocytosis was very mild in T. congolense infection of sheep (MacKenzie and Cruickshank, 1973) and T. brucei infection of goats (Igbokwe and Mohammed, 1991), moderate in T. vivax infection of cattle (Anosa et al., 1992) and absent in T. vivax infection of sheep and goats (Anosa and Isoun, 1980; Igbokwe and Anosa, 1989a). The low reticulocyte response reported in acute ruminant trypanosomiasis also suggested inadequate erythropoietic response (Anosa, 1988). Judging by the rates of plasma iron turnover and red cell iron utilization, erythropoiesis was reported to be increased during the parasitaemic phase of T. congolense infection, but the erythropoietic response was moderate in relation to the degree of anaemia and the number of red cells removed (Dargie et al., 1979a).

As trypanosomiasis progresses into the chronic phase, erythropoiesis is suppressed as suggested by the absence of reticulocytosis, appearance of microcytosis (Fiennes,

1970), normal or low erythroid cell population in the bone marrow (Dargie et al., 1979a,b; Valli and Mills, 1980) and hypoferraemia (Tartour and Idris, 1973).

The causes of bone marrow dyserythropoiesis are reviewed with focus on the following target sites in erythropoiesis:

(a) Erythroid cells

Phagocytosis of erythroid cells was demonstrated in the spleen of mice infected with T. brucei (Anosa and Kaneko, 1983c, 1984) and in the bone marrow of calves infected with T. vivax (Anosa et al., 1992); and it was thought to have been due to erythroid cell abnormalities or due to immunological mechanisms similar to those which predisposed red cells to phagocytosis.

(b) Erythropoietin

The plasma from sheep with acute T. vivax infection was found to be inadequately erythrogenic in mice (Igbokwe and Anosa, 1989a) and this finding suggested that the erythropoietin activity in the infected sheep plasma was depressed despite the anaemia (Igbokwe, 1986). Igbokwe (1989) had postulated that the trypanosome neuraminidase, produced by T. vivax (Esievo, 1979, 1983) during the parasitaemic phase, could desialylate plasma erythropoietin to produce asialoerythropoietin, which is biologically inactive as reported by Goldwasser et al. (1974). Furthermore, the production of erythropoietin may be depressed in the later phase of the disease because the liver and kidney involved in the production (Schuster et al., 1987) were damaged to some extent in

T. vivax infection of sheep and goats (Van den Ingh et al., 1976b; Anosa and Isoun, 1983) and cattle (Isoun and Esuruoso, 1972; Mwongela et al., 1981). Also, deficiencies of endocrine hormones of the pituitary, adrenal, thyroid and gonads, such as the androgens, cortisone, thyroxine, epinephrine and norepinephrine, depress erythropoietin production (Schalm et al., 1975). Hypothyroidism has been reported in T. congolense infection of goats (Mutayoba et al., 1988; Mutayoba and Gombe, 1989) and cattle (Ogwu et al., 1992; Abebe and Eley, 1992). There was a decrease in plasma cortisol level during the terminal phase of T. congolense infection of goats (Mutayoba et al., 1988) and cattle (Ogwu et al., 1992). Otesile et al., (1991) reported a decrease in testosterone concentrations in T. brucei infection of young pigs. Goodwin (1970) suggested that catecholamine metabolism was defective in trypanosomiasis because tyrosine, an important precursor of catecholamine was decreased by 50% of control levels in the sera of rabbits infected with T. brucei. These reports suggested that endocrine imbalance may impair erythropoietin production in trypanosomiasis.

(c) Haemoglobin synthesis

In trypanosomiasis, the presence of massive haemosiderin deposits in the mononuclear phagocytic system, hypoferraemia and reduced rate of $^{59}Fe$ clearance from the plasma (Dargie et al., 1979a) indicated that a proportion of the iron carried to the bone marrow was not incorporated into the erythrocytes because the iron was utilized extremely slowly or it was unavailable for haemoglobin synthesis after erythrophagocytosis. Testosterone, erythropoietin and ascorbic acid may be important in releasing iron from the reticuloendothelial system (Haurani and Green, 1967; Zucker et al., 1974; Zilva and Pannall, 1984; Murray and Dexter, 1988). Therefore the plasma level

of testosterone (Olesile et al., 1991) and possibly erythropoietin (Igbokwe, 1986) and ascorbic acid (Roskin and Nastiukova, 1948), which may be decreased in trypanosome-infected animals could play a role in the iron sequestration.

The decrease in the serum levels of certain free amino acids which occurred in trypanosomiasis (Goodwin and Guy, 1973; Newport et al., 1977; Isoun et al., 1978, 1979) may be responsible for the reduction in the rate of synthesis of the globin moiety of haemoglobin. Globin synthesis precedes haeme synthesis (Schalm et al., 1975) and it is possible that reduced globin synthesis may retard erythropoiesis and incorporation of iron into the red cells.

### 2.4.3 Haemodilution

While the red cell mass decreased , the plasma volume increased and the blood volume remained normal during the early phase of trypanosome infection, suggesting some measure of haemodilution (Anosa and Isoun, 1976; Holmes, 1976; Maxie and Valli, 1979). However, Dargie et al., (1979a,b) and Dargie (1980) concluded that haemodilution did not exist in infected animals because the plasma volume in the anaemic animal was overestimated. This was deduced from the fact that there was rapid transfer of $^{59}Fe$, used in the estimation, from the plasma such that the radiolabel did not equilibrate with the total plasma space. Other reports on this aspect have been reviewed by Murray and Dexter (1988)

### 2.4.4 Haemorrhage

In acute haemorrhagic T. vivax infections of cattle, sheep and goats, extensive petechial and ecchymotic haemorrhages have been described in various organs (Anosa, 1983b; Anosa et al., 1992) and such haemorrhages may contribute only slightly or insignificantly to the pathogenesis of the anaemia (Murray and Dexter, 1988).

## 2.5 FACTORS AFFECTING THE SEVERITY OF TRYPANOSOMIASIS

The severity of the anaemia caused by trypanosomiasis is a good indicator of the severity of the disease which is related to the virulence of the trypanosome as well as the breed, age and nutritional status of the host (Murray and Dexter, 1988).

### 2.5.1 Parasitaemia and virulence of trypanosome

The height of parasitaemia can determine the severity of the anaemia in T. congolense, T. vivax and T. brucei infections of cattle (Murray et al., 1979) and, perhaps other hosts. In a dose response experiment, it was shown that when the number of bloodstream forms of T. brucei inoculated subcutaneously into Ndama and Zebu cattle was increased, the prepatent period was shortened and the height of parasitaemia increased (Murray et al., 1979). However, the infective dose of T. congolense in cattle and mice affected the prepatent, but not the height of parasitaemia (Morrison and Murray, 1985).

The level of parasitaemia is related to the virulence of the trypanosome, a factor which is controlled by the capacity of the parasite to multiply and its ability to induce immune response (Roelants and Pinder, 1987; Murray and Dexter, 1988). The virulence of T. brucei was reported to be limited by the capacity of the parasite to become pleomorphic (Barry et al., 1979). The rapidly dividing monomorphic forms of T. brucei produce high parasitaemia and kill the host quickly; whereas the pleomorphic isolates produce lower parasitaemia and are less virulent (Murray and Dexter, 1988). Pleomorphism produces the stumpy forms of T. brucei which are responsible for the induction of immune response (Sendashonga and Black, 1982) capable of controlling the parasitaemia.

### 2.5.2 Breed and age of the host

The trypanotolerant Ndama and West African Shorthorn (Murray et al., 1982) and Orma Boran cattle (Ismael et al., 1985) and several species of wild Bovidae (Murray et al., 1981) are less susceptible to anaemia during trypanosome infections, because they have the ability to limit the intensity and duration of parasitaemia more than the trypanosusceptible Zebu or European breeds.

The relative tolerance of the Ndama breed of cattle may be attributable to the possession of erythrocyte surface sialic acids in amounts seven times greater than in trypanosusceptible Zebu breed (Esievo et al., 1986). The erythrocytes of both Zebu and Ndama breeds had N-acetylneuraminic acid, but the Ndama erythrocytes had an additional unidentified type of sialic acid (Esievo et al., 1990) which was considered responsible for the higher erythrocyte surface sialic acid concentrations in the Ndama.

Shugaba et al., (1994) showed that the Ndama erythrocyte sialic acids had more O-acetyl than glycolyl groups which may be relevant to trypanotolerance since O-acetyl groups in sialic acids might hinder rapid hydrolysis by trypanosome sialidase.

It has been reported that calves of less than one year are more resistant than adults to the effects of trypanosomiasis (Fiennes, 1970; Maxie and Valli, 1979; Wellde et al., 1981; Murray et al., 1982). It was suggested that this might be because the calves developed less severe parasitaemia (Wellde et al., 1981) or had superior erythropoietic response (Murray and Dexter, 1988) than the adults. However, young lambs and kids were reported to be more susceptible to T. congolense infection than the adults (Griffin and Allonby, 1981), which was contradictory to the earlier reports.

### 2.5.3 Nutrition

Trypanosomes depend on the host's supplies of carbohydrates, proteins, lipids and some micronutrients. During periods of high parasitaemia, the parasites may deplete the host's supplies. Trypanosomes have little polysaccharide stores and glucose is the most important exogenous substrate used by the bloodstream form for energy (Gutteridge and Coombs, 1977). Transitional form of the parasite have the ability to use α-ketoglutarate (Bienen et al., 1991). The parasites have been shown to take up and digest protein molecules (Brown et al., 1965; Langreth and Balber, 1975). They also take up free plasma amino acids (Gutteridge and Coombs, 1977) and exogenous lipid (Mellor and Samad, 1989). Some of the micronutrients which the parasite may take up from exogenous sources are p-aminobenzoic acid, vitamins such as thiamine, folic acid, riboflavin, cobalamine, ascorbic acid and nicotinamide (Von Brand, 1973) and nucleotide

precursors such as hypoxanthine and thymidine (Baltz et al., 1985). The situation may be compounded by the fact that trypanosomiasis causes a decrease in feed intake by the host (Verstegen et al., 1991; Zwart et al., 1991; Akinbamijo et al., 1990, 1992; Wassink et al., 1993).

The effect of trypanosome infection on the energy metabolism of the host has not been extensively studied. Seed and Hall (1985) reviewed the skeletal information available in mainly T. brucei group infections. The enzymes reported to be reduced in activity in trypanosome infections were fructose-1,6-diphosphatase, glucose-6-phosphatase and cytochrome oxidase (Lumsden et al., 1972; Gonzales et al., 1974; Shertzer et al., 1982). Fructose-1,6-diphosphatase and glucose-6-phosphatase are involved in gluconeogenesis while cytochrome oxidase is the terminal oxidase in the electron transport chain tightly coupled to oxidative phosphorylation (Conn and Stumpf, 1976).

In Microtus montanus infected with T. gambiense, radiolabelled phenylalanine was not converted to tyrosine, but was excreted in urine as phenylpyruvate because phenylalanine hydroxylase activity, which catalysed conversion of phenylalanine to tyrosine, was drastically reduced in the host (Seed et al., 1982, 1983). Also, trypanosomes catabolized phenylalanine to phenylpyruvate. Seed and Hall, (1985), therefore, suggested that the phenylpyruvate in circulation might inhibit pyruvate translocase (Halestrap et al., 1974) and pyruvate carboxylase (Patel et al., 1977) which were essential for gluconeogenesis.

During the metabolism of glucose by T. gambiense, it was reported that a dimer of pyruvate is formed (Seed and Hall, 1985) which was shown to inhibit the tricarboxylic

acid cycle in the mitochondria of rat heart (Montgomery and Webb, 1956; Montgomery et al., 1956). Ultrastructural changes were reported in the mitochondria of the liver of guinea pigs chronically infected with T. gambiense and such changes may be associated with alterations in mitochondrial functions (Lumsden et al., 1972).

Because of the alterations in energy metabolism in trypanosomiasis, energy-rich compounds such as pyruvate and α-ketoglutarate were not efficiently utilized; and were excreted in the urine of infected animals (Goodwin and Guy, 1973; Seed et al., 1982). The foregoing reports seem to suggest that the tricarboxylic acid cycle and oxidative phosphorylation may be inhibited, thus limiting the generation of energy from energy-rich compounds. This situation may lead to an energy deficit (Seed and Hall, 1985) since, for example, more than 90% of the energy available in glucose is released when pyruvate is oxidized to carbon dioxide and water through the tricarboxylic acid cycle and electron transport chain (Conn and Stumpf, 1976).

Increased glycogenolysis was suggested in chronic T. gambiense infection of guinea pigs and Microtus montanus when a large decrease in the glycogen content of the liver was observed (Lumsden et al., 1972; Ashman and Seed, 1973). Although the glycogen content of the liver was decreased, it was not exhausted (Mercado and Von Brand, 1960; Marciacq and Seed, 1970). However, the release of glucose into the extracellular fluid may be hampered by the decrease in the activity of fructose-1,6-diphosphatase and glucose-6-phosphatase (Lumsden et al., 1972; Gonzales et al., 1974). It has been hypothesized that the terminal hypoglycaemia (Andrews et al., 1930; Hoppe and Chapman, 1947) observed in some cases of trypanosomiasis may be due to a failure of the endocrine mechanisms controlling the mobilization of carbohydrate reserves (Von

Brand, 1973). Voorheis (1969) postulated that there may be a decrease in glucose metabolism in the host's peripheral tissues resembling the condition found in diabetes mellitus. This observation is consistent with hypercortisolism in acute T. congolense infection of goats (Mutayoba and Gombe, 1989) with the expected anti-insulin effect (Zilva and Pannall, 1984). When glucose is not readily available for metabolic energy in the peripheral tissues, there may be a tendency for gluconeogenesis to be accelerated leading to increased protein catabolism, muscle wasting and loss of body fat (Stephen, 1970).

Maclenan (1970) suggested that animals on low nutritional status could become less able to withstand the effects of trypanosomiasis. It has been shown that nutritional deprivation of the trypanosome-infected animal could result in more severe infection even among trypanotolerant breeds (Chandler, 1952; Stephen, 1966; Carlomagno et al., 1987; Reynolds and Ekwuruke, 1988). Starvation was reported to render mice more susceptible to T. duttoni infection (Sheppe and Adams, 1957). Semi-starvation was also observed to elicit higher parasitaemia in T. rhodesiense-infected rats (Singer, 1961).

Cattle under good husbandry were shown to be able to tolerate clinical infections (Hill, 1956). French and Hornby (1934, 1935) reported that levels of nutrition above maintenance had no effect on the course of T. congolense or T. brucei infection of cattle and sheep. Agyemang et al., (1990) showed that cattle on high plane of nutrition (grazing and supplemental feeding) developed anaemia to the same degree as those on low plane of nutrition (only grazing). Although sheep on high protein diet developed higher parasitaemia than those on a low protein diet after T. congolense infection, both groups developed similar degrees of anaemia (Katunguka-Rwakishaya et al., 1991) while the

sheep on high protein diet showed greater erythropoietic activity during infection and had more rapid recovery from the anaemia with chemotherapy 70 days after infection.

Supplemental feeding with maize bran and cotton seed significantly delayed the onset of trypanosomiasis among Djallonke sheep exposed to high tsetse challenge and the sheep also had significantly less incidence of infection than the sheep that received no supplemental feeding (Hecker et al., 1991). Fagbemi et al. (1990) placed growing pigs on high, medium, and low planes of dietary energy and subsequently infected them with T. brucei. They reported a drop in red blood cell values which was more severe with decreasing energy levels. Otesile et al. (1991) showed that the effect of T. brucei infection was more severe in young pigs on low energy diets than those on high energy diet, and after therapy (Otesile et al., 1992), recovery was slower in the pigs on low energy diet. They reported that serum total protein, albumin and testosterone concentrations decreased and aspartate and alanine aminotransferase increased to a greater extent in infected pigs on low energy diet. It was reported that glucose infusion increased the survival time of pigs infected with T. simiae (Anosa, 1988) and feeding of glucose to rats infected with T. equiperdum prolonged their lives for short period of time (Andrews et al., 1930; Hoppe and Chapman, 1947). It seems, therefore, that the plane of energy nutrition affects the pathogenesis of trypanosomiasis and can modulate the severity of the lesions.

Deficiencies of thiamine, pantothenic acid and pyridoxine have been reported to increase the susceptibility of rats to T. cruzi infection (Yaeger and Miller, 1960a,c,d). The vitamin-deficient rats showed more marked parasitaemia of longer duration and had more severe myocarditis. There was a higher incidence of spontaneous respiratory infections in T. cruzi-infected rats with pantothenic acid and pyridoxine deficiencies. Rats maintained

on riboflavin deficient diet did not seem to show significant increase in susceptibility to T. cruzi infection, although there was a slight increase in the severity of infection evidenced by cardiac damage which was attributed to inanition (Yaeger and Miller, 1960b).

The parasite multiplied at a higher rate and the parasitaemia was prolonged in pantothenic acid, biotin and thiamine deficient rats infected with T. lewisi (Caldwell and György, 1943, 1947; Becker et al., 1947; Lincicome, 1953). Vitamin B deficiency was shown to increase the susceptibility of pigeons to T. brucei infection (Sollazo, 1929). These reports seem to show that deficiencies of B vitamins may increase the severity of trypanosomiasis. However, there was a dissenting report which showed that deficiency of vitamin B did not affect prepatent period and multiplication rate of T. equiperdum in rats and indicated that the deficient rats lived 2-6 days longer (Reiner and Paton, 1932).

An excess of thiamine in a complete diet did not alter the resistance of rats to T. lewisi or T. brucei infection and when manganese was added to the diet, there was a reduction in the length of T. lewisi infection by 30-50% and some recoveries were recorded after T. brucei infection (Perla, 1941). Trypanosusceptible cattle have been shown to have abnormally high serum zinc levels which may inhibit macrophage activity and antigen presentation (Traore-Leroux et al., 1985) and reduce the ability of the cattle to control parasitaemia through immune response.

Trypanosoma congolense and T. vivax infections were markedly suppressed by feeding the host a diet containing cod-liver oil, but the same treatment was not observed to suppress T. cruzi and T. brucei infections (Godfrey, 1958). The suppression of infection by the diet was reversed by vitamin E supplementation. It was demonstrated that

the toxic factors in the cod-liver oil were polyunsaturated fatty acids. Ormerod and Venkatesan (1982) suggested that the action of the polyunsaturated fatty acids in inhibiting parasitaemia could be prevented by increasing dietary intake of vitamin E or other antioxidants which have the effect of preventing the formation of free radicals. However, vitamin E deficient diet fed with 5% menhaden oil offered no protection against T. cruzi and T. rhodesiense infections in mice (Levander et al., 1989). A diet of unextracted casein and cod-liver oil was reported to suppress T. congolense infection in mice (Keppie, 1953) and the degree of suppression was higher than that reported by Godfrey (1958).

From the review, it is deduced that low energy diets may predispose animals to more severe trypanosomiasis while protein or amino acid and vitamins B and C deficiencies may reduce immune and erythropoietic responses. Furthermore, high lipid diets may terminate parasitaemia.

## 2.6 ERYTHROCYTE GLUTATHIONE METABOLISM IN HEALTH, TRYPANOSOMIASIS AND OTHER ANAEMIAS

Glutathione (L-glutamyl-cysteinyl-glycine; GSH), a tripeptide synthesized from glutamate, cysteine and glycine (Elder and Mortensen, 1956), is the only important non-protein sulfhydryl compound in erythrocytes (Beutler et al., 1963). GSH reduces hydrogen peroxide and lipid hydroperoxide in the presence of GSH peroxidase to protect the erythrocytes from oxidative damage (Metzler, 1977) (Fig. 2.1). During normal erythrocyte metabolism, hydrogen peroxide is produced by mechanisms related to oxygen transport (Carrell et al., 1975). Both GSH peroxidase and catalase are important in

hydrogen peroxide detoxification in red cells, but GSH peroxidase is more important than catalase as the initial detoxifier of low concentrations of hydrogen peroxide (McCay and King, 1980).

GSH disulfide is formed after GSH oxidation and if the disulfide is not immediately reconverted to GSH by a reduction process, it readily diffuses out of the red cells into the plasma (Srivastava and Beutler, 1969). Therefore, GSH oxidation is coupled with the oxidation of glucose-6-phosphate and 6-phosphogluconate in the pentose phosphate pathway which provides NADPH for the reduction of the GSH disulfide by GSH reductase (Meiser and Anderson, 1983) (Fig. 2.1).

In T. brucei infection of mice, erythrocyte hexokinase, glucose-6-phosphate dehydrogenase, GSH peroxidase and reductase increased and it was interpreted that deficiencies of these enzymes were unlikely to precipitate anaemia in the infection (Anosa and Kaneko, 1983a). Blood GSH concentration decreased from 27.1 ± 1.0 mg/dl in controls to 24.1 ± 1.3 mg/dl on day 4 post-infection in rats infected with T. brucei gambiense (Ameh, 1984).

In man, hereditary erythrocyte GSH absence or deficiency and GSH instability due to deficiencies of GSH peroxidase and reductase and glucose-6-phosphate dehydrogenase have been reported to cause mild anaemia and to increase susceptibility to drug induced haemolysis (Beutler, 1957; Bauer, 1980). Hereditary GSH deficiency has been reported in sheep (Agar, 1975). Sheep have also been reported to have erythrocyte glucose-6-phosphate dehydrogenase activity similar to the level in deficient erythrocytes of man (Maronport, 1972).

The sheep reported to have erythrocyte GSH deficiency showed no evidence of anaemia (Agar, 1975) and no significant haemolysis was detected after administration of oxidizing drugs to such animals (Maronport, 1972). However, erythrocyte GSH decreased under oxidative stress caused by copper poisoning in sheep (Todd and Thompson, 1963) and in postparturient haemoglobinuria of buffaloes (Singari et al., 1989a,b; Rana and Bhardwaj, 1990), both associated with haemolytic anaemia.

An inverse relationship existed between erythrocyte GSH level and packed cell volume in man (Ibbott, 1974). During regenerative anaemias, erythrocyte GSH concentrations usually increased (Tucker and Kilgour, 1973; Agar et al., 1975). This was because the circulating young erythrocytes had higher GSH concentrations and greater activities of GSH metabolizing enzymes than older erythrocytes (Ibbott, 1974; Tucker and Kilgour, 1973; Agar et al., 1975; Gombe and Verjee, 1976).

## 2.7 FREE RADICALS IN RELATION TO TRYPANOSOMIASIS

A free radical is a highly reactive molecule or molecular fragment having an unpaired electron (Slater, 1984) and can be produced from compounds having relatively unstable bonds (Demopoulos, 1973). Free radicals are produced by homolytic bond fission through the absorption of radiation or by electron-transfer processes involving non-enzymic, metal-catalysed or enzyme-catalysed redox reactions (Slater, 1984). Redox systems in many cells produce superoxide anions (Fridovich, 1983). Activated macrophages and monocytes increase their oxygen uptake and release large amounts of superoxide anions into the extracellular fluid through the action of NADPH oxidase (Klebanoff, 1982; Schwacha and Loegering, 1992). During trypanosome infections, there

is a marked proliferation of activated macrophages in the reticuloendothelial systems of the infected animals (Ikede and Losos, 1972; MacKenzie and Cruickshank, 1973; MacKenzie et al., 1978; Anosa and Isoun, 1983; Anosa and Kaneko, 1983c), and it is expected that they may produce superoxide anions. These anions are not particularly reactive and, therefore, are capable of diffusing through relatively large distances through cells where, in the presence of copper or iron, they are converted to hydrogen peroxide and reactive hydroxyl radicals by superoxide dismutase (Slater, 1984) (Fig. 2.2). It is pertinent to note that reactive oxygen produced by activated macrophages and monocytes in malaria have been implicated in the causation of injury to adjacent tissues (Stocker et al., 1984) and the circulating blood components (Descamps-Latscha et al., 1982).

Because bloodstream forms of T. brucei lack catalase (Fulton and Spooner, 1956) and are without detectable GSH peroxidase activity (Meshnick et al., 1978), hydrogen peroxide accumulates in the trypanosome to a concentration of 70 μM (Meshnick et al., 1977), a value that is about 100-fold higher than that observed in mammalian cells (Chance et al., 1979). The cultivation of infective forms of T. brucei, T. equiperdum, T. evansi, T. rhodesiense and T. gambiense in a medium required the presence of a reducing agent such as mercaptoethanol or thioglycerol to stabilize glutathione which would eliminate activated oxygen species produced during parasite growth (Baltz et al., 1985). Therefore, hydrogen peroxide and activated oxygen may be discharged into the extracellular fluid by the parasite in infected animals. Production of hydrogen peroxide by T. vivax and T. congolense has not been reported.

Although GSH and GSH peroxidase are involved in detoxifying hydrogen peroxide in erythrocyte, the system may be overwhelmed (McCay and King, 1980). The

increase in the activities of erythrocyte GSH peroxidase and reductase and glucose-6-phosphate dehydrogenase in T. brucei-infected mice (Anosa and Kaneko, 1983a) could be an induction elicited by increased activity of the GSH system due to elevated peroxide load. This is consistent with the report of decreased blood GSH in T. brucei gambiense infection of rats (Ameh, 1984).

The hydroxyl radicals, generated from hydrogen peroxide which escape the GSH system, are scavenged by vitamin C in the aqueous phase and vitamin E in the lipid bilayer and the vitamin C may easily be depleted (Niki et al., 1988). In addition, vitamin C may participate in the regeneration of vitamin E and GSH (Willson, 1987). Vitamin C has been reported to decrease in the liver of T. brucei-infected guinea pigs (Roskin and Nastiukova, 1941) and in the liver, kidneys and adrenals of T. hippicum-infected rats (Nyden, 1948). The activated macrophages of the liver and the trypanosomes in the sinusoids may be responsible for the building up of a load of hydroxyl radicals which could have been responsible for the depletion of liver vitamin C in the above reports and the depletion of liver GSH in T. brucei gambiense-infected rats (Ameh, 1984). Furthermore, the increase in the susceptibility of erythrocytes to oxidative haemolysis in T. gambiense infection of rats (Ameh, 1984) was also an indication of the decline in the antioxidant capacity of the erythrocytes during infection.

If the antioxidant defense mechanisms are weakened, free hydroxyl radicals can abstract hydrogen atoms from deoxyribonucleic acid, nucleotide co-enzymes, carbohydrates, protein and lipids in the cell to alter cell metabolism (Slater, 1984). Polyunsaturated fatty acids which are major constituents of cell membranes are particularly susceptible to free-radical-mediated

oxidation and this process of lipid peroxidation (Fig. 2.3) could lead to disturbances in membrane structure and function (Slater, 1984; Duthie, 1991).

Although increased lipid peroxidation has not been reported in trypanosomiasis, the process has been associated with erythrocyte aging (Hoschstein and Jain, 1981) and various haemolytic anaemias such as thalassemia major, glucose-6-phosphate dehydrogenase deficiency, autoimmune haemolytic anaemia (Stocks et al., 1971, 1972), paroxysmal nocturnal haemoglobinuria (Mengel et al., 1967) and malaria (Clark et al., 1989). Lipid peroxidation has been evaluated in biological systems by measuring its by-products such as lipoperoxides, malondialdehyde, conjugated dienes, alkanals and fluorescent products (Yagi, 1976; Satoh, 1978; Santos et al., 1980; Bird et al., 1983; Humad et al., 1985; Wade and VanRij, 1985; Wong et al., 1987; Duthie et al., 1989).

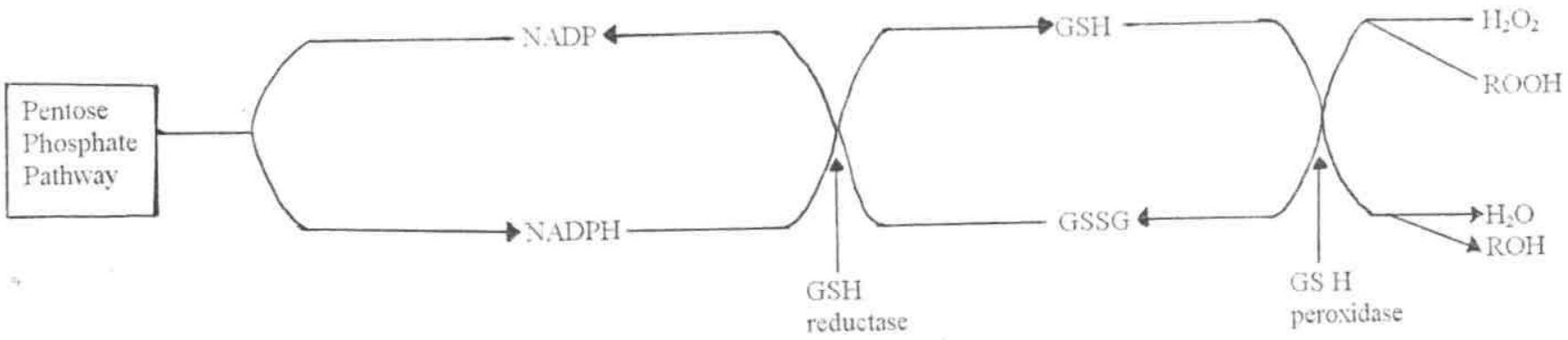



Figure 2.1 The glutathione system in erythrocytes (Metzler, 1977).

1) $O_2^- + O_2^- + 2H \xrightarrow{\text{Superoxide dismutase}} H_2O_2 + O_2$

Superoxide anions

2) $O_2^- + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$

3) $H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + HO^{\cdot} + OH^-$

Summary:

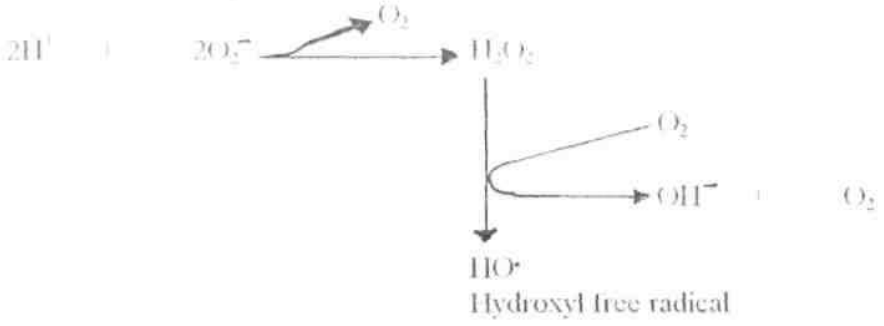



Figure 2.2 Formation of hydroxyl free radical (Slater, 1984).

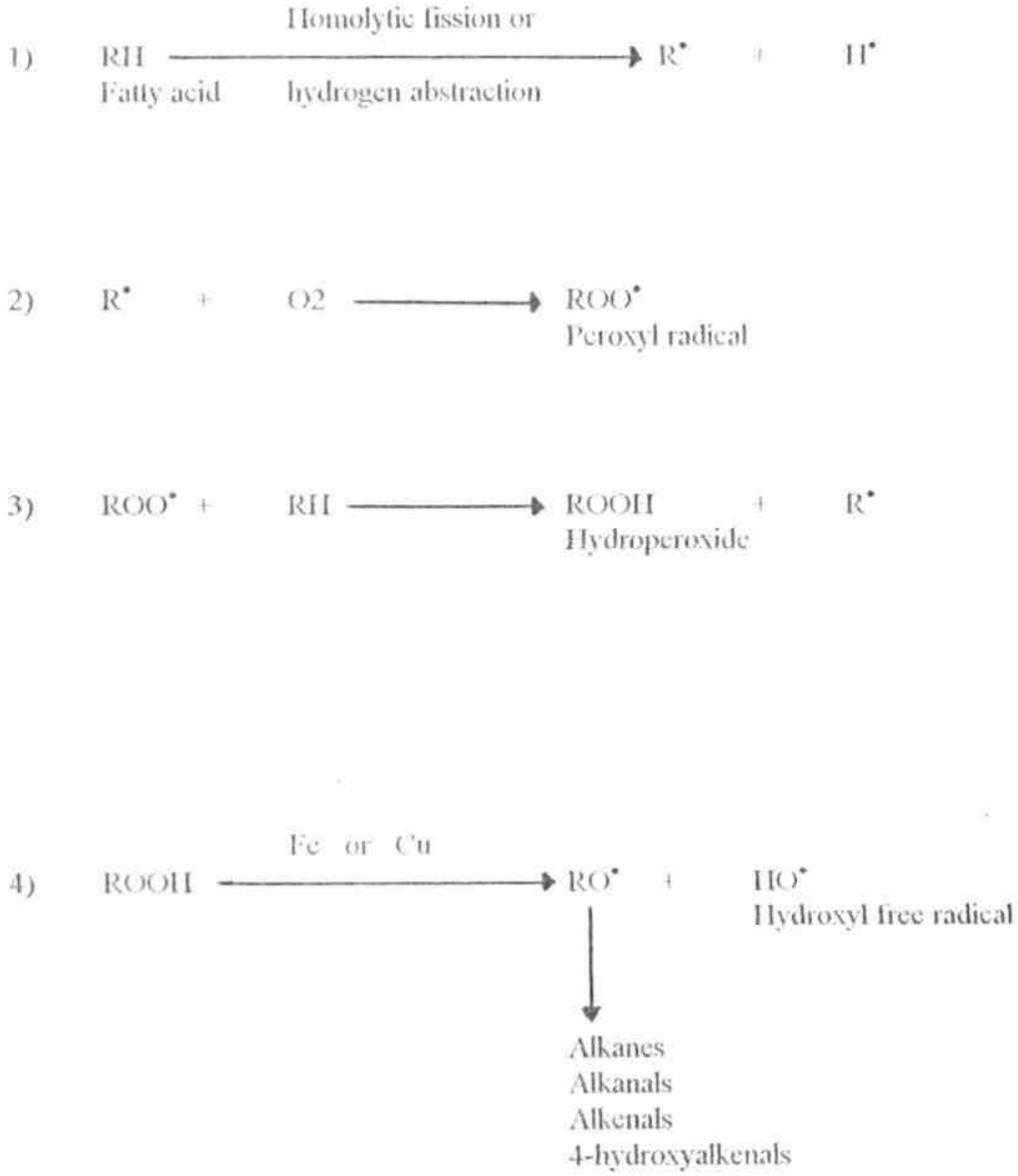



Figure 2.3 Peroxidation of polyunsaturated fatty acids (Duthie, 1991).

CHAPTER THREE

# EFFECT OF ACUTE *TRYPANOSOMA BRUCEI* INFECTION OF MICE ON THE SUSCEPTIBILITY OF ERYTHROCYTES TO *IN VITRO* PEROXIDATION

## 3.1 SUMMARY

*Trypanosoma brucei* produced an acute infection in mice and mortality was observed 2 days after the onset of parasitaemia which occurred 3-5 days post-infection. Anaemia was observed in the survivors, 5 and 8 days post-infection. When washed erythrocytes from the heart blood were incubated in physiological saline containing 1.5% hydrogen peroxide, the erythrocytes of the infected mice produced significantly ($P < 0.05$) greater amounts of by-products of lipid peroxidation, measured as thiobarbituric acid reactive substances, than the erythrocytes of the control mice. This observation suggested that the infected mice had reduced ability to prevent free radical-mediated lipid peroxidation in the erythrocyte membrane.

## 3.2 INTRODUCTION

Erythrokinetic studies in trypanosome-infected animals have shown that erythrocyte life-span is reduced due to various mechanisms which alter the erythrocyte cell membrane and predispose the cell to phagocytosis, lysis and fragmentation (Esievo et al., 1982; Suliman and Feldman, 1989). The immunological mechanisms have been reviewed by Esievo and Saror (1991). Although the onset of anaemia was related to the first wave of parasitaemia and rise in serum antibodies (Balber, 1971; MacKenzie et al., 1978), the anaemia may not

be due to only immunological mechanisms (Murray and Dexter, 1988). Anaemia occurred in Trypanosoma brucei-infected nude rats whose immune systems had been destroyed by irradiation (Murray and Dexter, 1988). After observing anaemia in mice within 4 days after infection with T. brucei, Jennings et al. (1974) suggested that the early anaemia might have been due to non-immunological mechanisms.

Trypanosome haemolysins appeared in the plasma within 2-3 days of infection in mice and rats infected with T. brucei (Murray, 1979; Murray et al., 1979). The nature of the haemolysins is yet to be fully elucidated. An active phospholipase A was reported to be present in high concentrations in T. brucei (Hambrey et al., 1984). It was shown that T. brucei contained proteases (Lonsdale-Eccles and Mpimbaza, 1986) and a peptidase of parasite origin was identified in the plasma of mice infected with T. brucei (Knowles et al., 1987). Whereas T. vivax was reported to produce neuraminidase (Esievo, 1979, 1983), bloodstream forms of T. brucei showed no neuraminidase activity while the insect-stage trypanosomes had sialidase (Engstler et al., 1991; Engstler and Schauer, 1993). Meshnick et al., (1977) showed that T. brucei produced enormous amounts of hydrogen peroxide. Normal mammalian erythrocytes produce hydrogen peroxide and increased lipid peroxidation has been observed in erythrocyte aging (Hochstein and Jain, 1981; Chiu et al., 1989).

The purpose of this study was to investigate the susceptibility of erythrocytes to in vitro peroxidation during the early acute phase of T. brucei infection in mice.

## 3.3 MATERIALS AND METHODS

### .3.3.1 Experimental animals

Thirty four male albino mice aged above 8 weeks and weighing 18.7 ± 3.2 were used. They were obtained from the experimental animal unit of the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria. They were housed in metal cages, fed with commercial pellets and watered ad libitum.

### 3.3.2 Trypanosome infection

A stabilate of Trypanosoma brucei, Wamba strain, was obtained from the Nigerian Institute of Trypanosomiasis Research, Vom. The parasite was originally isolated from cattle in Wamba, Plateau State, Nigeria. Twenty four mice were each infected by intraperitoneal injection with $10^3$ parasites suspended in diluted mouse blood. The other 10 mice were uninfected and served as control.

### 3.3.3 Blood sampling and analyses

The mice were bled from the tail to measure the packed cell volume (PCV) using the microhaematocrit method (Schalm et al., 1975). Blood (1.5ml) was obtained by cardiac puncture using ethylene diaminotetra-acetate (EDTA) as anticoagulant. The heart blood was used to measure PCV and in vitro erythrocyte peroxidation because it provided adequate sample quantity.

### 3.3.4 Peroxidation of erythrocytes in vitro

The *in vitro* peroxidation of erythrocytes was measured according to the method of Duthie et al., (1989). Washed erythrocytes were incubated in physiological saline containing 1.5% hydrogen peroxide (v/v) and subsequently, the by-products of lipid peroxidation were measured spectrophotometrically as thiobarbituric acid reactive substances (TBARS).

Aliquots (0.25ml) of the heart blood were washed twice with 2ml of saline solution (0.9% NaCl) and were finally resuspended in 2ml saline solution containing hydrogen peroxide at a concentration of 1.5% (v/v). The suspension was incubated at 37°C for 90 minutes. Thereafter, 0.5ml of 10% trichloracetic acid was added to the suspension to stop reactions. The suspension was centrifuged at 1,500G for 10 minutes. The supernatant was filtered with Whatman No. 1 filter paper and 0.75ml of 0.67% thiobarbituric acid was added to 0.5ml of the filtrate. The mixture was incubated at 100°C for 20 minutes,and after cooling to 20°C, was read spectrophotometrically at 535nm.

### 3.3.5 *Statistics*

Data were summarized as mean ± standard deviations. Statistical difference between means was assessed by paired Student's t-test and correlation coefficient between erythrocyte peroxidation value and PCV was calculated (Chatfield, 1983).

## 3.4 RESULTS

The infected mice were parasitaemic between days 3 and 5 post-infection (pi) and the parasitaemia was high within 2 days (that is, days 5 and 7 pi). They appeared lethargic

and droopy with starry haircoat. Nine infected mice died overnight before day 5 pi and another 4 died overnight before day 8 pi. The deaths occurred 2 days after the onset of parasitaemia. Among the survivors, 10 mice were bled by cardiac puncture on day 5 pi and one on day 8 pi. Four of the infected mice did not bleed freely from the tail while the others did.

The PCV and the in vitro erythrocyte peroxidation of the heart blood of the control and infected mice are shown in Table 3.1. Also displayed on Table 3.1 is the PCV of the tail blood which was significantly higher than the heart blood PCV in both control ($P<0.001$) and infected mice ($P<0.025$) (Table 3.2). The tail blood PCV decreased significantly ($P<0.025$) in the infected mice when compared with the control, but the heart blood PCV of the control and infected mice were not significantly different ($P>0.05$). The in vitro erythrocyte peroxidation increased significantly ($P<0.05$) in the infected mice when compared with the control.

The relationship between the erythrocyte peroxidation and PCV of the control and infected mice is shown in Fig. 3.1. In the control and infected mice, the correlation coefficients were -0.35 and -0.16 respectively indicating insignificant ($P>0.05$) low negative correlations. The scatter diagram of erythrocyte peroxidation in the control and infected mice showed a low data overlap and 73% of the infected mice had values (0.9-0.20) above the maximum value observed in the control (0.08).

TABLE 3.1

Packed cell volume and erythrocyte peroxidation (mean ± SD) of the blood of control and Trypanosoma brucei-infected mice.

| | Packed cell volume (%) | | Heart blood Erythrocyte peroxidation (Absorbance Units) |
|---|---|---|---|
| | Tail blood | Heart blood | |
| Control | $54.2 \pm 3.7^{cd}$ | $32.7 \pm 4.2^{c}$ | $0.05 \pm 0.01^{e}$ |
| | (n = 34[a]) | (n = 10) | (n = 10) |
| Infected[b] | $16.7 \pm 7.0^{d}$ | $32.1 \pm 5.0^{d}$ | $0.11 \pm 0.01^{e}$ |
| | (n = 7) | (n = 11) | (n = 11) |

[a]All mice before infection. Ten were uninfected controls and 24 were later infected

[b]Ten mice were bled at 5 days and one at 8 days post-infection. The 11 mice were survivors of 24 infected mice.

[c,d,e]Means bearing the same superscripts in columns and rows are significantly different c=P<0.001, d=P<0.025, e=P<0.05. (Two tailed t-test).

n, number of mice sampled to calculate mean

*TABLE 3.2*

Statistical differences between the paired means of the packed cell volume of control and *Trypanosoma brucei*-infected tail (CTB, ITB) and heart (CHB, IHB) blood of mice.

| Paired means | t-test (Two-tailed) |
|---|---|
| *CTB-CHB* | P 0.001, S |
| CTB-ITB | P 0.025, S |
| *CHB-IHB* | P 0.05, NS |
| ITB-IHB | P 0.025, S |

S. *significant*
NS. not significant

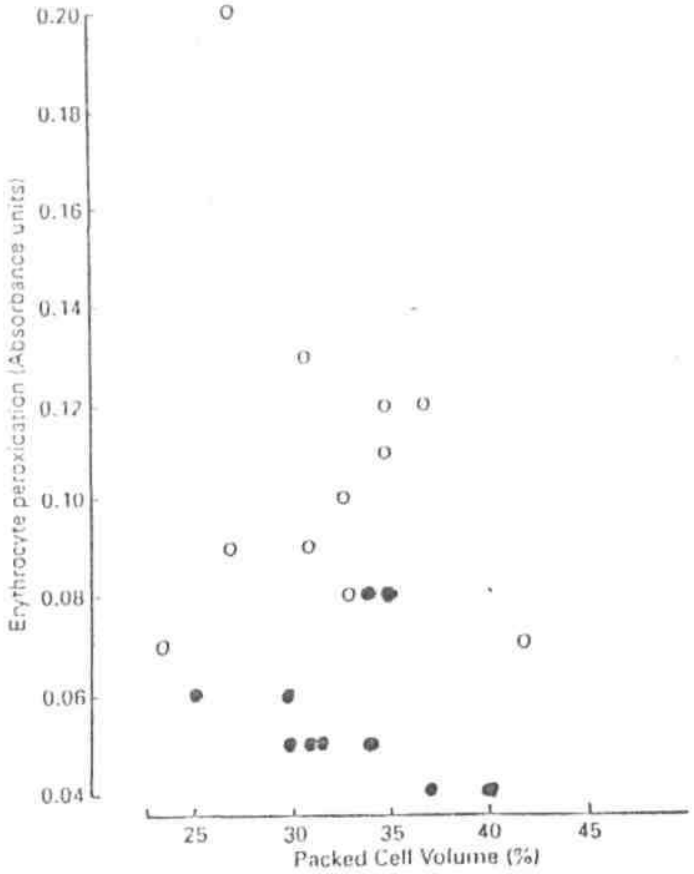



Figure 3.1 Scatter diagram showing the relationship between erythrocyte peroxidation and packed cell volume from the heart of control (•) and Trypanosoma brucei-infected (o) mice

## 3.5 DISCUSSION

The tail blood of the control mice in this study was comparable to the control tail blood PCV (50 ± 4%) reported in an earlier population of mice of the same species (Igbokwe, 1986). The mean tail blood PCV in both infected and control mice were significantly higher than the heart blood PCV, perhaps, because of sequestration of erythrocytes in the capillary beds (Schalm et al., 1975).

The infected mice had a short survival time, being unable to control the first wave of parasitaemia. Anaemia was observed in the infected mice after comparing the tail blood PCV with the control. But the heart blood PCV of the infected mice was not significantly different from the control. The reason for this observation is not readily clear. Heparinized capillary tubes were always blocked by the tail blood from the 4 infected mice that did not bleed freely from the tail and this observation suggested the presence of fibrin deposits in the microvasculature of the mice due to intravascular coagulation (Boreham and Facer, 1974; Suliman and Feldman, 1989).

*In vitro* erythrocyte peroxidation was significantly increased in the infected mice and a majority (73%) of the infected mice had values above the maximum value observed in the control showing that the test demonstrated a good discrimination between the infected and control mice. The correlation between the erythrocyte peroxidation and PCV was not significant in the control and infected mice. However, there was some tendency for the erythrocyte peroxidation to be lower in the blood samples with higher PCV (Fig. 3.1).

In the erythrocyte peroxidation test, the hydrogen peroxide was expected to promote the formation of free radicals. The hydroxyl free radicals would initiate a self-propagating reaction

of oxidative damage to the polyunsaturated fatty acid components of the erythrocyte cell membrane. The degradation products of the peroxidation such as lipid hydroperoxide and malonaldehyde (Slater, 1984), which were TBARS, were therefore estimated (Duthie et al., 1989). The increased production of TBARS by the erythrocytes in the infected mice indicated a reduced ability of the animal to prevent free radical-mediated lipid peroxidation in the erythrocyte membrane. Thus infected mice erythrocytes will be expected to exhibit increased susceptibility to oxidative haemolysis. The observation was in agreement with the report of depletion of erythrocyte and liver GSH and increased susceptibility of erythrocytes to oxidative haemolysis in acute T. brucei gambiense infection of rats (Ameh, 1984).

Anosa and Kaneko, (1983a) showed that erythrocyte peroxidase and reductase activities increased in T. brucei infection of deer mice. The increase in the activities of the erythrocyte enzymes suggested an increased demand for their function to maintain GSH in the reduced state, probably due to a free radical load. It has been reported that ascorbic acid was depleted in the tissues of T. brucei-infected guinea pigs (Roskin and Nastiukova, 1941) and T. evansi-infected rats (Nyden, 1948). Niki et al. (1988) showed that plasma ascorbic acid functioned as the primary defense against free radicals in the whole blood and the ascorbic acid was rapidly depleted in the process. The free radicals in the infected animals may arise from trypanosome metabolism (Meshnick et al., 1977; Baltz et al., 1985) and macrophage activation in the expanded mononuclear phagocytic system (Klebanoff, 1982; Schwacha and Loegering, 1992). Therefore, if there is reduction in the capacity of the erythrocytes of the infected mice to prevent free radical-mediated membrane damage in vivo, as has been observed in vitro, predisposition of erythrocytes to peroxidation may accelerate their aging, decrease their deformability and increase susceptibility to fragmentation (Hochstein and Jain, 1981; Knight et al., 1992). Erythrocyte fragmentation has already been confirmed in T. brucei-infected deer

mice (Anosa and Kaneko, 1983b). Thus, erythrocyte peroxidation may be one of the factors which plays a role in the pathogenesis of the anaemia in acute trypanosomiasis.

# CHAPTER FOUR

## ERYTHROCYTE GLUTATHIONE AND PLASMA ASCORBIC ACID CONCENTRATIONS IN APPARENTLY HEALTHY CATTLE

### 4.1 SUMMARY

Erythrocyte glutathione (GSH) concentrations of cattle in Zaria and Maiduguri were measured. In Zaria, Zebu (Sokoto Gudali) (n=30) and Ndama (n=9) cattle had erythrocyte GSH concentrations ranging from 40.8 to 135.1 mg/100ml RBC. The range was 40.8 to 127.3 mg/100ml RBC in the Zebu and 70.6-135.1 mg/100ml RBC in Ndama. There was a significant positive correlation (r=0.58, P<0.05) between the erythrocyte GSH concentrations and PCV of the cattle. In Maiduguri, the Zebu (Wadara) (n=32) cattle had erythrocyte GSH concentrations ranging from 21.7 to 60.8 mg/100ml RBC and the values were significantly (P<0.01) and positively correlated (r=0.59) with PCV. The plasma ascorbic acid concentrations ranged from 0.35 to 1.00 mg/dl in the Wadara cattle and no correlation (r=-0.01) existed between these values and the erythrocyte GSH concentrations, but there was a weak insignificant correlation (r=-0.35, P>0.05) with whole blood GSH concentrations.

### 4.2 INTRODUCTION

Erythrocytes produce hydrogen peroxide through mechanisms associated with oxygen transportation (Carrell et al., 1975; Chiu et al., 1989). In the presence of glutathione peroxidase, the hydrogen peroxide is rapidly converted to water and oxygen by glutathione (GSH) which is an antioxidant tripeptide (McCay and Ling, 1980). Catalase participates in

the neutralization of hydrogen peroxide only when the concentration in the erythrocyte is too great to be handled by the primary GSH-dependent system (McCay and King, 1980). When these processes are overwhelmed, the hydrogen peroxide interacts with iron to produce highly reactive hydroxyl free radicals which cause lipid peroxidation of the cellular constituents, with subsequent erythrocyte senescence and haemolysis (Chiu et al., 1989).

Ascorbic acid is an antioxidant in the blood plasma (Frei et al., 1989), but the addition of ascorbic acid to stored whole blood rather led to greatly increased lipid peroxidation of the blood (Knight et al., 1992), perhaps because of the prooxidant interaction of ascorbic acid with oxyhaemoglobin and heme iron (Gutteridge et al., 1979). Also, alpha-tocopherol did not protect erythrocytes in vitro from the effects of hydrogen peroxide and phenyl hydrazine (Diez Marques et al., 1986). However, addition of GSH to stored whole blood had a significant effect in reducing lipid peroxidation of the blood (Knight et al., 1992).

Erythrocyte GSH deficiency caused mild haemolytic anaemia in man (Bauer, 1980), but sheep reported to have low erythrocyte GSH showed no evidence of anaemia (Agar, 1975). Blood and erythrocyte GSH concentrations and their relationship with PCV in Nigerian cattle have not been reported. Also, there is no available report of the plasma ascorbic acid concentrations in Nigerian cattle. Ibbott (1974) reported that high ascorbic acid concentrations in samples reduced GSH determinations. Since cattle have adequate ascorbic acid levels because they synthesize the vitamin in their tissues (Blood and Radostits, 1989), it is desirable to find out whether the plasma ascorbic acid concentrations affect blood and erythrocyte GSH concentrations.

The aims of this investigation were

(a) to measure the normal concentrations of erythrocyte GSH in apparently healthy cattle,

(b) to determine the relationship of the erythrocyte GSH concentrations with PCV and age of the cattle;

(c) to measure the normal concentrations of plasma ascorbic acid in the cattle, and

(d) to determine the relationship of the plasma ascorbic acid concentrations with erythrocyte GSH concentrations in the cattle.

## 4.3 MATERIALS AND METHODS

### 4.3.1 Animals

Cattle in Zaria: Thirty nine apparently healthy cattle of both sexes, aged 1-5 years and weighing about 70-300 Kg were used. They comprised 30 Zebu (Sokoto Gudali) and 9 Ndama cattle which were located at Samaru and Shika in Zaria respectively. They were grazed daily, but only the Ndama received supplemental feeding which consisted of grain offals, cotton seeds and palm kernel cakes. PCV and blood GSH were estimated with blood samples from each cattle. The Ndama were bled twice at one week interval.

Cattle in Maiduguri: Thirty two apparently healthy Zebu (Wadara) cattle of both sexes, aged 8 months to 5 years and weighing about 60-300 Kg were used. Twenty two of them were located on the University of Maiduguri farm and 10 were presented for slaughter at the Maiduguri abattoir. The animals on the University farm grazed daily and occasionally received grain offals. PCV, blood GSH and plasma ascorbic acid were estimated with blood samples collected from each cattle.

### 4.3.2 Blood sampling and analyses

Blood samples were collected in November during the early harmattan in both Zaria and Maiduguri. Five millilitres of blood collected from the jugular vein from each cattle was placed in a container with EDTA as anticoagulant. The blood samples were preserved at 4°C and all the analyses were carried out within 24 hours of sample collection.

The PCV was determined by the microhaematocrit method. The GSH determination was done using whole blood according to the method of Beutler et al. (1963) and the erythrocyte GSH concentration was calculated from the PCV. The blood sample was centrifuged at 3,000G for 5 minutes and the plasma was collected for ascorbic acid estimation according to Roe (1937).

### 4.3.3 Blood GSH determination

Principle

The determination of blood GSH was based on the development of a relatively stable yellow colour when 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was added to sulfhydryl compounds.

Procedure

Two-tenth millilitre of whole blood was added to 1.8ml of distilled water. Three millilitres of the precipitating solution was mixed with the haemolysate. The mixture was allowed to stand for about 5 minutes and then filtered. Two millilitres of the filtrate was added to 8.0ml of the phosphate solution. One millilitre of the DTNB reagent was added. A blank was prepared with 8ml of the phosphate solution (3 parts to 2 parts of distilled water) and 1.0ml of DTNB reagent. The optical density of the sample was measured at 412 nm against the blank using a spectrophotometer.

*Standard curve preparation*

A serial dilution of the GSH stock solution was prepared in 1% metaphosphoric acid as in Appendix 4.1. Two millilitre aliquots, after the dilution, were used for GSH determination. The optical densities obtained were plotted against the GSH concentrations of the aliquots (Appendix 4.2).

Calculations

The whole blood GSH concentration (mg/dl) was calculated with the formula:

$$\frac{\text{Optical density (Blood)} \times 5 \times 25}{\text{Optical density (Standard)}}$$

The concentration of GSH standard was 5 mg/dl. Since 0.2ml of blood was used and the volume of the haemolysate was 5ml, the dilution of the blood was 1 to 25 and the correction was inserted in the formula by multiplying by 25.

The erythrocyte GSH concentration (mg/100ml RBC) was calculated with the formula:

$$\frac{\text{Blood GSH (mg/dl)} \times 100}{\text{PCV (\%)}}$$

### 4.3.4 Plasma ascorbic acid determination

Principle

Ascorbic acid would form a yellow coloured complex with dinitrophenyl hydrazine (DNPH) in strongly acid medium and it was very important that the reaction mixture was not allowed to become warm because heat would destroy the complex.

Procedure

One millilitre of plasma was added to 4ml of Norit suspension, the mixture was shaken vigorously for 2 minutes and centrifuged at 3,000G for 5 minutes. The supernatant was filtered with Whatman No.42 ashless paper. Two millilitres of the filtrate was mixed with 0.3ml of thiourea solution and 0.4ml DNPH reagent in tubes. The mixture was incubated in boiling water for 5 minutes and cooled in crushed ice bath. Slowly and with

constant agitation in the ice bath, 3ml of the sulphuric acid reagent was added, mixed thoroughly and allowed to stand for 10 minutes. The blank was prepared with 1.0ml of 5% trichloracetic acid and 0.3ml of thiourea solution, incubated in boiling water for 5 minutes and after cooling in ice bath, 3ml of sulphuric acid reagent and 0.4ml DNPH were added. The optical density of the sample was read at 520nm against the blank.

Standard curve preparation

A serial dilution of the ascorbic acid stock solution was prepared with 4% trichloracetic acid as in appendix 4.3. One millilitre aliquot, after dilution, was used for ascorbic acid determination. The optical densities obtained were plotted against the ascorbic acid concentrations in the aliquots (Appendix 4.4).

Calculation

The plasma ascorbic acid concentration (mg/dl) was calculated as follows:

$$\frac{\text{Optical density (Plasma)} \times \text{Concentration (Standard)}}{\text{Optical density (Standard)}}$$

### 4.3.5 Statistics

The data were summarized as mean ± standard deviations. Statistical differences were assessed by paired Student's t-test. Correlation and regression statistics were computed between PCV and blood or erythrocyte GSH concentrations (Chatfield, 1983). Correlation was also evaluated between plasma ascorbic acid concentrations and blood or erythrocyte GSH concentrations.

## 4.4 RESULTS

### 4.4.1 Blood and erythrocyte GSH concentrations of cattle in Zaria (Sokoto Gudali and Ndama)

The mean PCV, whole blood and erythrocyte GSH concentrations of Gudali Zebu and Ndama cattle in Zaria presented in Table 4.1 showed that Ndama had significantly ($P<0.05$) higher values than the Gudali. The erythrocyte GSH concentrations in both breeds was 40.8 to 135.1mg/100ml RBC. The range was 40.8-127.3mg/100ml RBC in Gudali and 70.6-135.1mg/100ml RBC in the Ndama.

There was a significant ($P<0.01$) positive correlation ($r=0.84$) between the blood GSH concentrations and the PCV of cattle (Fig. 4.1) and the regression equation was given as GSH (mg/dl) = 1.6 PCV (%) - 20.6. Also, a significant ($P<0.05$) and positive correlation ($r=0.58$) existed between the erythrocyte GSH concentrations and the PCV of the cattle (Fig. 4.2) and the regression equation was given as GSH (mg/100ml RBC) = 2.6 PCV (%) + 11.8.

From the scatter diagram in Fig. 4.2, it was clear that at the PCV range of 27-30%, the mean PCV and erythrocyte GSH concentrations of the Gudali and Ndama were not significantly different (P 0.05). The mean PCV were 28.7 ± 1.2% (n=6) and 28.4 ± 1.1% (n=5) and the mean erythrocyte GSH concentrations were 93.3 ± 24.8 and 97.7 ± 11.0mg/100ml RBC for Gudali and Ndama respectively. Table 4.2 showed that the adult Gudali had significantly (P 0.5) higher mean PCV, blood and erythrocyte GSH concentrations than the young ones.

### 4.4.2 Blood and erythrocyte GSH concentrations of cattle in Maiduguri (Wadara)

The mean PCV, blood and erythrocyte GSH concentrations of Wadara Zebu cattle in Maiduguri are presented in Table 4.3. The mean PCV and blood GSH concentrations were significantly (P<0.05) higher in the adult Wadara than the young ones. The mean erythrocyte GSH concentration of the adult Wadara was higher than that of the young ones but there was no significant difference (P>0.05). The erythrocyte GSH concentrations ranged from 21.7 to 60.8mg/100ml RBC. The blood GSH concentrations and PCV showed significant (P<0.01) and positive correlation (r=0.92) with regression equation given as GSH (mg/dl) = 0.7 PCV (%) - 7.8 (Fig. 4.3). The erythrocyte GSH concentrations and PCV were significantly (P<0.01) and positively correlated (r=0.59) with regression equation given as GSH (mg/100ml RBC) = 0.7 PCV (%) + 20.4 (Fig. 4.4).

### 4.4.3 Comparison of the blood and erythrocyte GSH concentrations of Gudali and Wadara Zebu cattle

In Table 4.4, the mean PCV, blood and erythrocyte GSH concentrations of the Gudali and Wadara Zebu cattle were compared. The wadara cattle had significantly

($P < 0.05$) higher mean PCV and lower mean blood and erythrocyte GSH concentrations than the Gudali cattle. The frequency distribution of the erythrocyte GSH concentrations in both types of Zebu cattle (Table 4.5) was positively skewed and unimodal. The skewness was more extreme among the cattle in Zaria than those in Maiduguri.

### 4.4.4 Plasma ascorbic acid concentrations of cattle

Table 4.6 shows the mean plasma ascorbic acid concentrations in adult and young Wadara Zebu cattle in Maiduguri. The overall mean was 0.65 ± 0.15mg/dl and the values ranged from 0.35 to 1.00mg/dl. There was no significant difference ($P > 0.05$) between the mean values in the adult and young cattle.

There was a weak insignificant ($P > 0.05$) negative correlation ($r = -0.35$) between the blood GSH and plasma ascorbic acid concentrations. No correlation ($r = 0.01$) existed between the erythrocyte GSH and plasma ascorbic acid concentrations.

TABLE 4.1

Packed cell volume, blood and erythrocyte glutathione concentration of Zebu (Sokoto Gudali) and Ndama cattle in Zaria.

| | PCV (%) | Blood GSH (mg/dl) | Erythrocyte GSH (mg/100ml RBC) |
|---|---|---|---|
| Gudali (n=30) | $24.7 \pm 4.1^{a}$ | $18.4 \pm 7.4^{a}$ | $73.4 \pm 23.5^{a}$ |
| Ndama (n=9*) | $33.1 \pm 4.2^{b}$ | $34.3 \pm 8.6^{b}$ | $102.6 \pm 16.8^{b}$ |
| All cattle | $27.8 \pm 5.8$ | $24.2 \pm 10.9$ | $84.0 \pm 25.8$ |

$^{a,b}$values with different superscripts are significantly different ($P < 0.05$)

n, number of animals

*Sampled twice; Mean based on 18 samples.

TABLE 4.2

Packed cell volume, blood and erythrocyte glutathione concentration of adult and young Gudali Zebu cattle in Zaria.

| | PCV (%) | Blood GSH (mg/dl) | Erythrocyte GSH (mg/100ml RBC) |
|---|---|---|---|
| Adult Gudali > 2 years (n=10) | $27.8 \pm 2.8^{a}$ | $25.5 \pm 5.6^{a}$ | $91.5 \pm 18.8^{a}$ |
| Young Gudali < 2 years (n=20) | $23.2 \pm 3.8^{b}$ | $14.9 \pm 5.4^{b}$ | $64.4 \pm 20.4^{b}$ |

$^{a,b}$ values with different superscripts are significantly different ($P < 0.05$)

TABLE 4.3

Packed cell volume, blood and erythrocyte glutathione concentrations of adult and young Wadara Zebu cattle in Maiduguri.

| | PCV (%) | Blood GSH (mg dl) | Erythrocyte GSH (mg/100ml RBC |
|---|---|---|---|
| Adult Wadara > 2 years (n=18) | 38.4 ± 8.7[a] | 18.6 ± 7.9[a] | 46.5 ± 12.6[a] |
| Young Wadara < 2 years (n=14) | 25.1 ± 3.0[b] | 10.3 ± 3.2[b] | 40.5 ± 9.8[b] |
| All cattle | 32.6 ± 9.5 | 15.0 ± 7.5 | 43.9 ± 11.7 |

[a,b] values with different superscripts are significantly different (P < 0.05)

TABLE 4.4

Comparison of the packed cell volume, blood and erythrocyte glutathione concentrations of Zebu cattle in Zaria (Sokoto Gudali) and Maiduguri (wadara).

| Age | Breed | n | PCV (%) | Blood GSH (mg/dl) | Erythrocyte GSH (mg/100ml RBC) |
|---|---|---|---|---|---|
| Adult > 2 years | Gudali | 10 | $27.8 \pm 2.8^{a}$ | $25.5 \pm 5.6^{a}$ | $91.5 \pm 18.8^{a}$ |
| | Wadara | 18 | $38.4 \pm 8.7^{b}$ | $18.6 \pm 7.9^{b}$ | $46.5 \pm 12.6^{b}$ |
| Young < 2 years | Gudali | 20 | $23.2 \pm 3.8^{b}$ | $14.9 \pm 5.4^{b}$ | $64.4 \pm 20.4^{b}$ |
| | Wadara | 14 | $25.1 \pm 3.0^{a}$ | $10.3 \pm 3.2^{b}$ | $40.5 \pm 9.8^{b}$ |
| All ages | Zaria | 30 | $24.7 \pm 4.1^{a}$ | $18.4 \pm 7.4^{a}$ | $73.4 \pm 23.5^{a}$ |
| | Maiduguri | 32 | $32.6 \pm 9.5^{b}$ | $15.0 \pm 7.5^{a}$ | $43.9 \pm 11.7^{b}$ |

$^{a,b}$values with different superscripts are significantly different ($P<0.05$)

TABLE 4.5

Frequency distribution of the erythrocyte glutathione concentrations of Zebu in Zaria and Maiduguri.

| Erythrocyte GSH (mg/100ml RBC) | Gudali cattle | Wadara cattle | All cattle |
|---|---|---|---|
| 20 - 39 | 0 | 12 | 12 |
| 40 - 59 | 12 | 17 | 29 |
| 60 - 79 | 7 | 3 | 10 |
| 80 - 99 | 7 | 0 | 7 |
| 100 - 129 | 4 | 0 | 4 |
| Total | 30 | 32 | 62 |

TABLE 4.6

Plasma and ascorbic acid concentrations in adult and young Wadara Zebu cattle in Maiduguri.

| Age | n | Plasma ascorbic acid (mg/dl) |
|---|---|---|
| Adult > 2 years | 18 | 0.69 ± 0.12[a] |
| Young < 2 years | 14 | 0.59 ±18[a] |
| All cattle | 32 | 0.65 ± 0.15 |

[a]No significant difference (P> 0.05) between means.

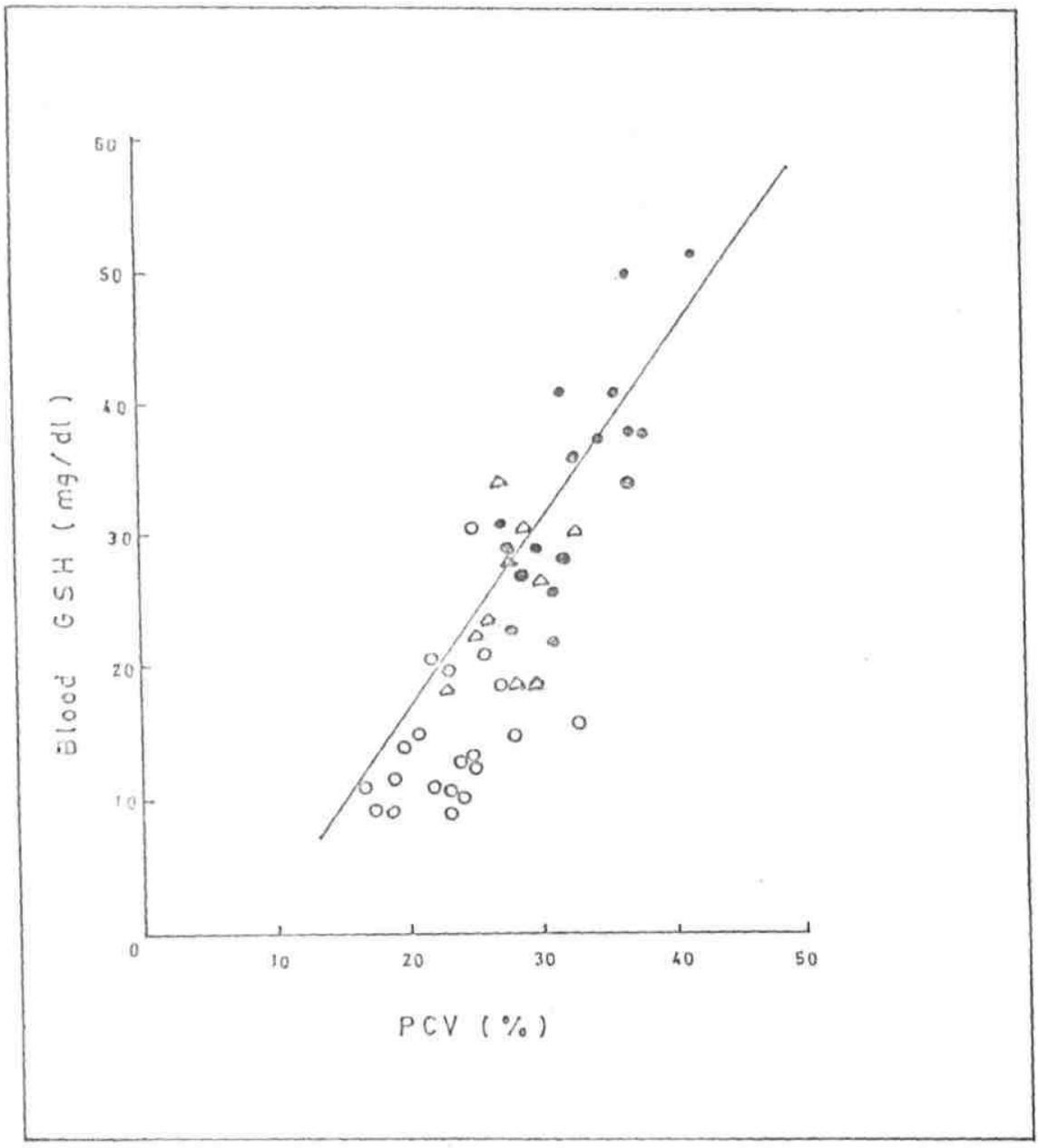



Figure 4.1 The relationship between blood glutathione concentrations and the packed cell volume of Sokoto Gudali Zebu and Ndama cattle in Zaria.

Young (o) and adult (Δ) Sokoto Gudali Zebu cattle

Ndama (•) cattle

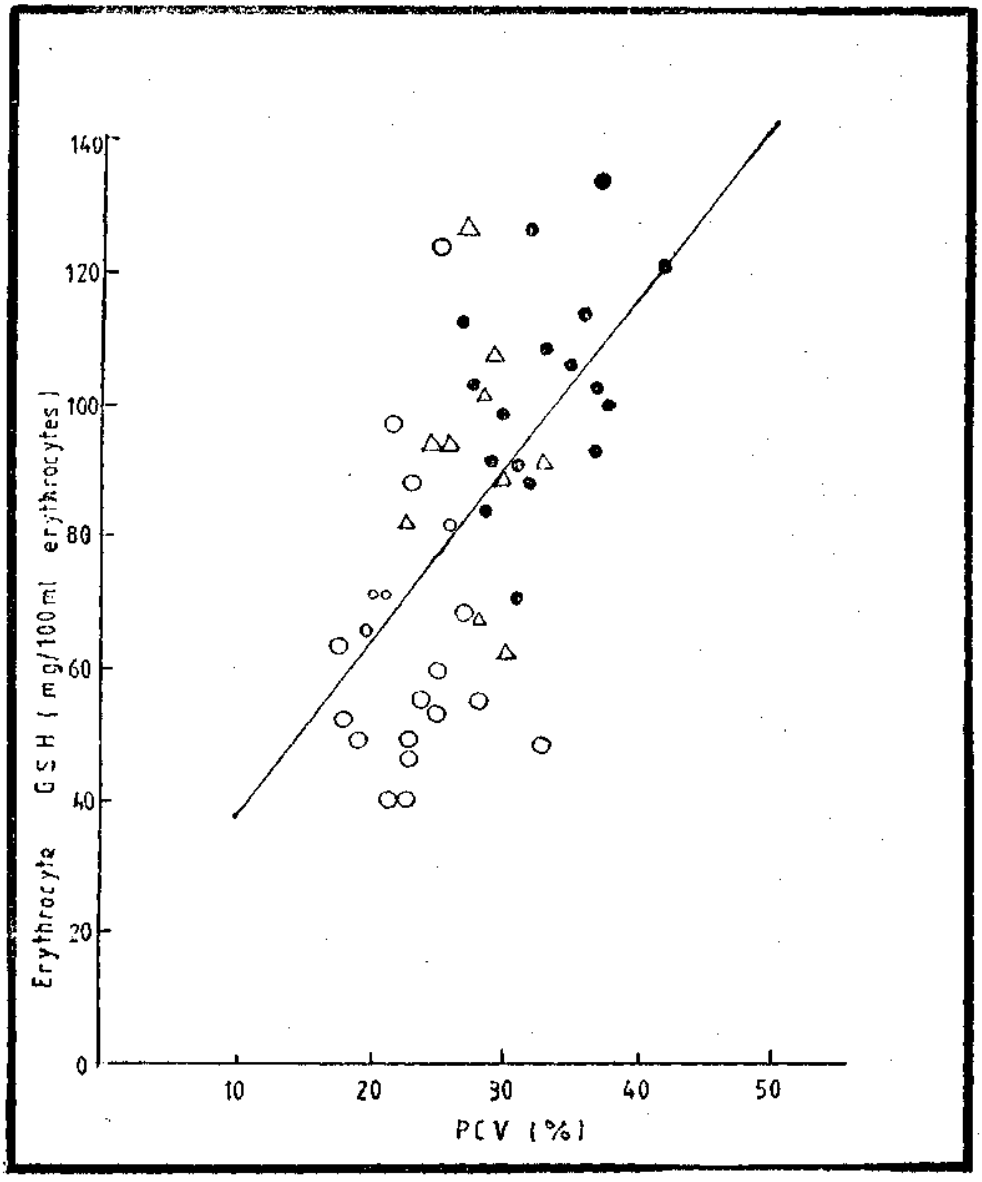



Figure 4.2 The relationship between erythrocyte glutathione concentrations and packed cell volume of Sokoto Gudali Zebu and Ndama cattle in Zaria.

Young (o) and adult (Δ) Sokoto Gudali Zebu cattle

Ndama (•) cattle

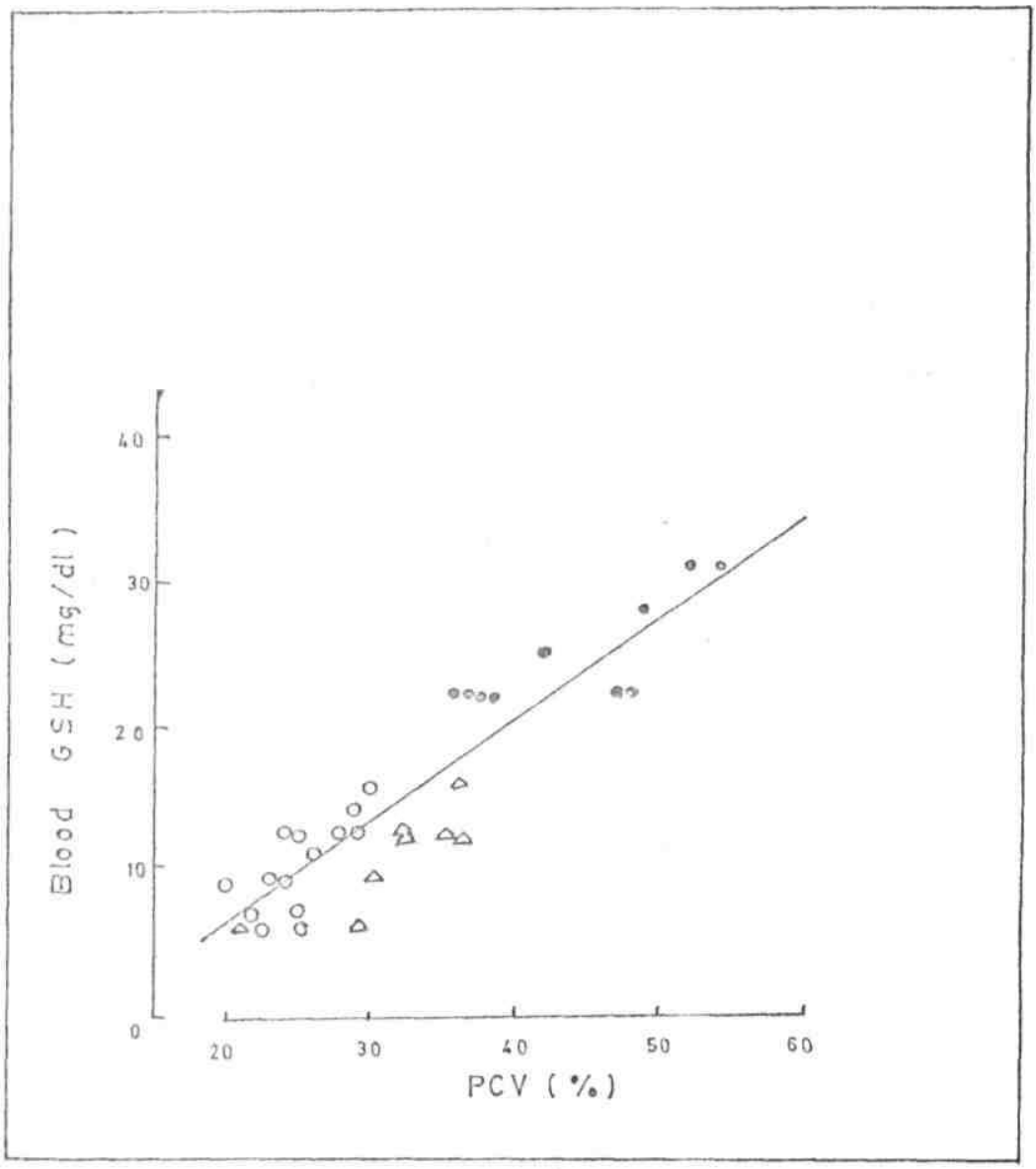



Figure 4.3 The relationship between blood glutathione concentrations and packed cell volume of Wadara Zebu cattle in Maiduguri.

Young (o) and adult ( ) Wadara Zebu cattle

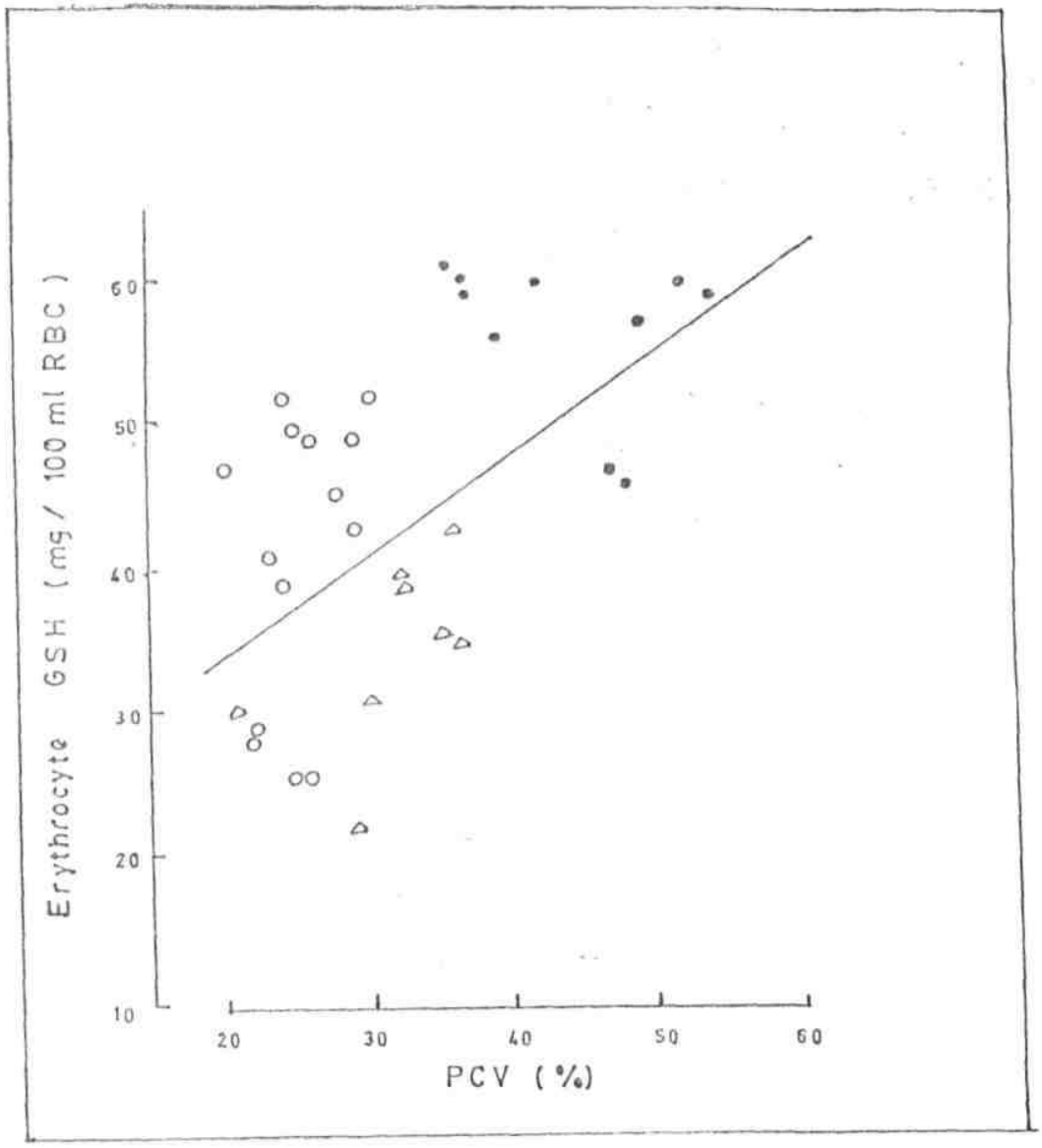



Figure 4.4 The relationship between erythrocyte glutathione concentrations and packed cell volume of Wadara Zebu cattle in Maiduguri.

Young (o) and adult ( ) Wadara Zebu cattle

## 4.5 DISCUSSION

The relationship between the blood and erythrocyte GSH concentrations and PCV in cattle had not been previously described (Agar et al., 1978). The present study showed that blood GSH concentrations and PCV had strong positive correlation while erythrocyte GSH concentrations and PCV had moderate positive correlation among the cattle in Zaria and Maiduguri separately considered. The correlations in Zaria cattle did not extend to the Maiduguri cattle in the sense that the higher PCV in Maiduguri cattle did not predict higher blood and erythrocyte GSH values when compared with the Zaria cattle. While the Gudali and Ndama cattle in Zaria had erythrocyte GSH values of 40.8-135.1mg/100ml RBC, the Wadara cattle in Maiduguri had values of 21.7-60.8mg/100ml RBC and the distribution of these values was unimodal and positively skewed. The range of values for cattle in Zaria was similar to 40-113mg/100ml RBC reported for Australian cattle where the distribution was rather normal and unimodal (Agar et al., 1978).

The mean erythrocyte GSH concentration in the Wadara Zebu (43.9 ± 11.7mg/100ml RBC) was significantly ($P<0.05$) lower when compared with the values in the Gudali Zebu (73.4 ± 23.5 mg/100ml RBC) and Australian cattle (72.0 ± 18.1 mg/100ml RBC) (Agar et al., 1978). However, the mean values in the Gudali Zebu and Australian cattle were not significantly ($P>0.05$) different. Although the frequency distribution of the erythrocyte GSH values in Gudali Zebu was skewed with mode at 40-59 mg/100ml RBC, only 40% of the population had values less than 60mg/100ml RBC. In contrast, 90.6% of the Wadara Zebu had values less than 60mg/100ml RBC.

Also, 37.5% of the Wadara Zebu had values less than the lower limit of 40mg/100ml RBC in Gudali Zebu and Australia cattle (Agar et al., 1978).

The higher PCV in Wadara Zebu compared to the PCV in Gudali Zebu did not predict a higher erythrocyte GSH in Wadara Zebu, indicating that the correlations between the PCV and erythrocyte GSH concentrations were exclusive for the different Zebu populations. The reason for this observation is yet unclear.

The Zaria and Maiduguri studies were conducted in November at the beginning of the harmattan and the difference in weather between the two locations might not have been very remarkable, although the locations are geographically sudan savanna and sahel savanna respectively. Board et al., (1976) had shown that environmental temperature and day length influenced erythrocyte GSH concentrations of sheep and reported that maximum concentrations occurred in midsummer and the minimum concentrations in winter.

Genetic factors might be involved in the variations of erythrocyte GSH values (Board et al., 1974). It had been shown that erythrocyte GSH concentration could be a highly heritable trait in cattle (Kunkel et al., 1954). Although the Ndama had higher mean erythrocyte GSH concentration than the Gudali Zebu in Zaria, when selected Ndama and Gudali Zebu with the same PCV values were compared, there was no difference in the erythrocyte GSH concentrations between the two breeds. Therefore, the Ndama might be having higher erythrocyte GSH concentrations because of their higher PCV values, perhaps arising from a better feeding regimen. Esievo et al., (1986) reported no difference in PCV of Zebu and Ndama cattle under the same management.

Nutrition might have contributed in causing the wide variation in blood and erythrocyte GSH concentrations. GSH is synthesized in erythrocytes from glutamic acid, cysteine and glycine and the extent to which the plasma concentrations of these amino acids influence the level of intra-erythrocytic GSH synthesis is yet to be determined (Board, et al., 1976). The erythrocyte concentrations of the substrates for GSH synthesis had been considered important in regulating erythrocyte GSH levels (Smith, 1977). The GSH in erythrocytes was reported to have a half-life of 2-3 days and its synthesis occurred continuously (Hochberg et al., 1964). Variations in the activities of erythrocyte GSH metabolizing enzymes might not have affected the erythrocyte GSH concentrations because it had been reported that activities of these enzymes did not differ significantly in cattle with high (102mg/100ml RBC) and low (56mg/100ml RBC) erythrocyte GSH concentrations (Agar et al., 1978).

The young cattle consistently had lower PCV and GSH levels than the adult ones in Zaria and Maiduguri. PCV and GSH concentrations were reported to decline with increasing age in calves between 1-12 weeks of age (Mabon, 1969; Smith and Morrill, 1974). Mabon (1969) reported that there was a rapid decline in GSH level because of greater efficiency of feed conversion. It was probable that the calves, in this study, had inadequate protein nutrition and were diverting their amino acids to anabolic processes necessary for rapid growth, thereby reducing the amount of amino acid substrates available for GSH synthesis and red cell production. Related reports have shown that high wool production in sheep may divert amino acids from GSH synthesis to wool growth (Hopkins et al., 1975; Kalla and Ghosh, 1975).

The mean plasma ascorbic acid concentrations of the Wadara cattle was comparable to the values of 0.5-2.0 mg/dl reported in man (Varley et al., 1976). The weak and insignificant negative correlations between blood or erythrocyte GSH and plasma ascorbic acid concentrations showed that plasma ascorbic acid concentrations were not likely to significantly influence GSH values. Although ascorbic acid concentrations in the cattle were adequate for normal individuals (Varley et al., 1976), under stress conditions when large amounts of ascorbate are released into circulation (McGilvery, 1979) there might be a tendency for blood GSH values to decrease because of the weak negative correlation ($r = -0.35$).

CHAPTER FIVE

# EFFECT OF ACUTE *TRYPANOSOMA VIVAX* INFECTION ON CATTLE ERYTHROCYTE GLUTATHIONE AND SUSCEPTIBILITY TO *IN VITRO* PEROXIDATION

## 5.1 SUMMARY

During acute Trypanosoma vivax experimental infection of calves, the mean PCV and red blood cell counts of the infected animals decreased significantly (P 0.05) between days 6 and 13 post-infection (pi). The moderately severe normocytic anaemia started to develop during the first wave of parasitaemia which occurred from day 2 pi and peaked between days 4 and 5 pi. The mean erythrocyte GSH concentration of the infected calves decreased significantly (P 0.05) from 58.4 ± 11.4mg/100ml RBC on day 0 pi to 44.5 ± 12.8 mg/100ml RBC on day 5 pi. As the GSH values recovered on day 6 pi and marginally increased thereafter, another slight decrease (P 0.05) in GSH concentration occurred on day 12 pi at the second peak of parasitaemia followed by a significant (P 0.05) increase to 79.1 ± 14.6mg/100ml RBC on day 13 pi. In the uninfected calves, the mean GSH values ranged from 47.7 ± 7.0 to 60.8 ± 6.8mg/100ml RBC. When washed erythrocytes of the infected and uninfected calves were separately challenged with hydrogen peroxide they produced comparable amounts of by-products of lipid peroxidation as measured by thiobarbituric acid reactive substances. This suggested that the ability of the erythrocytes to prevent peroxidative injury was not reduced, because GSH regeneration was probably enhanced and the antioxidant capacity of the erythrocytes was maintained.

## 5.2 INTRODUCTION

In the review by Murray and Dexter (1988), immunological and non-immunological mechanisms were implicated in the pathogenesis of the anaemia of bovine trypanosomiasis. During the early acute phase of trypanosome infection when the immune response of the host has not been fully mobilized, trypanosome protease (Londsdale-Eccles and Grab, 1986), phospholipases (Mellor, 1985) and sialidases (Esievo, 1979, 1983; Engstler and Schauer, 1993) may be involved in the non-immunological mechanisms of erythrocyte damage.

The erythrocytes of Trypanosoma brucei gambiense-infected rats showed increased susceptibility to in vitro oxidative haemolysis and the blood GSH concentration decreased between 2-3 days post-infection (Ameh, 1984). In chapter 3, the erythrocytes of mice with acute T. brucei infection produced greater amounts of by-products of lipid peroxidation than the uninfected control mice. These findings suggested that the trypanosome infections depleted the antioxidant capacity of the erythrocytes.

In Zebu cattle, T. brucei frequently causes a mild and chronic anaemia (Ikede and Losos, 1972b). However, T. vivax which is the most pathogenic trypanosome of cattle in Nigeria (Agu, 1983) often produces an acute infection. It is well recognized that the mechanisms of erythrocyte destruction in trypanosome infections may vary with the species of parasite, the host and the stage of infection (Ikede et al., 1977). This experiment was, therefore, designed to investigate the variations in the susceptibility of the erythrocytes to in vitro peroxidation during the early acute phase of T. vivax

infection of cattle, with the purpose of finding out whether the infection may reduce the ability of the erythrocytes to protect themselves against lipid peroxidation.

## 5.3 MATERIALS AND METHODS

### 5.3.1 Experimental animals

Nine male Zebu (Sokoto Gudali) calves aged 1-1½ years and weighing 70-90Kg were used. They were purchased from Talata-Mafara, an area known to be tsetse-free in the northwestern sahel region of Nigeria and were kept at the animal house of the Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. They were screened for haemoparasitism, helminthiasis and other common infections by blood and faecal examinations after which they were treated with thiabendazole (Merck, Sharp and Dohme, Ltd., U.K.), oxytetracycline, Long acting (Pfizer, Nigeria) and sprayed against ticks with chlorfenvinphos (Pfizona$^{R}$, Pfizer, Nigeria). They grazed for about 5 hours daily with supplemental feeding of mixed grain offals, and corn stalks. Salt licks and water were provided ad libitum. The animals were acclimatized for about 3 months and were on zero grazing one week before the commencement of the experiment.

### 5.3.2 Trypanosome infection

The Trypanosoma vivax was isolated from a pregnant cow in Samaru, Zaria, Nigeria. An adult male Sahel Brown goat weighing 12Kg was infected with this trypanosome by intravenous injection of $1.0 \times 10^4$ trypanosomes. The goat became

parasitaemic 3 days post-infection (pi) and on day 6 pi. when the parasitaemia was high, 5 ml of the infected goat blood containing $1.1 \times 10^7$ trypanosomes was injected intravenously into each of the 6 experimental calves. Three uninfected calves served as controls. The experiment was terminated on day 13 pi by treatment of the infected calves with homidium chloride (Novidium$^R$, Rhone Merieux, France).

### 5.3.3 Blood sampling

Five millilitres of blood was collected daily in the morning (09.00hr) before feeding from the infected and uninfected calves through the jugular vein and the blood was put into containers with EDTA as anticoagulant. The blood samples were stored at 4°C and analyses were carried out within 24 hours of sampling.

### 5.3.4 Haematological evaluation

PCV was estimated by a microhaematocrit method. Red blood cell (RBC) count was determined using a haemocytometer. Mean corpuscular volume was calculated using a standard formula (Schalm et al., 1975). Reticulocytes were counted from blood stained with 1% new methylene blue (Schalm et al., 1975).

### 5.3.5 Estimation of parasitaemia

Trypanosomes were detected in the blood samples by the microhaematocrit centrifuge technique and parasitaemia score was estimated as described by Woo (1969). The trypanosomes seen in the buffy coat area of the haematocrit were counted and the

parasitaemia scores were given as 1+ (occasional trypanosome), 2+ (1-2 trypanosomes), 3+ (3-20 trypanosomes) and 4+ (> 20 trypanosomes).

### 5.3.6 Determination of erythrocyte GSH concentration

The GSH determination was carried out on whole blood according to the method of Beutler et al. (1963) after reaction with DTNB as described in Chapter 4.

### 5.3.7 Peroxidation of erythrocytes in vitro

The in vitro peroxidation of erythrocytes was measured according to the method of Duthie et al. (1989) as described in Chapter 3.

### 5.3.8 Statistics

Data were summarized as means ± standard deviations and the means were plotted in graphs with their standard error bars. Statistical difference between means was assessed by paired Student's t-test (Chatfield, 1983).

## 5.4 RESULTS

### 5.4.1 Clinical observations

The infected calves maintained good appetite, but became weaker as the infection progressed and one of them was almost moribund on day 13 pi.

### 5.4.2 Parasitaemia

Parasitaemia was detected in the infected calves on day 2 pi. The parasitaemia reached a peak between days 4 and 5 pi, declined on day 7 pi and subsequently increased gradually to reach a second peak between days 11 and 13 pi (Fig. 5.1A).

### 5.4.3 Haematological changes

The mean PCV of the infected calves started to decrease on day 5 pi and between days 6 and 13 pi, the PCV values were significantly ($P<0.05$) lower than those of the controls (Fig. 5.1B). The mean RBC counts decreased significantly ($P<0.05$) between days 6 and 13 pi from the value on day 0 (Fig. 5.5A). Significant ($P<0.05$) differences in the mean RBC counts between the infected and control calves were observed on days 6 and 8 pi and between days 10 and 12 pi. The control calves maintained higher MCV values (41.0 - 52.5fl) than the infected ones (37.0 - 45.5fl) (Fig. 5.2B), and reticulocytes were rare in the blood films of both control and infected calves.

### 5.4.4 Changes in whole blood and erythrocyte GSH concentrations

The mean whole blood GSH concentrations of the control calves varied between 11.9 ± 3.1 and 15.1 ± 0.9mg/dl. In the infected calves, the GSH concentration decreased from 13.4 ± 3.6mg/dl on day 0 to 9.8 ± 3.1mg/dl ($P<0.05$) on day 5 pi and 10.2 ± 2.8mg/dl ($P<0.05$) on day 12 pi respectively (Fig. 5.3A). The blood GSH concentrations of the infected calves were lower than the uninfected control values

between days 4 and 12 pi but differences were significant (P 0.05) only on days 11 and 12 pi.

*The mean erythrocyte GSH concentrations of the control calves varied between* 47.7 ± 7.0 and 60.0 ± 6.8mg/100ml RBC. In the infected calves the erythrocyte GSH concentrations significantly (P 0.05) decreased from 58.4 ± 11.4mg/100ml RBC on day 0 to 44.5 ± 12.8mg/100ml RBC on day 5 pi and this value on day 5 pi was also significantly (P 0.05) lower than the uninfected control value on the same day (Fig. 5.3B), but not lower (P 0.05) than the control value of 47.7 ± 7.0mg/100ml RBC on day 0. The GSH value recovered to 57.2 ± 14.2mg/100ml RBC on day 6 pi. Between days 8 and 11 pi, the values were higher than that on day 0 as well as the uninfected control values, but significant (P 0.05) differences were only found between the values from the infected and controls on days 8 and 9 pi. The GSH value slightly decreased (P 0.05) from 66.0 ± 8.2mg/100ml RBC on day 11 pi to 56.0 ± 13.4 mg/100ml RBC on day 12 pi and subsequently rose to 79.1 ± 14.6mg/100ml RBC on day 13 pi. The GSH value of the infected group on day 13 pi was significantly ($P<0.05$) higher than the control values on all the days of the experiment.

### 5.4.5 Susceptibility of erythrocytes to peroxidation

The variations in the erythrocyte peroxidation values of the infected and control calves were not significantly ($P>0.05$) different (Fig. 5.4). The mean absorbance units of TBARS produced when the erythrocytes were challenged with hydrogen peroxide ranged from 0.02 to 0.03 in the control and 0.01 to 0.03 in the infected calves.

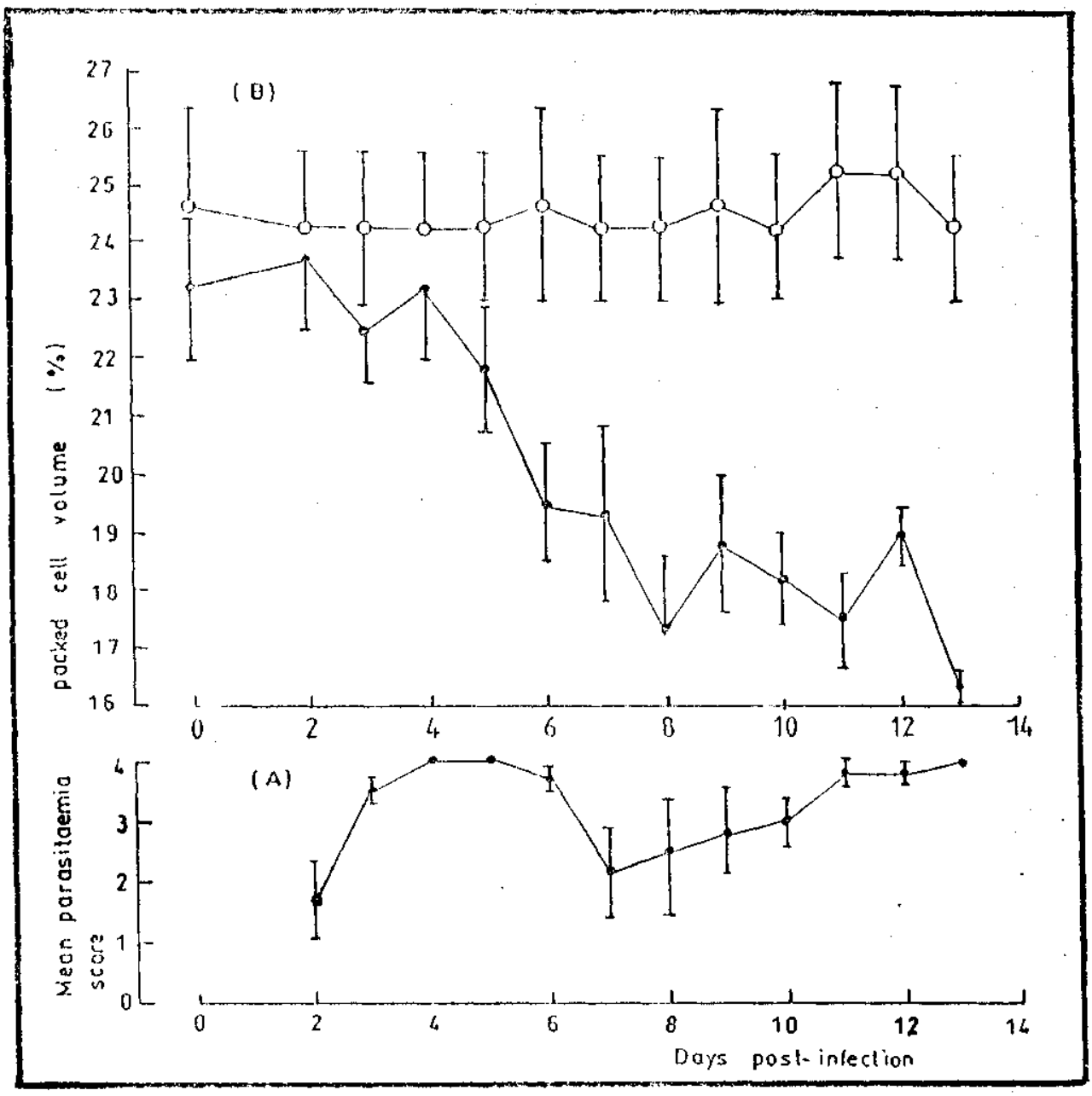



Figure 5.1 A,B Parasitaemia scores (A) and packed cell volume (B) of control and Trypanosoma vivax-infected calves.

Control (○) Infected (●)

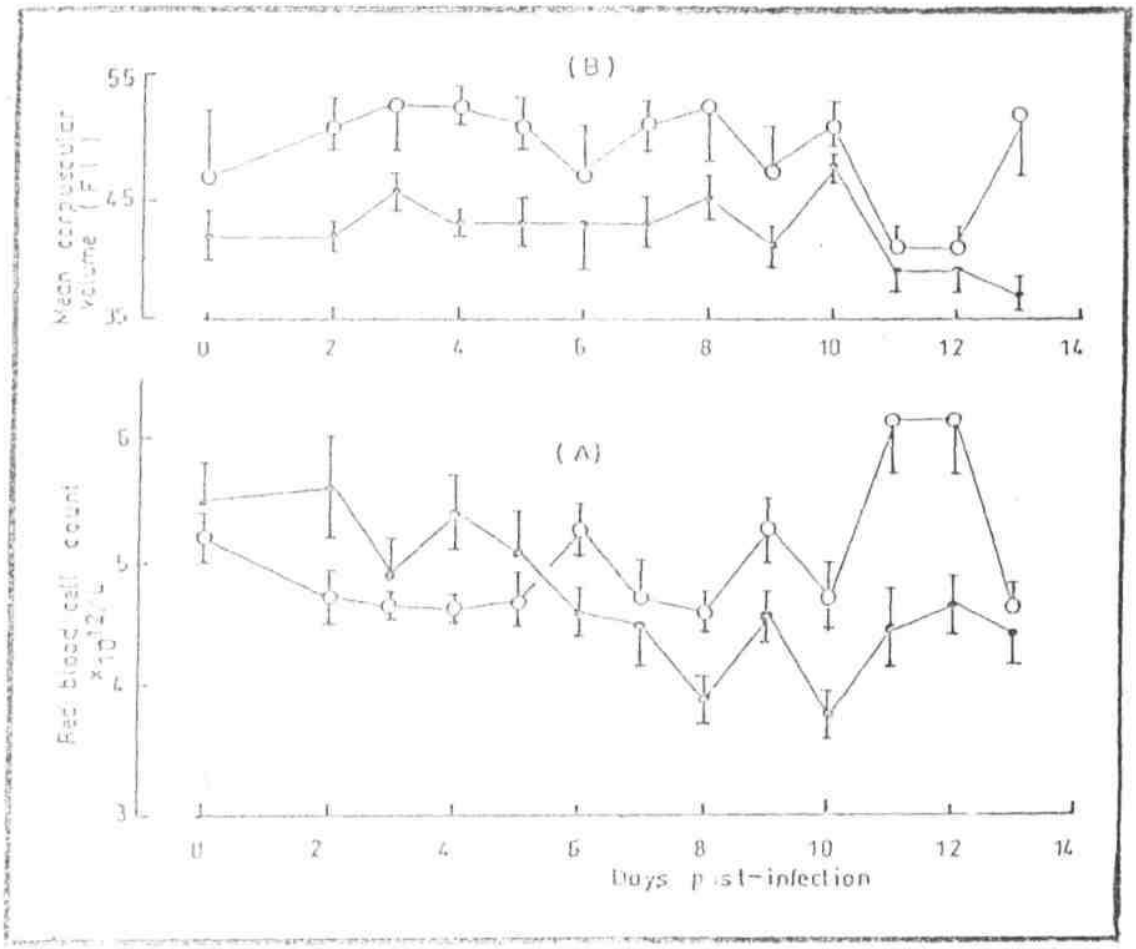



Figure 5.2 AB Red blood cell counts (A) and mean corpuscular volume (B) of control and Trypanosoma vivax-infected calves.

Control (O) Infected (●)

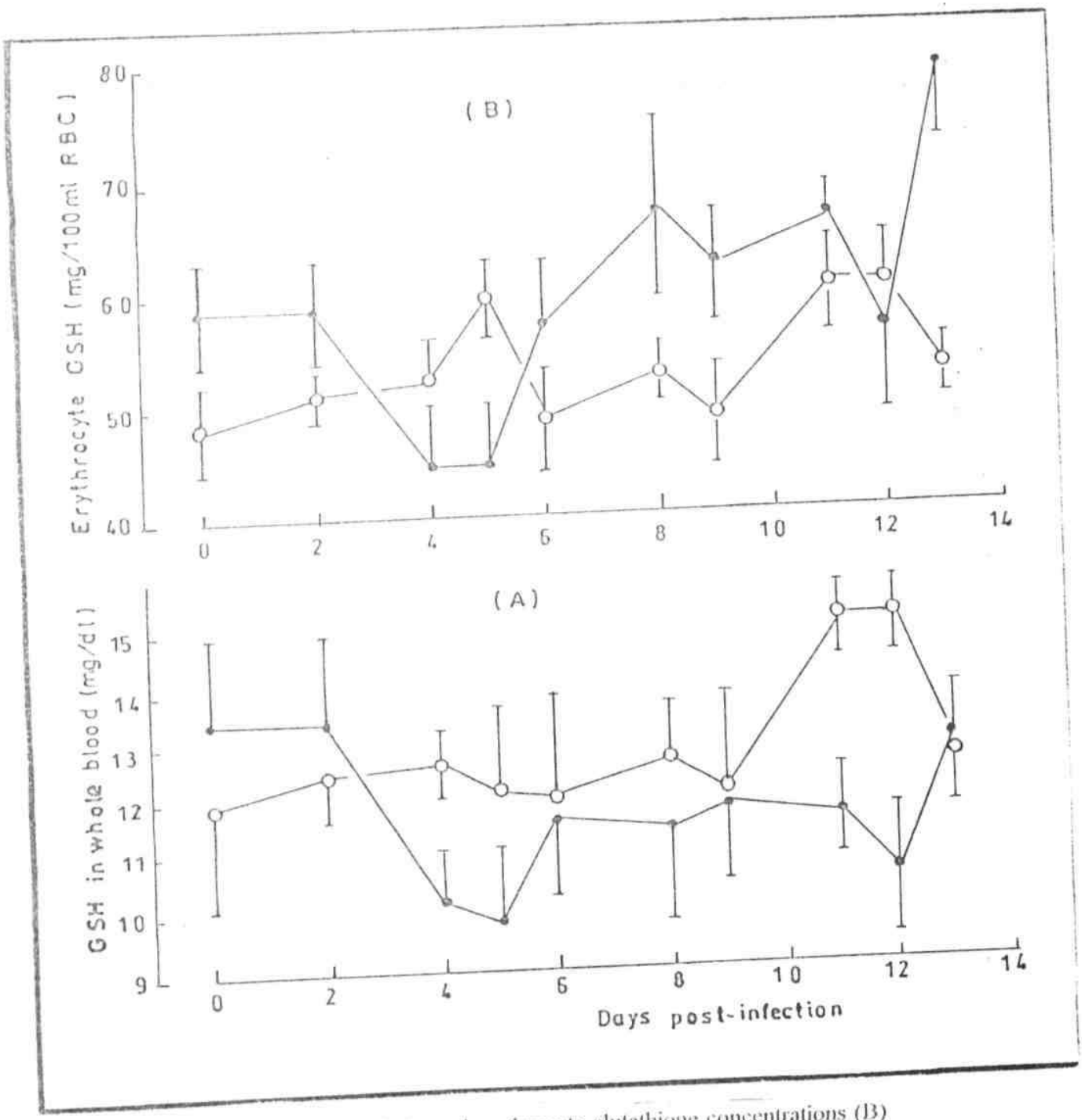



Figure 5.3 A,B Whole blood (A) and erythrocyte glutathione concentrations (B) of control and Trypanosoma vivax-infected calves.

Control (○) Infected (●)

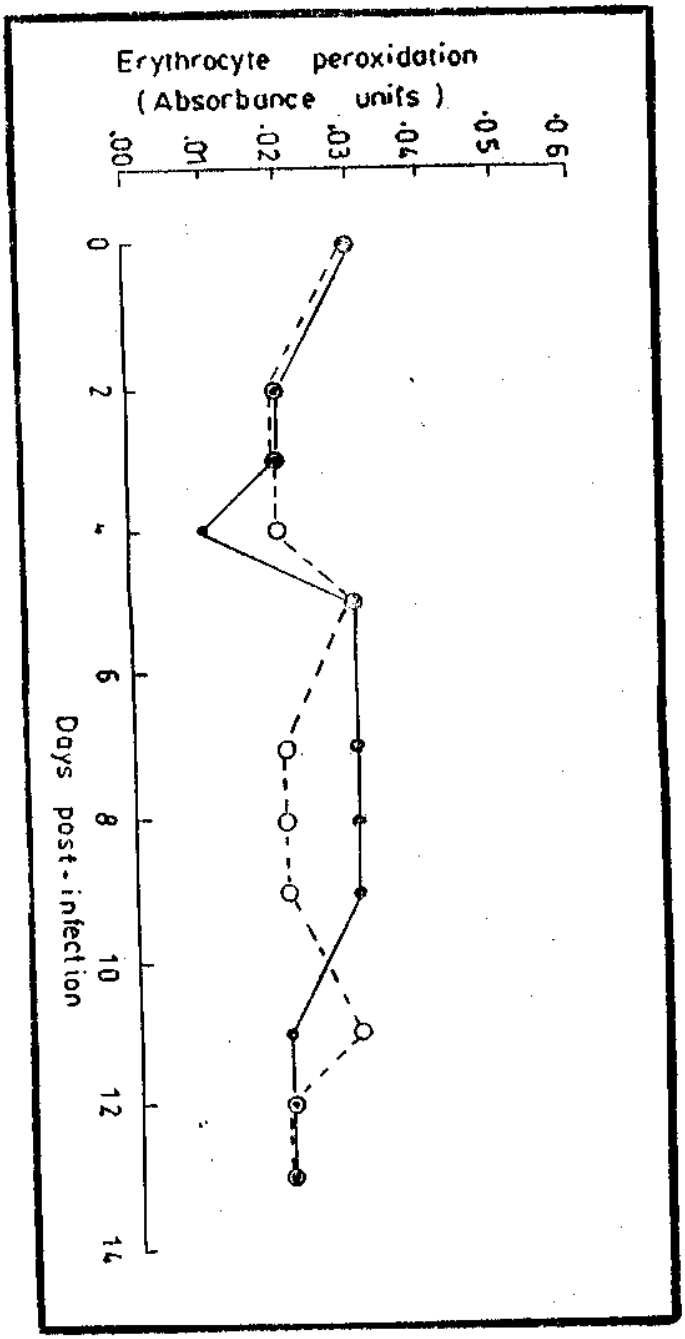



Figure 5.4 In vitro erythrocyte peroxidation values of control and Trypanosoma vivax-infected calves.

Control (○) Infected (●)

## 5.5 DISCUSSION

The *T. vivax* caused an acute infection in the calves. The prepatent period was very short, probably because of the high infective dose (Murray and Dexter, 1988). The anaemia which developed, judging from the decreases in the PCV and RBC counts, was moderately severe. During the peak of the first wave of parasitaemia, the erythrocyte GSH concentration decreased marginally when compared with the lowest mean value in the uninfected animals. As the parasitaemia declined, the erythrocyte GSH value recovered and tended to increase. In the middle of the peak of the second wave of parasitaemia, a slight drop in erythrocyte GSH concentration was again observed, which was followed by a significant increase in the GSH concentrations on day 13 pi.

*The drop in erythrocyte GSH concentration during the first wave of* parasitaemia might be related to the decrease in PCV since erythrocyte GSH and PCV were positively correlated in healthy cattle as shown in Chapter 4. It is noteworthy that oxidant stress could occur when the circulating blood passed through the expanded active mononuclear phagocytic systems (MPS) of the liver and spleen where the erythrocytes would be in close proximity to trypanosomes and activated macrophages. Trypanosoma brucei have been shown to produce enormous amounts of hydrogen peroxide (Meshnick et al., 1977) and activated oxygen species (Baltz et al., 1985). However, there was no available report to show that T. vivax produced such oxidants in comparable amounts with T. brucei. Moreover, the MPS in the infected animals may also be producing superoxide anions that diffuse into the extracellular fluid (Klebanoff, 1982; Schwacha and Loegering, 1992) and could permeate the erythrocytes where they

produce hydrogen peroxide through the action of superoxide dismutase (McCay and King, 1980). Since the size and activity of the MPS may be related to the parasitaemia (Murray and Dexter, 1989), the degree of oxidant stress could be expected to increase at peaks of parasitaemia. It was noted that the marginal decreases in erythrocyte GSH on days 5 and 12 pi (Fig. 3B) were followed by sharp decreases in PCV on days 6 and 13 pi (Fig. 1B). Therefore, the time of decrease in erythrocyte GSH seemed to have coincided with the period when intensified MPS activity was expected to have occurred to cause the subsequent decreases in PCV.

The tendency of the erythrocyte GSH concentrations to increase especially on day 13 pi despite the decrease in PCV was probably due to the emergence of circulating young mature erythrocytes which have been reported to have higher GSH concentrations than older erythrocytes (Tucker and Kilgour, 1973). Indeed, the erythrocyte GSH concentrations in dogs with haemolytic anaemia was 4-5 times higher than normal (Maede, 1977). During the acute phase of haemorrhagic T. vivax infection of calves, bone marrow erythropoiesis commenced in response to the anaemia with the release of reticulocytes into circulation (Anosa et al., 1992), but in the present study reticulocytes were rare in the infected blood even on day 13 pi. Saror (1979) showed that the anaemia in T. vivax infection of cattle was basically normocytic in the acute stage which was in agreement with our findings where MCV values were within the normal range (30-56fl) reported in cattle (Schalm et al., 1975). Therefore, any increase in erythropoietic response in the infected calves was not obvious. However, there might have been some young mature erythrocytes in circulation released from the bone marrow with enhanced capacity for GSH regeneration which could be responsible for the increase in erythrocyte GSH.

When the erythrocytes of the control and infected calves were challenged with hydrogen peroxide, the by-products would be thiobarbituric acid reactive substances (TBARS) (Duthie et al., 1989). The amounts of TBARS produced by the erythrocytes from control and infected calves did not differ significantly. This finding suggested that the antioxidant capacity of the erythrocytes of the infected calves was not deranged enough to allow for free radical damage of the erythrocytes in vivo. It was possible that T. vivax did not produce large amounts of oxidants and the extent of expansion and activation of the MPS did not elicit overwhelming production of oxidants, since it was reported that the capacity of activated macrophages to produce oxidants was depressed by phagocytosis of IgG-coated erythrocytes (Loegering and Schwacha, 1991).

In conclusion, erythrocyte GSH concentrations of T. vivax-infected calves tended to drop marginally at the first peak of parasitaemia and there was no significant increase in the susceptibility of the erythrocytes to in vitro peroxidation. Therefore, peroxidative injury may not be a mechanism of erythrocyte damage at the early phase of T. vivax infection of calves, because the antioxidant capacity of the erythrocytes in the infected animals was maintained.

CHAPTER SIX

# GENERAL DISCUSSION AND CONCLUSION

## 6.1 GENERAL DISCUSSION

During the acute phase of trypanosome infections, the pathogenesis of the anaemia may vary with each species of the parasite and the type of host involved (Ikede et al., 1977). In the present study, The T. brucei infection of mice was very acute with high parasitaemia, in contrast to the low level of parasitaemia usually reported in cattle with T. brucei infection (Ikede and Losos, 1972b; Murray et al., 1979). The T. vivax infection of calves also produced an acute infection with high parasitaemia, but this trypanosome species was reported not to be capable of ordinarily maintaining an infection in mice (Soulsby, 1982; Dirie et al., 1993) except the rodent-adapted strain.

The peroxidized erythrocytes of the T. brucei-infected mice produced greater amounts of TBARS than those of the uninfected controls. This observation suggested that the ability of the erythrocytes of the infected mice to prevent lipid peroxidation was reduced probably by oxidants produced by the trypanosomes as reported by Meshnick et al. (1977) and Baltz et al. (1985). The proliferation and activation of macrophages could also lead to the generation of oxidants through the respiratory burst (Klebanoff, 1982; Schwacha and Loegering, 1992), but the contribution made by the macrophages might be limited since the mononuclear phagocytic system would not be expected to have been fully mobilized until after 7 days pi (Jennings et al., 1974; Esievo and Saror, 1991). The predisposition of the erythrocytes of the infected mice to oxidative damage might accelerate their aging, decrease their deformability and increase susceptibility to fragmentation (Hochstein and Jain, 1981; Knight et al., 1992).

Haemolysins have been identified in the plasma of T. brucei-infected rats at 2-3 days pi (Murray, 1979). The nature of the haemolysins is yet to be fully investigated. The biologically active substances produced by T. brucei which were speculated by Murray and Dexter (1989) to be among the haemolysins were proteases (Londsdale-Eccles and Mpimbaza, 1986), peptidases (Knowles et al., 1987) and phospholipases (Hambrey et al., 1984). But trypanosome derived oxidants may well be part of these haemolysins and could contribute in the pathogenesis of the anaemia caused by T. brucei by increasing oxidative stress on the hosts and predisposing the erythrocytes to lipid peroxidation.

In the acute T. vivax infection of calves, the amounts of TBARS generated by the peroxidized erythrocytes of the control and infected calves did not differ significantly up to day 13 pi despite the sustained parasitaemia and decrease in PCV. Since the ability of bovine erythrocytes to protect themselves against lipid peroxidation did not seem to be affected by T. vivax infection, a number of possibilities were considered as being responsible for the difference in the experimental result from acute T. brucei infection of mice. Firstly, it was probable that T. vivax did not produce comparable amounts of oxidants as previously reported in T. brucei by Meshnick et al. (1977). Secondly, it was likely that the macrophages of the mononuclear phagocytic system in T. vivax infection would produce lower amounts of oxidants than in the T. brucei infection. This reason was supported by the report that the spleen was enlarged 25.9 times in T. brucei-infected mice (Anosa and Kaneko, 1984) and only 3 times in T. vivax infected sheep (Anosa and Isoun, 1983). It was also possible that the respiratory burst of the *macrophages in the T. vivax-infected calves could be depressed, as the infection progressed,* by the phagocytosis of IgG-coated erythrocytes (Loegering and Schwacha, 1991). Thirdly, it seemed that the erythrocytes of the calves had superior antioxidant capacity than those of the mice when they were uninfected. This was deduced from the fact that the erythrocytes of the healthy mice produced significantly ($P<0.05$) greater amounts of TBARS after peroxidation

than the erythrocytes of the healthy calves (Table 6.1). This meant that the ability of normal erythrocytes to prevent free radical-mediated damage was greater in the calves than in the mice.

The decline in erythrocyte GSH concentrations of the T. vivax-infected calves at the first peak of parasitaemia was marginal and indicated only a slight effect on the GSH system. Although the decline in erythrocyte GSH could have been associated with the onset of PCV decrease, because GSH concentrations and PCV correlate positively, the possible effect of the trypanosome infection need to be considered. In acute T. gambiense infection of rats, the blood GSH concentration was reported to be reduced concurrently with increased erythrocyte susceptibility to oxidative haemolysis (Ameh, 1984). Because of the depletion of liver antioxidants such as GSH in T. gambiense infection of rats (Ameh, 1984) and ascorbic acid in T. brucei infection of guinea pigs (Roskin and Nastiukova, 1941), it would seem that the reticuloendothelial systems of the liver and, perhaps, the spleen might be where high concentrations of the oxidants could be released by the trypanosomes and macrophages. The sequestration of erythrocytes in the liver and spleen due to hepatomegaly and splenomegaly respectively (Murray and Dexter, 1988), would lead to sustained exposure of the erythrocytes to the oxidants. The decline of the erythrocyte GSH concentration in T. vivax infection at peak parasitaemia might partially have been as a result of oxidation of GSH as peroxides were reduced by the GSH system. This event was expected to coincide with the peaks of parasitaemia when the macrophage activity in the MPS would be at the optimum because of high parasite particulate load.

The erythrocyte GSH concentrations in healthy Nigerian cattle showed a wide variation (21-.7-135.1mg/100ml RBC). It was presumed that the cattle with higher GSH concentrations might be more capable of handling oxidative stress than those with low GSH concentrations.

TABLE 6.1

Peroxidation values of normal erythrocytes of mice and calves

| Animal Species | Number of Samples | Erythrocyte Peroxidation (Absorbance Units) |
|---|---|---|
| Mice | 10 | $0.05 \pm 0.01^{a}$ |
| Calves | 33 | $0.02 \pm 0.01^{b}$ |

[a,b]Means with different superscripts are significantly different at $P<0.05$.

This was because Tucker and Kilgour, (1973) had reported that erythrocytes of sheep with low GSH concentrations were more prone to Heinz body formation than those with high GSH concentrations.

In the T. vivax-infected calves, there was a tendency for the erythrocytes to show enhanced GSH regeneration with the GSH increasing in concentration despite fall in PCV. The direct relationship between erythrocyte GSH concentrations and PCV in healthy cattle was modified during anaemia and was converted to a semblance of inverse relationship. That is, the erythrocyte GSH increased on days that the PCV decreased especially on day 13 pi. The enhanced GSH regeneration would improve the capacity of the erythrocytes to protect themselves against peroxide load.

Young erythrocytes in circulation were reported to have greater concentrations of GSH than old erythrocytes because of greater activities of GSH metabolizing enzymes (Tucker and Kilgour, 1973). Erythropoietic response was reported to invariably increase during the acute phase of bovine trypanosome infections (Murray and Dexter, 1988), but peripheral indications of such a response may be absent (Anosa, 1988) as was the case in this study where the anaemic calves showed neither reticulocytosis nor macrocytosis. Schalm et al., (1975) suggested that where the stimulus for erythropoiesis was not sudden and strong, reticulocytosis might not occur in cattle. Igbokwe (1986) also suggested that reticulocytosis did not occur in acute T. vivax infection of sheep because of possible splenic sequestration of reticulocytes due to hypersplenism (Anosa and Isoun, 1983) and Crosby (1963) had earlier reported that the spleen might normally sequester reticulocytes until they matured. Therefore young mature erythrocytes were expected to have been in circulation in the T. vivax-infected calves due to anaemic hypoxia that must have elicited some measure of bone marrow response.

The plasma ascorbic acid concentrations (0.35-1.00mg/dl) in healthy cattle as observed in the present study were adequate (Varley et al., 1976) and were not significantly correlated with either blood or erythrocyte GSH concentrations. Therefore, the plasma ascorbic acid concentrations were not expected to have significantly modified blood and erythrocyte GSH concentrations. However, because of the weak negative correlation ($r=-0.35$; $P>0.05$) between the plasma ascorbic acid and blood GSH concentrations, it could be possible for the plasma ascorbic acid concentration to increase when blood GSH decreased. Also, the stress of the infection in the acute disease could lead to large discharge of ascorbate from the adrenal cortex into circulation (McGilvery, 1979). However, if intravascular haemolysis occurred during the acute phase as suggested by Esievo et al., (1984), the ascorbic acid could become prooxidant in the presence of circulating iron (Knight et al., 1992) and promote oxidative processes in the circulating blood. In the infected calves, the interplay of these later events was unlikely since the susceptibility of the erythrocytes to in vitro peroxidation was not affected.

The non-immunological mechanisms in the pathogenesis of the early anaemia in trypanosomiasis might differ in acute T. brucei and T. vivax infections. While peroxidative injury to erythrocytes was possible in acute T. brucei infection of mice, it was unlikely in acute T. vivax infection of calves. The desialylation of erythrocytes by neuraminidase produced by T. vivax (Esievo, 1979, 1983) had been highlighted as an important non-immunological mechanism of erythrocyte damage in the early phase of acute T. vivax infection of cattle (Esievo et al., 1982), but this mechanism would not be expected in acute T. brucei infection because bloodstream forms of T. brucei did not produce neuraminidase (Engstler et al., 1991). These differences indicate the need for intensified research into the peculiarities of the pathogenesis of the anaemia in trypanosomiasis in different parasite-host systems.

## 6.2 CONCLUSIONS

The T. brucei, originally isolated from cattle, produced an acute infection in mice causing anaemia and 54.2% (13/24) mortality before day 8 pi. Infection of calves with T. vivax, isolated from a cow, also produced anaemia between days 6 and 13 pi with an infected animal that was almost moribund on day 13 pi.

Acute T. brucei infection of mice increased the susceptibility of the erythrocytes to in vitro peroxidation, but acute T. vivax infection of calves did not. These observations suggested that the ability of the infected mouse erythrocyte to prevent free radical-mediated lipid peroxidation was reduced whereas that of the infected bovine erythrocyte was not. Therefore, the possibility of peroxidative injury to erythrocytes existed in acute T. brucei infection of mice, but was absent in acute T. vivax infection of calves. In the uninfected controls, the bovine erythrocytes had superior ability to protect themselves against in vitro peroxidation than the mouse erythrocytes.

In apparently healthy cattle, erythrocyte GSH concentrations gave a very wide range. It was presumed that since the GSH system is the primary defense against peroxides (McCay and King, 1980), then the pathogenic effect of trypanosome infections that generate such oxidants may, perhaps, vary widely within the cattle population. The erythrocyte GSH concentrations in the cattle were positively correlated with PCV ($r=0.58, 0.59$). The cattle had adequate plasma ascorbic acid concentrations and there was no significant correlation between the plasma ascorbic acid and the blood or erythrocyte GSH concentrations.

In the T. vivax-infected calves, there was a marginal decline in the erythrocyte GSH concentrations at the first peak of parasitaemia and this was followed by an enhanced erythrocyte GSH regeneration because erythrocyte GSH concentrations tended to increase as the PCV decreased, thus improving the capacity of the erythrocytes to protect themselves against peroxide load.

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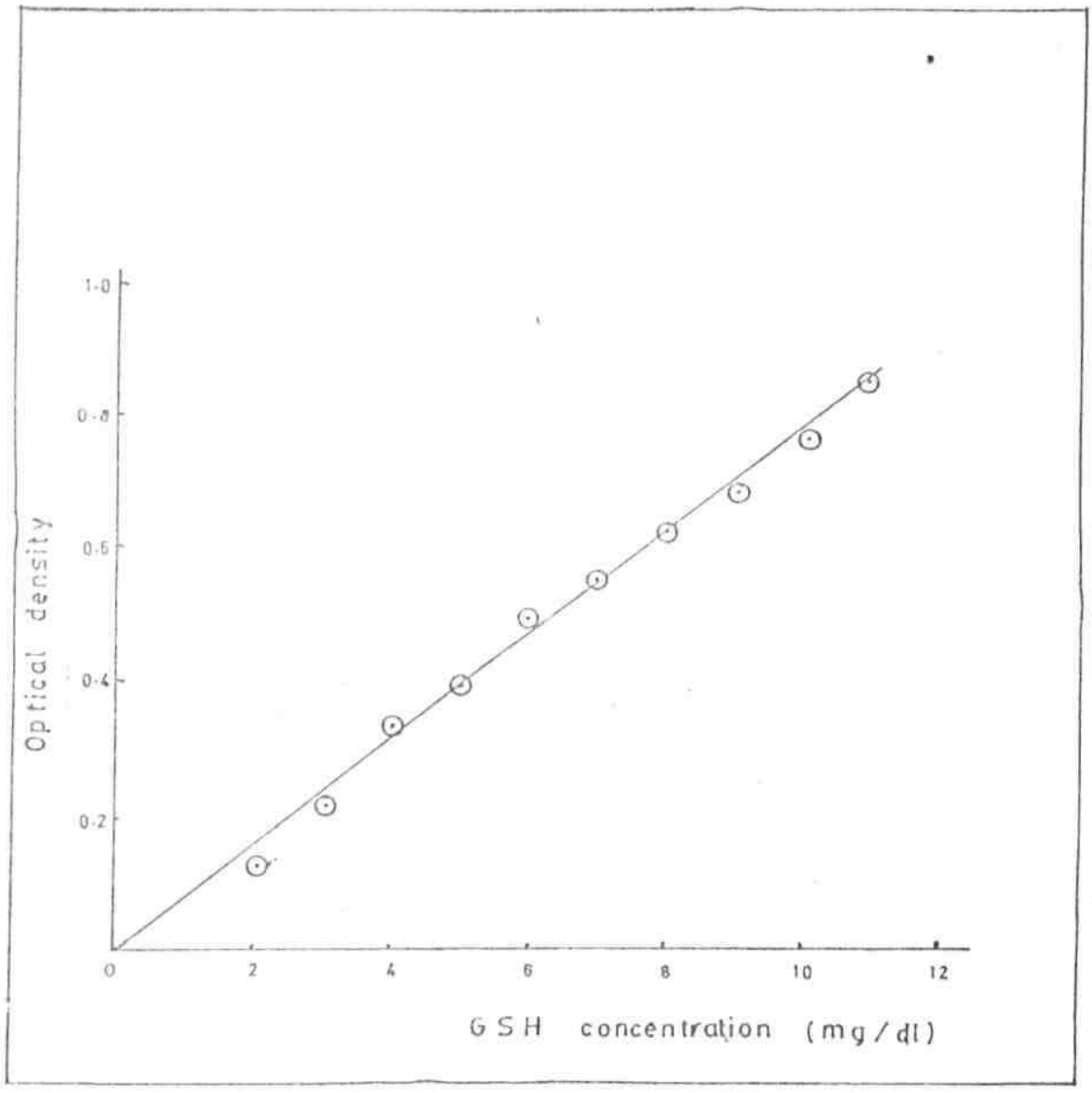
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APPENDIX 4.1

Serial dilution of glutathione (GSH) stock solution

| Tubes | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| GSH stock (ml) | 0.04 | 0.06 | 0.08 | 0.10 | 0.12 | 0.14 | 0.16 | 0.18 | 0.20 | 0.22 |
| Metaphosphoric acid (1%) (ml) | 1.96 | 1.94 | 1.92 | 1.90 | 1.88 | 1.86 | 1.84 | 1.82 | 1.80 | 1.78 |
| Tris buffer (ml) | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| GSH concentration (mg dl) | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| OD | 0.135 | 0.215 | 0.330 | 0.395 | 0.500 | 0.550 | 0.617 | 0.690 | 0.770 | 0.850 |



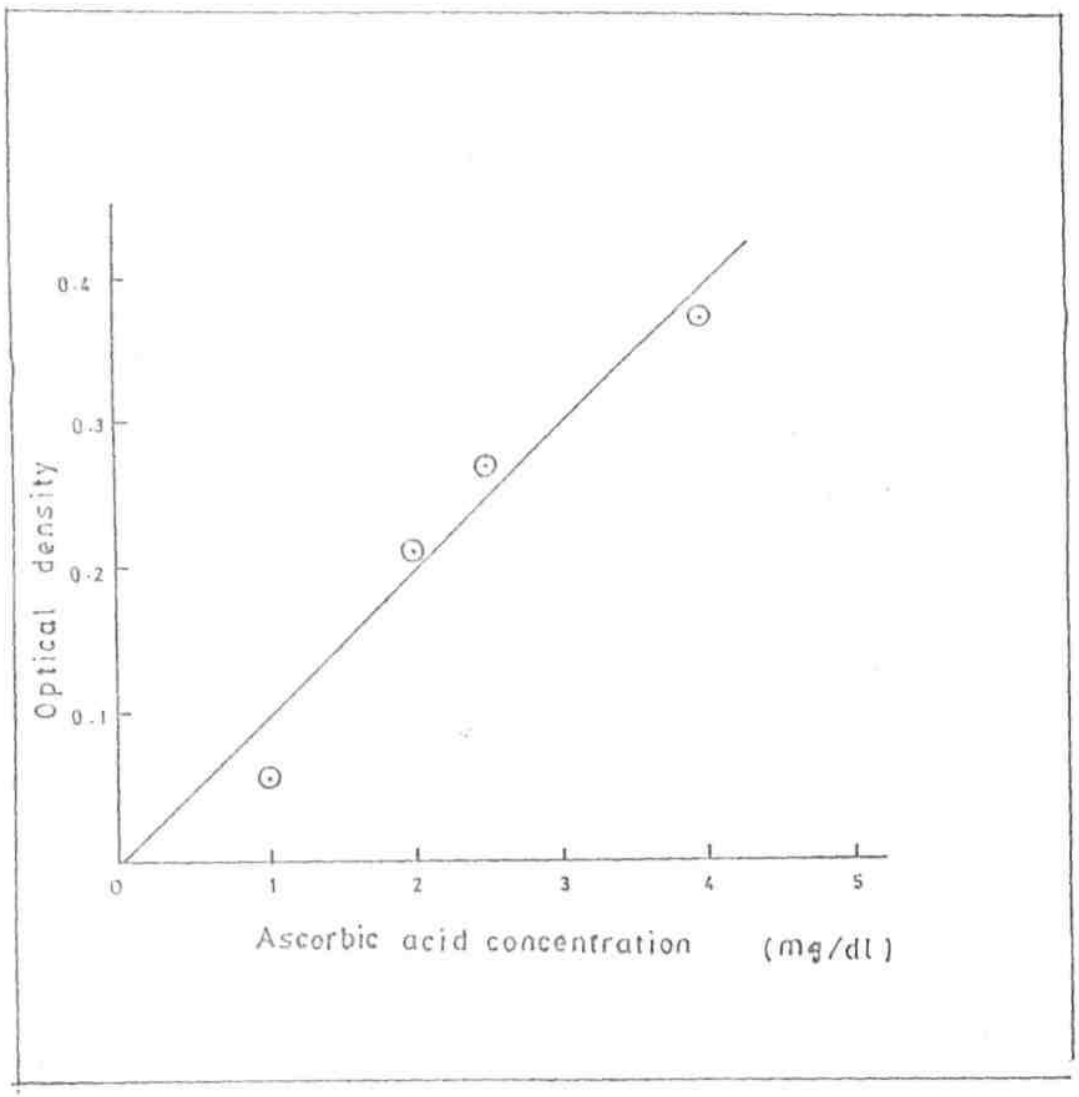

Appendix 4.2 Glutathione standard curve

APPENDIX 4.3

Serial dilution of ascorbic acid (AA) stock solution

| Tube | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| AA stock (ml) | 0.1 | 0.1 | 0.1 | 0.1 |
| 4% TCA* (ml) | 9.9 | 4.9 | 3.9 | 2.4 |
| Final volume | 10.0 | 5.0 | 4.0 | 2.5 |
| AA concentration (mg dl) | 1.0 | 2.0 | 2.5 | 4.0 |

*Trichloracetic acid




Appendix 1.1 Ascorbic acid standard curve

Appendix 4.5

Correlation coefficients (r) and regression equations in the relationship of blood and erythrocyte glutathione concentrations with packed cell volume and haemoglobin concentrations in Nigerian Wadara cattle.

| | r | t-test | Regression equations |
|---|---|---|---|
| Blood GSH Vs PCV | 0.92 | P 0.01 | GSH = -7.82 + 0.70 PCV |
| Erythrocyte GSH Vs PCV (mg 100ml RBC) | 0.59 | P 0.01 | GSH = 20.4 + 0.70 PCV |
| Blood GSH Vs Hb | 0.81 | P 0.01 | GSH = -5.61 + 2.63 Hb |
| Erythrocyte GSH Vs Hb (mg gHb) | 0.52 | P 0.05 | GSH = 0.76 + 0.13 Hb |
| PCV Vs Hb | 0.78 | P 0.01 | PCV = 10.71 + 3.01 Hb<br>Hb = 0.75 + 0.22 PCV |
| Erythrocyte GSH: | | | |
| mg 100ml RBC (A) Vs mg Hb (B) | 0.85 | P 0.01 | A = 9.28 + 18.39 B<br>B = 0.10 + 0.04 A |
| mg 100ml RBC (A) Vs Blood GSH (B) | 0.85 | P 0.01 | A = 24.00 + 1.33 B |
| mg gHb (A) Vs Blood GSH (B) | 0.91 | P 0.01 | A = 0.66 + 1.30 B |

APPENDIX 5.1

Mean erythrocyte parameters, blood and erythrocyte GSH concentrations and erythrocyte peroxidation values in 3 uninfected control calves.

| Days post-infection | PCV (%) | RBC $x10^{12}$ L | MCV (fl) | Blood GSH (mg dl) | Erythrocyte GSH (mg 100ml RBC) | Erythrocyte Peroxidation (Abs. unit) |
|---|---|---|---|---|---|---|
| 0 | 24.7 ± 2.9 | 5.25 ± 0.36 | 47.4 ± 9.0 | 11.9 ± 3.1 | 47.7 ± 7.0 | 0.03 ± 0.02 |
| 2 | 24.3 ± 2.3 | 4.73 ± 0.37 | 51.3 ± 3.3 | 12.4 ± 1.3 | 51.0 ± 3.3 | - |
| 3 | 24.3 ± 2.3 | 4.63 ± 0.11 | 52.5 ± 5.2 | - | - | 0.02 ± 0.00 |
| 4 | 24.3 ± 2.3 | 4.63 ± 0.75 | 52.5 ± 1.2 | 12.6 ± 1.6 | 51.8 ± 4.1 | 0.02 ± 0.01 |
| 5 | 24.3 ± 2.3 | 4.73 ± 0.37 | 51.3 ± 3.3 | 12.1 ± 2.4 | 58.8 ± 5.8 | 0.03 ± 0.02 |
| 6 | 24.7 ± 2.9 | 5.25 ± 0.36 | 47.4 ± 8.9 | 11.19 ± 3.1 | 47.7 ± 6.8 | - |
| 7 | 24.3 ± 2.3 | 4.73 ± 0.37 | 51.3 ± 3.3 | - | - | 0.02 ± 0.01 |
| 8 | 24.7 ± 2.9 | 4.63 ± 0.11 | 52.5 ± 5.2 | 12.6 ± 1.6 | 51.8 ± 4.1 | 0.02 ± 0.01 |
| 9 | 24.7 ± 2.9 | 5.25 ± 0.36 | 47.4 ± 9.0 | 11.9 ± 3.1 | 47.7 ± 6.8 | 0.02 ± 0.00 |
| 10 | 24.3 ± 2.3 | 4.73 ± 0.36 | 51.3 ± 3.3 | - | - | - |
| 11 | 25.3 ± 2.5 | 6.19 ± 0.75 | 41.0 ± 1.2 | 15.1 ± 0.9 | 60.0 ± 6.8 | 0.03 ± 0.02 |
| 12 | 25.3 ± 2.5 | 6.19 ± 0.75 | 41.0 ± 1.2 | 15.1 ± 0.9 | 60.0 ± 6.8 | 0.02 ± 0.00 |
| 13 | 24.3 ± 2.3 | 4.63 ± 0.12 | 52.5 ± 5.2 | 12.6 ± 1.6 | 51.8 ± 4.1 | 0.02 ± 0.01 |

APPENDIX 5.2

Mean erythrocyte parameters, blood and erythrocyte GSH concentrations and erythrocyte peroxidation values in 6 T. vivax-infected calves.

| Days post-infection | PCV (%) | RBC $\times 10^{12}$ L | MCV (fl) | Blood GSH (mg dl) | Erythrocyte GSH (mg 100ml RBC) | Erythrocyte Peroxidation (Abs. unit) | Parasitaemia score |
|---|---|---|---|---|---|---|---|
| 0 | 23.2 ± 3.1 | 5.52 ± 0.75 | 42.2 ± 4.7 | 13.4 ± 3.6 | 58.4 ± 11.4 | 0.03 ± 0.01 | 0 |
| 2 | 23.7 ± 3.1 | 5.64 ± 0.98 | 42.3 ± 2.8 | 13.4 ± 3.6 | 58.4 ± 11.4 | 0.02 ± 0.01 | 1.7 ± 1.4 |
| 3 | 22.5 ± 2.6 | 4.96 ± 0.57 | 45.5 ± 3.9 | - | - | 0.02 ± 0.01 | 3.5 ± 0.5 |
| 4 | 23.2 ± 3.1 | 5.42 ± 0.80 | 42.9 ± 2.5 | 10.2 ± 2.8 | 44.6 ± 14.3 | 0.01 ± 0.00 | 4.0 ± 0.0 |
| 5 | 21.8 ± 2.6 | 5.08 ± 0.71 | 43.3 ± 4.7 | 9.8 ± 3.1 | 44.5 ± 12.8 | 0.03 ± 0.02 | 4.0 ± 0.0 |
| 6 | 19.5 ± 2.4 | 4.62 ± 0.60 | 43.1 ± 5.2 | 11.3 ± 3.5 | 57.2 ± 14.2 | - | 3.7 ± 0.5 |
| 7 | 15.3 ± 3.8 | 4.48 ± 0.55 | 43.3 ± 4.3 | - | - | 0.03 ± 0.02 | 2.2 ± 1.7 |
| 8 | 17.3 ± 3.1 | 3.85 ± 0.57 | 45.0 ± 4.2 | 11.4 ± 3.8 | 66.5 ± 21.1 | 0.03 ± 0.02 | 2.5 ± 2.0 |
| 9 | 18.8 ± 2.8 | 4.61 ± 0.48 | 40.9 ± 4.5 | 11.8 ± 3.5 | 61.9 ± 12.8 | 0.03 ± 0.02 | 2.8 ± 1.5 |
| 10 | 18.2 ± 2.8 | 3.82 ± 0.48 | 47.7 ± 3.5 | - | - | - | 3.0 ± 0.9 |
| 11 | 17.5 ± 3.9 | 4.53 ± 0.84 | 39.3 ± 4.6 | 11.6 ± 2.0 | 66.0 ± 8.2 | 0.02 ± 0.01 | 3.8 ± 0.4 |
| 12 | 19.0 ± 1.9 | 4.68 ± 0.51 | 39.4 ± 4.8 | 10.5 ± 2.7 | 56.0 ± 13.4 | 0.02 ± 0.01 | 3.8 ± 0.4 |
| 13 | 16.3 ± 0.8 | 4.45 ± 0.48 | 37.0 ± 3.4 | 12.9 ± 2.2 | 79.1 ± 14.6 | 0.02 ± 0.01 | 4.0 ± 0.0 |

APPENDIX 6.1

Reagents

Precipitating solution: 1.67g glacial metaphosphoric acid, 0.2g disodium or dipotassium EDTA and 30g sodium chloride in 100ml of distilled water.

Phosphate solution: 0.3M disodium orthophosphate solution in distilled water.

DTNB reagent: 40mg DTNB in 100ml of 1% sodium citrate.

GSH stock solution: 100mg/100ml of 1% glacial metaphosphoric acid.

Norit suspension: 5% activated acid-washed charcoal solution prepared with 5% trichloracetic acid.

DNPH reagent: 2% DNPH solution prepared with 9.0N sulphuric acid to which 250mg of thiourea was added and dissolved. The reagent was stored in brown glass bottle and allowed to stand for 2 days before use.

Sulphuric acid reagent: 65% solution of concentrated sulphuric acid (w/v) was made by adding 70ml of the acid to 30ml of distilled water.

9.0N sulphuric acid solution: 25ml of concentrated sulphuric acid was dissolved in some distilled water and made up to 100ml with water.

10% thiourea solution: 10g of thiourea was dissolved in 50ml absolute ethanol and made up to 100ml with distilled water.

Ascorbic acid stock solution (100mg/dl): 50mg of ascorbic acid was dissolved in 50ml of 4% trichloracetic acid.