ANTIMALARIAL PROPERTIES OF RABBIT TUMOUR NECROSIS SERUM:

IN VIVO AND IN VITRO STUDIES.

(handwritten correction: VITRO above VIVO, VIVO above VITRO)

A Thesis submitted by

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TO MY FAMILY

ABSTRACT.

Plasmodium falciparum multiplication in culture, as measured by tritiated hypoxanthine incorporation, was inhibited in a dose dependent manner by rabbit tumour necrosis serum. The regimen by which tumour necrosis serum is produced caused significant increases in triglycerides and lipid peroxides, with the latter being indicated by the level of malondialdehyde in the serum. Tumour necrosis serum depleted of lipoproteins by aerosil (fumed silica), had no parasiticidal activity. This activity was recovered in the lipoproteins after preparative ultracentrifugation. This suggests that the *in vitro* activity of TNS was principally mediated by lipid peroxides. This was further confirmed by the parasiticidal activities of oxidised artificial lipoproteins made with unsaturated fats.

The multiplication of lethal *P.yoelii* parasites in mice was inhibited by i/p injection of rabbit tumour necrosis serum every other day from the day of infection. This activity was solely present in the non-lipid fraction of tumour necrosis serum, unlike the *in vitro* activity. Ion-exchange chromatography demonstrated that there were at least three different active factors in the serum whose combined activity is either additive or synergistic. Further separation of these factors was not achieved.

In vivo activity of tumour necrosis serum was almost totally reversed by vitamin C, vitamin E or superoxide dismutase, suggesting that the serum acts *in vivo* via oxygen radicals.

Antioxidants had little or no effect on parasite growth in primary infections, whereas they increased parasite growth in vaccinated animals, but did not prevent parasite clearance, therefore suggesting that oxygen radicals are not the main element responsible for controlling normal infections.

The control of parasite growth by tumour necrosis serum via oxygen radicals may be achieved by some or all of the following; increasing the quantity of radicals made, reducing the time for peak release to occur or increasing the length of time of the peak activity.

ACKNOWLEDGEMENTS.

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"Malarial fever or as it is often called, paludism, or intermittent fever is perhaps the most important of all diseases which afflict humanity. Broadly speaking, it is spread over almost the whole of the Tropics, and also extends into many countries which possess temperate climates-being found as far north as Sweden and Canada. Although, happily, it is not a very fatal disease, yet it is generally so prevalent in the countries in which it exists that the sum of the illness which it causes is immense."

Ross (1905)

1.1 BRIEF HISTORY OF MALARIA.

Pre-history: Fossilised mosquitos 30 million years old (Edwards 1923)
Prehistoric man may well have been malaria's first human victim!

1600 B.C.: Symptoms resembling those of malaria described by the
Egyptians in medical papyri. (Ebbell 1937, Leake 1952).

5th century B.C.: First clear description of malaria and prevalence
of the disease in Greece by Hippocrates. (Brock 1929). Description of
the disease being associated with marshes. Use of bednets. (Boyd-Ash
1934, 1941).

2nd century B.C.: Description of symptoms and clinical course of
quartan and tertiary malarias by Celsus and Galen. (Brock 1929,
Spencer 1935).
Malaria may have contributed to the downfall of the Greek Empire.
(Jones 1909).
Marcus Terentius Varro and Lucius Junius Moderatus Columnella both
write about marsh insects spreading diseases and they also describe
preventitive measures including drainage and flooding the marshes
with seawater. (Boyd-Ash 1934, 1941).

1137 A.D.: Emperor Lothar and his German army suffered badly from
malaria after their Roman campaign. (Bispham 1944).

1096-1798 A.D.: Holy crusaders suffered a similar fate. (Garnham
1966, Billings 1987).

1639 A.D.:The Count of Chinchòn (Viceroy of Peru) was treated for
malaria using the bark of a Peruvian tree. (Jarcho 1964). This was
then taken back to Europe.
Possible introduction of *P.falciparum* in S.America by African
slaves. (Sulzer et al 1975, 1978).

1696: Detailed description of the Peruvian bark. Revision of the
connection between marshes and malaria. (Morton 1696).

1717: Lancisi put forward the suggestion that mosquitos may spread
malaria during feeding. (Lama 1863).

1740: Peruvian tree named *Cinchona calsaya* by Linneus after the Count of Chinchòn. (Scott 1939). Bark used by physicians to treat malaria which was prevalent throughout Europe. Also enabled physicians to separate malaria from other disease with similar symptoms.

1820: Alkaloid quinine isolated from the cinchona bark by Pelletier and Caventon. (Scott 1939). Malaria shown to be essentially worldwide (Figure 1.1). Word 'malaria' coined from the Italian for 'bad air'.

1847: Meckel described malaria pigment in living patients. Pigment was later called haematozoin or melanin or paludic pigment. (Ross 1910).

20th October 1880: A. Laveran, a French military officer working in Bône in Algeria reported seeing crescent shaped bodies associated with the paludic pigment in patients blood. These were the gametocytes of the parasite now called *Plasmodium falciparum*. He also observed exflagellation. (Laveran 1880).

1884: Gerhardt proved that healthy persons could be infected by inoculation with blood from patients suffering from malaria.

1886: *P.vivax* and *P.malariae* described by Golgi, (Golgi 1903).

1888: Further blood stages described. (Marchiafava and Celli 1888).

1891: Romanowsky formulates a stain to enable easier recognition of the parasites.

1897:MacCullum observes macrogamete fertilisation by microgametes. Ross finds oocysts in the gut wall of *Anopheles* mosquitos fed on malaria infected people.

1898: *P.relictum,* an avian parasite, was transmitted to healthy sparrows by mosquitos fed on infected birds several days earlier (Ross 1905). Mosquito eradication reduced prevalence of malaria. The Panama Canal could not be built until this occurred.
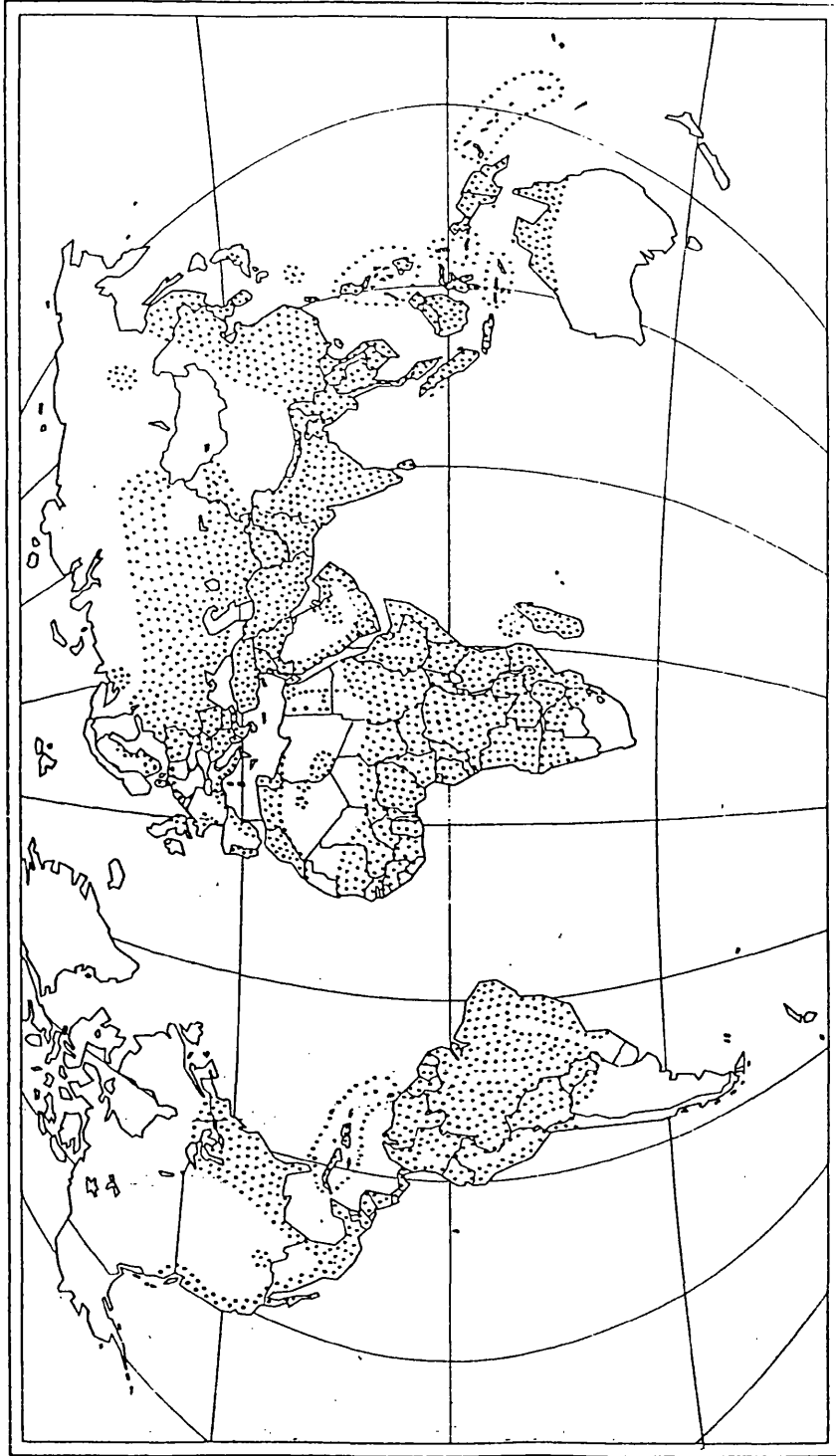
Figure 1.1: Worldwide distribution of malaria in the 19th Century.
(Wernesdorfer 1980)

1914-1918: The only anti-malarial drug was quinine. The shortcomings of this became apparent during the first world war when both sides ran short of the drug.

1922: The fourth human malaria parasite-(*P.ovale*)- was described by Stephens.

1925: Pamaquine synthesised. (Schulemann 1932).

1930: Mepacrine synthesised. (Mauss and Mietzsch 1933).

1934: Chloroquine developed in Germany (Bruce-Chwatt 1985).
Liver stage of malaria described. (James and Tate 1937).

1939: The insecticide properties of 1-1-1-trichloro 2-2-bis (p-chloro phenyl) ethane, (DDT) were discovered, even though it had first been synthesised in 1874. (Russel 1952).

1945: Proguanil produced.

1946: Amodiaquine synthesised.

1948: Liver stage of *P.cynomolgi* and *P.vivax* described by Shortt and Garnham.

1951: Liver stage of *P.falciparum* described. (Shortt et al 1951). Primaquine and pyrimethamine synthesised.

1955: World Health Organisation adopted malaria eradication programs. 30+ countries were cleared of malaria, but S.America, Africa and Asia are still endemic for the disease.

Drug resistance of the parasite increasingly becoming a problem. Insecticide resistance of the *Anopheles* mosquito also a severe problem.

1.2. MALARIA PARASITES.

1.2.1. TAXONOMIC CLASSIFICATION.

1.2.1.1. KINGDOM TO GENUS.

Malaria parasites can be classified according to the following scheme, (Baker 1977):

Kingdom:	Protista
Phylum:	Protozoa
Sub-Phylum:	Sporozoa
Class:	Teleosporea
Sub-Class:	Coccidia
Order:	Protococcidia
Sub-Order:	Haemospororina
Family:	Plasmodiidae
Genus:	Plasmodium

The family *Plasmodiidae* includes organisms undergoing asexual multiplication in a vertebrate host (Schizogony) and sexual multiplication in a mosquito host (Sporogony). The genus *Plasmodium* defines organisms on the basis of one type of asexual multiplication occurring in the parenchymal cells of the liver in the vertebrate host. The other characteristic of this genus is that the mosquito host is of the genus *Anopheles*.

1.2.1.2. SPECIES.

At present 136 species have been described and catalogued. This includes five species parasitic for man. *P.vivax* has a sub-species called *P.vivax hibernans* which is used to distinguish the far N.Hemisphere *P.vivax* from that elsewhere in the world. This is because it remained dormant within humans for the winter months of the year. This species was common in Europe and Russia.

Table 1.1 shows the distribution of the *Plasmodia* species within the animal kingdom. (Garnham and Duggan 1986).

GROUP	No. Species
Man	5
Apes	8
Monkeys	14
Murid Rodents	10
Other Mammals	14
Birds	35
Reptiles	50
Total number	136

Table 1.1: The Number of different species of *Plasmodia* which have been discovered in the different groups of animals. (Garnham and Duggan 1986).

1.2.2. LIFE CYCLE OF *PLASMODIUM.*

Figure 1.2: Generalised life cycle of *Plasmodium.*

1: The female *Anopheles* mosquito takes a blood meal and ingests malaria parasites. The asexual parasite stages are killed. and gametocytes mature into gametes.

2: Male gametes undergoes exflagellation to form about 8 microgametes.

3: Female gamete matures and undergoes the first meiotic division. The nucleus migrates to the cell surface which has a small groove along it.

4: Microgamete fuses with macrogamete via the groove. These form the zygote or ookinete. After 20 minutes the ookinete becomes motile.

5: The ookinete moves towards the stomach wall.

6: The ookinete crosses the peritropic membrane which has formed around the blood meal in the mosquito stomach (Freyvogel and Staübli 1965). It then crosses the the gut wall and rests just beneath the outer gut membrane.

7: The ookinete encysts, possibly with the aid of the crystalloid structures in its cytoplasm (Garnham 1966). The oocyst is now about 6μm in diameter. It then undergoes the second meiotic division within 48 hours of the ingestion of the blood meal.

8: Many mitotic divisions follow.

9: Each nucleus becomes parcelled up in its own amount of cytoplasm.

10:Within 4-15 days depending on the species, the oocyst is mature.

11:The oocysts diameter is now about 50μm and contains the mature sporozoites, (between 40 and 10000). The oocyst ruptures into the haemocoel. Some sporozoites reach the salivary glands where they bore through the outer membrane.

12:When the mosquito next feeds, the sporozoites bore through the gland cells into the ducts. They then become injected into the vertebrate host.

13:Some sporozoites reach the liver parenchymal cells. (Some non-mammalian species may also invade epithelial cells).

14:The sporozoite rounds up in the liver cells and undergoes repeated mitotic divisions.

15:The final schizont may contain between <100 and several million merozoites depending on the species. It ruptures releasing the merozoites into the blood. Some species' merozoites may reinvade the liver, (steps 16,17,18).

19:The merozoite invades a red blood cell via surface receptors and takes some red cell membrane with it into the cell.

20:The merozoite forms a large vacuole and pushes its cytoplasm and nucleus to the edge of its cell envelope. This is the ring form.

21:As the parasite grows it becomes amoeboid and more irregular in shape. This is the trophozoite. Heamoglobin is digested and residues are left which form the haemozoin pigment.

22:Amoeboid activities cease and the vacuole is totally lost.

23:Mitosis occurs and continues until a complete schizont is formed. This is a solid body with a number of nuclei varying between 4 and 72. Each nucleus is parcelled up with cytoplasm to form a merozoite.

24:The red cell disintegrates to release the merozoites into the blood. Upon reinvasion of the red cells some parasites may continue the asexual cycle, (steps 19-24).

25:For reasons not fully understood, the early ring stage may develop into either a male or female gametocyte. The mature gametocytes may completely fill the red cell. The female gametocyte has a compact, deep staining nucleus unlike the male gametocyte's, which is more diffuse and lighter staining.

(Garnhamm 1966, Bruce-Chwatt 1986, Garnham 1984)

MOSQUITO

VERTEBRATE HOST
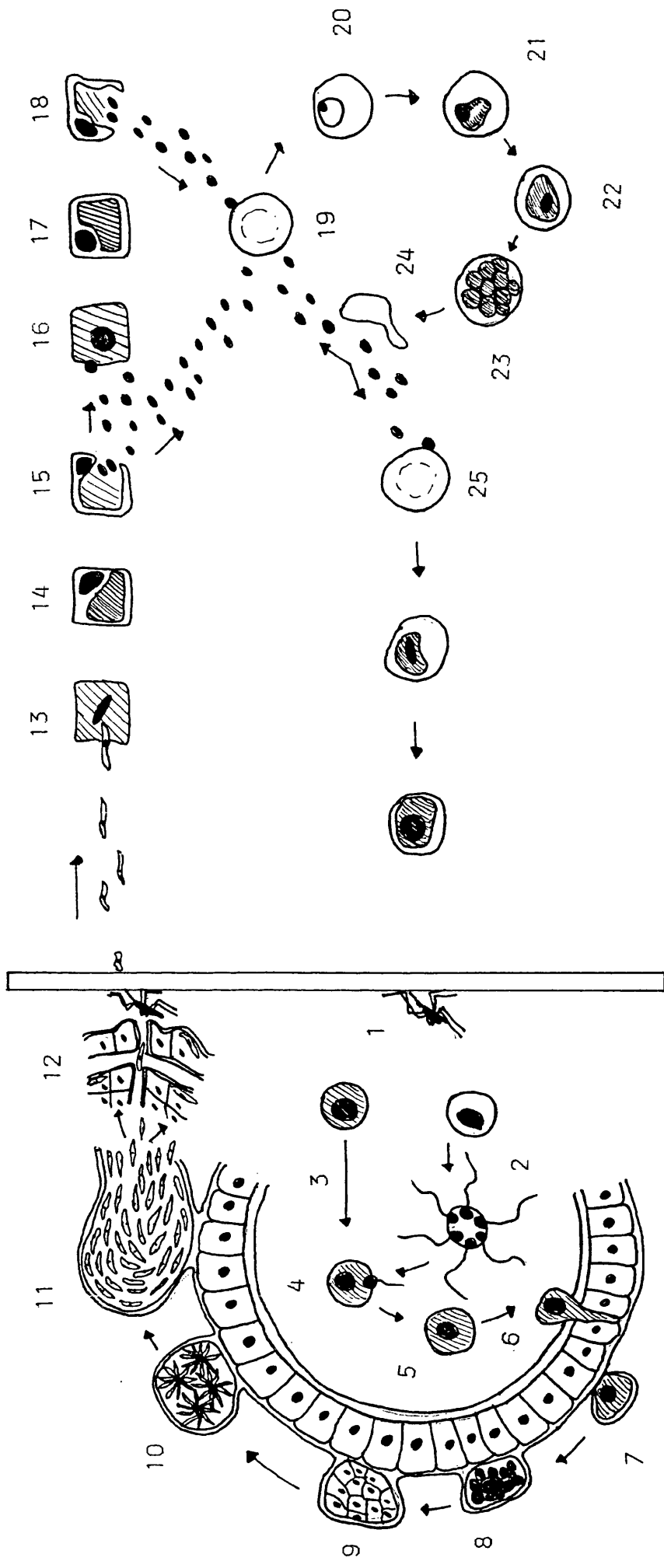
Figure 1.2:   Generalised life cycle of Plasmodium species.

1.2.3 Human Malarias.

1.2.3.1. Worldwide Distribution.

In 1982 the World Health Organisation published figures which showed that 28% of the World's population were living in areas where malaria has never existed or from where it has disappeared without specific antimalarial measures; 18% were living where the disease has been eliminated during recent decades due to antimalarial measures and changes in the environment and health care; 46% live in areas where antimalarial measures have reduced the disease incidence in degrees ranging from slight to almost complete. The other 8% of the population live in areas of nonspecific antimalarial measures. This means that at one time 72% of the World's population lived with malaria, (W.H.O. 1986).

300 years ago the whole of Europe was an endemic area and 100 years ago the U.S.A. was also an endemic area (figure 1.1). But now the disease is generally confined between the tropics of Capricorn and Cancer, except for a band from Turkey to China, (figure 1.3).

Within these areas the estimated number of cases of malaria per year may be more than the 100 million, which is usually quoted, (W.H.O. 1987). It may be as many as 489 million cases, with 234 million *P.falciparum* cases of which 2.4 million are fatal, (Stürcler 1989).

The disease incidence correlates with the distribution of the vector *Anopheles* mosquitos. Therefore endemic areas have been stratified into epidemiological zones depending on the; type of area, mosquito prevalence, living and working conditions and climate, (Kouznetsov et al 1985).

1.2.3.2. Human Parasites.

There are four species and one sub-species of *Plasmodia* which infect humans. They all have their exoerythrocytic stage within the liver of the vertebrate host. The non vertebrate host is the female *Anopheles* mosquito. The five species are: *P.malariae, P.falciparum, P.ovale, P.vivax* and *P.vivax hibernans.* Some of their characteristics are shown in Table 1.2.
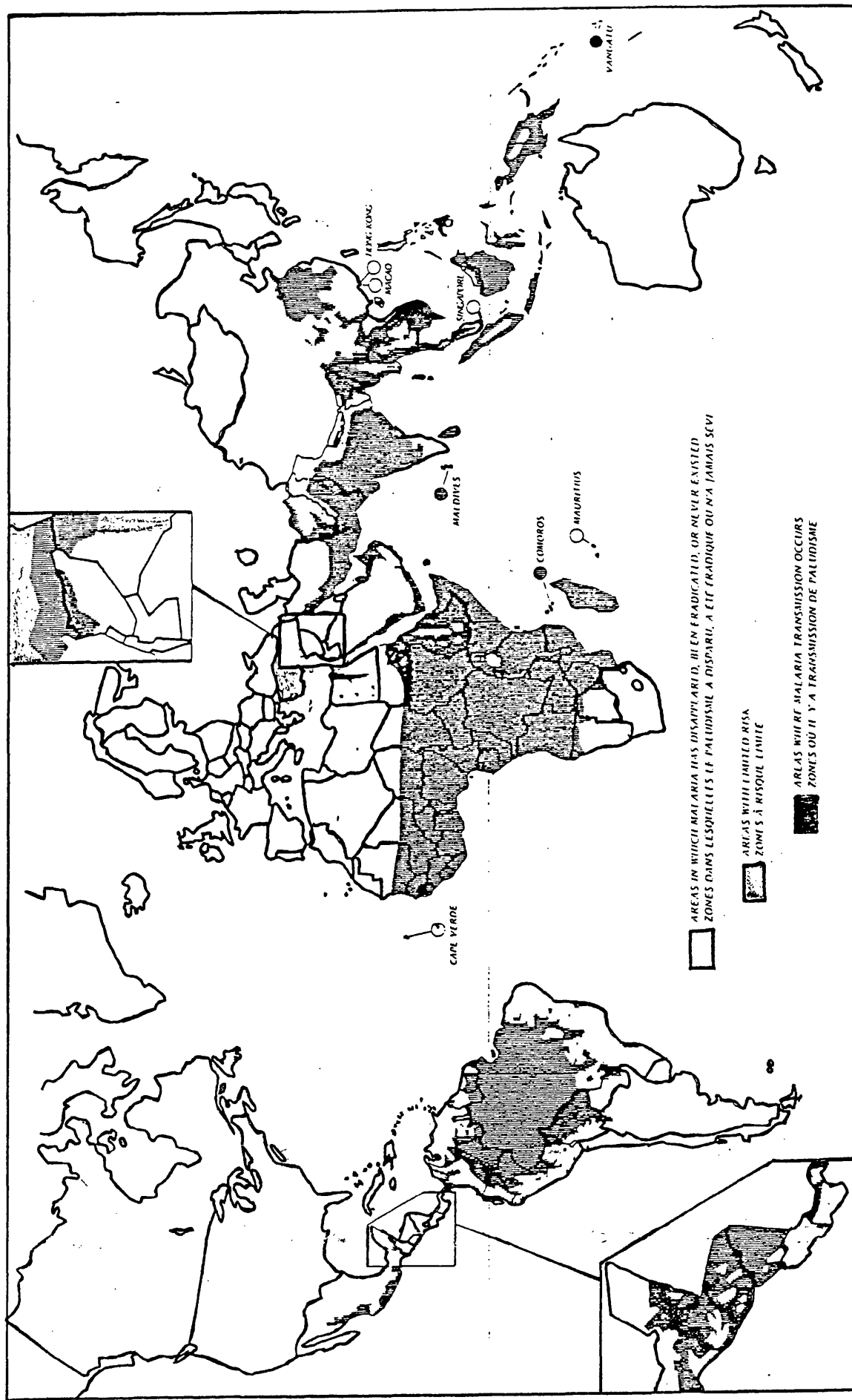
Figure 1.3: Epidemiological assessment of status of malaria in 1985. (W.H.O., 1987).

	P. vivax	P. ovale	P. malariae	P. falciparum
Occurrence	Temperate Zones, Tropics, not common in West Africa.	Mainly Africa	Tropics Sub-Tropics	Tropics Sub-Tropics
Illness	Mild to severe	Mild	Mild	Severe in non immunes
Name	Benign tertian	Tertian	Quartan	Malignant Tertian
Relapses	Frequent	Infrequent		Short
Duration of Disease	Long, 1.5 to 3yrs	Long, 1.5 to 3yrs	V.Long 1.5 to 50yrs	
Prepatent Period	11-13 days	10-14 days	15-16 days	9-10 days
Preerythrocytic	8 days	9 days	13 days	5-6 days
2° exoerythrocytic	Liver		Sometimes Liver	
No. liver merozoites per schizont	8-20 thousand	15 thousand	2 thousand	40 thousand
No. blood merozoites per schizont	12-18	8-10 sometimes 16	8	8-24
Length of asexual cycle	48 hours synchronous	49-50 hours synchronous	72 hours synchronous	<48 hours asynchronous
Red cell preference	Reticulocytes	Reticulocyte	Mature	Indifferent
Max' Parasitaemia	0.4 to 1%	0.18 to 0.6%	0.12 o 0.4%	0.4 to 10% possibly 40%
Time for appearance of gametocytes from first asexual in blood	3 days			10 days
Gametocyte shape	Round	Round	Round	Crescent
Length of cycle in mosquito	9 days 24°C	16 days 24°C	21 days 24°C	11 days 24°C
Length of fever	8 to hours	8 to 10 hours	8-10 hours	16 to 36 hours (or longer)

Table 1.2:   Selected characteristics of the human malaria parasites.

Occurrence: Main areas of the world where the parasites are found today.

Name: These were the names adopted on the basis of the fever periodicity. The first description to use these terms was documented by Hippocrates in the 5th century B.C.

Relapses: reappearance of symptoms of infection following the primary attack. These may be short, less than 8 weeks or as long as 24 weeks.

Duration of disease: Disease duration following a single infection and no reinfection. Parasites may not always be present in the blood all the time. Liver stages with long incubation times may be responsible.

Pre Patent Period: Time from inoculation with sporozoites to being able to detect asexual blood stages.

Pre Erythrocytic Period: Time from inoculation with sporozoites to the release of merozoites from the liver into the blood.

2° exoerythrocytic Stage: Some liver merozoite may reinvade the liver after release from schizonts.

No. of Liver Merozoites: Number of merozoites in 1 schizont produced from 1 sporozoite.

No. of Blood Merozoites: Number of merozoites in 1 schizont produced from 1 asexual stage.

Length of Asexual Cycle: Time from invasion of the red cell to the release of merozoites from the same cell. Synchronicity of the parasites with respect to each other and the stage of the asexual cycle.

Red Cell Preference: Type of red cell invaded as seen in blood films or *in vitro* culture (*P.falciparum*).

Maximum Parasitaemia: Maximum parasitaemias seen in patients. Calculated using 5×10^6 red blood cells per mm^{-3}.

Time for gametocyte appearance: Length of time from the end of the Pre erythrocytic period.

Gametocyte Shape: Apperance of the gametocytes in blood films.

Length of cycle in mosquitos: Time for the appearance of mature sporozoites in the salivary glands from the first ingestion of infected blood.

Length of fever: Length of the febrile symptoms of malaria following the release of merozoites from the red blood cells.

1.2.3.3. Clinical Course of Malaria.

1.2.3.3.1. General Description.

The clinical symptoms of malaria are associated with the blood forms of the parasites, the major symptom being fever. The incubation period from infection to clinical signs varies between the species(Table 1.2). The sometimes long incubation period for *P.vivax*, particularly in N. Europe where malaria was common before the end of the 19th century, may have been an over wintering mechanism. Some of these strains of parasite were classified under *P.vivax hibernans*.

The degree of illness and infection is very dependent on the immune status and drug treatment of the host. In all infections the periodic febrile response is related to the release of merozoites from the rupturing red blood cells. This was first clearly demonstrated in 1905 by Roseneau et al, (Ross 1910). In *P.vivax* and *P.ovale* this occurs every 48 hours or every 3rd day, hence its old name of tertian malaria, and 4th day or every 72 hours for *P.malaria* which was known as quartan malaria. *P.falciparum* is sometimes known as malignant tertian, because its periodicity is 48 hours. But the fever may not always regular because the parasites are not synchronous with each other. *P.falciparum* malaria is also very often fatal unlike the others.

1.2.3.3.2. *P.VIVAX, P.OVALE* AND *P.MALARIAE*.

The paroxysm accompanying these parasites follows the classic pattern of cold, hot and sweating stages, with a body temperature rise to 41°C. Accompanying symptoms include nausea, vomiting, drowsiness and dizziness. These may be more severe in *P.ovale* and *P.malariae*. Two weeks after the onset, the spleen has enlarged enough to be palpable. Anaemia develops as a result of the destruction of red blood cells and may be severe in children, although in *P.malariae* infection this is not usually a problem.

All the asexual stages can be found in blood films. Spontaneous

recovery can occur, but treatment is still necessary to prevent relapses, which can happen after several weeks. During the period between relapses, parasites may still be present in the blood even though there are no clinical symptoms. Relapses of fever occur in *P.ovale, P. vivax* and the former may persist for up to 3 years, without treatment. These usually are not fatal but are important because of the disability they cause during the paroxysms. This is also true for *P.malariae* which may persist for up to 50 years, although the relapses may not be very frequent.

1.2.3.3.3. *P.FALCIPARUM.*

Fever may not always be very high unlike the other human malarias, but accompanying symptoms may be severe, such as vomiting, sweating, nausea and diarrhoea. Coughing may occur because of pulmonary involvement. Both spleen and liver become palpable. Jaundice may accompany these signs.

Treatment can be very effective at this early stage, but if it is delayed, severe malaria may rapidly develop. This is more likely when the parasitaemia reaches 5% or so. Only the early asexual stages can be seen in blood films, because the schizont stage sequesters in organs, and this is what causes the majority of severe symptoms:

a) Cerebral malaria: Patient becomes drowsy and eventually comatose. Other neurological signs include; fits, delirium, intoxication and heatstroke. These have been attributed to the blocking of the brain capillaries by the parasitised erythrocytes. It is still not clear how this occurs but parasite proteins in the red cell membrane may be responsible for binding to the epithelium.

b) Algid malaria: This resembles surgical shock, with pale, cold and clammy skin, shallow breathing and a weak but rapid pulse. The blood pressure is low and vomiting and diarrhoea can occur. The face is drawn and pinched. Adrenal insufficiency may be responsible for death.

c) Gastro-intestinal: Symptoms may resemble cholera with bloody stools containing mucus and pus. Renal failure may occur leading to death from uraemia. Vascular collapse and pulmonary oedema may speed death.

Massive haemolysis can occur because of the high parasitaemia, which may reach 40%. This leads to the condition known as Blackwater Fever, because haemoglobin is passed in the urine. As a result of red cell destruction anoxia can lead to organ damage. In children this is especially pronounced and may be the major cause of death.

During pregnancy a the immune system is suppressed due to hormonal changes. This can result in a relapse, requiring treatment the mother It is also possible for parasites to cross the placenta. The mechanism is not known but pathological changes may be responsible. One important result of a relapse during pregnancy is to cause abortion, miscarriage, stillborn or underweight babies.

1.2.3.4. DIAGNOSIS OF MALARIA.

A definite diagnosis of malaria infection is established on finding parasites in the blood. Any fever in a person living in an endemic area or a visitor to such an area, even for several hours, should be suspected of having malaria.

A diagnosis cannot be made on symptoms alone because of their resemblance to other disease. Therefore thick and thin blood films are made from a finger prick. These are stained using one of several stains: (Giemsa, Wright's, Field's or Romanowsky).

A skilled person can determine the species of parasite which is important for treatment. Also they can detect very low numbers of parasites, which may be as few as 200 parasites per mm^3 of blood. (Approximately 0.004%).

(Bruce-Chwatt 1985).

1.2.3.5. TREATMENT OF MALARIA.

a) *P.vivax, P.malariae* and *P.ovale.*

Chloroquine is the preferred drug. An initial loading dose of 600mg is given to adults followed 6-8 hours later by 300mg and then 300mg every other day up to an accumulative dose of 25mg Kg^{-1}. In children the initial dose is 10mg Kg^{-1}, followed 6-8 hours later with 5mg Kg^{-1} and then 5mg Kg^{-1} every other day. This is adequate for *P.malaria* but for *P.ovale* and *P.vivax* Primaquine must also be given. 1mg must be taken each day for 14 to 21 days in adults to destroy the liver stages. In children the dose is 250μg Kg^{-1}.

However before primaquine administration, the enzyme glucose 6 phosphate dehydrogenase must be measured because in deficient disorders primaquine may cause haemolysis. Therefore the dose is adjusted to 30mg per week for 8 weeks.

b) *P.falciparum.*

Chloroquine sensitive strains: The dosage is the same as for the other human malarias, except in severe cases where 10mg Kg^{-1} is given i/v over 8 hours. This is followed by three 8 hour infusions of 5mg Kg^{-1}. Oral therapy is instituted as soon as possible up to an accumulative dose of 25mg Kg^{-1}. I/m administration can be used at 3.5mg Kg^{-1} every 6 hours or 2.5mg Kg^{-1} every 4 hours.

If chloroquine treatment fails then quinine is used, by mouth if possible. 600mg every 8 hours for 7 days. After this, adults are given 3 tablets of Fansidar (pyrimethamine).

Like chloroquine, parasites are appearing which are resistant to pyrimethamine; in these cases other drugs have to be used.

(British National Formulary 1989).

1.2.4. RODENT MALARIA.

The first species of *Plasmodium* to be found in rodents was discovered in thicket rats (*Thamnomys surdaster*) near Elizabethsville in Zaire by Drs. Vincke and Lips (Van den Berghe 1948). The parasitaemia reached 50% after 11-15 days post inoculation. Wild rats (*Rattus rattus*) and mice were also susceptible to this parasite which was named *P.berghei*. Further studies showed that this parasite could survive in a wide range of rodent hosts, although the hosts had different degrees of susceptibility. Mice were more susceptible and sometimes died.

In 1952 Vincke isolated a malaria parasite from rats. It was sent to J.Rodhain in Antwerp, who found that it was a new species. He named it *P.vinckei*, (Rodhain 1952). This was a lucky find as Garnham pointed out, because all other *Plasmodia* isolates from that region of Zaire proved to be *P.berghei*.

It wasn't until 1965 that Vanderbergh and Yoeli were able to describe fully the in laboratory transmission of *P.berghei*. This long lag period may have been due to difficulties in maintaining the mosquitos and achieving high enough sporozoite rates. In the same year the pre erythrocytic stages of *P.berghei* were described, (Yoeli and Most 1965) which mature in 50 hours.

At the same time Landau (1965) described a new parasite which he named *P.chabaudi*.

Because many rodent malaria isolates were made, in 1974 Killick-Kendrick made an attempt to tidy up the rodent parasite classification. He grouped them into 3 groups. To the original berghei and vinckei groups was added a new one made up of 3 sub species of berghei like parasites isolated from the lower Guinea forest called *P.yoelii*. A fourth group was also added, containing *P.chabaudi*. The discovery of these parasites has been invaluable to malaria research, especially in two areas: Immunology and chemotherapy.

Avian parasites were the standard model but rodent malaria was taxonomically closer to human malarias. However unlike human rodent malarias, rodent malaria is restricted to the continent of Africa, despite survey over the world. In Africa these parasites are limited to the central areas and the southern Sahara, (Figure 1.4).

Table 1.3 shows some of the characteristics of the parasites.
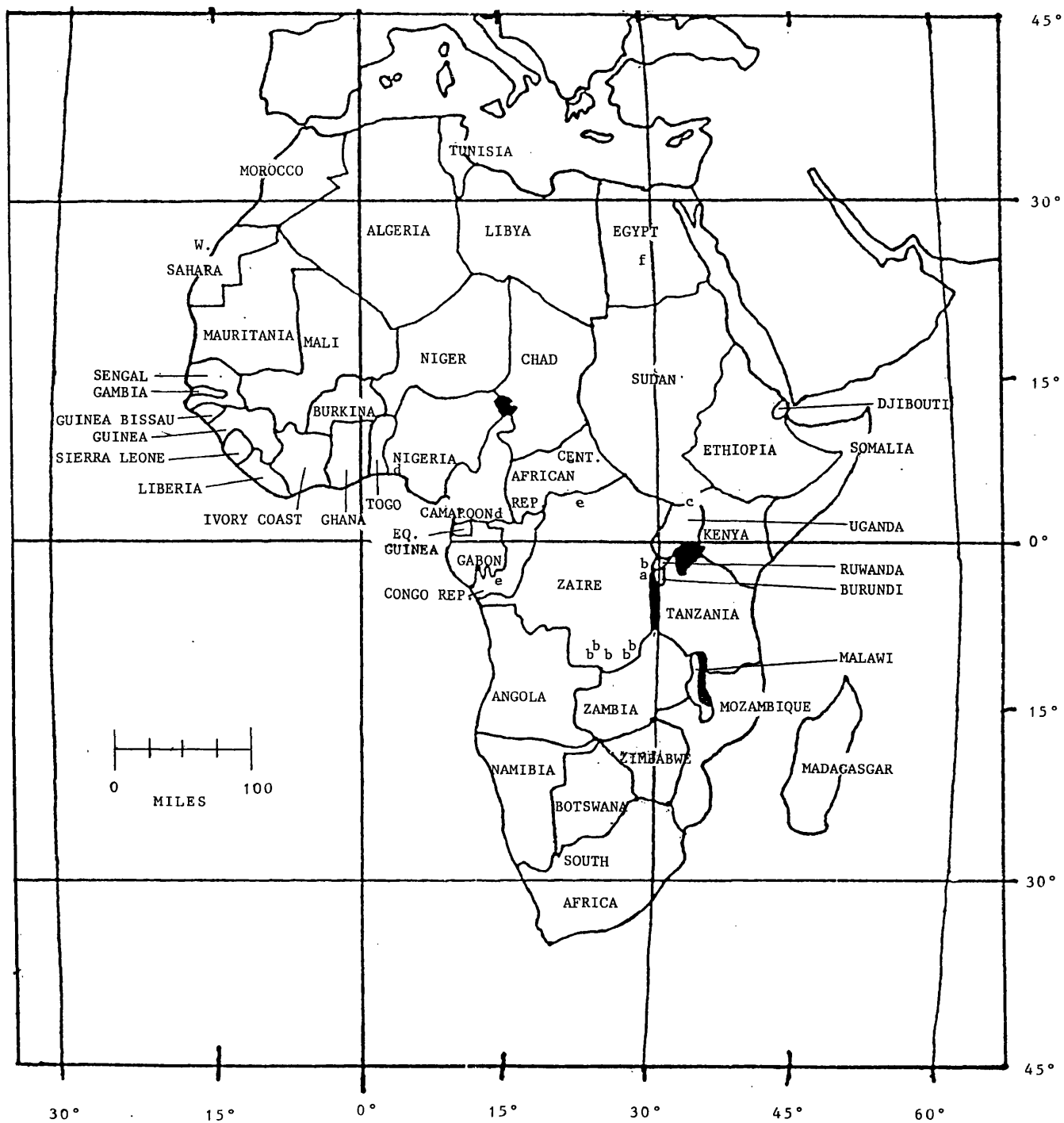
Figure 1.4: Primary locations in Africa where rodent malaria parasites have been isolated.
a: *P.berghei* and *P.vinckei*.
b: *P.berghei*.
c: *P.yoelii* (?).
d: *P.yoelii* and *P.vinckei*.
e: *P.yoelii*, *P.vinckei* and *P.chabaudi*.
f: *P.aegyptensis*.
from Killick-Kendrick (1978).

	P. berghei	P.vinckei	P.yoelii	P.chabaudi
Relapses	C H R O N I C I N F E C T I O N O F L O N G D U R A T I O N			
Duration of Disease	In the natural host (Thicket rat) the infection is life long. In the non-natural host the animals may quickly die or become immune.			
Preerythrocytic	50 hours	usually)60 hours	50 hours	)50 hours
No; liver merozoites per schizont	2-10 thousand	9 thousand	2-10 thousand	18-20thousand
No. blood merozoites per schizont	6-18	6-16	6-18	4-10
Length of asexual cycle	22-25 hours asynchronous	24 hours asynchronous	22-25 hours asynchronous	24 hours synchronous
Red cell preference	Reticulocytes	Mature	Reticulocytes	Mature
Gametocyte shape	Round	Round	Round	Round
Length of cycle in mosquito	14 days 19-21°C	9-10 days 22-24°C	10-13 days 24-26°C	10-11 days 24-26°C

Table 1.3: Selected characteristics of rodent malarias, for an explanation of terms see legend to table 1.2. (Landau and Boulard 1978)

1.2.4.1. COURSE OF INFECTION.

In the natural host the parasites may persist for 24 months, whereas in experimental animals (mice) it may be less than 6 months depending on the animal and strain.

Lethal *P.berghei* and *P.yoelii* parasitaemias grow rapidly and kill the host within 4-5 days from the detectable appearance of parasites in the blood. In the animals which survive, the parasites may persist at a high parasitaemia (approx 50%) for 10-20 days before clearing, (figure 1.5a,c). Non-lethal strains can take 15-20 days to reach 50% parasitaemia from their detectable appearance and before clearing their parasites, (figure 1.5b).

P.chabaudi infection increase similarly to lethal *P.yoelii* and P.berghei infections. Then within 48 hours the parasites are cleared from the blood. Some animals die at this point, but in survivors about 20 days later a recrudecence may occur, with much lower parasitaemias. This may or may not occur a second time, (Figure 1.5c).

Mice infected with any of these parasites gradually become shabby in appearance as the infection increase. Their coat becomes scruffy and they become less active and remain hunched together in one place. But soon after the parasites clear the mice become more active, although their coats may look scruffy for a few days. The eyes and skin of white mice loose their pink colouration, because of the parasite induced anaemia. They are also cold to the touch, (plate 1.1).

Plasmodium yoelii (YM) (lethal variant)
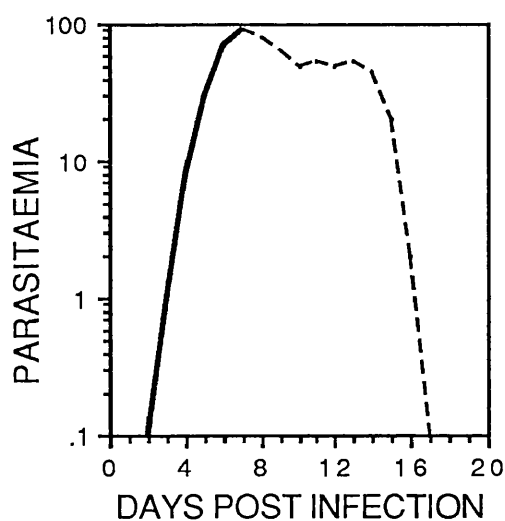
Plasmodium yoelii 17X (non-lethal variant)
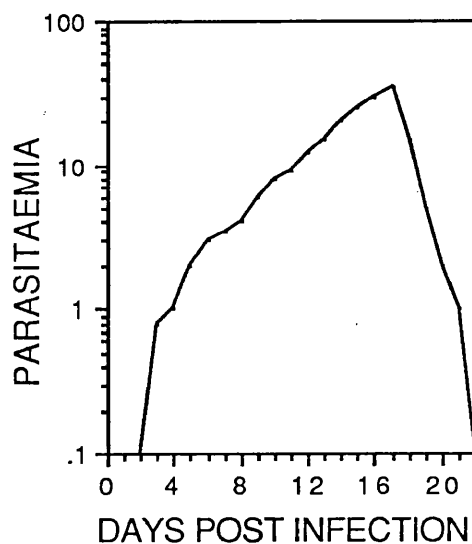
Plasmodium berghei Anka strain
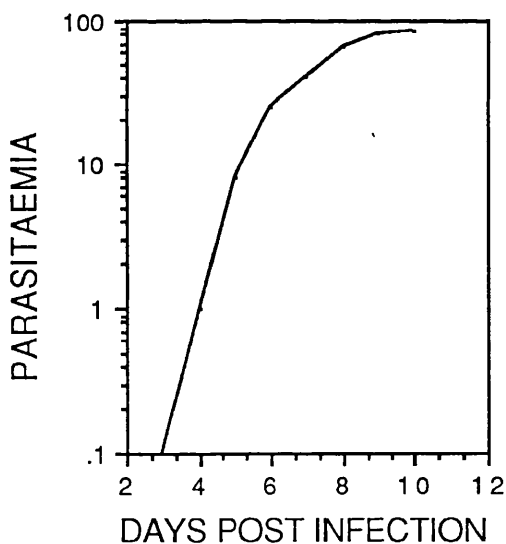
Plasmodium chabaudi
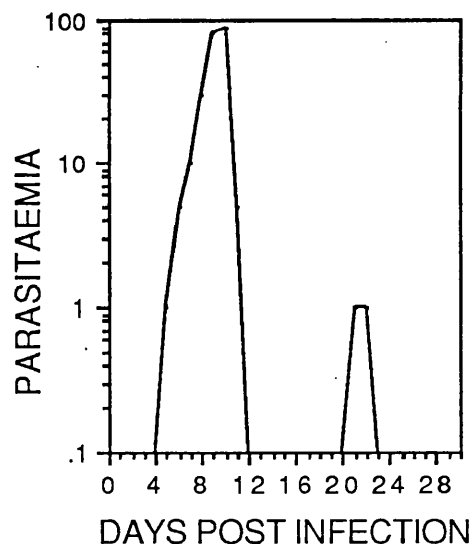
Figure 1.5: Course of *Plasmodia* infections in mice injected i/v
with 10⁴ parasitised red blood cells.

a: Course of infection in mice infected with a lethal strain of
P.yoelii 17X. The bold line indicates the parasitaemia for all
animals. The following dotted line shows the parasitaemia in mice
which survive the infection.

b: Course of infection in mice injected with a non-lethal strain of
P.yoelii 17X.

c: Course of infection in mice injected with *P.berghei* Anka.

d: Course of infection in mice injected with *P.chabaudi*.

(TAVERNE et al 1986 Fig a,b,c ; TARGETT 1984)

Plate 1.1: Appearance of Tuck No.1 mice during the course of a lethal *P.yoelii* infection.

a: Before infection and up to 5 days following infection with 10^4 parasitised red blood cells i/v. Mice are very active.

b: Day 6 to day 10 of infection. Mice are very inactive. Ears and tails become white because of anaemia. Death may occur at this stage.

c: From day 10 onwards the mice begin to recover and become more active.

1.3. Resistance to Malaria.

1.3.1. Non-Immunological Mechanisms.

P.vivax and *P.knowlesi* cannot invade red blood cells lacking Duffy blood group molecules, (Miller et al 1976, Spencer et al 1978).

Other red cell membrane molecules have been shown to be involved in parasite invasion (Pasvol 1984, Breuer 1985), and soluble forms can block parasite entry (Hermentin et al 1987a,b)

Persons homozygous for the Sickle cell trait (Hb S/S) are protected against malaria. This can be demonstrated *in vitro* (Pasvol et al 1978). Two other heamoglobins can also protect against malaria: HbF (foetal haemoglobin) (Weatherall et al 1979) and HbE (Livingstone 1971).

Glucose-6-phosphate dehydrogenase (G6PD) variants can affect parasite growth (Siniscalo 1961, Friedman and Trager 1981) especially if the enzyme is deficient (Etkin and Eaton 1975).

Thalassaemia leads to premature lysis of the red cells which interrupts parasite growth, (Siniscalo 1961, Oppenheimer et al 1984).

Hereditary elliptocytosis red blood cells are also unable to support optimal parasite growth (Kidson et al 1981). Epidemiological evidence supports this claim (Serjentson 1977).

1.3.2. Immune System

Robert Koch in 1900 described how the prevalence and density of parasitaemia waned as age increased in people living in endemic areas (Ross 1910). From the age of 6 months to 5 years, humans are most at risk of dying from malaria, from then on the severity of the disease gradually decreases despite continual exposure to the parasites (McGregor et al 1956). Only after this stage does the parasitaemia decrease, so that by adult life residents in endemic areas may have an undetectable parasitaemia or no clinical signs for much of the time. This two stage "immunity" was first described in 1924 by Christophers who considered the first stage to be characterised by a high parasite load and severe illness lasting about two years, and then a decrease in parasitaemia and illness which then persists for many years. The first stage was considered by Sinton in 1939 to be controlled by an antitoxic immunity and the second stage by an antiparasitic immunity which is unstable. If an immune person moves from an endemic area to a non endemic area for as little as 6 months or they are drug treated then they become susceptible to severe malaria (Maegraith 1974).

This can be contrasted with rodent malaria where true sterilising immunity can be achieved by treating a lethal infection (Cox 1964), by vaccination (Playfair et al 1977) or by allowing, a non lethal infection to take its course.

The immunity is both species specific and stage specific.

1.3.2.1. Species Immunity.

This can be best demonstrated in rodents or monkeys where complete protection can be achieved with some *Plasmodium* species. Mice infected with *P.chabaudi* would survive an otherwise lethal infection with *P.vickei* but were not protected against *P.berghei*.

P.berghei would protect against *P.yoelii* but the reciprocal did not give complete protection (Cox and Voller 1966). These observations correlate to some extent with the cross reactivity patterns of antibody (Nussenzweig et al 1978).

This diversity between species is expected. More importantly for vaccines it occurs within species (Hommel et al 1983, Howard

1984). If *P.berghei* infections were suppressed by drug treatment, parasites from repeated recrudescences could be isolated from the animals to be injected into recovered or naive mice. The resulting infections showed that *P.berghei* was immunogenically variable (Cox 1957,1959,1962). Some antigens are highly geographically diverse (Coppel et al 1984) whereas some are not. One example of conservation is a portion of the circumsporozoite proteins, which is claimed to be present worldwide in all isolates (Zavala et al 1984).

Now that some of the immunodominant antigens have been sequenced at the genomic and amino acid levels, the basis for antibody reactivity diversity is beginning to be understood.

In view of the above, it will be appreciated that a "universal" vaccine is a fairly unlikely possibility.

1.3.2.2. STAGE IMMUNITY.

Mice vaccinated with sporozoites are resistant to infection with sporozoites, but not with blood stages (Nussenzweig et al 1969). Similarly immunity can develop to gametocytes (as shown by decreased infectivity to mosquitos) without affecting the asexual parasites (Hawking et al 1966). Antibodies to surface antigens of merozoites can block reinvasion of red blood cells. This can be demonstrated both *in vivo* and *in vitro*. These antibodies do not affect the intra-erythrocytic stages but only the inter-erythrocytic stages (Cohen and McGregor 1963).

1.3.3. CHANGES WITHIN THE IMMUNE SYSTEM.

1.3.3.1. ANTIBODY.

IgM, IgG, and IgA plasma concentrations increase following infection with malaria parasites (Collins et al 1971). Most of the antibody is parasite specific but antibodies to red cells, lymphocytes, complement, nuclear components and immunoglobulin can also be demonstrated (Deans and Cohen 1983). IgM and IgA responses

are transient whereas the IgG response is persistent and involves all the four subclasses in humans (Facer 1980).

Although the protective effect of immunoglobulin was first reported in 1917 by Sotiriadès it was not until 1961 that Cohen et al convincingly demonstrated that immunoglobulin could protect after passive transfer of maternal immunogl$\overset{b}{\wedge}$ulin to sick children. This has also been demonstrated in rodents. Passive transfer of immune sera to sporozoites can increase their clearance rate (Nussenzweig et al 1972) and decrease their infectivty (Nussenzweig et al 1969). The primary antigen is the coat protein or circumsporozoite protein (CSP). A monoclonal antibody raised to this molecule can protect mice (Yoshida et al 1980). The CSP is being investigated as a possible vaccine candidate.

Merozoites also stimulate protective antibodies (Cohen et al 1961, Diggs and Osler 1975). *In vitro* studies have shown that the antimerozoite antibodies do not inhibit the intracellular growth of the parasite but block the entry of the merozoites into the red cell (Miller et al 1975, Mitchell et al 1976). This immunity is not complement dependent (Williams et al 1975). In the field, antimerozoite antibody inhibitory titres correlate with immunity in 70-80% of sera studied (Cohen and Butcher 1970).

Inhibitory antibodies have also been demonstrated to other stages of the parasite. Many antigens have been identified and cloned and used to immunise experimental animals, either as whole molecules or as peptides. Table 1.4 shows some of the major antigens used in this way.

Apart from parasite agglutination, antibody causes complement fixation, during a malaria infection without affecting the parasites (Fogel et al 1966). Another possible role of antibody is in antibody dependent cellular cytotoxicity. The cells involved may be killer cells, monocytes or polymorphonuclear cells (Greenwood et al 1977). Opsonisation is more likely (Tosta and Wedderburn 1980) and like complement it may not be an important parasiticidal mechanism. Some parasite antigens stimulate the production of antibodies which upon passive transfer of antibodies do not confer protection. The 230KD *P.yoelii* merozoite antigen is a good example (Freeman and Holder 1983). Nevertheless this antigen is an effective vaccine in mice and will protect them (Holder and Freeman 1981). This protection correlates with T-cell immunity (Playfair et al 1985).

Antigen	Stage	reference
40 KD (CSP)	Sporozoite	Nussenzweig et al 1969 Nussenzweig et al 1972 Yoshida et al 1980
235 KD	Merozoite	Freeman et al 1980
185-200KD	Merozoite	Holder and Freeman 1984 Siddiqui et al 1987
155 KD (RESA)	Merozoite	Collins et al 1986
Knobs	Trophozoite/ Schizont	David et al 1983
155Kd, 83KD, 55KD, 35Kd	Trophozoite/ Schizont	Patarroyo et al 1987
220 KD (S-antigens)	Schizont	Anders et al 1983 Wilson 1980
	Schizont	Vincent and Wilson 1980
230KD, 48/45KD	Gametocytes	Carter et al 1988
25KD	Ookinete	Carter et al 1988

Table 1.4: Some of the major antigens from *Plasmodia* species which
have been isolated and shown to stimulate protective antibodies.
CSP: circumsporozoite antigen; RESA: ring infected erythrocyte
surface antigen.

1.3.3.2. T-CELLS.

Many changes have been reported in T-cell numbers in malaria infections (Playfair 1982).

The production of antibody in most circumstances involves the interactions of B-cells with T helper cells. A role for T-cells via delayed type hypersensitivity (DTH) has also been proposed (Weinbaum et al 1976, Finerty and Krehl 1976). This implies that T-cells are directly parasiticidal. Indeed recent data supports this hypothesis in mice depleted of either T-helper cells or T-suppressor/cytotoxic cells (Süss et al 1988). Further to this it has been shown that T-cell clones can transfer immunity (Brake et al 1986). The antigens involved are not as well defined as with antibodies but they are being described. The 230 KD merozoite as described above confers immunity correlating with DTH (Playfair et al 1985).

Sporozoite immunity is dependent on CD8+ cells especially against the intrahepatic stage (Schofield et al 1987, Weiss et al 1988, Romero et al 1989).

The specificity of the CD8+ cells has been studied against the CSP. The epitope is located within the variable region of the molecule. Therefore one form of the molecule will not vaccinate against all the isolates and strains of *P. falciparum*. (Kumar et al 1988).

T-cell clones have also been described which can transfer immunity against gametocytes (Carter et al 1988).

All these experiments have been done in mice with intact B-cells, although some experiments have showed that they are not involved. This has been further strengthened by using B-cell deficient mice, who could still control an infection (Grun and Weidanz 1981). In this case T-cell responses are evidently more than just T helper cell responses. Apart from T cytotoxic cell responses, T cells can release many cytokines. These could act on other cells which in turn become cytotoxic to the malaria parasites.

1.3.3.3. NON-SPECIFIC EFFECTOR MECHANISMS.

1.3.3.3.1. GENERAL.

The first real description of a non-specific mechanism was reported by Taliaferro and Taliaferro in 1944, who described the death of *P.brasilianum* within red cells before phagocytosis. These parasites were called "Crisis forms".

This effect could be induced by agents unrelated to malaria but which could act on the immune system. These agents are related by being mostly microbial in origin (Strickland and Hunter 1980). They can be divided into two categories; whole microorganisms which are administered a week or more before infection with malaria parasites or purified products of organisms which can be given 24 hours or less before infection. Some plant mitogens can have a similar effect.

Commonly used organisms are *Propionibacterium acnes* (formerly *Corynebacterium parvum*), (Nussenzweig 1967, Cottrell et al 1977) or *Mycobacterium bovis* BCG, (Clark et al 1976). These can protect the animals against a range of organisms, including malaria. Protection against *Babesia* is perhaps expected, because they are in the same family as malaria parasites (Clark et al 1976), but protection extends against other microorganisms including bacteria (Shilo 1959).

The effect of these microorganisms was to delay the onset of parasitaemia by 1 to 2 days and reduce the mortality of the mice in lethal infections. The maximum parasitaemia was not necessarily reduced, nor was the length of infection.

Concanavalin-A (Con-A) and lipopolysaccharide (LPS) given i/p at sub lethal doses 24 hours or less before the parasites had similar effects to the above (Cottrell et al 1977, MacGregor et al 1969, Martin et al 1967). The questions raised were; how are these organisms/molecules affecting the animal so that it is protected against infection?

They might stimulate polyclonal antibody production and specific antibody production. However antibody is not detected against malaria parasites during the period of greatest effect (Clark et al 1976) and more importantly the passive transfer of serum from animals treated with endotoxin alone, does not protect against the parasites (MacGregor et al 1969). The role of T-cells cannot be totally ruled out, even though nude mice are protected with *P.acnes* but not Con-A (Cottrell et al 1977).

All this points to the reticulo-endothelial system, especially macrophages. Macrophages have a direct effect on malaria parasites, which is important in non-immune animals. Blocking macrophage activity in *P.yolii* infction with silica increases the parasitaemia, whilst enhancing macrophage activity with C.parvum delays the parasitaemia(Playfair 1979, Playfair and DeSouza 1979). BCG also has a similar effect. This can be reduced by using silica (Clark et al 1976). The remaining activity may be due to soluble factor release because silica does not immediately kill macrophages but blocks phagocytosis (Levy and Wheelock 1975). Soluble factors released from activated macrophages may be more important than antibody in a primary malaria infection because specific antimalarial antibody is not detected in BCG treated, malaria infected mice (Clark et al 1976). However in vaccinated this this may not be true, because silica can stimulate an increase in antibody levels and cause faster clearance of the parasites, whilst *C.parvum* treatment reduces the antibody levels and delays clearance (Playfair and De Souza 1979). Clearly the macrophages are important in malaria infections in a dual role, by direct action on the parasite and by the effects on the immune system. In normal mice the former is more important and non specific stimuli such as *C.parvum* and BCG increase this activity.

This regimen for protecting mice against microorganisms has also been employed in mice with certain forms of tumour. The effect of LPS *in vivo* on tumours was known since 1944 (Shear), but in 1975, Carswell et al described a serum factor which was optimally produced in animals 2 hours following LPS injection, 14-21 days after inoculation with BCG. This dose of LPS would normally be non-lethal but BCG primed animals died in shock about 2 hours after the LPS.

The factor present in the serum from these animals was called Tumour Necrosis Factor (TNF) and the serum, Tumour Necrosis Serum (TNS). In 1981 TNS was first shown to be toxic to *P.yoelii in vitro* (Taverne et al 1981), as well as *in vivo* (Clark et al 1981, Taverne et al 1982). *P.falciparum* is also succeptible to TNS *in vitro* (Haidaris et al 1983, Wozencraft et al 1984). TNF was the favoured candidate molecule, especially after separation experiments appeared to correlate tumour killing activity *in vitro* with parasite killing *in vitro* (Taverne et al 1984). Once the TNF molecule was cloned (Pennica et al 1984, Fransen et al 1985) it became possible to know whether TNF is parasiticidal. The answer was very simple, TNF did not have any effect on *P.falciparum in vitro* (Jensen et al 1987, Hviid

et al 1988), or *P.yoelii in vitro* (Taverne et al 1987) even at
concentrations exceeding those in TNS. However TNF did have an effect
in vivo (Taverne et al 1987) which was similar to the effects of
BCG or *P.acnes in vivo*, but not as strong as that of TNS. Therefore
it appeared that TNS contained other molecules which were
parasiticidal *in vitro* and *in vivo* or stimulated the production
of parasiticidal molecules *in vivo*.

1.3.4. NATURE OF THE PARASITICIDAL MOLECULE IN TNS.

Because of the method used to make TNS, the parasiticidal
molecules must be synthesised or released into the blood within the
two hours following LPS stimulation. This would probably mean that
only those cells directly acted upon by the LPS will be involved. As
the evidence suggests, these will primarily be those cells of the
reticuloendothelial system especially macrophages. So far about 100
substances have been described to be released from macrophages
following stimulation (Nathan 1987a). These range in size from 32D
(superoxide) to 440 KD (fibronectin) and in biological activity from
cell death to cell growth.

Table 1.5 list some of the molecules released by macrophages.

Table 1.5: Some molecules which may be present in TNS.

CYTOKINES.

Interleukin-1 (IL-1) (Auron and Webb 1987): Three forms; IL-1α, IL-1β, and membrane bound IL-1α. Soluble forms 17KD. All share 20% homology and are non glycosylated. Primarily produced by macrophages. Resting cells have little or no IL-1 mRNA. Upon stimulation immediate production of message which is maximal after 1 hours. Precursor protein present after 30 mins and membrane forms after 30 to 60 mins. Soluble IL-1 detectable after 1 hour and peaks at 4 hours. IL-1β is the major component of IL-1 activity. Functions include; T-cell activation, antibody secretion (in synergy with B-cell factors), induction of fever by inducing prostaglandins which then act on the hypothalamus. If IL-1 is present in TNS it could augment a response *in vivo* by quickening the time for the response and also increase the response. TNS given over a period of time would ensure a continuous supply of this molecule.

IL-6 (Wong and Clark 1988): 19-30KD glycosylated molecule made by monocytes/macrophages, phagocytes, fibroblasts and T-cells. Involved in B-cell differentiation and stimulating antibody secretion. Activation of T-cells and induction of acute phase reactant release by hepatocytes. No species specificity.

IL-8 (Westwick et al 1989): 8KD heparin binding protein secreted by macrophages and fibroblasts. Under physiological conditions exists as a multimer up to 3000KD. Neutrophils possess 20,000 receptors for IL-8. Stimulates the oxidative burst and locomotion of neutrophils. IL-8 injections cause a short lived granulocytosis (Van Damme et al 1988). These effects could be important *in vivo* in a malaria infection.

IL-2,3,4,5 (Smith 1984, Garland 1986, Sideras et al 1988 respectively). All T-cell factors and therefore unlikely to be present in TNS because of the short time involved. However factors in TNS may trigger earlier release of these molecules so that their effects on T-cells and B-cells happen sooner.

Interferon-γ (IFN-γ)(Trincieri et al 1987): Another T-cell product, unlikely to be present in TNS. Species specific, therefore if present in rabbit TNS, would not be active in mice. However factors in TNS could stimulate its *in vivo* production. Functions include activation of phagocytes to release reactive oxygen radicals.

TNF (Trincieri et al 1987): First isolated from TNS. No *in vitro* antimalarial activity. Modest *in vivo* antimalarial activity. 17KD molecule existing as a 55KD trimer *in vivo*. Two forms TNFα and TNFβ (lymphotoxin). Latter produced by T-cells and NK cells and may not be present in TNS. Apart from some tumour killing activity its major function is a regulatory cytokine with many functions similar to IL-1 (Nathan 1987a). Potent neutrophil stimulator in the presence of fibronectin, vitronectin and laminin (Nathan 1987b).

Granulocte monocyte-colony stimulating factor (GM-CSF) (Clark-Lewis et al 1988): Macrophage product but with strict species specificity. Functions include promotion of monocyte/granulocyte growth and activation of cytocidal mechanisms.

COMPLEMENT COMPONENTS

(Nathan 1987a): Normal component in serum. Their synthesis and relese would only increase the serum concentrations. However heat inactivation of complement in TNS does not change the serum's activity and as described earlier the evidence is against complement being important in malaria infections.

COAGULATION FACTORS

(Nathan 1987a): Some of these are made by macrophages but would be removed from the serum during clotting.

ENZYMES

(Nathan 1987a): A number of enzymes are released by macrophages upon stimulation. Polyamine Oxidase has been shown to have antimalarial activity (Egan et al 1986, Ferrante et al 1983). This may be mediated through the products of the enzymes action. Babesia (Morgan et al 1981), Trypanosomes (Ferrante et al 1982) and bacteria (Tabor and Rosenthal 1956) are all killed by polyamine oxidase. But this enzyme has different activities depending on the species of origin (Egan et al 1986). Lysosomal enzymes are also made by macrophages. During *P.berghei* infection, recovering albino rats have elevated serum levels of these enzymes, unlike Thicket rats which do not and which die (Gupta et al 1980). *M.tuberculosis* or *M.bovis* infection in rabbits increases these enzyme serum concentrations, especially acid phosphatases (Allison et al 1961, Saito and Suter 1964a/b)Serine protease is released after LPS stimulation especially after BCG priming. These enzymes have tumouracidal activity (Johnson et al 1981), and synergise with hydrogen peroxide (Adams et al 1981).

ENZYME INHIBITORS

Released by macrophages. May block *in vitro* activities but may be removed *in vivo* by other regulatory mechanisms. An IL-1 inhibitor is also released in response to stimuli (Liao et al 1985, Rodgers et al 1985, Roberts et al 1986). Therefore the balance between IL-1 and the inhibitor may dictate whether any IL-1 in TNS is active or not.

BINDING PROTEINS.

One such molecule that has created much interest in malaria is transferrin. This is a carrier molecule for Fe^{++} ions in the body. Several studies have shown the parasiticidal effects of iron-chelators *in vitro* and *in vivo*, (Fritsch et al 1987, Fritsch et al 1985). These molecules may act by inducing oxygen radicals or by starving the parasite of iron.

BIOACTIVE LIPIDS

(Nathan 1987a): These include prostaglandins and leukotrienes, which act as stimuli or depressants for a number of cell types. Probably not directly toxic to malaria parasites *in vitro*, but may be important *in vivo*. Some may be dialyseable.

POLYMORPHONUCLEAR LEUKOCYTE PRODUCTS.

Although eosinophils have not been associated with immunity to malaria, rodent malaria parasites have been shown to be killed by myeloid cells rich in eosinophils (Taverne et al 1982). Eosinophils mediate their cytotoxic activities through the release of toxic granules upon stimulation. Purified granule contents kill *P. falciparum in vitro* (Waters et al 1987). Antibody to the granule contents partially reversed the parasiticidal activity. Granule release requires an activating factor, TNF being one factor which can do this (Silberstein and David 1986), although it is unlikely that theTNF in TNS would have time to act in this way. Eosinophils can also mount an effective oxidative burst (Spry 1985). Neutrophils are also a major source of of oxygen radicals. This does not rule out other mechanisms because neutrophils from chronic granulomatous disease patients, which cannot mount an oxygen burst, are still said to be parasiticidal (Kharazmi et al 1984).

REACTIVE OXYGEN SPECIES.

See section 1.3.4.1.

REACTIVE NITROGEN SPECIES.

See section 1.3.4.4.

1.3.4.1. REACTIVE OXYGEN INTERMEDIATES.

Upon appropriate stimuli such as; C5a, IFN-γ, IL-1, TNF, IL-8 and mitogens, monocytes and polymorphonuclear cells are capable of producing large quantities of highly reactive oxygen-derived radicals.

"Reactive oxygen intermediates" or ROI, which include superoxide (O_2^-), peroxide (O_2^-), hydroxyl ($°OH$) and hypochlorous (ClO^-).

Other radicals are produced but these are generally produced as a result of one of the above radicals reacting with other molecules. Their primary function is the destruction of foreign, material especially microorganisms. The radicals are produced within the cells by enzymes. Superoxide is the primary radical produced. It formed by reacting NADPH with oxygen in the presence of an oxidase:

The superoxide radical can then be modified to produce the other radicals (Table 1.6). Monocytes, as they mature into macrophages lose their myeloperoxidase and therefore do not produce the hypochlorous radical. However neutrophils are very good at producing this radical. (Halliwell and Gutteridge 1985).

Humans with chronic granulomatous disease (CGD) cannot produce an oxidative burst. There are several forms of the disease, each characterised by a different difficiency of the oxidative pathway. For example cytochrome b_{558}, which is important for the oxidation of O_2 to O_2^- may be absent. The phosphorylation of various proteins in this pathway can be abnormal (Babior 1988).

The radicals are released into phagosomes and then into the environment. Because of the highly reactive nature of the ROI, to ensure preferential target damage rather than host damage the radical producing cell must be in close proximity to the target, or, ideally, engulf it, as in phagocytosis.

Some of the molecules regulating the release of ROI have already been discussed. ROI themselves will not be present in TNS, especially after dialysis, because of their very short half lives. However their reaction products may be present, in particular oxidised proteins and lipids. Because of the strong reactivity of the ROI, mechanisms exist to protect the host, (see section 2.4).

The susceptibility of parasites to ROI is well documented, even in such places as the gut lumen; *Nippostrongylus brasiliensis* (Smith and Bryant 1987a,b). Successful parasites may defend themselves

SUPEROXIDE

$$2O_2 + NADPH \xrightarrow{\text{OXIDASE}} 2O_2^- + NADP + H^+$$

PEROXIDE

$$2O_2^- + 2H^+ \xrightarrow[\text{DISMUTASE}]{\text{SUPEROXIDE}} H_2O_2 + O_2$$

HYPOCHLOROUS

$$H_2O_2 + CL^- \xrightarrow{\text{MYELOPEROXIDASE}} CLO^- + OH^-$$

HYDROXYL

$$H_2O_2 + Fe^{++} \longrightarrow OH^- + OH^* + Fe^{+++}$$
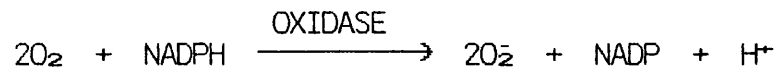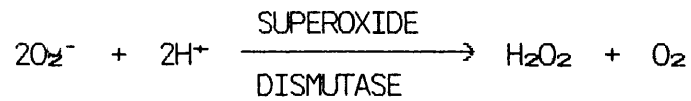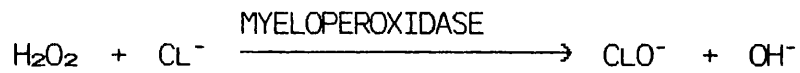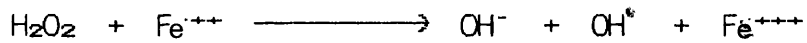
Table 1.6: Production of the four primary reactive oxygen intermediates. Superoxide is the species made initially and subsequently the others are formed by either catalysts or enzymes.

against ROI by producing enzymes such as superoxide dismutase, catalase, or by an intracellular habitat. However, even *Trypanosoma cruzi*, living in muscle cells can be killed by eosinophil products including a peroxidase (Molina et al 1988).

Intra erythrocytic parasites are also susceptible to ROI, probably mediated via the red cell membrane. This may be why G6PD deficiency is protective against malaria (Friedman 1979, Eaton et al 1976) as discussed in section 1.3.1. and section 2.4.3.

The susceptibility of malaria parasites to ROI was shown by several groups in 1983. The experiments were done both *in vivo* and *in vitro*. Ian Clark in Australia injected malaria infected mice with alloxan. This chemical generates ROI and results in a reduction in parasitaemia from >30% to <10% within an hour. Further injections could completely cure the mice (Clark and Hunt 1983). Similar results were obtained by directly introducing strong oxidising agents such as t-butyl hydroperoxide (Clark et al 1983). Further strenghtening the oxidant sensitivity of malaria parasites were experiments *in vitro* using hydrogen peroxide. *P.yoelii* was sensitive down to 10^{-5}M Hydrogen peroxide. Added uninfected red cells blocked this activity, which was thought to be due to excess catalase (Dockrell and Playfair 1983), though it could also be due simply to the hydrogen peroxide to red cell ratio being less. Direct injection of hydrogen peroxide into *P.yoelii* infected mice reduced the parasitaemia within two hours (Dockrell and Playfair 1983).

Other ROI are also parasiticidal. Superoxide produced by Xanthine/Xanthine Oxidase is toxic, as is peroxide generated by Glucose/Glucose Oxidase (Dockrell and Playfair 1984). Further evidence that ROI may be important *in vivo* comes from experiments using macrophages. Activated macrophages could inhibit *P. falciparum* growth by 60% or more *in vitro* (Ockenhouse et al 1984a). Hydrogen peroxide has been said to be the most important ROI, based on the activities of the scavengers; Catalase, SOD, histidine, mannitol and sodium benzoate: The latter four did not affect parasite killing, whereas catalase did (Dockrell and Playfair 1984). Monocytes from CGD patients are unable to mount an oxidative burst and also could not kill *P.falciparum* infected erythroctes as well as monocytes from normal individuals monocytes (Ockenhouse et al 1984a).

Factors affecting the susceptibility of malaria parasites include red cell age. *P.berghei* parasites were more susceptible in mature red cells than in reticulocytes (Cox 1983). However the difference

between a non-lethal *P.berghei* variant and a lethal variant, could not be explained in this way, as both are equally sensitive to ROI (Waki et al 1985).

Stimulants of macrophages *in vivo* such as microbial molecules may act via ROI, and this could be an explanation of the results discussed in section 1.3.3.3.

It is not clear whether ROI act directly on the parasite or via red cell damage. The latter seems most likely because for the ROI to reach the parasite they would have to cross two membranes and the red cell cytoplasm, which means that if they do not react with the membrane they will be destroyed by the red cell antioxidants. (Antioxidants are discussed later).

Several forms of damage occur within red cells following exposure to ROI. Methaemoglobin is produced which ultimately forms carboxyhaemoglobin (Weiss 1982). This may itself be toxic to the parasite or simply be unavailable for digestion by the parasite possibly because the methylhaemoglobin is insoluble. It also forms part of the haemozoin pigment (Sherman 1979).

Parasite death may simply occur due to red cell lysis resulting from membrane damage (Vercellotti et al 1985). Membrane lipid damage can be detected in malaria infected red blood cells, by looking for lipid peroxidation products (Wozencraft 1986, Buffington et al 1988). Some of the products of lipid peroxidation are toxic to the parasite at micromolar concentrations, in particular aldehydes (Clark et al 1987)

1.3.4.2. CRISIS FORMING FACTOR.

Jensen et al (1983) working in the Sudan found that sera from malaria infected individuals, particularly those with no clinical history of malaria, contained a strong parasiticidal factor. This factor was not antibody, as purified antibody had no effect and the activity was present in the antibody depleted serum fraction. Umbilical cord sera could produce the same results. These inhibitory sera were postulated to contain a molecule called crisis forming factor because of the appearance of the parasites incubated with these sera.

The activity of the sera was highest during the wet season, when malaria transmission was highest (Van de Waa et al 1984). Therefore it appeared that crisis forming factor was an adaptive response rather than constitutive.

People have looked in other endemic areas for a similar factor without much success, though Nkuo and Deas (1988) described a similar effect with sera from the Cameroon. Butcher et al (1987) published a report of crisis sera in Papua New Guinea, however unlike those from the Sudan, activity was lost after dialysis.

Sera from patients with tuberculosis could also induce crisis forms just as TNS can (Carlin et al 1985).

From the available data, the crisis factor is most prevalent in individuals non-immune to malaria. When antibody titres increase and clinical immunity develops crisis factor was not present. Thus in malaria individuals, as with TNS, a non specific factor is produced. The nature of this crisis forming factor is not understood yet, although *in vitro*, the factor does not appear to act by creating oxygen radicals [(Geary et al 1986)]. The same is true for the tuberculosis sera and tumour necrosis sera.

1.3.4.3. ACUTE PHASE PROTEINS. (Gitlin and Colten 1987)

These are molecules released by the body undergoing trauma. The first discovery occurred in 1930 with the C-reactive protein, during infection. A number have now been isolated and studied, with the liver as the major source.

Table 1.7 list the major acute phase proteins and some of their properties.

TNS would contain several of these proteins because of the time course involved in making this serum. It is unlikely that LPS alone would induce acute phase proteins within 2 hours. However BCG infection would within 3 weeks. But after this time albumin, transferrin, prealbumin, CRP and SAA have returned to normal levels. All others are still high, and LPS stimulation at this point may well increase their levels by upregulating their production.

α_1-acid glycoprotein has been reported to be inhibitory to

P.falciparum in vitro, possibly because this molecule competes for the red blood cell glycophorins with the merozoites thus blocking reinvasion, (Friedman 1983).

Table 1.7: Some acute phase proteins and their properties.

C-reactive protein:
   First described in 1930. Pentamer in plasma. 206 $\alpha\alpha$. No S-S bonds. Induced by monokines and primarily synthesised by the liver. Increases by up to 100 fold during an infection. Function unknown but binds to bacterial phosphorylcholine.

Serum amyloid-P-component:
   First described in 1965. Decamer in plasma (5x2). 204 $\alpha\alpha$. Induced by monokines and primarily synthesised by the liver. No change in humans only in lower animals. Increases up to 100 fold during infection. Function unknown.

Serum amyloid-A-component:
   First described in 1971. Complexes with high density lipoproteins. 12kD. Major acute phase reactant. Increases up to 100 fold during infection within 18h. Can be induced by monokines.

Fibrinogen:
   Part of the clotting cascade. 340kD. 2 to 5 fold increase during infection. Remains high for up to 3 weeks. Induced by monokines.

α_1-acid glycoprotein:
   40kD, 181 $\alpha\alpha$. Normal plasma protein, increases during infection. Highly glycosylated and sialated. May be toxic to malaria parasites by blocking reinvasion. Functions still (Schmid 1975, Friedman 1983).

α_1-proteinase inhibitor (α_1-antitrypsin):
   Major-α-globulin in plasma. Synthesised by macrophages/monocytes. Induced by monokines.

Prealbumin:
   127 $\alpha\alpha$. Occurs as a tetramer. Transports thyroid hormones and vitamin A. Synthesised by the liver. Decreases by up to 50% during infection.

Albumin:
   Most abundant plasma protein (65%). 586 $\alpha\alpha$. Acts is a transport molecule. Decreases during infection, possibly IL-1 dependent route.

Complement:
   Various components increase and decrease during infection.

1.3.4.4 Reactive Nitrogen Intermediates.

Current interest is mainly centred around ROI (section 1.3.4.1) which is odd because reactive nitrogen intermediates (RNI) are not a new phenomenon. As Table 1.8 shows, the bactericidal action of RNI has been known since well before 1926, but it is only in the last few years that macrophages have been shown to synthesise these molecules, and epithelium to synthesise nitric oxide (NO°). RNI are toxic to a wide range of cell types which is perhaps not suprising because of the molecular groups that they attack. Two contrasting facts about RNI compared to ROI are:

a) RNI have much longer half lives than ROI, especially at neutral pH.

b) Many lymphokines can induce ROI release but only Y-IFN can induce high RNI release. However microbial products will stimulate the production of both RNI and ROI.

This has two implications for TNS. Both RNI and ROI will be released in the animal. TNS injected into a normal animal may initially stimulate ROI release via TNF. Y-IFN release may also be stimulated, therefore RNI will be induced but at a later time than ROI.

Table 1.8: A brief chronology of nitrogen radical research.

18th Century: Studies on bacterial growth in gases.
(Hatton 1881)

By 1926: Sodium Nitrite used in the U.S.A. for curing meat.
(Kerr et al 1926)

By 1940: Sodium Nitrite used in the U.K. for curing meat.
(Brooks et at 1940)
        Bactericidal action mediated through Nitric Oxide Radical (NO°) due to pH < 7.0 in cured meats.   (Tarr 1941)

1954: Bacterial hydrolases containing Fe^{++} blocked by NO°
(Krasna and Rittenberg 1954)

1958: Tobacco mosaic virus rendered uninfective by NO°, because conversion of adenine to hypoxanthine, guanine to xanthine and cytosine to uracil causes lethal mutations in TMV genomic RNA.
(Greier and Mundey 1958)

1959: Other viruses rendered uninfective by NO° due to nucleic acid mutation.   (Teissman 1959)

1962: Nitrous acid (HNO$_2$) acts on bacterial cell walls.
(Shank et al 1970)

1970: Bacteriophage ϕX174 rendered uninfective by submutagenic doses of NO°. Action attributed to damage of viral protein coat.
(Lytle and Ginoza 1970)

1975: NO° reacts with SH groups in bacterial cell walls.
(Riha and Solberg 1975)

1976: NO° can react with intracellular SH groups.
(O'Leary and Solberg 1976)

1980: Evidence supports bactericidal action of RNI via direct toxicity on bacterial cell walls, nucleic acids and blocking metabolic enzymes. Many bacteria and viruses are recorded as being susceptible.
        Postulation of an endothelium derived relaxing factor (EDRF).   (Furchgott and Zawadski 1980)

1981: NO° is one molecule capable of relaxing epithelium.
(Gruetter et al 1981)

1983: Endotoxin stimulated reticuloendothelial system may synthesise nitrate from reduced nitrogen via ROI *in vivo*.
(Wagner et al 1983)

1984: Further data on the *in vivo* production of nitrate.
(Saul and Archer 1984)
Xanthine/Xanthine Oxidase in presence of H_2O_2 and NH_3
produces NO_3^- (Dull and Hothkin 1984)
Generation of Nitrogen-Chlorine oxidants from phagocytes.
(Test et al 1984)

1985: Endotoxin stimulates macrophages to produce NO_2^- and NO_3

(Stuehr and Marletta 1985)
Superoxide reacts with NO_2^- to form peroxonitrite at
pH7.0 (Blough and Zafiriou 1985)

1986: Superoxide destroys EDRF. (Gryglewski et al 1986)

1987: EDRF properties very similar to NO° (Ignarro et al 1987)
Synthesis of NO_2^- and NO_3^- in macrophages and
macrophage cell lines by a number of inducers, including, BCG,
cytokines and mitogens. (Stuehr and Marletta 1987a,b)
Arginine is essential for RNI synthesis by macrophages.
(Iyengar et al 1987)
Macrophage toxicity to cell lines can be inhibited by
arginine depletion. (Hibbs et al 1987)
Arginine is catalysed to citrulline and NO_2^- by a
deaminase and an oxidase. (Hibbs et al 1987)

1988: Macrophages require arginine to be cytostatic to
Cryptococcus neoformans . (Granger et al 1988)
EDRF release and NO° release kinetics from endothelium are
the same. (Kelm et al 1988)
NO° is the active RNI released by macrophages.
(Hibbs et al 1988)
Compared to the release of ROI, γ-IFN is the only cytokine
to induce the release of RNI. (Ding et al 1988)
NO° may be an intermediate in NO_2^- and NO_3^- catalysis
of arginine. (Marletta et al 1988)

1989: Became accepted that EDRF is NO° (Ignarro 1989)
One effect of the release of RNI by macrophages is to
relax endothelium. (Stuehr et al 1989)
Although RNI are released by macrophages, it is likely that
NO_2^- and NO_3^- are the actively secreted molecules and
the local conditions if <pH7.0 form NO° from these two species.
NO° is then the toxic molecule. Tumour cells are sensitive to
RNI. (Stuehr and Nathan 1989)

2. LIPID BIOCHEMISTRY.

The word lipid has long been used to denote a chemically heterogeneous group of substances, having in common the property of insolubility in water, but solubility in non-polar solvents such as chloroform, hydrocarbons or alcohols. I shall concentrate on the long chain fatty acids and where relevant, associated molecules, as these constitute the majority of body fats.

2.1. CLASSES OF FATTY ACID.

In general fatty acids do not occur as free carboxylic acids because of their affinity for proteins, which for enzymes leads to their inactivation. However when they do occur as free carboxylic acids they are found bound to albumin in mammalian blood.

Table 2.1 shows the major fatty acids utilised by mammals. These are by no means all the fatty acids found in nature. Most have an even number of carbon atoms, because of their method of synthesis. But odd numbered carbon chain fatty acids do exist.

The short chain fatty acids of up to C_8 are all water soluble although associated with each other rather than as single molecules. Above C_8 the fatty acids become increasingly less soluble in water unless they are converted to alkali metal salts to make soaps and detergents. The non-polar carbon chains associate with each other leaving the ionised polar end in the water, in effect making the molecules soluble.

2.2. FATTY ACID SYNTHESIS.

2.2.1. ESSENTIAL FATTY ACIDS.

Although pathways exist for making fatty acids either from acetate or by modifying dietary fatty acids, mammals are unable to synthesize those fatty acids containing a double bond at positions C_{12} or C_{13}. The function of these fatty acids which can be readily obtained from plants, seems to be in the manufacture of

C_n	SYSTEMATIC NAME	COMMON NAME	OCCURRENCE
	SATURATED FATTY ACIDS		
8	n-octanoic	caprylic	minor in animal and plant fats.
10	n-decanoic	capric	minor, widespread.
12	n-dodecanoic	lauric	widespread.
14	n-tetradecanoic	myristic	widespread, occasionally major component.
16	n-hexadecanoic	palmitic	major, widspread.
18	n-octadecanoic	stearic	major,widespread.
20	n-eicosanoic	arachidic	minor,widespread.
	MONO-UNSATURATED FATTY ACIDS		
16	cis-9-hexadecanoic	palmitoleic	widespread.
18	cis-9-octadecanoic	oleic	possibly commonest.
	DI-UNSATURATED FATTY ACIDS		
16	cis,cis,6,9-octadecadienoic		minor.
18	cis,cis,6,9-octadecadienoic		minor.
	cis,cis,9,12-octadecadienoic	linoleic	major, essential.
	TRI-UNSATURATED FATTY ACIDS		
18	all cis,6,9,12-octadecatrienoic	γ-linolenic	minor.
	TETRA-UNSATURATED FATTY ACIDS		
20	all cis,5,8,11,14-eicosatetraenoic	arachidonic	major.
	PENTA-UNSATURATED FATTY ACIDS		
22	all cis,7,10,13,16,19-docosapentanoic	dupanodonic	major, especially in phospholipids.
	HEXA-UNSATURATED FATTY ACIDS		
22	all cis,4,7,10,15,16,19-docosahexanoic		major, especially in phospholipids

Table 2.1: Fatty acids commonly found in mammals unless otherwise stated.
C_n, refers to the length of the carbon chain.

prostaglandins. Some of the effects of essential fatty acid deficiency are shown in table 2.2. These can be completely reversed by feeding with linoleic acid (Gurr and James 1980, Mayes et al (1988).

2.2.2. SATURATED FATTY ACID SYNTHESIS.

The first step in fatty acid synthesis is the carboxylation of acetyl-CoA to malonyl-CoA, by Acetyl-CoA:Carbon Dioxide Ligase-ADP. The fatty acid is then made up by condensation reaction between malonyl-CoA molecules. CO_2 ions are removed at each condensation step. The carbon is the same carbon initially reacted with acetyl-CoA, (Figure 2.1). The primer molecule to begin the chain is either acetate or butyrate. Other precursors can be; odd carbon primers which give rise to odd carbon fatty acids, and branched primers which give rise to a terminally branched fatty acid. These reactions primarily occur in the cytoplasm. This is a *de novo* pathway which can be altered to accept existing fatty acids for modification and elongation.

2.2.3. UNSATURATED FATTY ACID SYNTHESIS.

These are made by directly introducing a double bond into the carbon chain; this primarily occurs at the C_9 position. A desaturase enzyme, oxygen and NADPH are used used to remove two hydrogens from the carbon chain to make water and the double bond. Further double bonds can be introduced but only on the carboxyl side of the existing double bond. Hence only fatty acids with double bonds up to C_9 can be made, unless dietary unsaturated fatty acids are used. The most important of these are oleic (also made by the animal), linoleic acid and linolenic acid, all of which compete for the same desturase enzyme. Oleic acid gives rise to a family of acids with a double bond at C_9 whilst linoleic and linolenic acids give rise to a family with a double bond at C_{12}. These can be used to make prostaglandins and leukotrienes and for the formation of membranes. Prostaglandins and leukotrienes are involved in platelet

Affected Organ	Symptoms resulting from deficiency
Skin	Increased water permeability Decreased sebum secretion Epithelial hyperplasia
Weight	Decreased
Circulation	Increased heart size Decreased capillary resistance Increased capillary permeability
Lung	Cholesterol accumulation
Endocrine Glands	Adrenals:Females—↑ hormone prod'n Males —↓ hormone prod'n Thyroid: ↓ body weight
Reproduction	Females—irregular oestrus Males —degeneneration of seminiferous tubules
Metabolism	Fatty acid composition changes Increaed cholesterol levels Decreased plasma cholesterol Oxidative phosphorylation impaired Increased triglyceride synthesis

Table 2.2: Major effects of essential fatty acid deficiency in rats.

Biotin

$$ATP + HCO_3^- + Biotin \longleftrightarrow CO_2^--BCC + ADP + Pi$$

Carboxylase

Acetyl-CoA ——————

Carboxy
transferase

Malonyl-CoA + Biotin + BC

Primer ——————

Malonyl-CoA ——————————→ C_n + CO_2^-

6 enzymes

$$CH_3(CH_2)_nCOOH$$
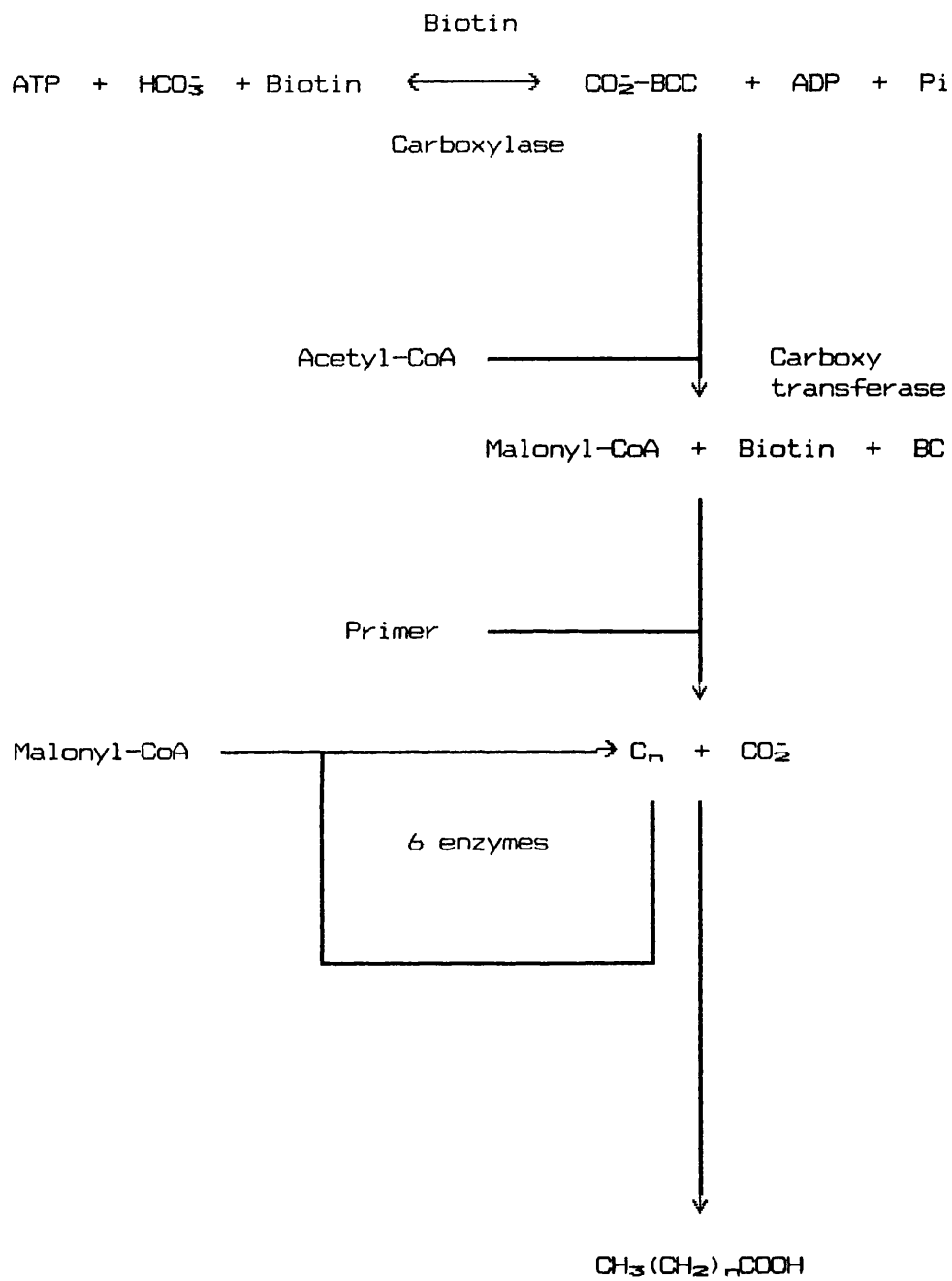
Figure 2.1: Schematic diagram to show the major cycle of the fatty acid synthesis pathway.
ATP, Adenosine triphosphate; ADP, adenosine diphosphate; Pi, inorganic phosphate. CO_2^--BCC, Carboxy-Biotin carboxylase Complex; BC, Biotin Carboxylase

function and can stimulate smooth muscle contraction. A lack of these does not give rise to the symptoms of fatty acid deficiency as in table 2.2, rather these symptoms relate to fatty acids as structural molecules in the membrane.

2.2.4. OTHER FATTY ACID SYNTHESES.

Branched chain fatty acids are not very common in animals and are formed by introducing a branched chain fatty acid into the synthesis pathway.

Hydroxyl fatty acids are formed as by-products of α-oxidation of fatty acids. A hydroxyl group is inserted into the carbon chain. Common in plants and bacteria.

Fatty acids are not always made by condensation reactions between smaller molecules. They can also be made by shortening long acids. This involves a limited number of cycles of the β-oxidation pathway.

2.3. DEGRADATION OF FATTY ACIDS.

2.3.1. β-OXIDATION.

Fatty acids acids are stored as triglycerides (fats) and provide the major energy store within the body. The enzymes for β-oxidation reside in either peroxisomes or mitochondria, which preferentially catabolise long and short chain fatty acids respectively. The carboxyl end is activated by attaching a CoA group to them. A double bond is inserted at the end of the chain across which water is added. The hydroxyl group is oxidised to a keto group and finally the carbon-carbon bond is broken to give acetyl-CoA and a new fatty acid, but two carbon atoms shorter. This cycle continues until the fatty acid is completely broken down. The products are then fed into the Krebs cycle.

Odd carbon fatty acids end up with a propionic acid residue which is oxidised by the liver to succinate.

Branched chain fatty acids are oxidised as above. The problem

arises with unsaturated fatty acids. Some cycles of β-oxidation can occur up to the double bond but stop there because the bond is the wrong conformation for the hydroxylase enzyme (cis as opposed to trans). An isomerase enzyme is required to change the conformation.

2.3.2. α-OXIDATION.

This pathway is mainly confined to plants and the brain in animals. The pathway involves cleavage of one carbon group at a time. The enzymes are located within microsomes and the pathway does not require the fatty acids to be activated with CoA. This is a useful pathway for disposing of long chain fatty acids in the brain which are difficult to transport, and in aiding the degradation of odd carbon chain fatty acids and branched chain fatty acids.

2.3.3. ω-OXIDATION.

This process occurs in the endoplasmic reticulum, and attacks the fatty acid chain away from the carboxyl group. The process stops on the formation of a dicarboylic acid residue. Thus complete degradation of fatty acids never occurs in the pathway.

2.3.4. PEROXIDATION OF FATTY ACIDS.

On exposure to oxygen, lipids form peroxides. This can lead to tissue damage *in vivo* and is also responsible for the spoilage of foods- in fact it is the limiting factor for the storage of frozen goods. A good example is butter turning rancid. Peroxidation can be caused by two processes: enzymatic and non-enzymatic, the latter being an auto- catalytic pathway or chemical oxidation. Either way, it is the unsaturated fatty acids which are the most susceptible, and the more unsaturated the fatty acid the more susceptible it becomes.

2.3.4.1. ENZYMATIC OXIDATION.

The enzymes acyclooxigenase and lipoxygenase catalyse enzymatic peroxidation. The resulting peroxides and hydroperoxides may only be present within the enzyme complex and are quickly detoxified. The function of these may not be clear, but they do give rise to the characteristic smells and flavours of plants.

2.3.4.2. NON-ENZYMATIC OXIDATION.

A radical that has sufficient energy to abstract a hydrogen atom from a methylene carbon of an unsaturated fatty acid can initiate a chain reaction in bulk lipids (1). The resulting carbon centered radical reacts rapidly with molecular oxygen to form a peroxy radical (2), which itself is then able to abstract a hydrogen atom from an unsaturated fatty acid, leaving a carbon centered radical and a hydroperoxide (3), Thus;
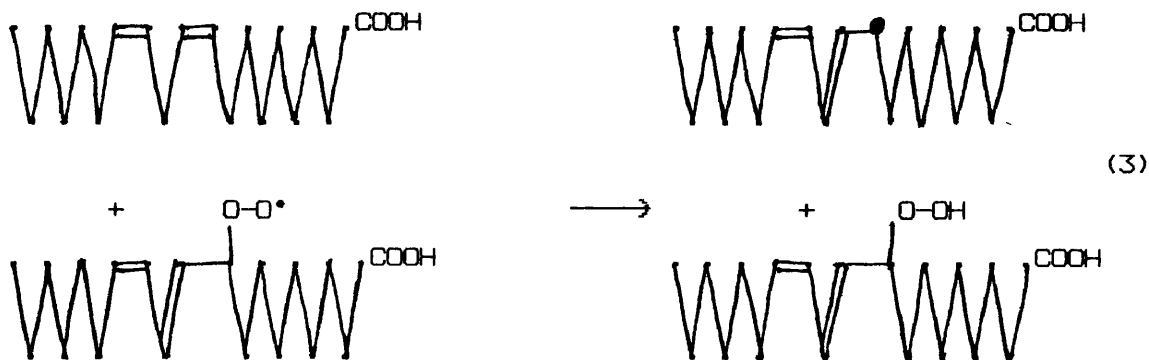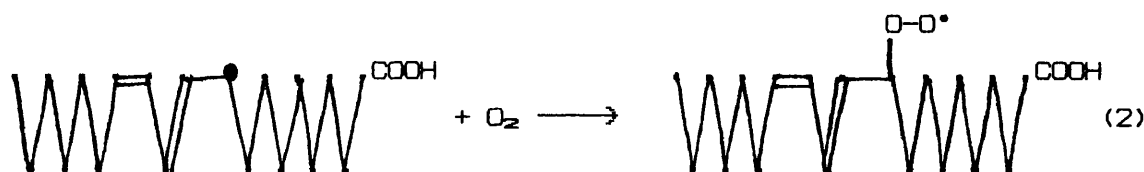
The reaction terminates if two radicals react with each other:

$$L^\circ \quad + \quad L^\circ \quad \longrightarrow \quad LL$$

$$LO_2^\circ \quad + \quad LO_2^\circ \quad \longrightarrow \quad LOOL \quad + \quad O_2$$

$$LO_2^\circ \quad + \quad L^\circ \quad \longrightarrow \quad LOOL$$
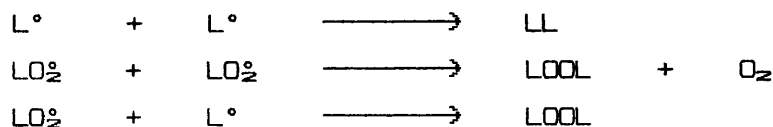
Antioxidants are very important in preventing initiation and propagation of these reactions. When the fatty acids have more then one double bond, peroxidation can lead to the formation of a multiperoxide (1) or can cause the shortening of the chain with malondialdehyde as a by-product (2), which can be measured.

(1)

(2)
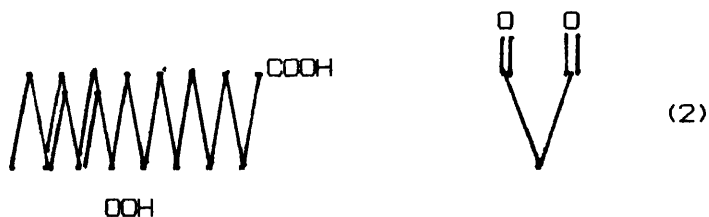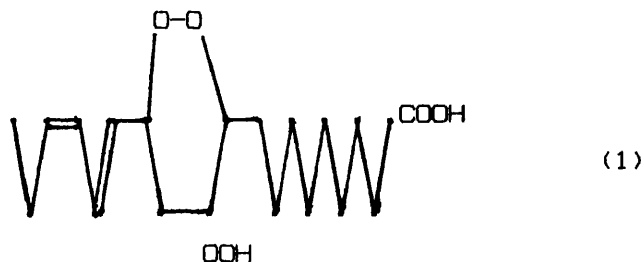
In the polyunsaturated fatty acids where the double bonds are separated by a methyl group, on peroxidation one double bond is shifted towards the other to form a conjugated diene, with strong absorbtion at 233nm.

(1)
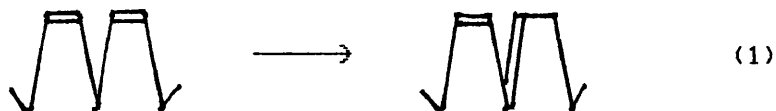
Other products of lipid peroxidation include; low molecular weight hydrocarbon gases, fluorescent products and aldehydes . Because of

this complex mixture of products there is no single method for accurately determining the extent of lipid peroxidation. Some of the products (e.g. malondialdehyde and dienes) can be used as an indication of lipid peroxidation, but not a measurement.

Figure 2.2 summarises the peroxidation of unsaturated fatty acids.

2.3.4.2.1. INITIATORS OF LIPID PEROXIDATION.

As stated above lipid peroxides can initiate and cause propagation of peroxidation, and indeed these are probably the most important molecule for maintaining the process, via autocatalysis. Any lipid preparation, however prepared, will eventually oxidise, providing of course that oxygen is available.

In body tissues an important initiator is iron. It is involved in the production of several oxygen radicals which can all initiate lipid peroxidation.

$$Fe^{2+} + O_2 \longleftrightarrow Fe^{3+} + O_2^-$$

$$2O_2 + 2H^+ \longrightarrow H_2O_2 + O_2$$

$$Fe^{2+} + H_2O_2 \longrightarrow OH^- + Fe^{3+} + {}^\circ OH$$
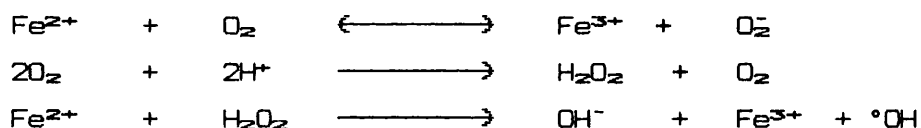
Mammals posses the perfect environment for these processes; Haemoglobin contains both Fe^{2+} ions and oxygen. Ferritin, an iron storage protein with 4500 moles of iron per mole of protein, stimulates lipid peroxidation. In order to prevent peroxidation of lipids, antioxidants are needed which compete for oxygen and radicals with the lipids (see section 2.4).

FREE RADICAL

↓

UNSATURATED FATTY ACID

H° ABSTRACTION
OXYGEN (RADICAL) — (MOLECULAR REARRANGEMENT)

↓

DIENE CONJUGATION

PEROXIDES

↓

ALDEHYDES —— (HYDRAZONES)

POLYMERISATION
PRODUCTS

TRIENE CONJUGATION

HYDROCARBONS
ETHANE
PENTANE

EXCITED CARBONYLS
MALONDIALDEHYDE
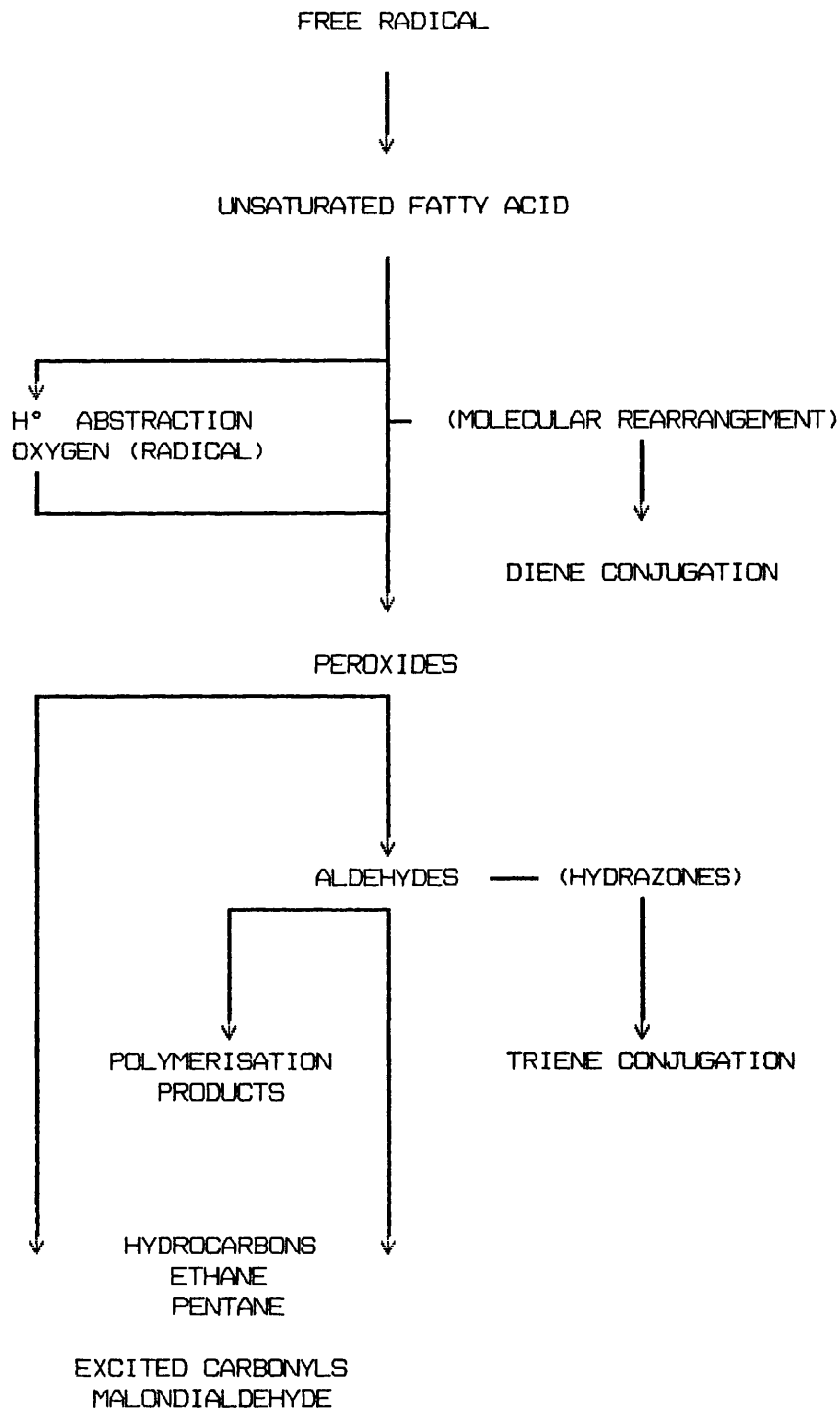
Figure 2.2: Schematic diagram to summarise the degradation of unsaturated lipids by non-enzymatic peroxidation.

2.3.5. TOXICITY OF LIPID PEROXIDATION PRODUCTS.

Lipid peroxidation does not only occur during infection or conditions of macrophage stimulation, since oxygen radicals are continuously being generated via aerobic respiration. Malondialdehyde levels in urine increase in response to increases in lipid peroxidation due to a number of factors, including high dietary polyunsaturated fatty acids, CCl_4 and some hormones. (Draper et al 1984, Dhanakoti and Draper 1987, Piché et al 1988).

Table 2.3 shows some of the known effects of lipid peroxides *in vivo* and *in vitro*. Most workers take care to prevent unintentional *in vitro* autooxidation of the lipids, which would otherwise give false results.

Common features amongst the data include: high levels of polyunsaturated fats in the diet and therefore in the body, and release of lipid into the blood stream due to infection and disease or starvation. Peroxides form even in the absence of an oxidative burst.

It is generally considered that the damage caused by lipid peroxides is at the level of cell membranes. This can take several forms: changes in membrane rigidity, solvency and permeability, protein and nucleic acid damage. In many instances antioxidants can be shown to prevent these effects by blocking lipid peroxides forming.

The antipsychotic drug family of phenothiazines increase the level of cerebrospinal fluid diene conjugates and malondialdehyde, especially in patients with severe side effects to the drugs (Pall et al 1987).

Sera from patients with various connective tissue diseases are toxic to cultured fibroblasts. The activity is likely to be assoc iated with low density lipoproteins (Blake et al 1985).

Endotoxin induces lipid peroxidation within the liver which is reversed by antioxidants (Sugino et al 1987).

High density lipoproteins in normal human sera can be toxic to *Trypanasoma brucei* (Rifkin 1978a,b).

Deoxycholic acid binds covalently to DNA and RNA in the presence of methyl linoleate hydroperoxide, therefore increasing the risk of mutagenesis. This may explain why increased colon cancer correlates with high fat diets (Kodama et al 1987)

Very low density lipoprotein from diabetic rats serum is toxic to porcine aortic endothelial cells (Arbogast et al 1982).

A very low density lipoprotein associated cytotoxic factor can be found in pregnant rats (Chan and Pollard 1978, 1981).

Low density lipoprotein cytotoxicity can be induced by free radical peroxidation of lipids (Morel et al 1983).

In vivo hyperoxia leads to peroxidation of erythrocyte lipid (Mengel and Kann 1966).

Lipid peroxidation products can be found in malaria infected red cells and these products can be toxic to malaria parasites (Wozencraft 1986, Clark et al 1987, Buffington et al 1988)

Atheroscleratic plaques are formed when blood lipid levels stay high for long periods. Lipid laden macrophages can be found in these lesions. Oxidised lipoproteins are preferentially taken up. Destruction of the lipids by oxidation may cause further external lipid oxidation. The blood vessel lesion may worsen due to the action of these peroxides (Mitchinson 1983, Ball et al 1986, Ball et al 1987)

Table 2.3: Some of the detrimental effects of lipid peroxides.

2.4. ANTIOXIDANTS.

There are a number of molecules whose role is primarily to block lipid peroxidation. They may be protein in form and act either directly or indirectly, or they may be small molecules which become oxidised in preference to other molecules.

The first group include iron binding proteins, apart from those mentioned in Table 1.5. These proteins act by binding any free iron in the serum. Once the iron is bound it is unable to take part in redox reactions.

Therefore these proteins are acting indirectly to remove an initiator of oxidation. Two proteins which do this are transferrin and lactoferrin. Being only 30% loaded with iron they can quite easily take up any free iron should it occur. This effect should increase the malaria parasites' chance of survival if it is under oxidative stress. However the parasite does have a requirement for iron and so too much iron binding protein may be indirectly fatal to the parasites (Fritsch et al 1987).

A number of enzymes exist intracellularly and extracellularly whose function is to detoxify oxygen radicals, namely superoxide dismutase (SOD), catalase, and glutathione peroxidase. The former enzymes remove superoxide and hydrogen peroxide respectively and the latter enzyme acts by oxidising reduced glutathione which is the sacrificial antioxidant. Other examples of sacrificial antioxidants include vitamins A, C and E. All these have a higher affinity for ROI than proteins or lipids, and therefore will be oxidised first.

2.4.1. SUPEROXIDE DISMUTASE (EC 1.15.1.1.).

This is a 32KD intracellular enzyme composed of two 16KD subunits. Depending on the compartment within the cell, Zn^{2+}, Cu^{2+} or Mn^{2+} is present in the enzyme (Dixon et al 1979). The reaction catalysed is:

$$2O_2^- + 2H^+ \xrightarrow{\quad SOD \quad} H_2O_2 + O_2$$
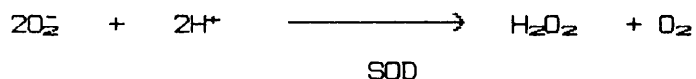
However the reaction produces another radical in the form of hydrogen peroxide which is detoxified by catalase.

2.4.2. CATALASE (EC 1.11.1.6.).

This is an iron containing enzyme with four subunit haem groups of total molecular weight of 232KD (Dixon et al 1979). The reaction catalysed is:

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$
$$\text{catalase}$$
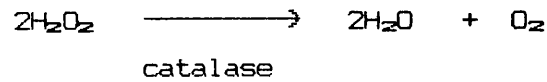
2.4.3. GLUTATHIONE PEROXIDASE.

This is a selenium containing enzyme of molecular weight 84KD. The reaction catalysed is similar to catalase, except that glutathione is used as the proton donor (Fohé and Günzler 1975).

$$H_2O_2 + 2 \text{ Glu-Cys-Gly} \longrightarrow 2H_2O + \text{Glu-Cys-Gly}$$

```
                    |          glutathione              |
                    SH         peroxidase               S
                    ↑                                   |
                    |               2AA or 2NADPH        S
                    |_____Glu-Cys-Gly
                              Glutathione reductase
```
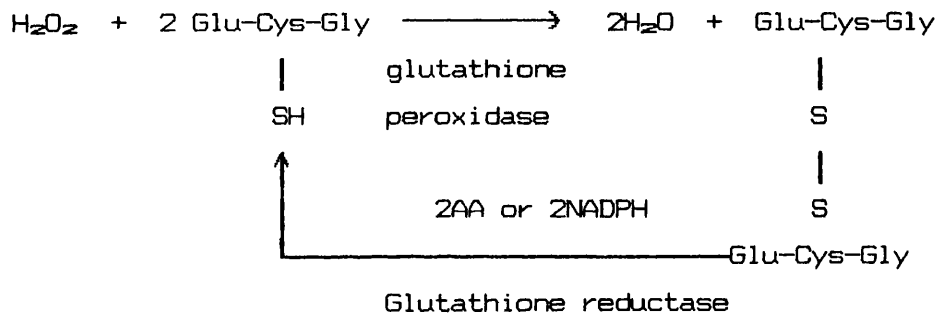
Reduced glutathione is regenerated from the oxidised state by glutathione reductase. These reactions occur at the membrane in the cytochrome chain. The reaction uses NADPH as the proton donor. Glutathione dehydrogenase which may be the same enzyme as glutathione reductase can use ascorbic acid (AA) as the proton donor (Dixon et al 1979).

2.4.4. VITAMIN-A.

Vitamin-A or retinol is made from β-carotene which is the active antioxidant molecule. In mammals the major function of β-carotene is not as an antioxidant, though since it is fat soluble it may be particularly important in fat stores, especially in plant seeds.

2.4.5. VITAMIN C (ASCORBIC ACID).

This is a water soluble vitamin which is essential for the synthesis of collagen and intracellular material. One deficiency state is well known; scurvey. Apart from this it is an important antioxidant. The oxidation of ascorbic acid is only reversible within a short time period, then internal rearrangemnt of the molecule occurs and ascorbic acid can no longer be made:

Ascorbic Acid

```
          HO   H    OH   OH
          |    |    |    |
HOCH₂  -  C  - C  - C  = C  - CO
          |    |              |
          H    |____ O _____|
```


Dehydroascorbic acid

```
          HO   H    O    O
          |    |    ‖    ‖
HOCH₃  -  C  - C  - C  - C  - CO            ─────────→ Oxalic acid
          |    |              |          ┌──
          H    |____ O _____|          │
                                        └───────────→ Ascorbate-2-sulphate
```
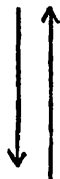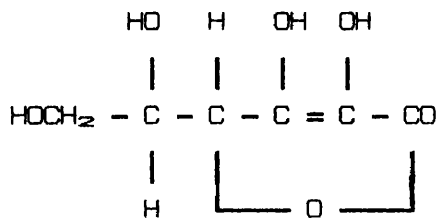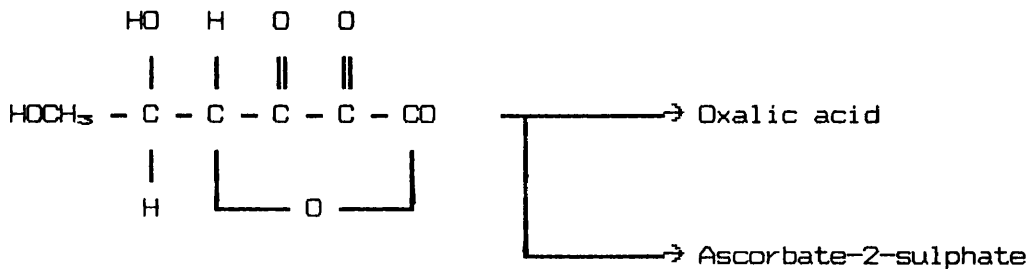
Two metabolites are then made which are excreted in the urine. Humans need a constant dietary supply of vitamin C because unlike most other animals they are unable to synthesise the vitamin. Humans require 30-60mg of vitamin C per day. This is taken up by the intestines. About 25% of the 1.5g body stock is bound to plasma proteins. Leucocytes contain a higher concentration than erythrocytes. Very high doses of vitamin C are well tolerated

although some intestinal disturbance may occur, and calcium oxalate renal calculi may develop. Tolerance may develop with prolonged use. The half life of vitamin C in the body is about 3.37 hours in humans although this may be artifactual because of the test conditions used.

As already shown vitamin C can be used to regenerate reduced glutathione. Also glutathione can be used to regenerate ascorbic acid from dihydroascorbate (Fohé and Günzler 1975).

2.4.6. VITAMIN E (TOCOPHEROL).

This is a fat soluble vitamin whose main function is to protect unsaturated fats from oxidation, though another possible function is during the development of the foetal gonads. The primary form of vitamin E is α-tocopherol. β, γ and δ tocopherols exist but do not have the antioxidant potential of α-tocopherol.
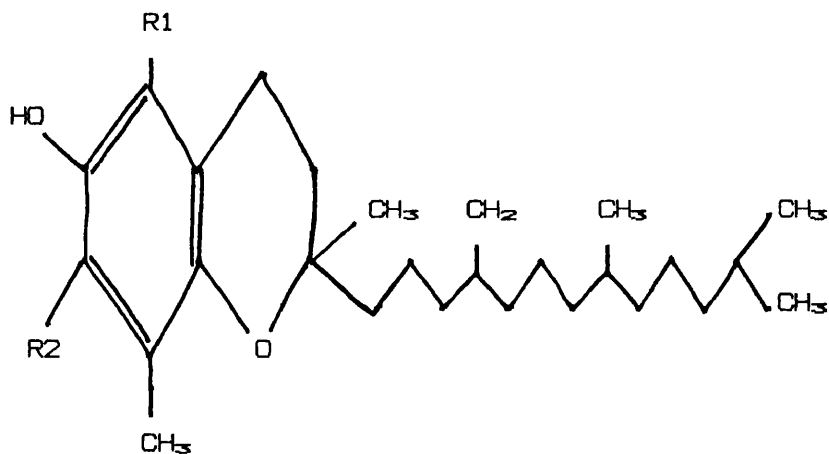
R1,R2 =	CH₃, CH₃	α-tocopherol
	CH₃, H	β-tocopherol
	H, CH₃	γ-tocopherol
	H, H	δ-tocopherol

Vitamin E is well tolerated in large doses but may cause instestinal disturbances. It is taken up by the intestines into the lymph where it associates with the chylomicrons. It is stored in all cell membranes and primarily in the fat stores. The daily requirement is somewhat dependent on the amount of body fat but is between 2 and 4mg per day.

Metabolism and oxidation of vitamin E results in glucaronids and tocopheronic acid and its γ-lactone which are all excreted in the urine. Excess vitamin E is excreted in the bile. Deficiency results in red cell oxygen sensitivity and eventually anaemia, as well as numerous other symptoms related to membrane damage.

Vitamin E has a very important interaction with vitamin C, that of regeneration of vitamin E. Thus membrane vitamin E can remain in the membrane for a much longer time because it need not be replaced once it has been oxidised. Also because vitamin C can be regenerated from its oxidised form, they form a very efficient antioxidant mechanism.

Figure 2.3 shows an overall summary of these antioxidant actions and interactions.

They will now be discussed further along with the lipids in the context of the red cell, malaria parasites and TNS.

It should be stated that vitamin E is probably the most important fat antioxidant because it is fat soluble. Therefore it will prevent propagation of fatty acid autooxidation as well as preventing initial oxidant attack. The water soluble antioxidants (vitamin C and enzymes) will primarily remove the oxygen radicals before they can reach the fats, even capture them as they are made and prevent oxidation of proteins. They can also play a role in regeneration of antioxidants (vitamin C on vitamin E, Glutathione reductase on oxidised glutathione).

Other references used for sections 2.1 to 2.4

Gurr and James	1980
Logani and Davies	1980
Halliwell and Gutteridge	1985
Elsas and McCormick	1986
Gutteridge	1987
Machlin and Bendich	1987
Murray et al	1988
Reynolds	1989

SOD ⟍ O_2^-

Catalase ⟍ H_2O_2

H_2O

GSH — GSSG

G.R.

Vit E ⟵ Vit E°
    Vit C

Vit C ⟵ Vit C°
   DHA reduct

REACTIVE
OXYGEN
RADICALS
(e.g. SO, PER,
HYDR, SingOx)

β-carotene°   β-carotene

ENERGY

ALDEHYDES

GSSG — NADPH

Glutathione   Glutathione
Peroxidase   Reductase

GSH ⟵   NADP

LIPID PEROXIDES — Vit E ⟵   Vit C°
AND   RADICALS

E.C
1
10
2,1

UNSATURATED ⟵ Vit E° —   Vit C ⟵
FATTY ACIDS

OXALATE

ASCORBATE
-2-
SULPHATE

GLUCARONOIDS
γ-LACTONES

DIET

Figure 2.3: Micronutrient interactions in the antioxidant defence
system.
GSH, reduced glutathione; GSSG, oxidised glutathione; DHA Reduct,
Dihydroascorbate Reductase; Vit E°, oxidised vitamin E; Vit E,
vitamin E; SO, superoxide; Per, peroxide; HYDR, hydroxyl; SingOx,
singlet oxygen.

2.5. THE RED CELL.

2.5.1. MEMBRANE ORGANISATION.

The mature red cell is devoid of *de novo* synthesis pathway. Therefore the membrane cannot be changed or repaired except for serum/extracellular molecules which can exchange with those in the membrane. The most important of these as *far as* this work is concerned are the lipids and antioxidants. Figure 2.4 shows a cross section of the membrane. It can be divided in to two parts, the cytoskeleton controlling cell shape and rigidity; and the lipid layer which controls the exchange of material between the outer and inner surfaces of the cell.

Table 2.4 shows the major fatty acids which make up the lipids of the membrane from several animal species. These fatty acids are mostly bound up in phospholipids (>70% of membrane lipids). The other (30% is made up from di and tri glycerides, unesterified fatty acids and cholesterol. The proteins embedded in the membrane include those involved in ion transport to ensure correct intracellular osmotic concentration, and those concerned with the uptake of glutathione from the plasma.

2.5.2. ANTIOXIDANTS OF THE RED CELL.

Table 2.5 shows the relative levels of some antioxidant enzymes and vitamin E in human and mouse red blood cells both normal and malaria-infected. The enzymes are located in the cytoplasm of the red cell whereas the vitamin E is contained in the membrane.

All these are required to protect the red cell membrane from oxidative damage, but perhaps the single most important one is vitamin E. This has been demonstrated many times. Animals starved of vitamin E in their diet have very oxygen sensitive red cells, and a shorter red cell half life, which ultimately results in anaemia. This can be corrected by replacing the vitamin E.

However a red cell deficient in the other antioxidants also becomes susceptible to oxidative haemolysis, and this is also true if the enzyme G6PD is hereditarily absent. This enzyme reacts with glucose
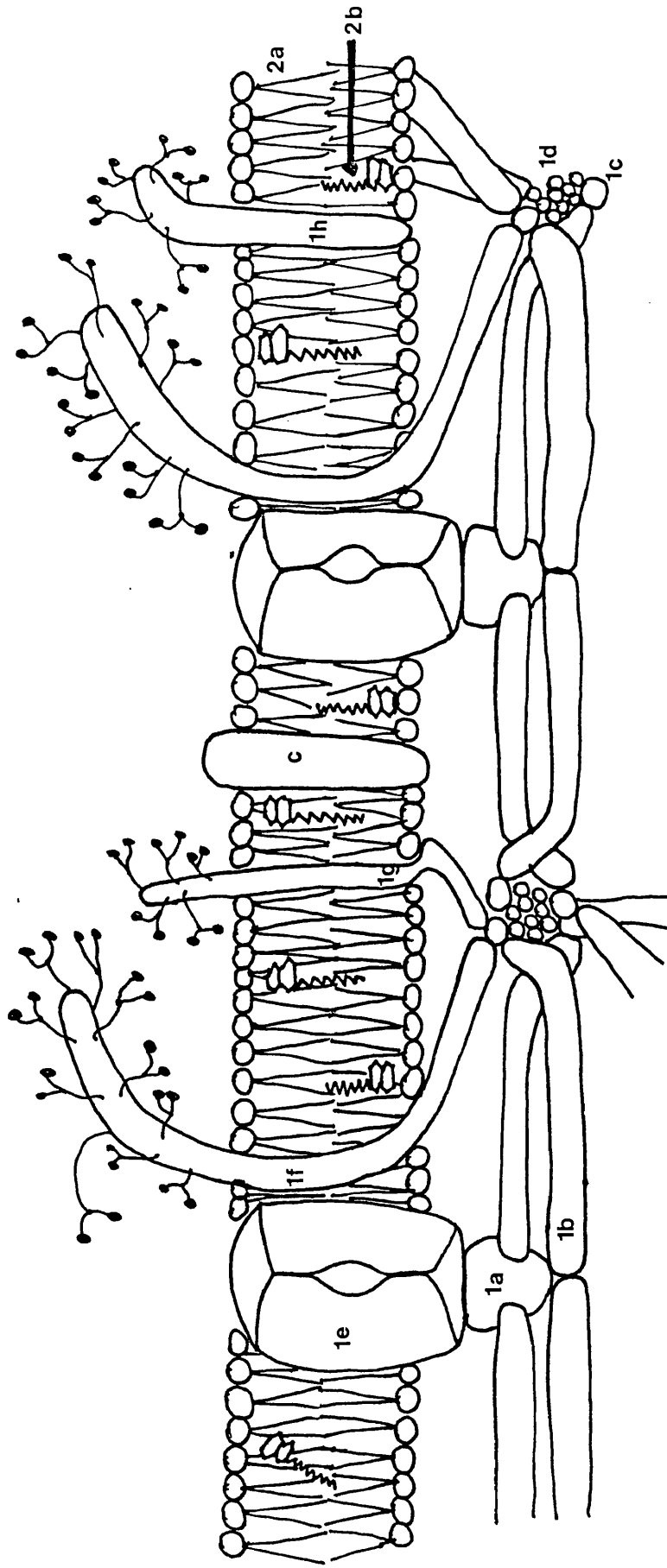
Figure 2.4: Cross-section of erythrocyte membrane showing: 1, cytoskeleton; 1a, ankyrin; 1b, spectrin; 1c, 1d, actin; 1e, band 3 protein; 1f, 1g, 1h, glycophorins with sialic acid and carbohydrate residues. 2, membrane proper; 2a, phospholipids; 2b, vitamin E. Embedded in this membrane would also be proteins some of which transport molecules between the exterior and interior of the cell or vise versa

(Modified from Hermentin 1987)

TOTAL FATTY ACIDS (%)								
	DUCK *p.lopurae*		MONKEY *P.knowlesi*		RAT *P.berghei*		MOUSE *P.vinckei*	
FATTY ACID	UN	IN	UN	IN	UN	IN	UN	IN
14:0	1	<1	1	<1	—	2	1.8	1.4
14:1	nd	nd	nd	nd	nd	nd	0.3	0.6
16:0	24	26	22	34	24	42	36	30.9
16:1	1	2	—	—	<1	4	nd	nd
18:0	10	16	15	9	17	15	8.3	4.2
18:1	18	33	18	36	8	21	12.8	10.3
18.2	21	12	15	15	11	7	23.1	31.9
18:3	1	1	1	1	—	2	nd	nd
20:2	1	<1	<1	—	—	—	nd	nd
20:3	1	1	2	<1	—	—	nd	nd
20:4	10	3	17	2	31	5	9.3	15
20:5	1	1	2	<1	—	—	nd	nd
22:0	nd	nd	nd	nd	nd	nd	0.7	2.3
22:5	2	1	2	<1	2	—	nd	nd
22:6	7	3	2	<1	—	—	4	2.8

Table 2.4: Fatty acid composition of uninfected (UN) and malaria infected (MI) red blood cell membranes. These results do not include the parasite membranes. Fatty acid numbers; e.g. 16:1 refers to a molecule with 16 carbon atoms and 1 C=C bond; nd, not done (Holz 1977, Stocker · et al 1987)

to form a new metabolite and NADPH. NADPH is important for the regeneration of oxidised antioxidants because without it the antioxidant pool would soon be consumed. Vitamin E in high doses (800mg per person per day for 90 days) could relieve this condition, and also other related enzyme deficiencies (Corash et al 1980, Schulman et al 1980).

These enzymes are constitutive in the red cell and cannot be replaced. Vitamin E levels however can be changed, increased or decreased by exchange between the membrane and plasma lipoproteins.

The red cell vitamin E concentration is dependent on the plasma lipid concentration rather than that of plasma vitamin E itself. If plasma lipid concentrations change, the red cell membrane vitamin E concentration can equilibrate with the plasma concentration within 4 hours (Bieri et al 1977, Kayden 1978).

The vitamin C concentration within red cells is subject to similar alterations.

2.5.3. Effect of Malaria Parasites on the Red Cell.

Malaria parasites do not have a *de novo* fatty acid synthesis pathway, although they do possess limited mechanisms for altering existing fatty acids (Sherman 1979), thus the parasite must obtain its lipid requirements for growth and division from the host.

Fatty acids increase by as much as 500% in infected red blood cells. This is true for a number of *Plasmodium* species from human, simian, bird and rodent. As table 2.4 shows, this is not just a general increase since some fatty acids increase in more in proportion to others, and this also differs betwee species. This is perhaps why there is conflict in the literature regarding the increase in fatty acids, therefore there is a general consensus that unsaturated fatty acids increase, (Holz 1977, Sherman 1979, Beaumelle and Vial 1986, Stocker et al 1987).

These lipid studies were done using red cell ghosts so that they do reflect the red cell membrane lipid composition. Studies on the parasite membrane are not as detailed as the above although they do show that it contains a higher percentage of unesterified fatty acids

and some classes of phospholipids than the red cell membrane. Overall the parasite membrane contains 90% unsaturated lipid compared with 75% for the red cell membrane (Sherman 1979).

The high unsaturated lipid content may allow the membrane to be more flexible and therefore aid its passage through capillaries. However this increase does mean that the membrane is more oxidant sensitive. The red cell is normally protected against oxidative attack by several antioxidant mechanisms as shown in table 2.5. However after invasion by the malaria parasite and the changes in membrane lipids along with immune responses to the cell, the constitutive antioxidant mechanism may no longer be sufficient protection for the cell. This may be a reason why the parasite changes the antioxidant content of the cell. It can synthsis its own SOD, catalase, glutathione peroxidase and glutathione reductase (GR) (see table 2.5). SOD may also be obtained from the host (Fairfield et al 1984, Fairfield et al 1988).

Vitamin E increase markedly, although this may simply be, due to the membrane lipid increase. This would mean that this is a passive process unlike the antioxidant enzymes, whose concentrations are actively increased.

Reduced glutathione (GSH) is also important to the parasite. This is reflected by the increase in red cell GSH and GR. The GR in the parasitised red cell has a higher affinity for oxidised glutathione (GSSG) then an uninfected red cell (Eckman 1984). to reduce GSSG to GSH requires NADPH. This may explain why the parasite cannot survive in G6PD deficient red cells, which cannot make NADPH (Etkin and Eaton 1975).

	SOURCE OF RED BLOOD CELLS			
	HUMAN UNINFECTED	HUMAN *P.falciparum*	MOUSE UNINFECTED	MOUSE *P.berghei*
CATALASE	22.2^5 μmol Min^{-1} (protein)$^{-1}$	$17\text{--}24^5$	15.92^4 IU (g Hb)$^{-1}$	12.22^4
GLUTATHIONE PEROXIDASE	12.4^5 μmol Min^{-1} (protein)$^{-1}$	$2\text{--}7.7^5$	78.87^4 IU (g Hb)$^{-1}$	41.4^4
SUPEROXIDE DISMUTASE	2.2^5 μmol Min^{-1} (protein)$^{-1}$	$6.3\text{--}25^5$	167^4 IU (g Hb)$^{-1}$	541^4
GLUTATHIONE REDUCTASE			12.9^4 14.7^2 IU (g Hb)$^{-1}$	28^4 35.4^2
VITAMIN E	2.44^3 μg/ml	4.97^3	1.71^3 μg/ml	11.97^3
GLUTATHIONE	2.45^1 mg (g Hb)$^{-1}$		5.6^2 4.48^4 μM (g Hb)$^{-1}$	16.3^2 8.4^4

Table 2.5: Concentrations of antioxidants found to be either made by the parasite or to be taken up by the parasite. Hb, Haemoglobin. (Altman and Dittmer 1972 (1), Eckman and Eaton 1979 (2), Roth et al 1984 (3), Seth et al 1985 (4), Fairfield et al 1988 (5))

2.6. Summary of Section 2.5.

The parasite invades and inhibits a red cell, where it requires a lipid source to build new membranes for cellular division. These lipids are obtained from the host, and the red cell/parasite has a 3 to 5 fold increase in these lipids compared to unparasitised red cells. There is a preference for unsaturated fatty acids. This will ultimately affect the oxygen sensitivity of the red cell, which is further diminished because the parasite produces oxygen radicals which deplete host oxygen defences. This is partly compensated for by antioxidant mechanisms of parasite origin, but there appears to be a fine balance because the parasitised red cells are very sensitive to external oxygen stress, which could be mediated through lipid peroxidation of membrane protein damage, leading to premature red cell lysis.

2.7. AIMS OF THE PROJECT.

a) To determine some basic properties of the *in vitro* parasiticidal activity of TNS against *Plasmodium falciparum*. Namely inhibition of proteases, denaturation of disulphide bonds and temperature sensitity.

b) To separate the serum into lipid and non-lipid fractions, and then to follow up on the active fractions by;

   1) If the lipids are parasiticidal:- How are they parasiticidal? Can this effect be made *in vitro*?

   2) If the non-lipid fraction is parasiticidal:- Can the activity be separated out by chromatography?

c) To determine the nature of the *in vivo* parasiticidal activity against lethal *P.yoelii* in mice.

d) see b)

e) To determine the importance of TNF in the activity of TNS.

f) Can IL-1 or γ-IFN alone be responsible for *in vivo* activity of TNS?

3. MATERIALS AND METHODS.

3.1. RABBIT SERA

Male and female half lop rabbits bred in the University College and Middlesex School of Medicine's (UCMSM) animal house, aged between 8 months and 4 years were used. They all had had no previous treatments of any kind. The animals were fasted overnight for at least 16 hours before being bled. 10 to 20 ml of blood was taken by ear venupucture into a plastic tube and allowed to clot. This was the pre-treatment serum (Normal rabbit serum-NRS).

5×10^7 live *Mycobacterium bovis* BCG organisms (Glaxo Pharmaceuticals, Greenford, U.K.), freshly prepared in 1ml of 0.9% saline, were injected intravenously (i/v) into the ear vein. 14 to 21 days later the animals were fasted overnight as before and a blood sample taken, as before (BCG serum). Then $80\mu g$ of bacterial lipopolysaccharide (LPS), (*Escherichia coli*, Glaxo), prepared in 1ml of saline was injected i/v into the ear vein. Following this the animals were observed closely and between 1.5 to 2 hours after LPS administration, depending on the animal's condition, they were anaesthetised using Hypnorm (Janssen (Animal Health) Pharmaceutical Ltd, Oxford, U.K.), intramuscularly (i/m) (0.5ml/Kg body weight). The rabbits were then bled out by cardiac puncture using a 16 gauge needle (Sabre, Reading, U.K.) connected to a vacuum pump and glass flask. The blood was allowed to clot as before (Tumour necrosis serum-TNS).

In order to control for the LPS; untreated rabbits were fasted overnight and 10 ml of blood taken for NRS. Then 80 μg LPS was injected i/v into the ear. After 2 hours 10 to 20 ml of blood was taken by ear venupuncture, and allowed to clot for serum (LPS serum).

All sera were stored in 2ml aliquots at -20°C until used.

3.2. SERUM LIPIDS.

3.2.1. SERA TRIGLYCERIDE CONCENTRATIONS.

Triglycerides were measured by using an enzymatic method (kit 676519, Boehringer Mannheim Biochemicals, Indianapolis, Ind, U.S.A.), and an Encore Chemical System (Baker Instruments Group, Allentown, Pa, U.S.A.). These assays were carried out by the Middlesex Hospital Routine Chemical Pathology Department.

3.2.2. SERUM LIPID PEROXIDATION MEASUREMENT. (Buege and Aust 1978).

To 1ml of serum in a glass bijou (Scientific Supplies Ltd, London) were added 5µl of 0.3% butylated hydroxytoulene (Sigma, Poole, Dorset, U.K.) to prevent iron catalysed peroxidation during the assay, and 2ml of a solution containing 15% trichloroacetic acid (BDH, Poole, Dorset, U.K), 0.375% thiobarbituric acid (Sigma) and 0.25N HCl (BDH). The container was then placed into boiling water for 15 minutes. The sample was cooled and centrifuged at 1000 xG for 15 minutes after which the optical density of the supernatant was measured at 535nm, and the concentration of malondialdehyde was calculated by using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}. The amount detected in phosphate buffered saline (0.16M, pH7.2)(PBS) after similar treatment was taken as the zero value in each assay.

3.2.3. SERUM LIPID DEPLETION.

3.2.3.1. AEROSIL (FUMED SILICA). (Stephan 1971).

Aerosil (particle size, 0.008µm) (Sigma), was used to deplete serum of lipoproteins. 20mg aerosil was added to 2ml of serum in a glass Medical Universals (Payne Scientific, Slough, Berks) and then stirred in a water bath at 45°C for 4 hours. The serum was then cooled and centrifuged at 2500 xG for 20 minutes. The supernatant was decanted and dialysed against PBS-5mM glutathione (reduced [GR]) (Sigma) before testing for parasiticidal activity. The glutathione was used

as a water soluble antioxidant.

3.2.3.2. ULTRACENTRIFUGATION.

Serum dialysed against PBS-5mM GR was adjusted to a density of 1.21g/ml with NaBr-5mM GR solution (density 1.51 g/ml). 1ml of NaBr-5mM GR (density 1.21 g/ml) was layered onto the serum preparation in capless 13.5ml double walled polyallomer ultracentrifuge tubes (Kontron Int. Ltd, Watford, Herts). The samples were centrifuged for 44 hours at 5°C in a 50Ti rotor (Beckman, Palo Alto, Ca, U.S.A.) at 105,000 xG in a Beckman ultracentrifuge.

After centrifugation the top 1ml lipoprotein containing layer was removed using glass pipettes (J.Poulten Ltd, Barking, Essex). The technique was to just place the tip of the pipette onto the surface of the lipoprotein layer and aspirate it so that air bubbles were drawn up. This ensured that the lipoproteins were removed in the minimum volume.

The serum protein pellet was resuspended in the remaining volume. All samples were extensively dialysed against PBS-5mM GR and concentrated with polyethyleneglycol (20KD) (BDH) as necessary to adjust volumes back to the starting serum volume. Samples were tested for parasiticidal activity within 48 hours of separation.

3.2.3.2.1. NABR SOLUTIONS.

NaBr solutions were made up in distilled water and the density of the solution was measured using an ABBE refractometer (Carl Zeiss Jena, Borehamwood, Herts, U.K.). The relationship between density and refractive index was calculated from table 69 in the Handbook of Chemistry and Physics 39th Edition, (1981). The glutathione was then added. Solutions were millipore filtered through 0.22µm pores (Millipore, Harrow, Middx, U.K.) and kept at 4°C.

Volume of NaBr (density 1.51g/ml) added to serum was calculated as follows:

The refractive index of the serum was measured and the density calculated (Weast and Astle 1981).

$$\text{Volume} = \left[\frac{\text{Serum volume} \times (\text{Serum density} - 1.51)}{-0.3}\right] - \text{Serum Volume}$$

3.3. CULTURE OF *PLASMODIUM FALCIPARUM.* (Trager and Jensen 1976)

3.3.1. STRAINS OF *P.FALCIPARUM.*

Two strains of *P.falciparum* were used throughout this study: The Ugandan strain Palo Alto (Dubois et al 1984) and the Dutch strain Nijmegen Falciparum 54 (NF54) (Ponnudurai et al 1982).

3.3.2. MAINTENANCE CULTURES *IN VITRO.*

3.3.2.1. CULTURE MEDIUM.

Powdered RPMI 1640 (Gibco, Uxbridege, Middx, U.K.) was rehydrated with double distilled water. 25mM HEPES (N-2-hydroxyethylpiperazine-N-2 ethanosulphonic acid), (Sigma), was added and the pH was adjusted to between 7.25 and 7.4 with 5M NaOH solution. The medium was filtered and stored frozen at -20°C in 1l aliquots until used. Before the medium was used for cultures, sterile sodium bicarbonate solution (5% in water) was added to give a final concentration of 0.2%. Streptomycin (Evans, Glaxo) was added to a final concentration of 40U/ml. This is Incomplete medium.

To make complete culture medium; glutamine (Gibco) and heat inactivated human A+ serum (North London Blood Transfusion Centre, Edgeware, Middx, U.K.) were added so that the final concentrations were 0.03% and 10% respectively.

3.3.2.2. RED BLOOD CELLS.

Whole blood (O+ or A+) (North London Blood Transfusion Centre) between 2 and 7 days old was centrifuged at 2000 xG for 10 mins. The plasma and buffy coat were removed and discarded. The red cells were resuspended in incomplete medium and centrifuged at 2000 xG for 10 minutes. The supernatant was discarded and the red cells washed twice more. The red cells were finally left packed at 4°C until used or up to 7 days before being discarded.

3.3.2.3. Reviving *P.FALCIPARUM* from Liquid Nitrogen.

The vial was thawed in a water bath at 37°C and then immediately placed on ice. The cells were transferred to a 15ml test tube (Falcon, Becton Dickinson Ltd, Oxford, U.K.) and 2 volumes of ice cold Sorbitol (27% in water)(Sigma) was added dropwise with gentle shaking. After 8 mins a further two volumes of ice cold sorbitol (5% in water) were added and then left for 5 minutes. The red blood cells were centrifuged for 5 minutes at 1000 xG and the supernatant discarded. The pellet was resuspended in two volumes of 5% sorbitol and then centrifuged at 1000 xG for 5 minutes. The supernatant was discarded and the pellet was resuspended in complete culture medium. The haematocrit was adjusted to 5% with fresh red blood cells, (250µl per 5ml). 5ml of culture was pipetted into a 50ml culture flask (Nunc, Gibco), and then gassed using a blunt sterile needle (Monoject, Sherwood Medical Ind. Ltd, Crawley, W.Sussex) for 30 seconds with 5% CO_2, 5% O_2, 90% N_2 (BOC Ltd, Guildford, Surrey, U.K.). The flask was then laid on its large flat side with the cap secured tight, and the incubator at 37°C.

3.3.2.4. Culture Maintenance.

This was carried out every 24 hours. The culture flasks were rested at an angle of approximately 45° for 30 minutes in the incubator to allow the red cells to settle into the corner of the flask. At the same time complete medium was warmed to 37°C. The old culture medium was carefully removed using a sterile 1ml plastic pipette (Alpha Laboratories, Eastleigh, Hants, U.K.), so as not to disturb the red cells. A tiny volume of the red cells was then removed to make a smear. 4.5ml of complete medium was added to the flasks and it was gassed and incubated as described above.

3.3.2.5. STAINING OF SMEARS.

After air drying the smears were fixed briefly with methanol (BDH) and then stained with Giemsa (BDH). The Giemsa was diluted 1:10 with Sörenson's buffer (72ml 0.95% NaH_2PO_4 + 28 ml 0.907% KH_2PO_4 + 900ml distilled water), immediately prior to staining the smears.

After 20 minutes the smears were washed with water and air dried. The parasitaemia was assessed using a x100 oil immersion objective and x10 eye pieces on a Zeiss Microscope (Carl Zeiss Jena). Sufficient fields were counted so that approximately 2000 red blood cells were seen.

3.3.2.6. SUB-CULTURING.

When the parasitaemia reached more than 5%, fresh flasks were prepared. These contained 5ml complete medium and $250\mu l$ red blood cells. Sufficient parasitised culture was added to give a new parasitaemia of between 0.001% and 1% depending on how soon parasites were required.

3.3.2.7. SYNCHRONISING CULTURES. (Lambros and Vanderberg 1979)

A post schizogony culture was centrifuged at 1000 xG for 5 minutes and the supernatant discarded. The pellet was resuspended in 2.5 ml of 5% sorbotol in water for 5 minutes at room temperature. This was then centrifuged as above and the pellet resuspended in complete medium. A drop of fresh red cells was added and the culture re-established as before. A smear was made to assess the parasitaemia and to ensure synchrony.

3.3.2.8. Freezing *P.falciparum* Cultures.

The culture was centrifuged at 1000 xG for 5 minutes. The pellet was mixed with glycerolyte solution. (1:3 blood:glycerolyte (Fenwal, Trevanol labs Inc, Doefield, Il, U.S.A)) and put in to freezing vials (Nunc) and frozen directly into liquid nitrogen (BOC).

3.4. Assay for *P.falciparum* Growth.

All sera samples were heat inactivated for 45 minutes at 56°C and dialysed against PBS to remove serum hypoxanthine and xanthine. All samples containing lipid were dialysed against PBS containing GR unless otherwise stated. Dilutions of samples were plated out at 50μl per well in a 96 well flat bottom plate (Nunc) in triplicates. Control wells were of a sample of the dialysate PBS or complete medium. Synchronised cultures at the ring stage were adjusted to a parasitaemia of between 0.5 and 1% at a haematocrit of 5% in complete medium. This was plated out at 50μl per well on to the samples.

The plates were incubated at 37°C in an atmosphere of 5% CO_2, 5% O_2, 90% N_2 in a small Modular Incubating Chamber (Billups, Rothenberg, Del Mar, Ca, U.S.A), containing damp tissue to provide the humidity.

Except for three wells used to make smears, the rest of the plate was pulsed after 24 hours with tritiated hypoxanthine (Amersham Int PLC, Amersham, Bucks) at 10μl per well giving a final concentration of 0.4μCi per well. The plates were incubated for a further 24 hours. A control well was smeared and stained as before. The parasitaemia was assessed to ensure the parasites had grown as normal. The plate was then harvested onto glass fibre paper (Skatron Ltd, Newmarket, Suffolk) using a semi automatic cell harvester (Skatron) with cycle times of: 2 second pre wash with saline, 9 second wash with saline, 9 second wash with water and a final 9 second period to dry the paper. The filter papers were dried on a hot plate at 40°C. The small discs were then put into 4ml scintillation vials (Canberra Packard, Pangbourne, Bucks, U.K.)) and 2ml of scintillant was added to each vial. (Ecosint, National Diagnostics, Manville, NJ, U.S.A). These small vials were placed into larger 10 ml vials (Griffith and Nielson Plastics Ltd, Billingshurst, Sussex, U.K.). This allowed the to

samples to be counted in a Tricarb 574 scintillation counter (Packard Instrument Co., Rockville, Md, U.S.A.). Each sample was counted for 2 or more minutes.

3.4.1. MODIFICATION OF PROCEDURE TO TEST FOR PH DEPENDANCE OF NITROGEN TOXICITY.

The parasite culture were adjusted to a parasitaemia of 1% and then aliquoted into sterile 1.5ml Conicle ependorf tubes (Alpha Labs Ltd, Eastleigh, Hants) at 150μl per tube. They were centrifuged at 2000 xG for 10 seconds and then the supernatant was discarded. The pellets were then resuspended in 250μl of the following: PBS at different pH's, PBS+NaNO$_2$, PBS+NaNO$_2$ at different pH'S, PBS, NaNO$_3$ in complete medium, NaNO$_2$ in complete medium or complete medium. The tubes were incubated in the incubation chamber set up as before. The lids were left loose.

After various times at 37°C the tubes were sealed and centrifuged at 2000 xG for 10 seconds. The supernatant was discarded and replaced by complete medium (300μl per tube.) Each sample was plated out into 96 well flat bottom plates at 100μl per well and the assay continued as in section 3.4.

NaNO$_2$ and NaNO$_3$ (Sigma).

3.4.2. CALCULATION OF RESULTS.

The Means ± Standard Errors of the Means (SEM) were calculated for each triplicate. The mean for uninfected red blood cells was subtracted from each sample. The SEM's remain unchanged. The PBS control was used as the 100% growth sample. All the other samples were then calculated as a percentage of this control sample. This was done for means and SEM's alike.

Individual experiments were expressed as a figure showing percent parasite growth on the y-axis and sample dilution/concentration on the x-axis.

In order to compare one experiment to another and to pool results,

the dilution to inhibit parasite growth by 50% was calculated by regression analysis on the straight part of the curve. A mean ± SEM could be calculated for these results and these were then expressed as a histogram; Samples on the x-axis and dilution/concentration to inhibit parasite growth by 50% on the y-axis. Statistical analysis was carried out on these results using Student's t-test. This was done using a computer programmed to give exact values of probability from the calculated t values and degrees of freedom.

3.4.2.1. EQUATIONS USED. (Neave 1981, Casio Calculator Co.)

$$SEM = Standard\ Deviation/\ \sqrt{n} = \sigma/\sqrt{n}$$

$$\bar{\sigma} = \left[\frac{[(n1-1)\sigma1^2] + [(n2-1)\sigma2^2]}{(N1 + N2 - 2)} \right]^{1/2}$$

$$t = \frac{(mean1 - mean2)}{[\bar{\sigma}\sqrt{((1/n1) + (1/n2))}]}$$

$$Y = n1 + n2 - 2 \qquad\qquad degrees\ of\ freedom$$

Calculation of probability for any give value of t and Υ.

Probability = $p(t, \Upsilon)$ $\Theta = \tan^{-1}(t/\sqrt{\Upsilon})$ in radians

$p(t, \Upsilon\ 1) = 2\Theta/\pi$
$p(t, \Upsilon\ \text{odd})$

$$\frac{2\Theta}{\pi} + \frac{2}{\pi}\cos\Theta\left[\sin\Theta\left[1 + \frac{2}{3}\cos^{2}\Theta + .. \frac{2 * 4 .. (\Upsilon-3)}{3 * 5 .. (\Upsilon-2)}\cos^{\Upsilon-3}\Theta\right]\right]$$

$p(t, \Upsilon\ \text{even})$

$$\sin\Theta\left[1 + \frac{1}{2}\cos^{2}\Theta + \frac{1 * 3}{2 * 4}\cos^{4}\Theta + .. \frac{1 * 3 * 5 .. (\Upsilon-3)}{2 * 4 * 6 .. (\Upsilon-2)}\cos^{\Upsilon-3}\Theta\right]$$

The computer programs for using these equations are shown in the appendix.

3.5. ARTIFICIAL LIPOPROTEINS. (Werb and Cohn 1972)

Four sources of lipid were used; stearic acid (Sigma), linoleic acid (Sigma), corn oil (Sigma) and coconut oil (Sigma).

20mg of lipid was dissolved in 1ml acetone (BDH) and added quickly to 4ml of 1% bovine serum albumin (Sigma) in saline. The mixture was vortexed for 10 seconds followed by sonication for 1 minute in a water bath sonicator (Sonicleaner, Lucus Ultrasonics Ltd, London).

1ml aliquots were put into dialysis tubing (Medicell Int Ltd, London, U.K.) with or without the addition of $50\mu l/ml$ vitamin E acetate (Sigma) at 40 mg/ml in acetone. Half of the aliquots were put into a saline solution and the others into saline plus 0.4mM $FeCl_2$ (BDH). Both groups were incubated overnight at 4°C and then the dialysate was replaced for fresh saline only. The samples were further dialysed for 24 hours with one change of saline. During this period the whole container was sealed in a bag and flushed with nitrogen gas (BOC). The samples were immediately tested for parasiticidal activity. Similar treatment was performed on separated human lipoproteins.

3.6. THIN LAYER CHROMATOGRAPHY.

Lipoprotein samples were spotted onto Silica Gel 60A G plates (Whatman Ltd, Maidstone, Kent) at $2\mu l$ per spot. The plate was put into a glass developing tank containing 100 ml of Petroleum ether (40-60°):Diethyl ether:Acetic acid (90:10:1) (BDH). The lid was sealed in place and the chromatogram run at room temperature until the solvent front reached 1cm from the top of the plate, (approximately 1.5 hours). The plate was removed and left to dry at room temperature for 15 minutes.

The unsaturated fatty acids were developed by spraying the plate with 50% sulphuric acid (BDH) and then baking it in a dry oven for 15 minutes at 140°C.

3.7. Ion Exchange Chromatography.

The gel matrix used was DE-52 (Whatman Ltd, Maidstone, Kent, U.K.). which is positively charged and therefore separates proteins on the basis of their negative charge, (Pharmacia information booklet). The column dimensions were; radius 12.5mm, height 132.5mm and volume 65ml. The column was stored at 4°C with the gel equilibrated with 0.04M NaCl 0.01% NaN$_3$ (Sigma) pH 7.2. The apparatus was arranged as in figure 3.1.

The column was equilibrated with 0.04M PBS (1:4 dilution of 0.16M PBS) at room temperature. The serum sample was dialysed overnight against the same buffer.

10ml serum was pumped onto the column at 42ml h^{-1}, and then proteins were eluted using 0.04M PBS until the absorbance at 280nm returned to zero. The remaining bound proteins were sequentially eluted using the following buffers; 0.08M PBS, 0.16M PBS and 1.5M NaCl respectively. The buffer being changed. when the absorbance returned to zero. The pump speed was maintained at 42ml h^{-1} and 10ml fractions were collected.

The 1.5M NaCl eluted all the remaining bound proteins from the column. After this buffer the column was equilibrated with 0.04M PBS plus 0.01% NaN$_3$ for storage.

Like fractions were pooled respective to the elution buffer and concentrated with PEG 20KD back to the original serum volume. The four fractions were then dialysed against PBS and stored at -20°C until used.

3.8. Molecular Weight Chromatography.

The gel matrix used was Sephacryl S-200 superfine (Pharmacia LKB Biotechnology), which separates molecules on the basis of size, between 10KD and 250KD for dialysed samples (Pharmacia information booklet).
The column dimensions were; radius 7.5mm, height 840mm and volume 148.4 ml. The column was stored at room temperature with the gel equilibrated with PBS 0.01% NaN$_3$ pH7.2. The apparatus was arranged as in figure 3.2. Before running a sample it was washed through with PBS.

6ml of ion exchange fraction was concentrated to 1ml using
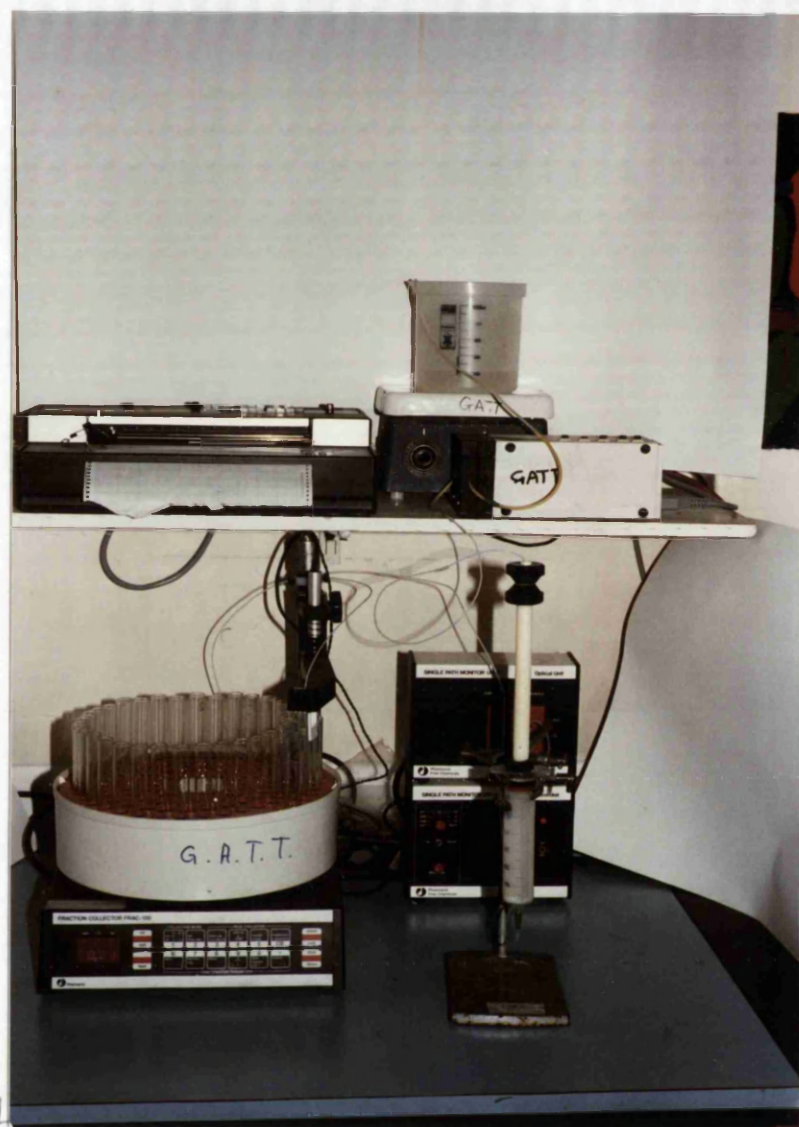
Plate 3.1: Apparatus for ion-exchange chromatography; pump, column (70 ml syringe (Sabre)) with column adaptors, single path UV monitor unit and power supply, Frac-100 fraction collector and a flat bed recorder. All apparatus was from Pharmacia-LKB Biotechnology, unless otherwise stated.

Plate 3.2: Apparatus set up for molecular weight chromatography; pump, 1m x 1.5cm diameter coulumn, and an Ultrorac fraction collector. All apparatus was from Pharmacia-LKB biotechnology.

PEG 20KD. The sample was centrifuged briefly at 16Krpm (minifuge, MSE, Crawley, W.Sussex) to remove any precipitated protein. The supernatant was pumped onto the column and the fraction collector started. Each fraction was collected for 10 minutes which was equivalent to 3.5 ml at a speed of 21 ml h^{-1} or 6.5 ml at 39 ml h^{-1}.

After the sample separation the column was re-equilibrated with PBS 0.01% NaN$_3$. The absorbance 280nm was measured for each fraction in a spectrophotometer (Pharmacia LKB Biotechnology). From the absorbance trace, 3-5 pools were made and concentrated back to 6ml with PEG 20KD. These were stored at -20°C until used.

3.8.1: MEASUREMENT OF PROTEIN CONCENTRATIONS.

The Bio-Rad protein assay kit No. 500-0002 was used (Bio-Rad) . The assay was carried out as per the instruction manual (Bio-Rad 1986)

3.8.2: MEASUREMENT OF LIPOPOLYSACCHARIDE.

This was carried out as using a chromogenic assay as per the kit's own instructions (QCL-1000 LPS assay kit, Whittaker Bioproducts Inc. U.S.A.)
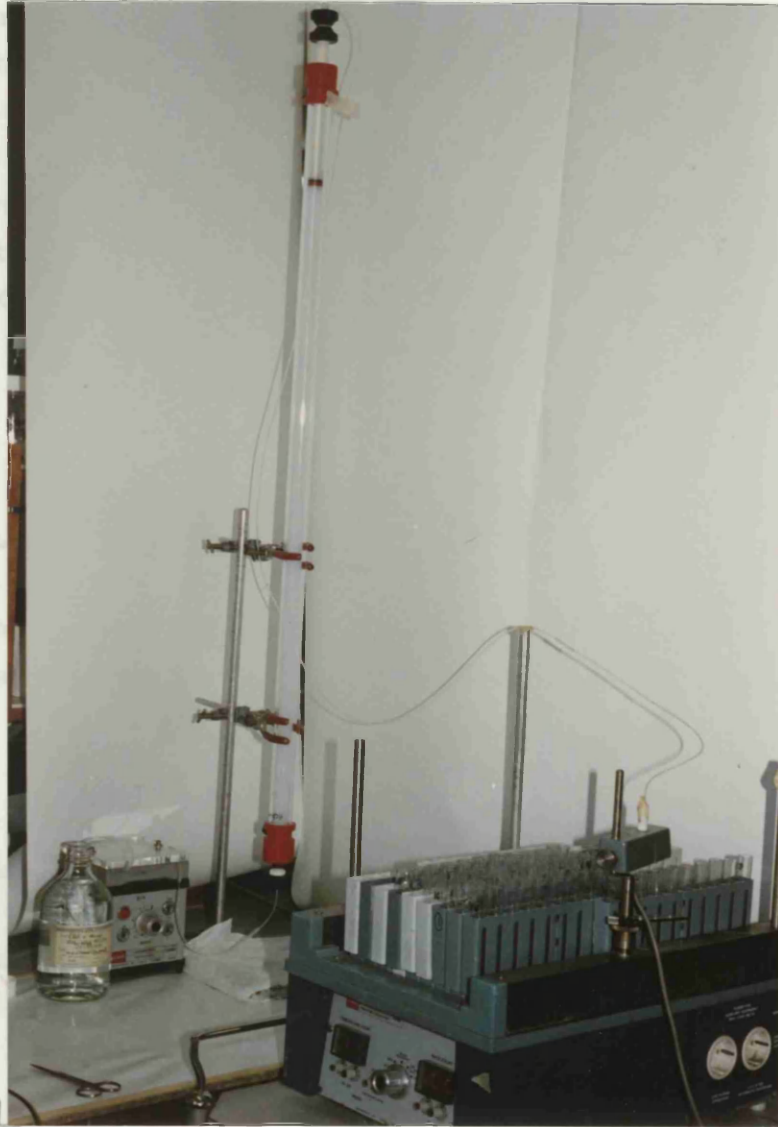
3.9. GEL ELECTROPHORESIS.

3.9.1. AGAROSE GEL ELECTROPHORESIS.

23 ml of 1% agarose (Sigma) in 0.075M barbital buffer (Sigma), 2mM calcium lactate (sigma), pH8.6 was poured onto a glass plate 11cm x 20.5cm to give a gel 1-2mm thick. This was placed into a flat bed gel electrophoresis apparatus (Biorad, Hemel Hempstead, Herts) with an electrode solution the same as the gel buffer. Serum samples were used neat and placed into wells at the anode end of the gel (5μl per well). Bromophenol blue solution (BDH) was put into a separate well

as a marker to show the run progress. The gel was run for 1.5 hours at 15V/cm by which time the bromophenol blue front had travelled approximately 3/4 of the gel length. When the run was finished the gel was fixed in 5% trichloroacetic acid in water for 20 minutes and then washed with water. The lipids were stained with the following:

Sudan black (BDH) solution for 2 hours (0.4g sudan black in 120ml ethanol plus 4g zinc acetate (sigma) and 80 ml distilled water. This was brought to the boil and filtered). The gel was destained in ethanol:water (1:1).

3.9.2. SDS-Polyacrylamide Gel Electrophoresis.

This was used to determine the molecular weight content of any active fractions.

3.9.2.1. Gel Solutions.

10% acrylamide (Sigma) plus 0.25% Bisacrylamide (Sigma) in 0.375M Tris/HCL buffer pH8.8 plus 0.1% sodium dodecal sulphate (SDS) (Sigma) and 0.02% ammonium persulphate (Sigma).

3.9.2.2. Gels.

Prior to pouring the gel solution into the moulds (Biorad). TEMED (N,N,N',N' Tetramethlethylenediamine), (Sigma) was added (20μl/60ml gel). Final gel size was 15cm x 10cm x 0.2cm. Butanol (BDH) was overlayed onto the gel in the mould to allow it to polymerise in the absence of oxygen. When the gel was set the butanol was washed off and a 5% stacking gel measuring 15 x 3 x 0.2cm was poured on top. A 15 well comb was inserted and the gel left to set for 2 hours. The comb was removed and the gel clamped into the running apparatus.

3.9.3. RUNNING BUFFER.

0.05M Tris, 0.38M Glycine (Sigma) pH8.7 plus 0.1% SDS.

3.9.4. SAMPLE BUFFER.

The samples were made up to 75% v/v in 10% SDS, 50% Glycerol (BDH) in 0.128M Tris pH6.8 plus 100μl/ml bromophenol blue solution (BDH).

3.9.5. RUNNING CONDITIONS.

40 μl of each sample was loaded per well and 10μl of molecular weight markers were used (prestained low and high standards- Biorad). per gel.

Markers; Tri-isophosphate isomerase (26.6KD), Lactic Dehydrogenase (36.6KD), Fumerase (48.5KD), Pyruvate kinase (58KD), Fructose-6-phosphate kinase (84KD) and β galactosidase (116KD).

The running time for the gel was 4hours at 45mA constant current.
After completing the run the gel was removed and stained in 0.25% Coomassie blue (Sigma) w/v in 50% methanol, 10% acetic acid, 40% water. The gel was destained in 50% methanol, 10% acetic acid and 40% water.

3.10. Tumour Necrosis Factor Activity of Sera.

As tumour necrosis factor (TNF) has been implicated as part of the anti malarial activity of TNS, the TNF activity of whole or separated sera was measured. This was very kindly done by Dr. J. Taverne and Mr. C. Bate of the Dept of Immunology, UCMSM, using the L929 tumour cell line bioassay.

3.11. Inhibiting Protease Activity and Denaturing Disulphide Bonds.

TNS dialysed against PBS was used. TNS was pipetted into glass bijoux and the following were added. Final concentrations are quoted.

a) TLCK (N -p-Tosyl-L-lysine Chloromethyl Ketone)
at 10^{-4}M (Sigma). Incubated for 60 mins at 4°C.

b) Protease inhibitor mixture (PIM):- PMSF (Phenylmethylsulfonyl Fluoride) at 5mM (Sigma), Pepstatin 1μg/ml (Sigma), Leupeptin 1μg/ml (Sigma) and Trasylol 6.25 U/ml (Sigma). These were incubated at 4°C for 30 minutes.

c) Dithiothreitol (DTT) 10^{-4}M (Sigma). Incubate at 30°C for 30 minutes. Untreated TNS was also incubated for 30 minutes at 30°C.

All sera were dialysed against PBS before being tested for parasiticidal activity.

3.12. MICE.

Mice were housed in polypropylene cages and fed a standard rat and mouse No1 maintenance diet (SDS Ltd, Witham, Essex, U.K.), *ad libitum* and water *ad libitum*.

Two strains of mice were used; Tuck No1 (Tuck and sons Ltd, Battlebridge, Essex, U.K.) outbred strain. This strain was used for most experiments except for vaccination experiments where an F1 hybrid between C57/black and Balb/c (UCMSM) was used. The Tuck mice were females between 3 and 15 weeks old. The F1 hydrids were males between 10 and 20 weeks old.

3.13. MAINTENANCE OF *PLASMODIUM IN VIVO.*

The species of choice was a lehal strain of *Plasmodium yoelii* (YM) (LPY) (Freeman and Holder 1983) in the strains of mice used. The parasites were maintained by blood passage.

An untreated mouse was used as the recipient. The donor was also an untreated mouse to ensure there were no selective pressures put on the parasites. This mouse's tail was snipped at the very end and a drop of blood was taken to make a smear. Two or three more drops were put into a tube containing ice cold PBS. The smear was treated as in section 3.3.2.5. A red blood cell count was made using a haemocytometer (Arnold R. Horwell Ltd, W.Hampstead, London) using a microscope with a x 40 objective and x 10 eye pieces (Carl Zeiss Jena).

From the parasitaemia and red cell count the number of parasites per ml was calculated. From this an appropriate dilution was made to give a final parasite count of 5×10^4 per ml. 200μl (10^4 parasites was injected i/v into the tail vein of the recipient mouse. This regimen meant that a 5 to 10% parasitaemia occurred after 4 days. This was also the regime used to infect all the mice used in the experiments.

3.13.1 Other Species of Rodent Malaria.

A non-lethal strain of *P.yoelii* (17X) (NLPY) (Taverne et al 1982) was maintained and used as above.
A strain of *P.chabaudi* (PC7/F1 from The Pasteur Institute, Paris, France) was also maintained in the same way but experiments were only done in the F1 hybrid mice.

3.13.2. Vaccination Regimen. (Playfair et al 1977).

Blood from a donor mouse with a parasitaemia >50% was collected using a 1ml syringe (Sabre) and a 21 gauge needle (Sabre) by cardiac puncture following neck dislocation. The blood was immediately put into 5 ml of ice cold PBS. The blood was washed twice with ice cold PBS. After the last centrifuge (2000 xG for 10 mins), the pellet was resuspended in 0.01% saponin (w/v) (BDH) for 30 minutes at 4°C to lyse the red cells. The blood was then washed twice with ice cold PBS as before. The pellet of released parasites was resuspended in 0.6% formalin solution (BDH) for 10 minutes at room temperature. The parasites were washed three times in PBS. A parasite count was made using a haemocytometer and then the parasites were resuspended at 2×10^8/ml and mixed with 0.33 ml pertussis toxin (Wellcome)/ml of parasite suspension. Mice were immunised with 0.4ml i/v with the above mixture (final dosage per mouse-6 x 10^7 parasites + 0.8 EIU pertussis). The mice were ready for challange with a live infection 14 days later.

3.14. *IN VIVO* ACTIVITY OF TNS AND FRACTIONATED TNS.

Three mice per group were used unless otherwise stated. Each mouse was infected with 10^4 lethal *P.yoelii* (YM) parasitised red blood cells as in the above section.

The mice were then injected intraperitoneally (i/p) with 0.5ml PBS, serum or fractions and then every other day up to day 4 or 8.

Blood films were made from tail snips from day four as described above and stained with Giemsa.

3.15. *IN VIVO* ACTIVITY OF TNS INCUBATED WITH AN ANTISERUM MADE TO TNF.

A goat anti rabbit TNF antiserum (No. 6688) kindly provided by Dr. N.Rapson (Wellcome, Beckenham, Kent), was mixed with TNS at a dilution known to inhibit TNF activity as shown by the L929 bioassay.

The serum/antiserum mixture was left at room temperature for 1 hour to ensure complexing of the antibody to the TNF. Normal goat serum from a pre bleed of the animal was used as a control, at the same dilution.

3.16. RECOMBINANT CYTOKINES.

The following cytokines were tested:

50 000 U/dose recombinant murine IL-1α (Hoffman La Roche, Inc, ß-Sclave, Sienna, Italy)

10 000 U/dose recombinant murine IFN-γ (Gift of Dr. Adolf, Vienna, Austria).

Each was given in 1ml PBS i/p on days 0,2 and 4 after LPY infection.

3.17. Antioxidants.

3.17.1. Supplemented Food and Water.

3.17.1.1. Vitamin E in Food.

Mouse diet pellets were minced into a powder. The powder was divided into portions. All portions were treated in the same way except that one portion was mixed with vitamin E.

2% Vitamin E oil (Sigma) in acetone (BDH) was added to powdered mouse diet at 50ml to 250g respectively. The food was mixed thoroughly and left for 48 hours to allow the acetone to evaporate.

3% gelatine (Davis gelatine, Leamington Spa, Warks, U.K.) was made up in hot distilled water as directed on the packet. This was added to the powdered food at 100ml per 100g. The food was well mixed and then lightly pressed down. The food was then allowed to cool and set before being cut into approximately 25g blocks and frozen at −20°C to prevent fungal growth. The food was presented to the mice in glass Kilner jars (Ravenshead Glassware, U.K.) with special wire inserts to allow access to the food. (figure 4.1). The feeding regime allowed for 10g of the gelled food per mouse per day which was adequate enough without much waste. The food was changed each day and the jar rinsed out each day. This method prevented the animals scattering the food around the cage and allowed for easy measurement of daily consumption. This diet was started from the day of infection.

3.17.1.2. Vitamin C in Water.

L-ascorbic acid (Vitamin C) (Sigma) at 10mg per ml in distilled water was millipore filtered (0.22μm) and put into autoclaved drinking bottles. Otherwise it was found that fungi grew in the water within 2 days. The mice were allowed to drink *ad libitum* and the consumption was measured each day.

For the experiments, mice were injected i/p with 20mg Vitamin C in PBS 24 hours before infection with the parasites. They were then given the supplemented drinking water.
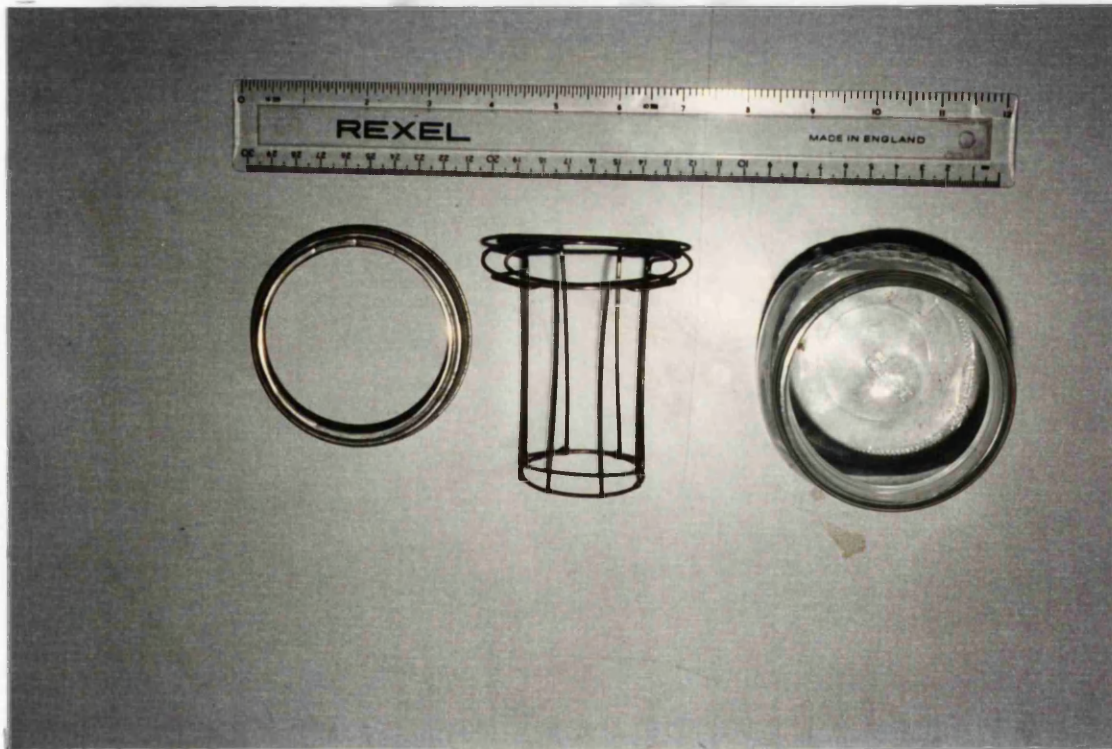
Plate 3.3a: Jar used for feeding vitamin E supplemented and control
diets to the mice.
Left; plan view of jar.
Right; Side view of jar.
Plate 3.3b: Individual components of the jar in plate 3.3a.
Left to right; Cap, wire insert and glass jar.
The scale is shown in both inches and centimetres.

3.17.1.3. Antioxidants Injected Directly.

Three antioxidants were used. These were Superoxide Dismutase, Trolox (active part of vitamin E) and nordihydroguariaretic acid. (Sigma, Aldrich (Gillingham, Dorset), Sigma respectively). These were made up in PBS to 5000 U/ml, 40mg/ml and 20mg/ml respectively. All were made up and stored in 1.5 ml aliquots at -20°C prior to use. The mice were injected with 0.5 ml of these antioxidants each day from 24 hours before infection with the parasites.

4. RESULTS. (*IN VITRO* KILLING OF *P.FALCIPARUM*).

4.1. TWO SIGNAL REQUIREMENT TO INDUCE A PARASITICIDAL FACTOR.

Rabbits were injected with BCG, LPS or both. From figure 4.1 it can be seen that only the combination of BCG and LPS produces a serum factor which can be more than 50% parasiticidal. These effects are not due to complement as all the sera were heat inactivated before the assay. They are not due to competition by unlabelled hypoxanthine in the sera because all the sera were dialysed before the assay. This also means that either the parasiticidal factor(s) are >10KD in size under the conditions of the assay, or that they are associated with larger molecules.

Of the control sera, the LPS serum may be more parasiticidal than the others but it still does not inhibit parasite growth by more than 40%.

Plates 4.1 to 4.6 show the appearance of the parasite after incubation for 24 hours with TNS and NRS. At this point in the assay, trophozoites predominate because the assay started with synhronised ring forms. Plate 4.3 shows trophozoites in normal rabbit serum at a dilution of 1:2. They are large and healthy looking, and go on to develop into normal schizonts and take up labelled hypoxanthine. The parasites incubated with a 1:2 dilution of TNS (plates 4.5 and 4.6) are much smaller and denser. After 24 hours very few parasites, if any, were seen. These should be compared to the control parasites in plates 4.1 and 4.2.

Similar inhibition of *P.falciparum* growth was seen when asynchronous cultures were used. Thus the inhibitory effect of TNS was not stage specific.

One other important feature of the TNS parasiticidal activity *in vitro*, is that it dilutes out very quickly. In any given TNS tested the activity was lost at a dilution of between 1:32 and 1:64.
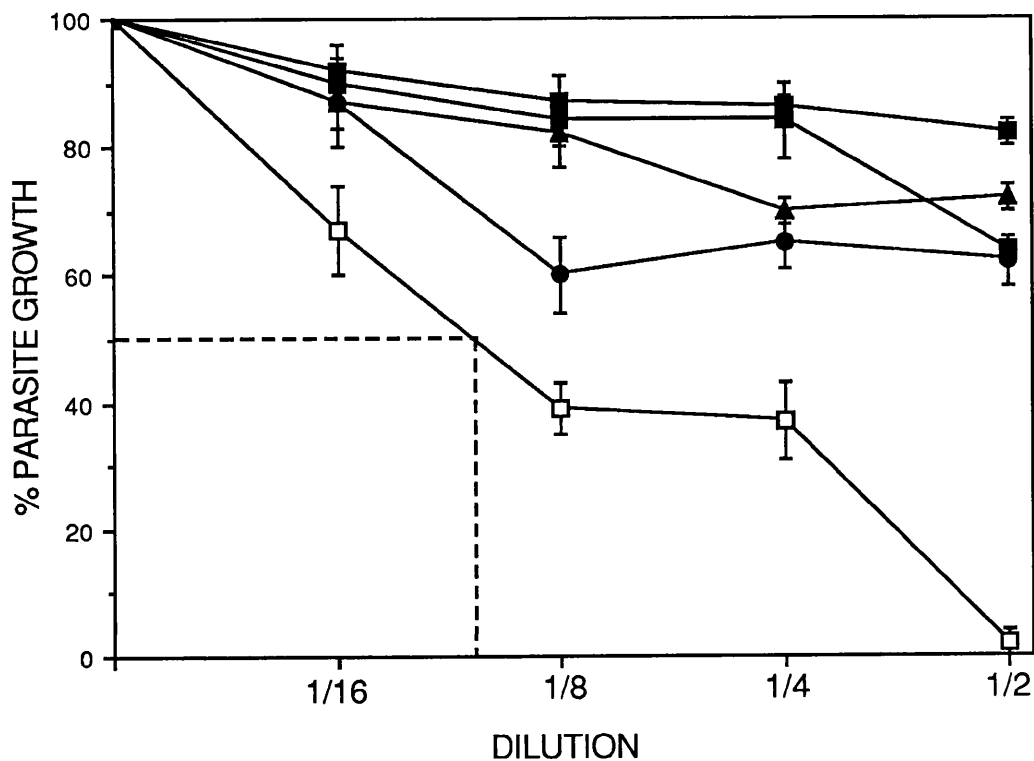
Figure 4.1: Inhibition of *P. falciparum* growth by serum samples from rabbits treated with BCG alone (—▲—), LPS alone (—●—), BCG followed 14 days later by LPS (—□—), or serum samples from two normal rabbits (—■—). The data given are from a single experiment in which all sera were tested at the same time. The point used to compare parasiticidal activity in experiments is the dilution of serum required to inhibit parasite growth by 50% (———). Each datum point is the mean ± standard error of the mean for the sample tested in triplicate.
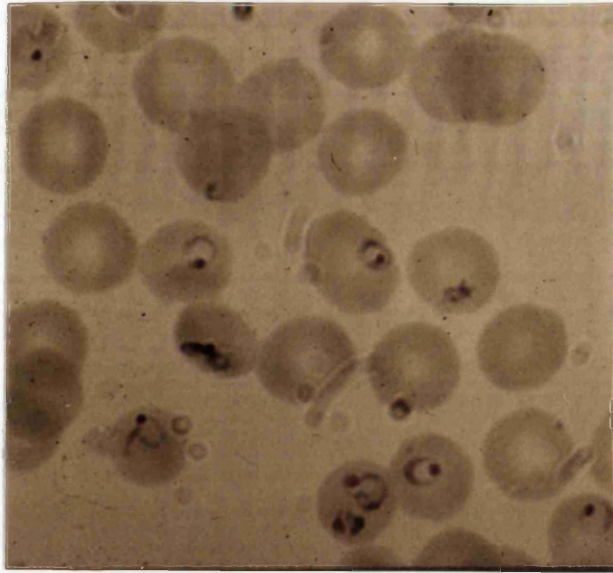
Plate 4.1: Appearance of *P. falciparum* parasites before the start of
the assay. The parasites have not been diluted as yet. Giemsa stain.
Magnification x1000. Ring stages.
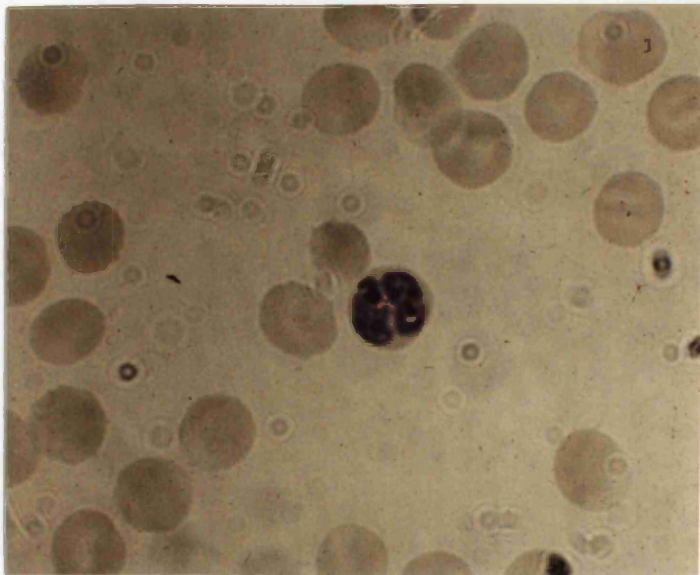
Plate 4.2: Appearance of *P. falciparum* parasites 24 hours after the
start of the assay. Giemsa stain. Magnification x1000.
Double Trophozoite infection.
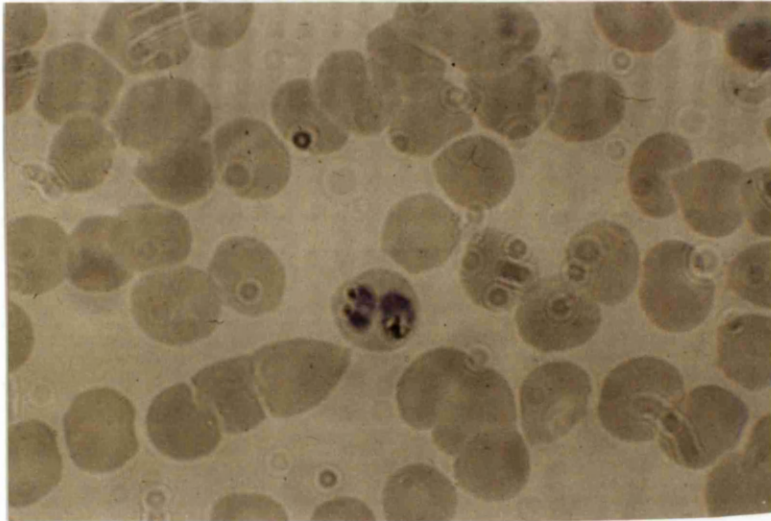
Plate 4.3: Appearance of *P.falciparum* parasites after 24 hours in *in vitro* culture with 1:2 NRS. Giemsa stain. Magnification x1000. Trophozoites. Double infection.
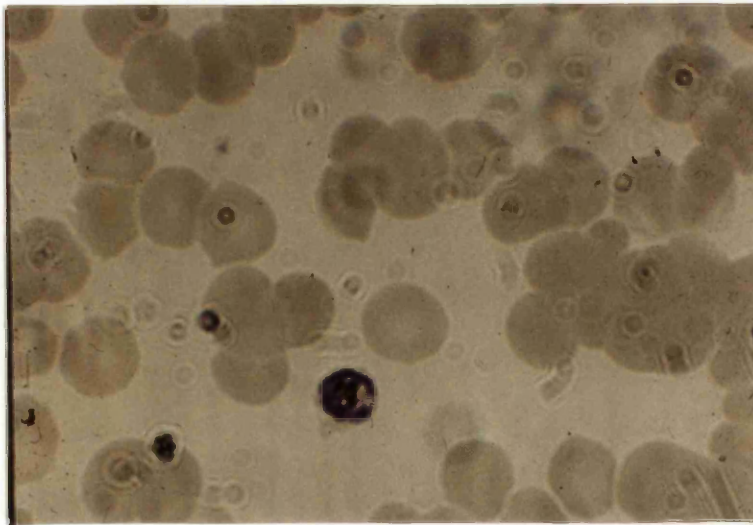
Plate 4.4: Appearance of *P.falciparum* parasites after 24 hours in *in vitro* culture with 1:2 TNS. Giemsa stain. Magnification x1000. Young schizont.
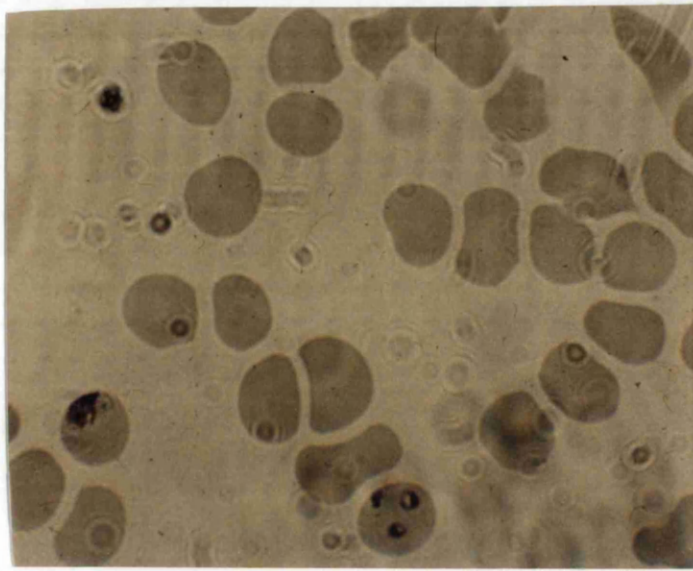
Plate 4.5: Appearance of *P. falciparum* parasites after 24 hours in *in vitro* culture with 1:2 TNS. Giemsa stain. Magnification x1000. Schrivelled rings and trophozoites.
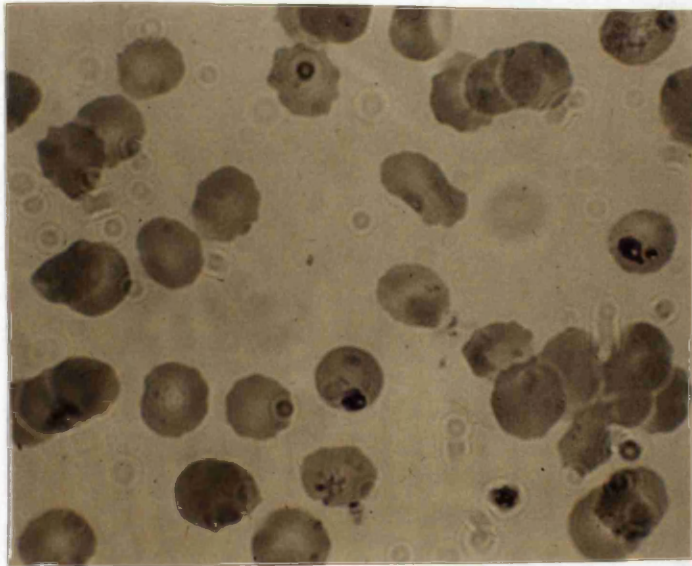
Plate 4.6: Appearance of *P. falciparum* parasites after 24 hours in *in vitro* culture with 1:2 TNS. Giemsa stain. Magnification x1000. Schrivelled rings.

4.2. SOME BASIC PROPERTIES OF THE *IN VITRO* PARASITICIDAL FACTOR.

TNS was incubated with either protease inhibitors, a disulphide bond denaturing agent, or at different temperatures.

After dialysis the serum was tested for parasite killing. Table 4.1 shows the data collected. *P.falciparum* growth was inhibited by 50% by 14% TNS (1:7 dilution) as compared to 85.7% (1:1.7 dilution) for NRS (p=0.00082).

Destroying protein tertiary or quaternary structure by breaking disulphide bonds had little effect on TNS activity, (TNS 14.09% to TNS+DTT 18.91%). Similarly neither the different protease inhibitors, nor the protease inhibitor mixture had any effect on the parasiticidal activity of TNS, (TNS 14.09% to TNS-TLCK 18.91% or TNS-protease inhibitor mixture 15.71%).

DTT was incubated at 30°C with TNS, therefore a 30°C control was done for this incubation. However neither this incubation nor the DTT decreased the activity of TNS, (TNS 14.09% to TNS 30°C 8.85% or TNS-DTT 18.91%).

The higher incubation temperature chosen, was 65°C as at this temperature proteins may be inactivated without visible denaturation such as precipitation, although it was noted that after a longer incubation some precipitation did occur. It can be seen from table 4.1 that this temperature had no effect on the TNS parasiticidal activity, (TNS 14.09% to TNS 68°C 12.50%).

These results suggest that the parasiticidal factor(s) in TNS are neither a protease nor a protein requiring disulphide bonds for its activity, and that the molecule is not heat sensitive under the conditions tested.

SERUM SAMPLE	% serum mean ± SEM (n) to inhibit parasite growth by 50%	
NRS	85.70 ± 7.34	(4)
TNS	14.09 ± 5.90*	(3)
TNS+DTT	18.91	(2)
TNS+TLCK	18.91	(2)
TNS+PIM	15.71	(1)
TNS 30°C	8.85	(1)
TNS 65°C	12.50	(1)

Table 4.1: Effect of protease inhibitors, disulphide bond denaturing agents and temperature on the parasiticidal effect of TNS *in vitro*. DTT 10^{-4}M, TLCK 10^{-4}M and PIM (protease inhibitor mixture - see section 3.11). TNS 30°C is the control for DTT. The control sera (TNS and NRS are those used for these experiments). *, p=0.00082 compared with normal rabbit serum.

4.3. Effect of Aerosil on the Parasiticidal Activity of TNS *in Vitro*.

Aerosil has been shown to almost exclusively remove the lipids from serum. To check this, sera were separated by agarose gel electrophoresis and then stained for lipid. Plate 4.7 shows the two different patterns of lipid in TNS and NRS. The NRS has a uniform lipid band whereas the lipid pattern for TNS is concentrated more towards the β region of lipoproteins (corresponding to the β globulins). The minor band of TNS is nearer the α region of lipoproteins, corresponding to the α globulins. Albumin, seen as a small band about half way between the dye front and origin, stains because of bound fatty acids. This is the only band in aerosil treated serum, and is slightly smaller than the corresponding band in untreated serum. This confirms that aerosil removed all the lipid from the serum except for that associated with albumin.

Figure 4.2 shows the parasiticidal activity of these sera expressed as the mean dilution to inhibit parasite growth by 50% ± standard error of the mean. For TNS this is 1:4.19 (23.89% ± 2.9%) and for NRS is 1:1.19 (60.69 ± 6.43%). These are highly significantly different by Student's t-test, (t=4.96, Y=59, p=6.28 x 10^{-6}).

When the sera were incubated at 45°C to control for the aerosil incubation, little change in activity was observed (TNS 1:4.19 to 1:5.22, not significant, and NRS 1:1.19 to 1:1.28 not significant).

After incubation with aerosil, the parasiticidal activity was markedly reduced in TNS, (1:4.19 to 1:1.36) by 89% (p=0.0000254) compared with untreated TNS. The weaker activity of NRS was also lost. These results are highly suggestive that the TNS parasiticidal factor is lipid in nature.

Tritiated hypoxanthine was used to assess parasite growth in the above assays, however this assay could not be used to test the aerosil serum fraction because the aerosil particles clogged the cell harvesting apparatus. This was overcome by making smears and counting the parasitaemia visually.

Over the 24 hours the parasitaemia increased from 3% to 5%. Figure 4.3 shows that the TNS is more toxic than NRS. This would look similar to figure 4.1 after 48 hours. However the aerosil alone was 100% parasiticidal after 48 hours, perhaps because it interferes with uptake of nutrients or oxygen, or it physically prevents parasite

reinvasion. It can be seen that the aerosil treated TNS had lost all its activity, while the aerosil fraction had similar activity to whole TNS. Therefore these results support the nucleotide uptake results in figure 4.2.

This assay was awkward to use and control, therefore another method of separation the lipoprotein fraction of TNS was tried, namely ultracentrifugation.
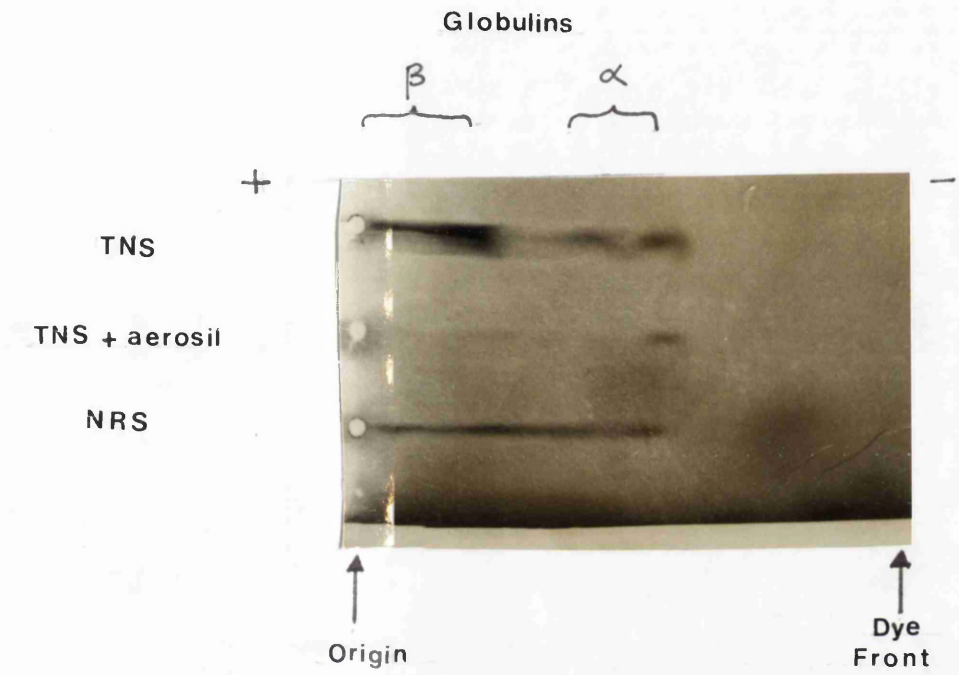
Plate 4.7: Agarose gel electrophoresis of TNS, NRS and aerosil treated TNS. The gel was fixed with trichloroacetic acid and then stained with sudan black. Lipids stain black.
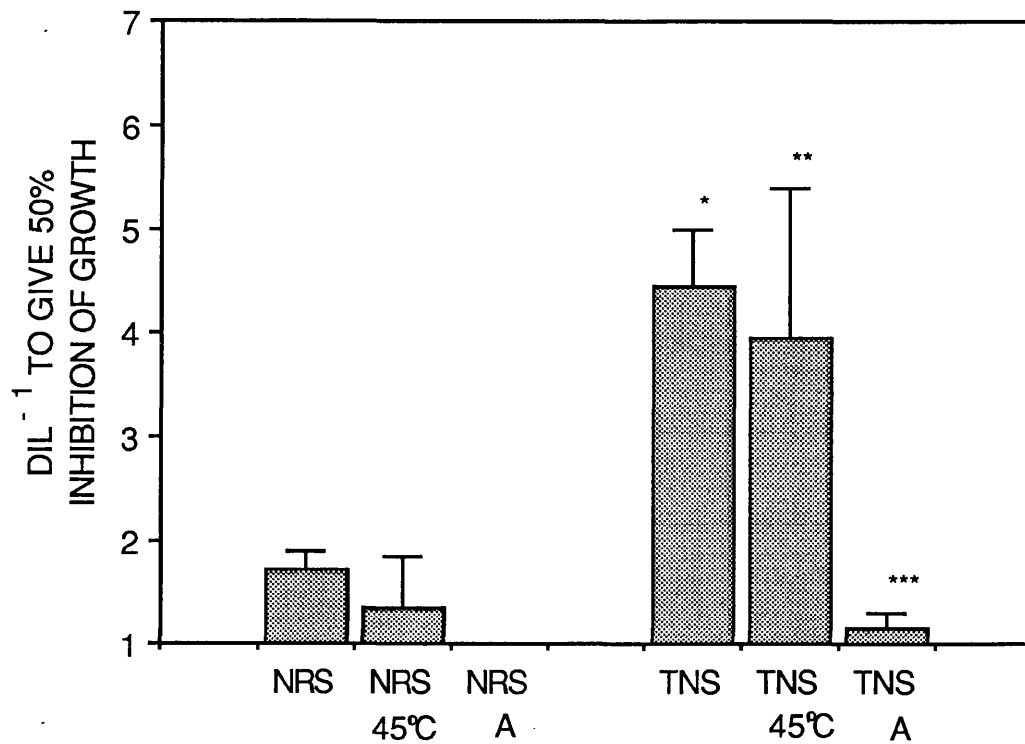
Figure 4.2: Inhibition of *P.falciparum* growth by TNS and effect of depleting serum lipoproteins with aerosil. The values were calculated as in the legend to figure 4.1. Abbreviations: 45°C, serum incubated for 4h at 45°C; A, serum after treatment with aerosil at 45°C; Dil^{-1}, dilution^{-1}; NRS, normal rabbit serum; TNS, tumour necrosis serum. Each bar is the mean ± Standard error of the mean for four to six experiments. Symbols: *, P<0.001 compared with normal rabbit serum; **, not significant compared with TNS; ***, P<0.001 compared with TNS.
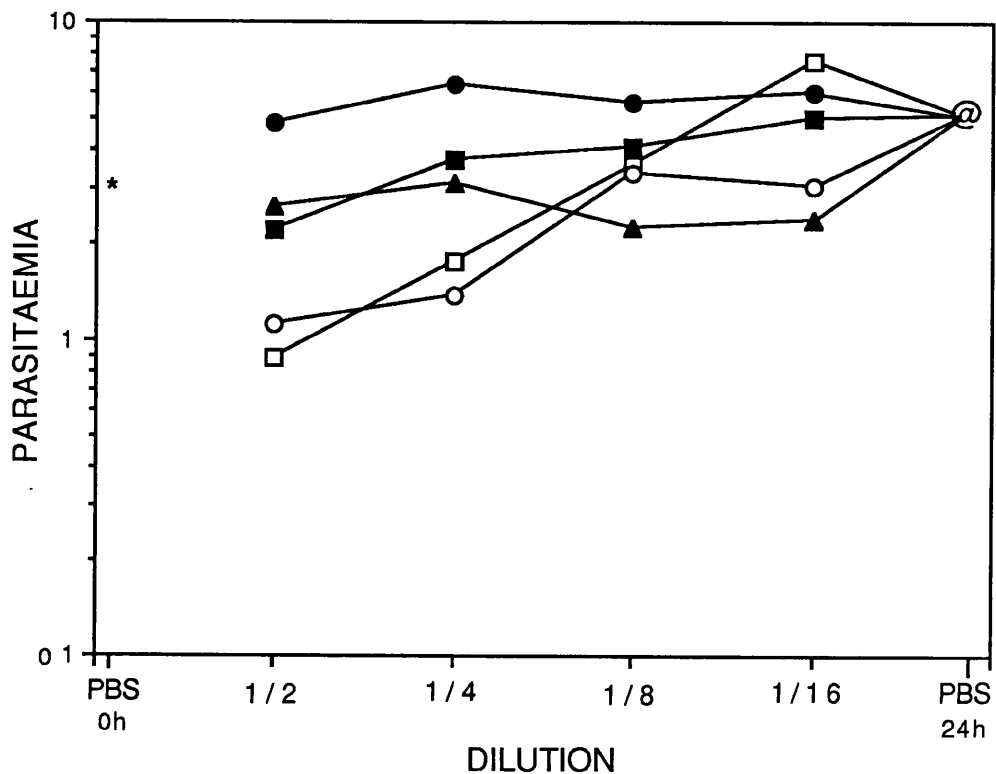
Figure 4.3: Inhibition of *P.falciparum* growth by TNS and the effect of depleting serum lipoproteins with aerosil. Instead of using ³H-hypoxanthine to estimate parasite growth, smears were made from each well and the parasitaemia assessed by microscope examination after staining the smears with giemsa. Each point is the mean of two smears.

Symbols: —■—, Normal rabbit serum; —□—, tumour necrosis serum, —●—, TNS after treatment with aerosil; —○—, TNS lipoproteins bound to aerosil; —▲—, aerosil alone; *, stating parasitaemia (t=o); @, control parasiteamia after 24h incubation (t=24h).

4.4. EFFECT OF ULTRACENTRIFUGATION ON THE *IN VITRO* PARASITICIDAL PROPERTIES OF RABBIT TNS.

TNS and NRS were made up to a density of 1.21 g/ml with NaBr. They were centrifuged at 105 000 xG for 44h at 5°C. The centrifuged sera are shown in plate 4.8. The most noticeable difference is the thick white lipoprotein layer on TNS and not NRS, which occurred with every separation. The top 1ml from each tube was removed and put into glass tubes (plate 4.9). This gives a better idea of the relative amounts of lipoprotein in the sera. Because the lipids are sensitive to oxidative attack, antioxidants were added during all steps of the separation and assay.

When assayed for parasiticidal activity by tritiated hypoxanthine incorporation, the unseparated and separated NRS were not significantly different. However when the TNS was tested, the lipoprotein depleted serum's activity was greatly reduced (figure 4.4) (TNS 1:4.19 to TNS lipoprotein depleted 1:2.18). This is equivalent to a 63% reduction in activity, somewhat less than that for aerosil (89% reduction).

The lipoprotein fraction of TNS had similar activity to whole TNS (p=0.00397 compared with NRS).

Two effects may be responsible for these observations; triglyceride concentration and lipid peroxidation.

T   N

Lipoproteins ⟶

Plate 4.8: Appearance of sera after preparative separation of lipoproteins by ultracentrifugation on a NaBr gradiant of 1.21 g/ml and 105 000 xG for 40h at 5°C. T, Tumour necrosis serum; N, Normal rabbit serum.

Plate 4.9: Appearance of the top 1ml of lipoproteins from the sera samples in plate 4.8 after aspirating into clean glass test tubes. Left hand tube-Tumour necrosis serum, Right hand tube-Normal rabbit serum.

Figure 4.4: Inhibition of *P. falciparum* growth by TNS and the effect
of preparative separation of lipoproteins by ultracentrifugation of
sera at a density of 1.21 g/ml for 40h at 105 000 xG and 5°C. The
values were calculated as described in the legend to figure 4.1.
Abbreviations: LP, lipoprotein fraction of serum; -LP, serum depleted
of lipoproteins; Dil^{-1}, dilution^{-1}; NRS, normal rabbit serum; TNS,
tumour necrosis serum. Each bar represents the mean ± standard error
of the mean for between 6 and 9 experiments.
Symbols: *, p6.28 x 10^{-6} compared with NRS; **, p=0.00397 compared
with NRS; ***, p=0.033 compared with TNS.

4.5. CORRELATION OF PARASITICIDAL ACTIVITY WITH LIPID PEROXIDATION.

The three control rabbit sera and the tumour necrosis serum were tested for lipid peroxidation by measuring the malondialdehyde (MDA) concentration in the sera. This provides an indication of lipid peroxidation rather than an absolute measurement. The serum triglyceride concentrations were also measured.

The qualitative triglyceride measurements seen after ultracentrifugation (plates 4.8 and 4.9) were confirmed in table 4.2. There was a significant increase in triglycerides in the BCG sera and TNS sera. LPS induced a greater increse in the concentration of serum trigyceride. The increase in triglycerides were 2.28 fold, 2.92 fold and 3.43 fold respectively. All the animals were fasted overnight before being bled which means that the increases were induced by the treatments.

When these levels were compared to the 50% parasite growth inhibition data, there was no correlation with parasiticidal activity. LPS and BCG sera, although having as high a triglyceride concentration as TNS, were not as toxic to the parasites. However the serum MDA concentrations did correlate with the parasiticidal activity of the sera. There was no increase in MDA concentration in the BCG sera, and only a small rise in the LPS sera, while in TNS there was a 2.3 fold increase (p=3.15 x 10^{-6}).

If these data are taken together with the aerosil and ultracentrifugation data, they suggest that lipid peroxides are toxic to the malaria parasites. The lipid peroxides are actively generated within the rabbit when TNS is induced. The rabbits can have high serum triglycerides without lipid peroxidation (BCG sera). The administration of LPS to unprimed animals caused a small increase in lipid peroxidation which is not suprising since LPS triggers an oxidative burst.

	Serum [Concentration ± SEM]		
Rabbit Treatment	Trigyceride [mM]	Malondialdehyde [μM]	% serum (mean ± SEM) to inhibit parasite growth by 50%
NRS	0.53 ± 0.06 (8)	2.43 ± 0.34 (12)	60.96 ± 6.43 (33)
LPS	1.82 (2)	3.31 (2)	75.00 ± 25.00 (3)
BCG	1.21 ± 0.09 (3)	2.36 ± 1.03 (3)	46.40 ± 17.90 (5)
TNS	1.55 ± 0.26 (7)	5.62 ± 0.29 (8)	23.86 ± 2.9 (28)

Table 4.2: Rabbit serum concentrations of triglyceride, malondialdehyde and % serum to inhibit parasite growth by 50% *in vitro*. Data are means ± standard error of the means. Numbers in brackets are the number of experiments for each sample. Abbreviations: NRS, normal rabbit serum; LPS, serum from rabbits given LPS alone; BCG, rabbits given BCG alone and TNS, tumour necrosis serum (rabbits given BCG followed 14 days later by LPS). Symbols: a: p=0.0002; b: p=0.0015; c: p=3.15 x 10⁻⁶; d: p=6.28 x 10⁻⁶
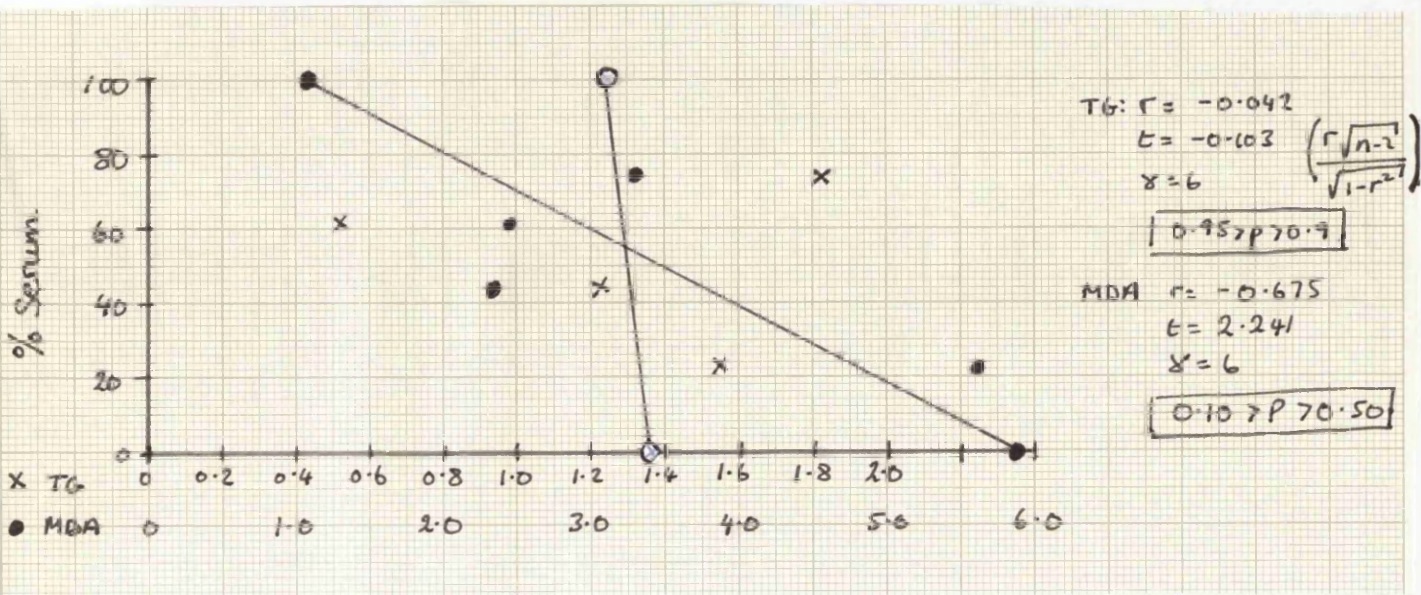
4.6. LIPID PEROXIDATION IN TNS OCCURS *IN VIVO* AND NOT *IN VITRO*.

If an *in vivo* oxidative burst occurred after LPS administration, this may have depleted the serum antioxidants so that the serum lipids could oxidise when exposed to the atmosphere after bleeding the animal. To test this a primed rabbit was injected with 5mg of vitamin E (i/v) at the same time as injection of the LPS. After 2 hours the animal was bled out. If *ex vivo* oxidation was occurring the addition of 5mg vitamin E to the blood collection flask should have prevented this process.

From figure 4.5 it can be seen that the TNS collected into a flask containing vitamin E was still active, therefore lipid peroxidation must have occurred prior to collection. However the injection of 5mg vitamin E i/v to a rabbit did block the parasiticidal activity of TNS, suggesting that the lipid peroxidation was occurring *in vivo*.
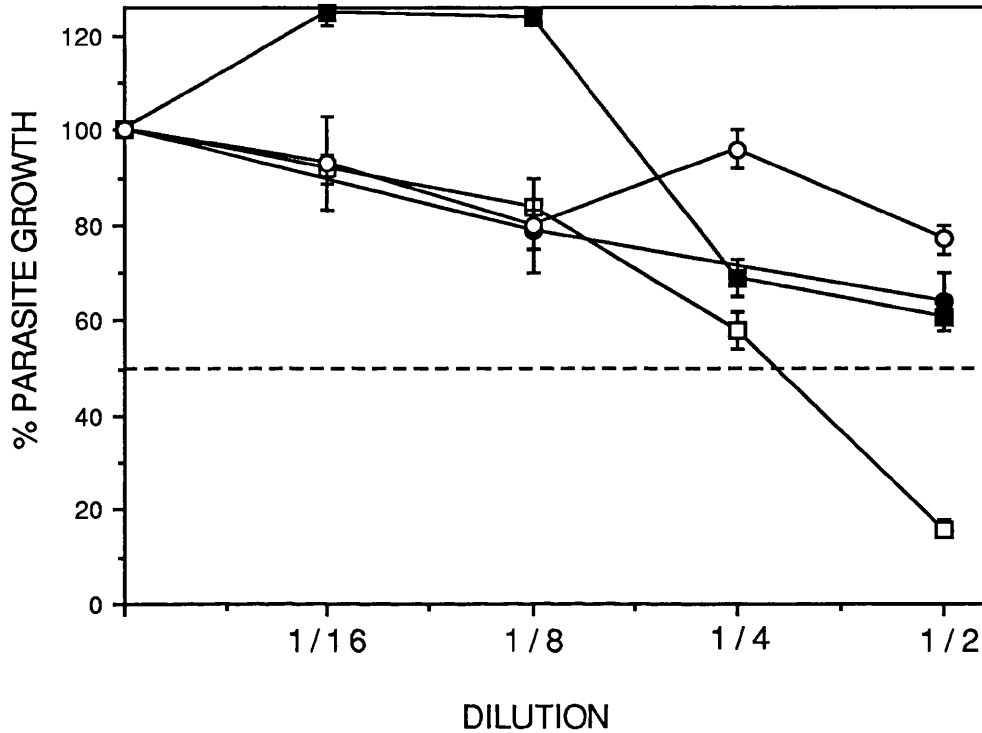
Figure 4.5: Inhibition of *P.falciparum* growth by serum samples from
rabbits treated with; BCG alone (—●—),   BCG followed 14 days later by
LPS, either with vitamin E given i/v at the time of LPS injection
(—■—) or with vitamin E in the serum collection flask (—□—). Normal
rabbit serum (—○—). The data given are for a single experiment in
which all the sera were tested at the same time. Each point
represents the mean ± standard error of the mean for the samples
tested in triplicate. (———) 50% inhibition of parasite growth.

4.7. Parasiticidal Effect of Oxidised Lipoproteins.

4.7.1. Human Lipoproteins.

Human lipoproteins were separated by density gradient ultracentrifugation and then dialysed overnight against 0.4mM ferrous chloride in saline. Another sample was treated in the same way except that 100µM of vitamin E was added before oxidation. Ferrous ions were used rather than enzyme systems which generate oxygen radicals because these would still operate during the assay for parasiticidal activity (e.g Xanthine oxidase would metabolise hypoxanthine, and glucose oxidase would metabolise glucose). Ferrous ions, however, are removed by dialysis against saline. Saline was used because the ferrous ions would react with phosphate in PBS. Ferrous phosphate is insoluble (Reynolds 1989). The lipoproteins were tested for parasiticidal activity and the results expressed as percent sample relative to the original serum (table 4.3).

Normal human lipoproteins were not inhibitory to the parasites. However overnight exposure to ferrous ions significantly increased their parasiticidal activity ($p=0.037$, $\gamma=4$). This activity was comparable to the activity of TNS lipoproteins (29.46% compared with 19.6%, although significantly less $p=0.022$, $\gamma=9$). This activity was prevented if vitamin E was added to the lipoproteins prior to exposure to ferrous ions.

SAMPLE	% Sample (mean ± SEM (n)) To Inhibit *P. falciparum* Growth by 50%
Human Lipoproteins	75.75 ± 17.18 (3)
Human Lipoproteins Fe^{++}	29.46 ± 6.43 * (3)
Human Lipoproteins + 100 μM Vitamin E Fe^{++}	65.43 (2)

Table 4.3: Inhibition of *P. falciparum* in vitro by human lipoproteins separated by ultracentrifugation.
Human lipoproteins were oxidised by dialysis overnight at 4°C in 0.4mM ferrous chloride in 0.9% saline and then dialysed against 0.9% saline for 24 hours. Vitamin E (100 μM) was added to one sample before oxidation. The control sample was dialysed against saline only. Results were calculated as in the legend to figure 4.1.
*, p=0.037 when compared with untreated lipoproteins.

4.7.2. ARTIFICIAL LIPOPROTEINS MADE USING PURE FATTY ACIDS.

Artificial lipoproteins were made using pure linoleic acid (C18:3) or pure stearic acid (C18:0). These were combined with bovine serum albumin and vitamin E was added to some samples as an antioxidant.

The lipids were oxidised using ferrous ions in 0.9% saline overnight at 4°C and then dialysed for 24 hours against PBS.

The albumin alone was not toxic to the parasite at the concentrations tested, neither was it toxic after the oxidation by ferrous ions with/without vitamin E (figure 4.6a).
Stearic acid lipoproteins were equally toxic to the parasite whether they contained vitamin E or not, or had been exposed to ferrous ions, (50% activity at 50 μg/ml Stearic acid), and they were uniformly toxic (figure 4.6c).

Vitamin E did not affect the ferrous oxidation of linoleic acid lipoproteins. The activity of the ferrous exposed lipoproteins was similar to the stearic acid lipoproteins. However with the samples not exposed to ferrous ions it can be seen that (figure 4.6b) the vitamin E sample is less toxic than the control sample, suggesting that the vitamin E has protected the fatty acid in some way.

Its clear that the toxicity of saturated and unsaturated fatty acid lipoproteins was not simply due to oxidation of the fatty acids. A more natural system is described in the next section where fats are used rather than pure fatty acids. This presents the parasite with lipids in the form that it would encounter within the body, and also with a mixture of fats.
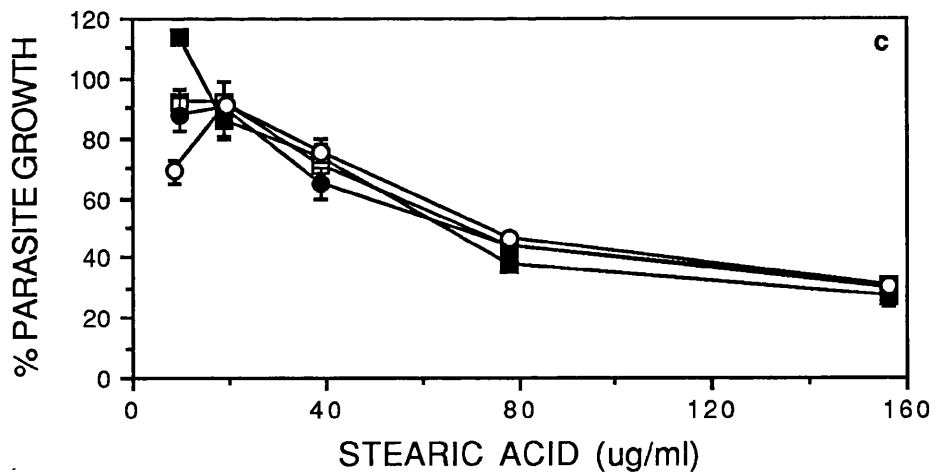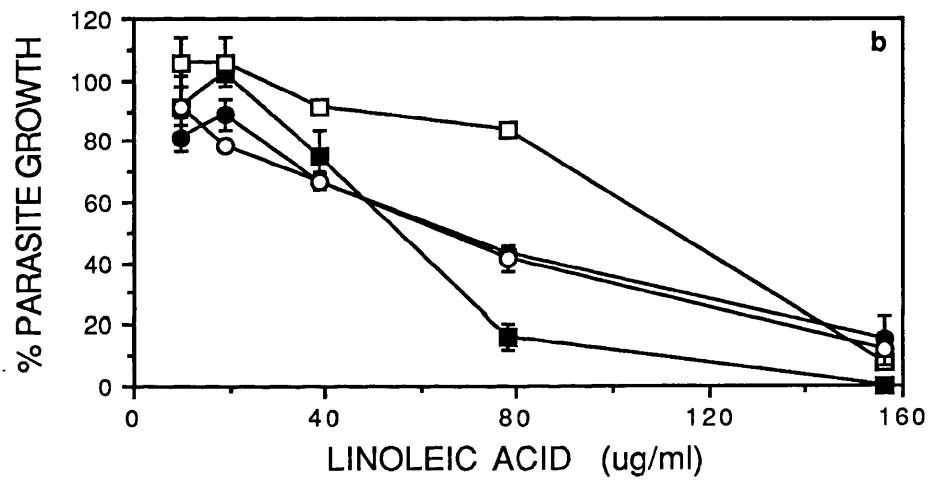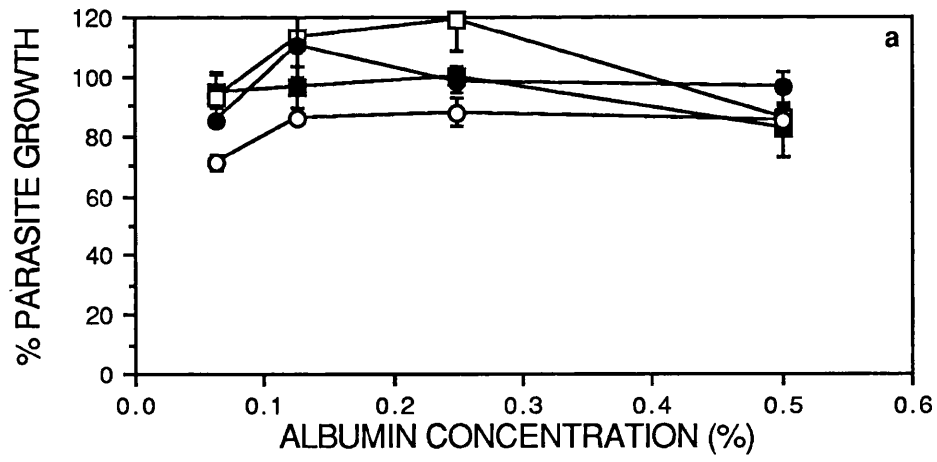
Figure 4.6: Inhibition of *P.falciparum* growth by artificial lipoproteins made using albumin and pure fatty acids. Each figure represents the mean ± standard error of the mean of triplicate samples treated in the folowing ways:

—▣—, dialysed against 0.9% saline for 36h at 4°C; —□—, with 100μM of vitamin E acetate, dilysed against 0.9% saline for 36h at 4°C; —●—, dialysed against 0.4mM Fe^{++} for 12h at 4°C and 24h at 4°C against 0.9% Saline; —○—, with 100μM vitamin E acetate , dialysed against 0.4mM Fe^{++} for 12h at 4°C and 24h at 4°C against 0.9% saline.

Figure 4.6a: Albumin alone. The concentration points are those associated with the concentrations of fatty acid shown in figures 4.6b and 4.6c and similarly figures 4.7a and 4.7b.

Figure 4.6b: Lipoproteins made using albumin and pure linoleic acid (C18:3).

Figure 4.6c: Lipoproteins made using albumin and pure stearic acid (C18:0)

4.7.3. ARTIFICIAL LIPOPROTEINS MADE USING VEGETABLE OILS.

Artificial lipoproteins were made using corn oil which contains predominantly unsaturated fatty acids, and coconut oil which contains predominantly saturated fatty acids (table 4.4). The lipoproteins were made in exactly the same way as those in the previous section.

By using oils rather than fatty acids the general toxicity to *P.falciparum in vitro* was much reduced. Parasite growth was not inhibited by concentrations of oil up to 2.5mg/ml. (figure 4.7). If unsaturated fatty acids (corn oil) were used (figure 4.7a) vitamin E prevented the lipoproteins being parasiticidal whether they had been exposed to ferrous ions or not. However without this protection they were toxic. Ferrous ions increased this toxicity to a small extent. But if saturated fatty acids were used (coconut oil) then no parasiticidal activity could be detected (figure 4.7b).

When fatty acids acids are oxidised they may become smaller. Therefore the di- or triglyceride that they are a part of would have a different mobility on a thin layer chromatography plate. This is shown in plate 4.10. The above preparations were separated by petroleum ether:diethyl ether:acetic acid (see section 3.6).

The corn oil has more unsaturated fats than the coconut oil. When these were oxidised by ferrous ions there was a shift towards the origin, demonstrating that the fats had become smaller (size is proportional to solubility in petroleum ether and therefore mobility).

These results show that the saturated/unsaturated nature of the lipoproteins is important in oxidative environments whether catalysed or not. They also further support the hypothesis that serum lipoproteins when oxidised are toxic to malaria parasites.

Fatty Acid	g fatty acid/100g oil	
	Coconut Oil	Corn Oil (Maize)
Saturated		
8:0	7.5	0
10:0	7.1	0
12:0	47.7 (44–51)	0
14:0	15.8	0.8
16:0	9.0	14.0 (6–22)
18:0	2.4	2.3
20:0	1.0	0.3
22:0	0	Trace
24:0	0	Trace
Mono-Unsaturated		
16:1	0.4	0.3
18:1	6.6	30.0 (19–50)
20:1	0	0.2
22:1	0	0.2
Poly-Unsaturated		
18:2	1.8	50.0 (34–62)
18:3	0	1.6

Table 4.4: Fatty acid composition of coconut oil and corn oil. The fatty acids are listed by carbon chain length and number of carbon-carbon double bonds. Figures in brackets show the composition range between batches of oil (Paul and Southgate 1978).
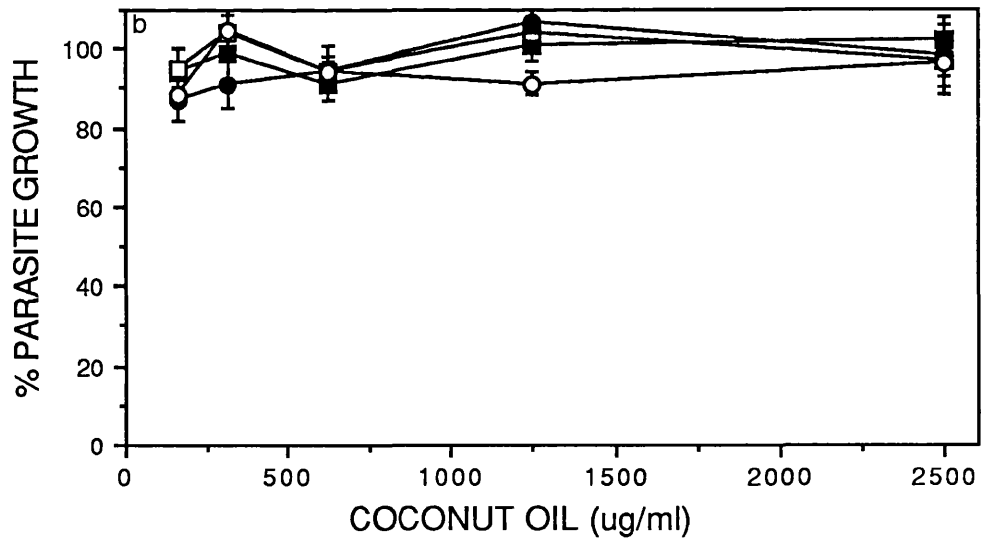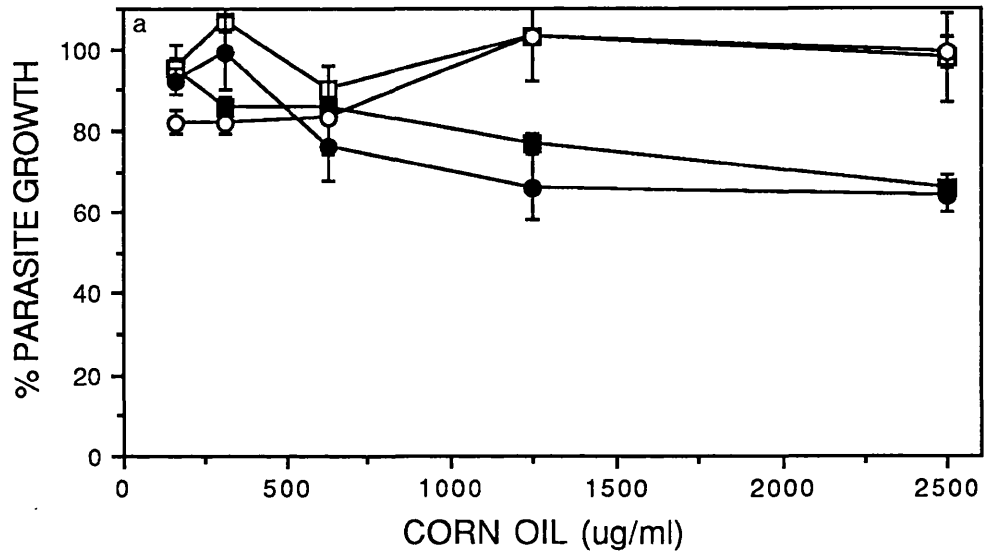
Figure 4.7: Inhibition of *P. falciparum* growth by artificial lipoproteins made using bovine serum albumin and corn or coconut oils. Each figure represents the mean ± standard error of the mean of triplicate samples treated in the following ways:

—■—, dialysed against 0.9% saline for 36 hours at 4°C; —□—, with 100μM of vitamin E acetate, dialysed against 0.9% saline for 36 hours at 4°C; —●—, dialysed against 0.4mM Fe^{++} for 12 hours at 4°C and 24 hours at 4°C against 0.9% saline; —○—, with 100μM vitamin E acetate, dialysed against 0.4mM Fe^{++} for 12 hours at 4μC and 24 hours at 4μC against 0.9% saline.

Figure 4.7a: Lipoproteins made using albumin and pure corn oil.

Figure 4.7b: Lipoproteins made using albumin and pure coconut oil.
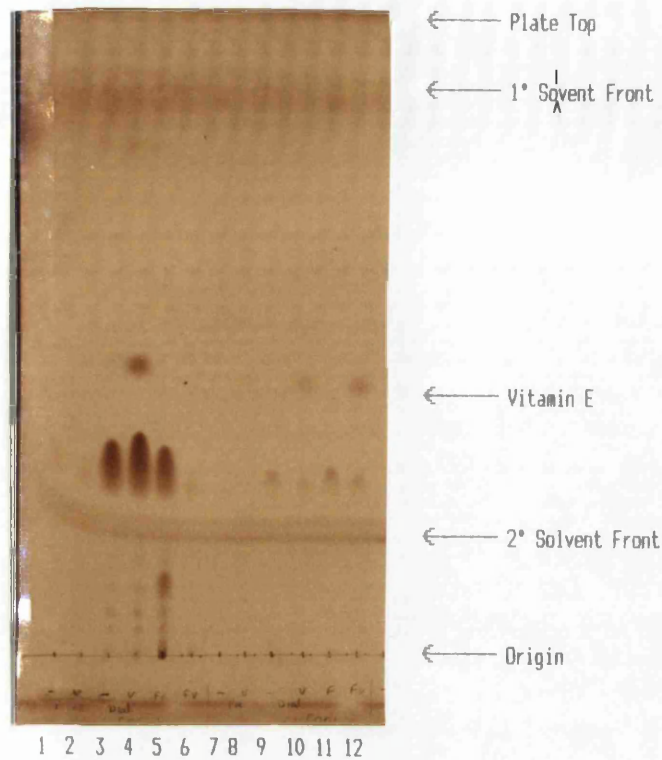
Plate 4.10: Thin layer chromatography of oxidised artificial lipoproteins made using corn and coconut oils.
Solvent: Petroleum ether:diethyl ether:acetic acid (90:10:1).
The solvent front had moved 6 inches from the origin at the end of the run. 2μl of sample was spotted on the plate on the origin.
Lanes 1 to 6 corn oil. Lanes 7 to 12 coconut oil.

1 and 7 freshly prepared lipoprotein.
2 and 8 freshly prepared lipoprotein + 100 μM vitamin E.
3 and 9 lipoproteins after overnight incubation in saline at 4°C.
4 and 10 lipoproteins after overnight incubation in saline at 4°C
        + 100 μM vitamin E.
5 and 11 lipoproteins after overnight incubation in saline + 0.4mM
        Fe++ at 4°C + 100 μM vitamin E.
6 and 12 lipoproteins after overnight incubation in saline + 0.4 mM
        Fe++ at 4°C + 100 μM vitamin E.

The chromatogram was air dried and then sprayed with 50% sulphuric acid. It was then baked at 140°C for 15 minutes.

4.8. INHIBITION OF *P.FALCIPARUM* GROWTH *IN VITRO* BY NITRIC. NITRITE AND NITRATE IONS.

Parasites were incubated with different concentrations of sodium nitrate ($NaNO_3$) and sodium nitrite ($NaNO_2$). Nitrate and nitrite have both been shown to be produced by macrophages after stimulation.

Nitrate was not toxic to the malaria parasites over the concentrations tested (0.05mM to 5mM, figure 4.8). However nitrite was parasiticidal in a dose response manner with a 50% growth inhibition concentration of 0.9mM by regression.

Under these conditions at pH 7.2 the nitrite ions should remain as nitrite. But under acidic conditions (pH <6.8) the evidence supports the breakdown of nitrite to nitric (NO), i.e the Nitrite/nitric equilibrium moves towards the right. However the parasites cannot survive for long periods at these acidic pH's. Therefore the parasite killing assay was modified. Parasites were incubated in 100% phosphate buffered saline with or without nitrite and at different pH's.

Figure 4.9 shows the effect of pH and time in this system. Although for up to 6 hours the pH had no effect, the time without nutrients or serum did have an effect. Therefore a maximum of 6 hours was chosen for the assay and a pH of 6.0 was chosen to maximise the effect of nitrite ions. After the incubation in these conditions the parasites were put into complete medium and the assay continued as normal. The former part of the assay was carried out in nutrient-free medium because the nitric radicals would be mopped up by the components of the complete medium.

The results are expressed as the percent growth compared to the control buffer and are shown in figure 4.10.

Up to 2 hours and in concentrations of nitrite up to 0.8mM the parasites recovered. But after 6 hours exposure there was still 50% inhibition of growth with 0.08mM nitrite, and this was not pH related.

It is impossible to say from these results whether nitric oxide is involved in the killing of *P.falciparum*, but it cannot be ruled out. The assay conditions may be the important factor in these experiments. However it can be said that nitrate is non toxic and that nitrite is toxic (ID50 0.9mM).
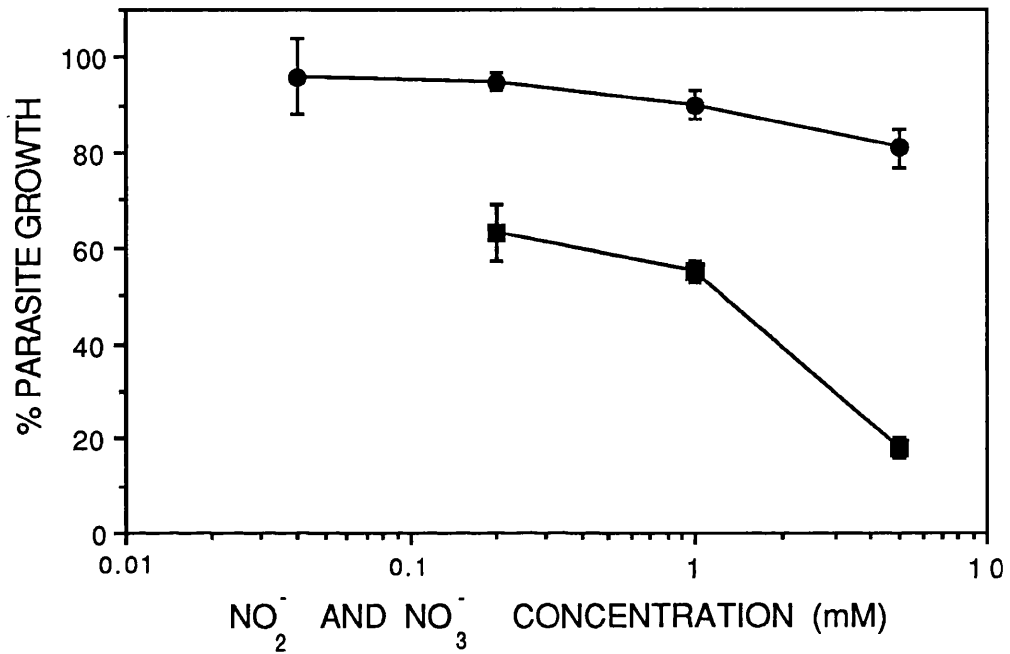
Figure 4.8: Inhibition of *P.falciparum* growth *in vitro* by NaNO₃
(—●—) and NaNO₂ (—■—). Parasites were incubated in complete medium
plus nitrate or nitrite for 24 hours. This medium was replaced for
fresh medium containing tritiated hypoxanthine for a further 24 hours
before being harvested.
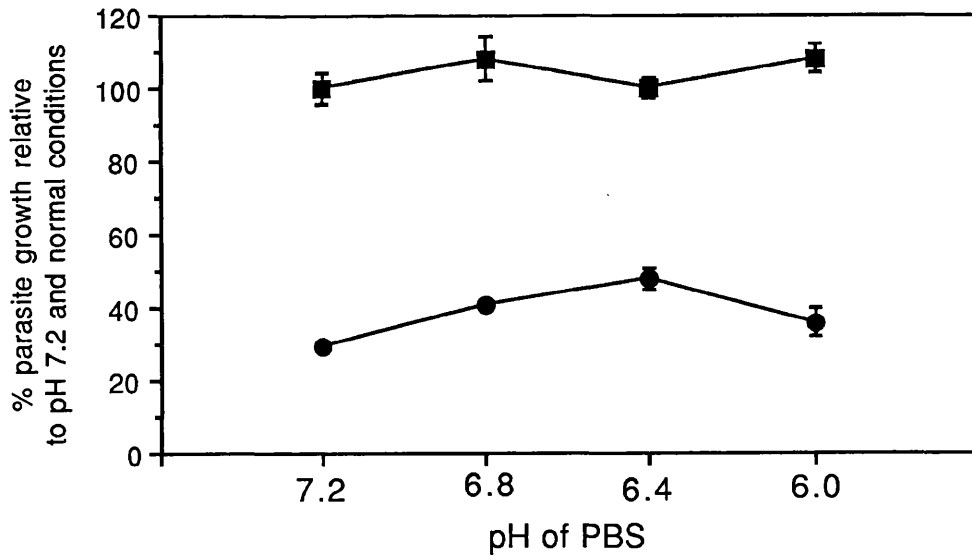
Figure 4.9: Survival of *P.falciparum* parasites in 100% 0.16M phosphate buffered saline at different pH's for 2 hours (—■—) or for 6 hours (—●—). After these incubation times the parasites were returned to complete medium for the rest of the assay.
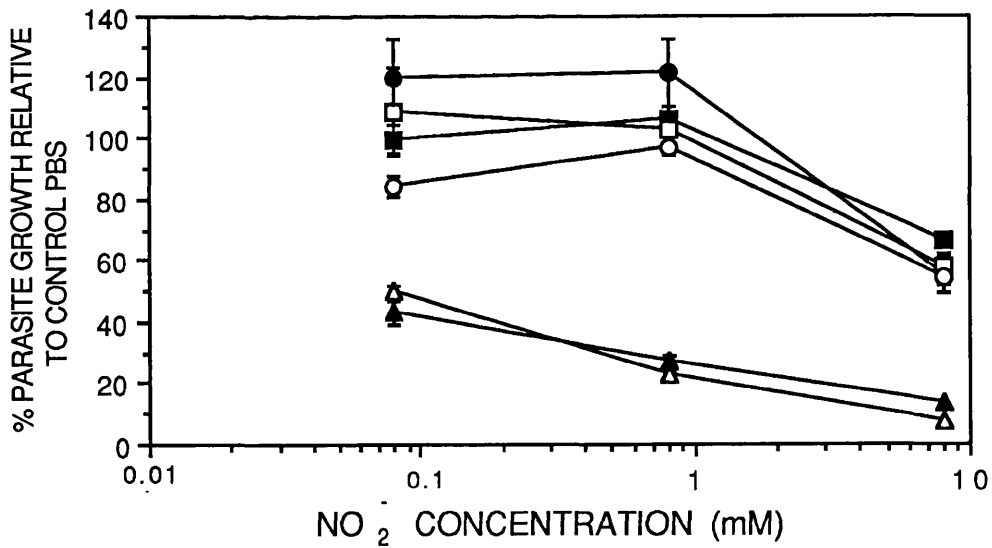
Figure 4.10: Inhibition of *P.falciparum* growth by acidified nitrite in phosphate buffered saline over different incubation times. The results are expressed as a percentage of the parasite growth in phosphate buffered saline alone at the appropriate pH and incubation time. At the end of the incubation the parasites were returned to complete medium for the remainder of the assay time.

Symbols: —■—, pH 7.2 for 1 hour; —●—, pH 7.2 for 2 hours; —▲—, pH 7.2 for 6 hours; —□—, pH 6.0 for 1 hour; —○—, pH 6.0 for 2 hours; —△— pH

4.9. SUMMARY OF *IN VITRO* RESULTS.

a) Only the combination of BCG and LPS produced a parasiticidal serum factor.

b) The parasiticidal activity of the sera correlated with lipid peroxidation and not with triglyceride concentration.

c) Removal of the lipoprotein fraction depleted the activity of TNS. This effect was greater when aerosil was used than when ultracentrifugation was used.

d) The ultracentrifuge-separated lipoproteins of TNS were parasiticidal.

e) Lipid peroxidation occurred *in vivo*, and was not an *in vitro* artefact.

f) Various lipid preparations could be oxidised *in vitro* with ferrous ions and became parasiticidal. It was necessary for the preparations to be a mixture of lipids rather than purified fatty acids.

g) Nitrite ions were toxic to *P. falciparum in vitro*.

5. DISCUSSION I.

5.1. NATURE OF THE PARASITICIDAL ACTIVITY OF TNS *IN VITRO.*

In section 4, I have demonstrated that the administration of BCG followed 14 to 21 days later by LPS leads to an increase in serum triglycerides in rabbits, and also to an increase in serum lipid peroxidation as indicated by the thiobarbituric acid test. This high triglyceride concentration was also measured in animals given BCG or LPS alone.

A number of bacterial infections (including *M.bovis*) have been shown to increase serum triglycerides (Thoen et al 1972), and maintain raised levels for 21 to 28 days. In contrast, LPS has been shown to do this for only about 12 hours following administration. The peak occurs at 8 hours, but starts almost immediately following LPS injection. By 2 hours there is an increase of 2 to 3 fold (Kaufmann et al 1976), which is in agreement with my results. The mechanism responsible for this has two components. Firstly, the stimulation of hepatic lipogenesis occurs, therefore increasing the serum triglycerides. This can be induced by TNFα (Kawakami et al 1982, Feingold et al 1987), which is released after LPS stimulation. TNFα also has been shown to interfere with the enzyme lipoprotein lipase. This enzyme removes triglycerides from the plasma and is very important for removing excess lipid (Krause and Hartman 1984).

Secondly BCG may work in a similar way to LPS through its effects on macrophages. But it may also affect another mechanism, as postulated by Thoen et al (1972), viz the destruction of mast cells. Heparin, one source of which is mast cells, which can stimulate lipoprotein lipase. *Mycobacterium* infections in the lungs cause destruction of the tissues and mast cells, and therefore a down regulation of lipoprotein lipase. The administration of LPS at this point would trigger the first mechanisms, and the combination of all these mechanisms may be responsible for the further increase in lipids observed (table 4.2)

The increase of triglycerides under the above conditions may be a defence mechanism against microbial endotoxin. It has been shown that endotoxin binds to triglycerides and is rendered inactive (Shaw Warren et al 1988). But if an oxidative burst should occur in the presence of high levels of lipid, lipid peroxidation will be initiated. If LPS is given to a normal animal, the oxidative burst

triggered is less than that in BCG primed animals (Nathan and Root 1977) and therefore less lipid peroxidation would occur (table 4.2).

The aerosil and ultracentrifugation results demonstrate that these lipids in TNS are parasiticidal and the thiobarbituric acid test results suggest that it is lipid peroxides that are the toxic molecules, because the parasiticidal activity correlates with lipid peroxidation and not with plasma triglyceride concentration.

In both these separations there is some residual activity in the non-lipid fraction of TNS. That this was not due to any remaining lipids, is suggested by the agarose gel electrophoresis results after aerosil treatment (plate 4.7), and also the ultracentrifuge separated serum as measured by the enzymatic method.

The aerosil depletion removes more activity than the ultracentrifuge which suggests that there is a parasiticidal molecule which binds aerosil but which does not associate with the lipoproteins. In fact this molecule may be common to both NRS and TNS. After ultracentrifugation the non-lipid fractions of NRS and TNS may have similar activities but after aerosil treatment even these are depleted.

If there is another *in vitro* parasiticidal factor, it is either not very active or it is at a low concentration in the serum. It was not a protease or a molecule dependent on disulphide bonds as shown by the results in table 4.1.

When an oxidative burst occurs *in vivo* antioxidants would be consumed. Therefore it is reasonable to assume that the antioxidative capacity of the serum would be reduced. This may be sufficient to allow lipid peroxidation to occur in the serum *in vitro*. However this does not explain the activity of TNS, since primed rabbits injected with vitamin E at the time of LPS administration produced TNS, which was shown to have no parasiticidal activity, unlike TNS made in the usual way but with vitamin E added when the blood was collected. Thus lipid peroxidation was occurring not *in vitro* but *in vivo*.

There was always the possibility that a non-lipid molecule which associates with lipids could be responsible for the parasiticidal activity of TNS. Either its release or oxidation could be induced by BCG/LPS treatment. Various lipoprotein preparations were made to try and answer this question.

Normal human lipoproteins were separated by ultracentrifugation and shown not to be inherently parasiticidal. These were oxidised using ferrous ions as a catalyst with and without vitamin E, used as an antioxidant. The lipoproteins were only parasiticidal after oxidation but this was blocked by vitamin E. Therefore parasiticidal activity must be due to the oxidation of a molecule and not to the induction and increase in a non-lipid, lipid-associating molecule. Also the parasiticidal activity cannot be due to small by-products of oxidation as these should all have been dialysed out.

The action of lipid peroxides was further confirmed by preparing artificial lipoproteins made using bovine serum albumin and pure fats. Albumin alone was not toxic to *P.falciparum in vitro* even after exposure to vitamin E or ferrous ions.

Lipoproteins were first made using pure fatty acids. The two fatty acids used were identical in carbon chain length and structure apart from the fact that linoleic acid possesses two unsaturated double bonds and stearic acid does not. The concentration range over which the fatty acids were tested against the parasites was about 100 times less than the serum triglyceride concentration (8-156μg/ml compared with 125-4000μg/ml). Under the conditions tested, stearic acid lipoproteins were parasiticidal before and after oxidation. Each form was equally toxic. Lipoproteins made using linoleic acid were similarly parasiticidal although vitamin E did reduce their activity.

With hindsight these results are also perhaps not surprising because the fatty acids would exchange with the red cell membrane. The parasite requires fats from its surroundings because it lacks a *de novo* synthesis pathway (Sherman 1979). Therefore if these fats are predominantly of one species they would compete with other fats for parasite enzymes more efficiently and result in a limited number of products.

With stearic acid, which is totally saturated, the parasite must desaturate it, since its requirements are for unsaturated fats. This may not be very efficient (Sherman 1979), and some essential double bonds (those above C_9) may be impossible to make (section 2.2.3). Therefore the parasite may not be able to properly form new membranes. Similarly if the number of modified fat products are limited. This latter reason may be important for the toxicity of

linoleic acid. This fatty acid may also be parasiticidal via peroxidation, which could explain the effect of vitamin E.

The only problem with using pure fatty acids is that they are artefactual, and would not occur *in vivo*. Therefore pure vegetable oils were used which contain a mixture of fats and therefore fatty acids. The first observation was that the oils could be used at similar concentrations to serum lipid without having inherant parasiticidal activity (250-2500μg/ml compared with 125-4000μ/ml). Coconut oil (primarily saturated fats-table 4.4) was not parasiticidal after exposure to ferrous ions, unlike corn oil (primarily unsaturated fats-table 4.4) which was parasiticidal. This latter activity could be prevented by vitamin E.

5.2.1. MECHANISM OF LIPID PEROXIDE TOXICITY.

In retrospect it is not surprising that lipid peroxides were toxic to the parasite, since malaria parasites do not posses a *de novo* synthesis pathway for fatty acids; therefore all their lipid requirements must come from the the plasma, so that as well as an equilibrium being maintained between the red cell membrane and plasma lipids there will also be a nett influx of lipids from the plasma to the parasite. I have already described some of the membrane lipid changes and in summary there is up to 500% increase in the red cell lipids and an increase from 75% to 90% in its unsaturated fats. Therefore if oxidised lipids are added to the parasites they would be taken up by the parasite because they are primarily unsaturated fats. Eventually these peroxides would destroy both red cell and parasite. The peroxides can act as initiators for further peroxidation of unsaturated fats (section 2.3.4.2). The parasitised red cells would be more susceptible to peroxidation because they have a greater unsaturated fat content, not only in quantity but in percentage of the total fats.

Membrane peroxidation will alter membrane fluidity because fluidity is dependent on unsaturated fats. Membrane fluidity may not be very important *in vitro*, however it may have consequences *in vivo*. For example a reduction in the red cell's ability to squeeze through narrow capillaries may increase the chance of it being removed in the spleen.

Should sufficient peroxidation occur in the membrane, it will fall apart, resulting in parasite death or the inhibition of parasite reinvasion.

Lipid peroxides can also react with proteins and DNA (Logani and Davies 1980, Schaich and Black 1980). Thus damage to membrane proteins such as ion channels and enzymes would occur. This could explain why parasites are seen as as denser darker shrivelled forms before they disappear: they may be slowely dieing of starvation. This would not, of course, occur if the red cell membrane were simply being destroyed.

Some of the above effects can be prevented by antioxidants and indeed, these are raised in parasitised red cells (table 2.5). But even these will have a limit of oxidation that they can cope with. This threshold concept may explain why LPS serum did not have a parasiticidal effect. TNS, however, may provide too great a stress. Even in the presence of high concentrations of antioxidants, lipid peroxides would still be toxic. Further membrane peroxidation may not be possible but if sufficient pre-formed peroxides get into the membrane, then the membrane may be disrupted. Vitamin E would be the only antioxidant to prevent further lipid peroxidation.

This may explain why antioxidants could not block the parasiticidal action of TNS against *P. falciparum* (Geary et al 1986).

5.3. PARASITICIDAL EFFECTS OF RNI.

Up to now attention has been focused on the effects of oxidative stress on the malaria parasite, or on molecules which subsequently become parasiticidal. However the cells, especially macrophages, which produce these oxygen radicals can also produce RNI.

Malaria parasites were incubated with sodium nitrate or sodium nitrite as both nitrate and nitrite have been shown to be released by macrophages after stimulation (section 1.3.4.4.). Under the conditions tested, only nitrite inhibited *P. falciparum* growth *in vitro* (50% inhibition at 0.9mM nitrite). This may be directly due to the nitrite or the nitrite could have been reduced to nitric oxide under slightly acid conditions (pH <7.0) (Stuehr and Nathan 1988). This was tested by incubating the parasite at different pH's. Nitric oxide is the radical produced by macrophages and nitrite and nitrate

are formed from it. However the change in pH did not alter the toxicity of nitrite.

The only way to determine whether nitric oxide is parasiticidal would be to incubate the parasites in an atmosphere containing nitric oxide gas.

One interesting preliminary result has been found (data not shown) with macrophages from lethal *P.yoelii* infected mice. Spleen macrophages were tested separately for superoxide and nitric oxide release after stimulation by phorbol myristic acetate and bradykinin respectively. Vaccinated animals with an infection just before clearance produced 20 times more nitric oxide than superoxide. Further work in this area is clearly called for.

5.4. SUMMARY.

a) Only the combination of BCG followed by LPS induces a parasiticidal serm factor.

b) The factor is neither a protease nor a protein relying on disulphide bond for structure and function.

c) Aerosil depletes serum lipids and the parasiticidal factor. Lipids separated by ultracentrifugation contain the parasiticidal activity of TNS. Lipid peroxidation correlates with parasiticidal activity and not lipid concentration. Lipid peroxides may kill the parasite by destroying the red cell membrane and damaging essential membrane proteins such as enzymes and trans-membrane carrier proteins.

d) Normal human lipoproteins are not inherently parasiticidal neither are artificial lipoproteins made using vegetable oils. These can be made parasiticidal by oxidising them with ferrous ions.

6.1. INHIBITION OF LETHAL *P.YOELII* (YM) GROWTH BY RABBIT TUMOUR NECROSIS SERUM.

10^4 lethal *P.yoelii* (YM) parasites (LPY) were injected i/v into Tuck No1 mice. From this inoculum parasites become detectable in blood films by day 3 or day 4. They increase to parasitaemias in excess of 50% on day 6 and the mice start to die from day 7 onwards. Though lethal in most inbred strains, this parasite does not kill 100% of Tuck mice. For example, in the experiment shown in figure 6.1, the mortality for day 8 of the control infection was 40% (4/10). The remaining mice eventually recovered.

RTNS was injected into a group of mice at a dose of 0.5ml serum i/p per mouse on days 0,2,4,6 and 8 following LPY injection. This group of mice maintained low parasitaemias (<0.1%, p<0.0001), with a further decrease following each TNS injection.

The previous experiments *in vitro* suggested that the lipoprotein fraction in TNS was parasiticidal. However the results *in vivo* were quite different. The separated TNS lipoprotein fraction was injected into mice, the regimen being the same as for TNS except that the injections were i/p in one experiment and i/v in another. The resulting parasitaemias for these two groups were identical and are pooled in figure 6.1. Parasitaemias were not significantly different from the control infection although the mice began to die 1 day earlier than the controls, 2/10 on day 7. On day 9 there was 60% mortality compared to the control of 40%.

In contrast, the lipoprotein depleted serum showed the same antiparasitic activity as the whole serum, demonstrating that *in vivo* the TNS activity is in this fraction and not in the lipoproteins. (p<0.0001).

One other difference was noted from day 4 onwards. In the mice injected with TNS or lipoprotein deple ted TNS (TNS-lp), the number of polymorphonuclear cells per field was between 5 and 10 times more than the control infection or the mice injected with TNS lp, for a similar density of red cells.
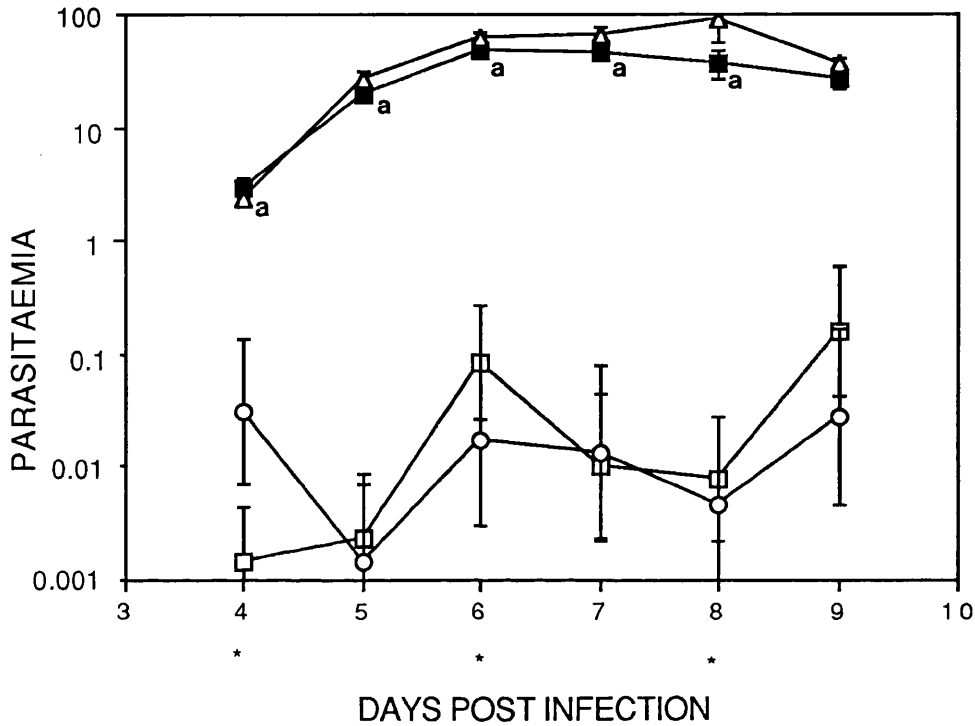
Figure 6.1: Inhibition of lethal *P.yoelii* (YM) infection *in vivo* by whole and fractionated rabbit tumour necrosis serum.
10^4 parasitised red blood cells injected i/v on day 0. 0.5 ml TNS injected i/p on days 0,2,4,6 and 8.
Symbols: —■—, control infection;   —□—, TNS i/p; —○—, TNS minus lipoproteins i/p after ultracentrifugation;   —△—, TNS lipoproteins i/v after ultracentrifugation; *, days of serum sample injection.

a:p<0.0001, control infection compared with either TNS or TNS-lp.

The mean TNF activity of rabbit TNS was $18.96 \pm 1 \times 10^4$U/ml. A goat anti rabbit TNF antiserum at a dilution of 1:5 blocked the TNF activity in a bioassay, (L929 cell cytotoxicity).

TNS was incubated with the antiserum before injecting into mice. The pre-bleed serum from the goat was used as a control and incubated with TNS in the same way as the antiserum. Tuck No1 mice were injected with 0.5ml serum i/p on days 0,2 and 4 after infection with 10^4 LPY i/v.

Figure 6.2 shows that by day 4 the control parasitaemia was well established $(5.24 \pm 1.41\%)$, but in the TNS injected mice, parasites were only just detectable. The control parasitaemia increased to 50% by day 6 where it remained level for the rest of the experiment, 2/3 mice died on day 8. After the TNS injections were stopped the parasitaemia began to rise so that by day 8 it reached the control parasitaemia. The number of TNS injections was reduced to 3 because a large difference in parasitaemias is already evident after 4 days in treated mice and remains for another 2 to 3 days. Once the TNS injection have been stopped the resulting increase in parasitaemia is similar in gradient to a normal infection, which suggests that the factor responsible for holding down the parasitaemia must either be a component of TNS or a factor induced by TNS.

Pretreatment with anti-TNF antibody did not significantly reduce the antiparasitic effect of TNS (figure 6.2), suggesting that TNF itself is not the only active factor.
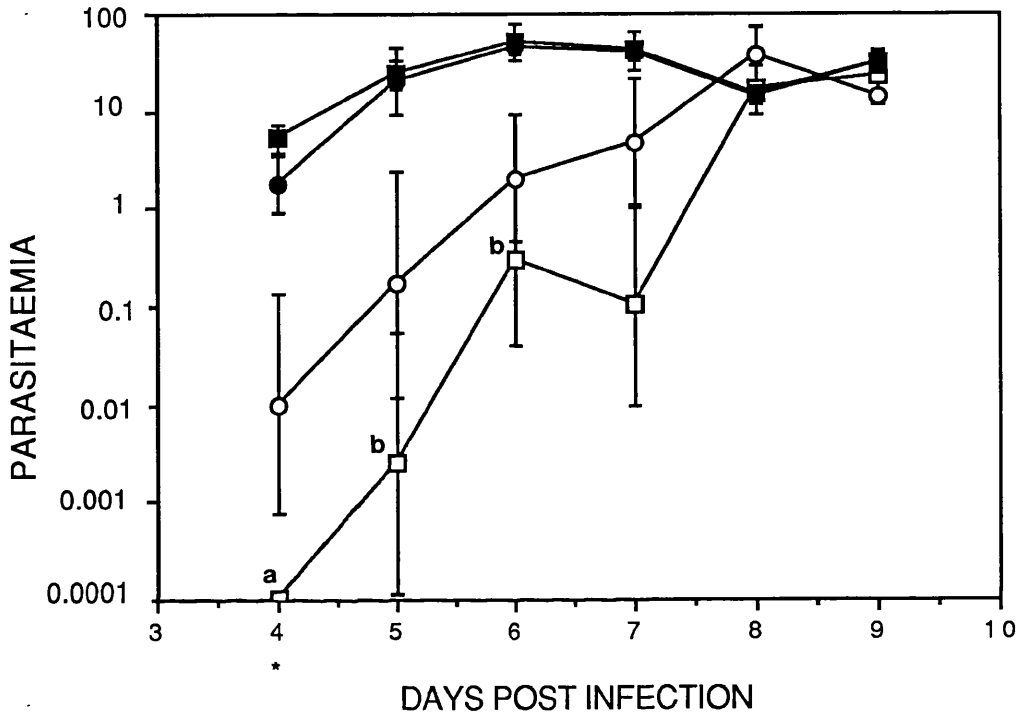
Figure 6.2: Inhibition of lethal *P.yoelii* (YM) infection *in vivo* by TNS and the effect of a polyclonal goat antiserum made against rabbit TNF. The antiserum was used at 1:5 and pre-incubated with TNS. TNS was also incubated with the goat pre-bleed serum at the same dilution. All sera were injected i/p at 0.5ml per mouse on days 0,2 and 4.

10^4 parasitised red blood cells i/v on day 0.

Symbols: —■—, control infection; —●—, Normal rabbit serum i/p; —□—, TNS pre-incubated with goat pre-bleed serum at 5:1 (TNS:Goat) i/p; —○—, TNS pre-incubated with goat polyclonal antiserum against rabbit TNF at 5:1 (TNS:Goat) i/p; *, days of serum sample injection.

a, $p < 10^{-5}$ compared with the control infection; b, $p < 0.04$ compared with the control infection.

6.3. Ion-Exchange Chromatography of TNS.

TNS was separated on DE-52 anion exchange resin into four fractions. (0.04M PBS, 0.08M PBS, 0.16M PBS and 1.5M NaCl). These fractions were tested *in vivo* against a LPY infection as described for TNS in the previous sections.

The control infection followed the same course as before with animals dying from day 9. Whole TNS controlled the infection as before except that after stopping TNS injections the parasitaemia rose slower than before, (figure 6.3)

A pooled fraction of the separated TNS was tested to make sure activity was retained. As figure 6.3 shows the activity of the pooled fraction was not significantly different from TNS.

One fraction (0.04M PBS) did not have any activity compared with the control infection. The other three fractions were not as effective as whole TNS but did control the infection, preventing parasitaemias increasing above 10%.

Assuming that one component was not spread across all three active fractions this result suggests the additive, or perhaps synergistic, activity of two or more components of TNS.

6.4. MOLECULAR WEIGHT SEPARATION OF THE ION-EXCHANGE FRACTIONS.

The three active fractions were separated by sephacryl-200 superfine beads, using PBS as the separation buffer. The absorbance profiles at 280nm for the separations are shown in figures 6.4a, 6.5a and 6.6a. Fractions were then recombined as shown by the vertical lines in these figures since the mouse assay was impractical for large numbers of samples.

Assay of fraction activity was carried out as before. From figures 6.4b,6.5b and 6.6b it can be seen that there was only one fraction with any activity (figure 6.5b, fraction 2). This was a fraction of the 0.16M ion-exchange fraction. However this activity was not statistically significant. These fractions were recombined but lost any of the activity that they had had in figure 6.3. One of the major problems with these separations was that a large amount of protein had to be separated by the column to satisfy the assay requirements, and therefore some proteins would most certainly be present in several fractions. This is shown in plate 6.1. The 0.16M PBS ion-exchange fraction at two dilutions are on the right hand side and the S-200 separated fraction in the left hand lanes. Lane 4 corresponds to the slightly active fraction and should have only contained high molecular weight proteins, because this is from the early part of the column effluent. Several bands can be seen, some of which are present in the other fractions. This would reduce the activity of any parasiticidal proteins present although the pooled fraction should then be active, which it was not.

Even though these assays did not work as hoped, several important points did arise:

a) Activity was lost after molecular weight separation although the protein concentrations of some of the fractions was substantial. (Table 6.1) showing that foreign protein was not responsible for the activity of TNS.

b) The LPS concentration in the fractions was greater than that in the whole TNS. Presumably this LPS came from the separation columns and handling procedures, but these concentrations of LPS clearly do not affect the parasitaemia. (table 6.2).

Overall the separation results suggest that the mouse assay is not the best way to test fractionated TNS.
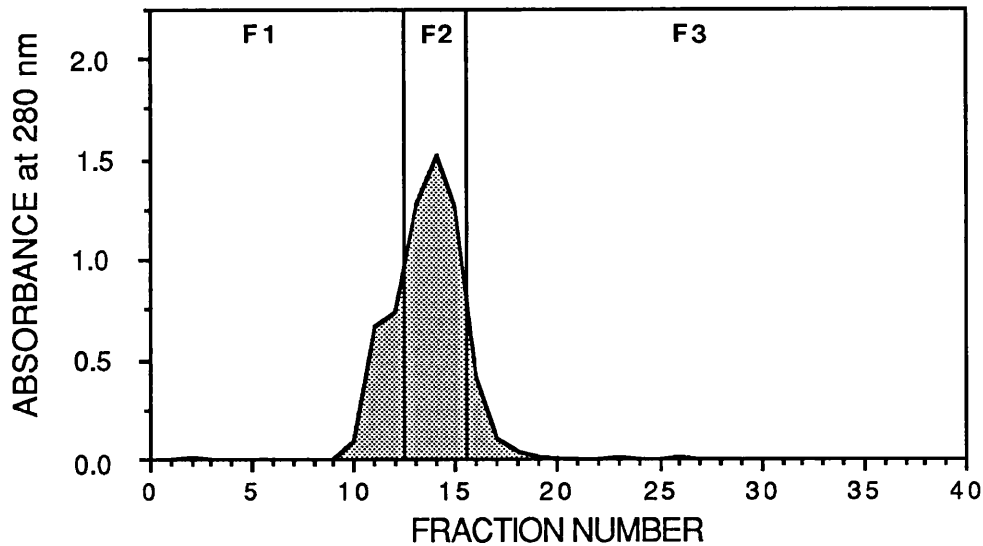
Figure 6.4a: Separation of the 0.08M PBS ion exchange fraction of
TNS, by S-200 molecular weight sieving gel. 1 ml of 5 times
concentrated sample was applied to the column and 5 ml fractions were
collected. The vertical lines indicate fractions which were pooled
together. F1=fractions 5 to 12; F2=fractions 13 to 15; F3=fractions16
to 28.
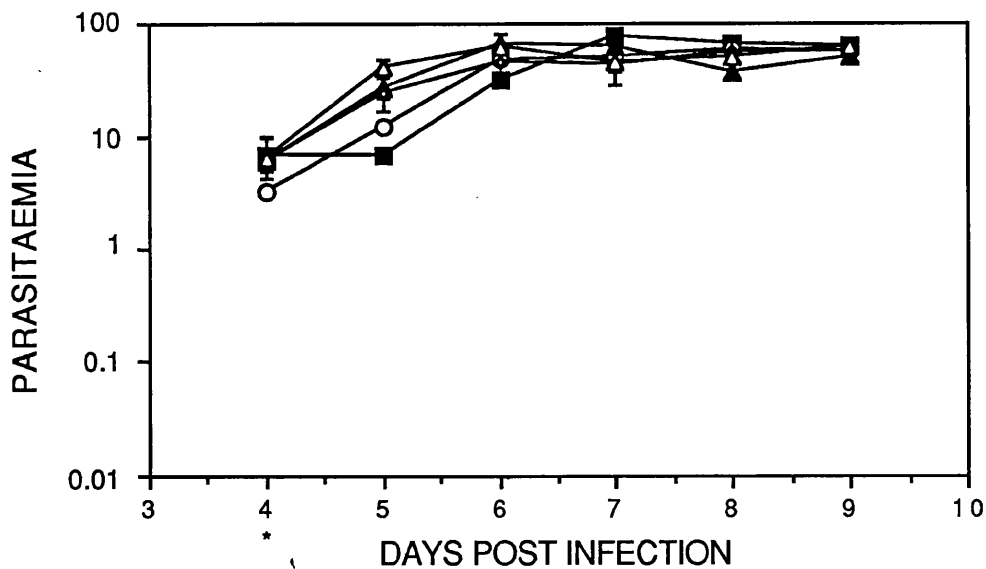
Figure 6.4b: Lethal *P.yoelii* (YM) infection *in vivo* and the
effect of of the fraction from figure 6.4a.
10⁴ parasitised red blood cells i/v on day 0 and 0.5ml of
fractionated serum i/p on days 0,2 and 4.
Symbols: —■—, Control infection; —O—, pooled fractions
(equivalent to 0.08M PBS ion exchange fraction); —●—, F1; —▲—, F2;
—△—, F3; *, days of sample injection.
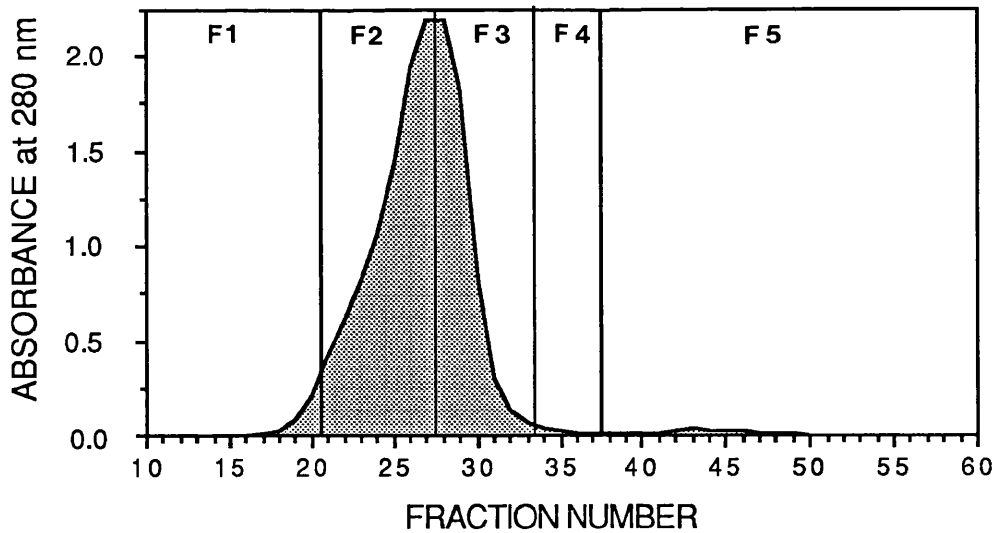
Figure 6.5a: Separation of the 0.16M PBS ion exchange fraction of
TNS, by S-200 molecular weight sieving gel. 1 ml of 5 times
concentrated sample was applied to the column and 5 ml fractions were
collected. The vertical lines indicate fractions which were pooled
together. F1=fractions 15 to 20; F2=fractions 21 to 27; F3=fractions
28 to 33; F4=fractions 34 to 37; F5=fractions 38 to 53.
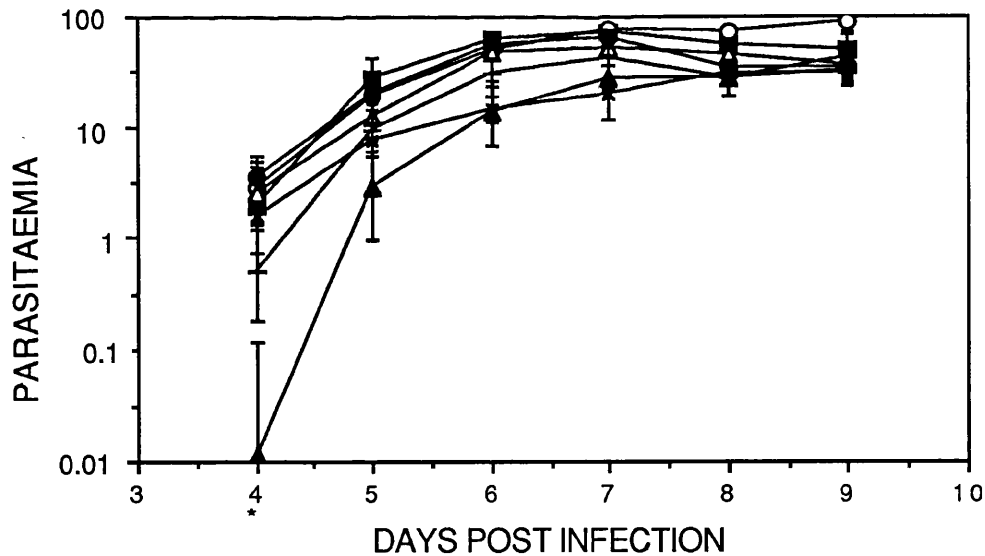
Figure 6.5b: Lethal P.yoelii (YM) infection in vivo and the
effect of of the fraction from figure 6.5a.
10^4 parasitised red blood cells i/v on day 0 and 0.5ml of
fractionated serum i/p on days 0,2 and 4.
Symbols: —■—, Control infection; —O—, pooled fractions
(equivalent to 0.16M PBS ion exchange fraction); —●—, F1; —▲—, F2;
—△—, F3; —+—, F4; —X—, F5, *, days of sample injection.
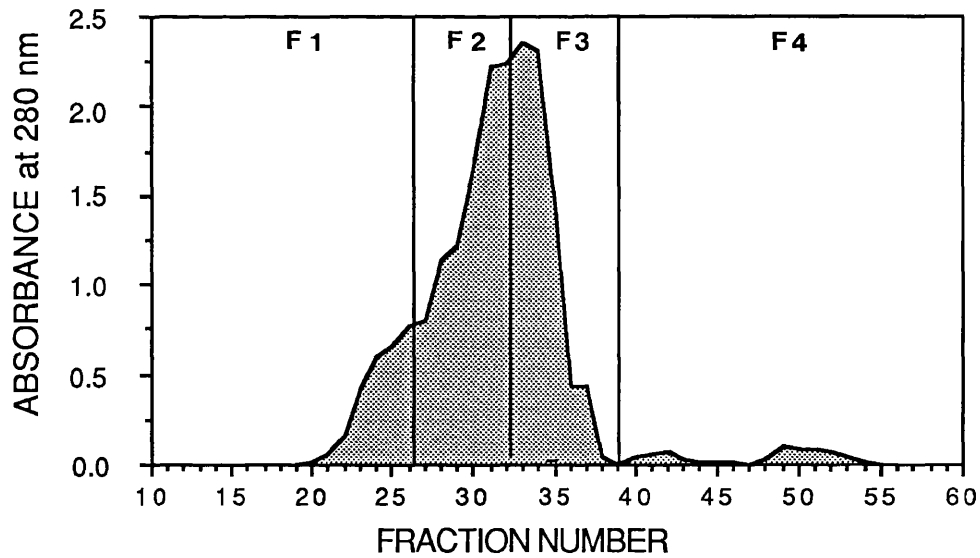
Figure 6.6a: Separation of the 1.5M NaCl ion exchange fraction of TNS, by S-200 molecular weight sieving gel. 1 ml of 5 times concentrated sample was applied to the column and 5 ml fractions were collected. The vertical lines indicate fractions which were pooled together. F1=fractions 15 to 26; F2=fractions 27 to 32; F3=fractions 33 to 39; F4=fractions 40 to 56.
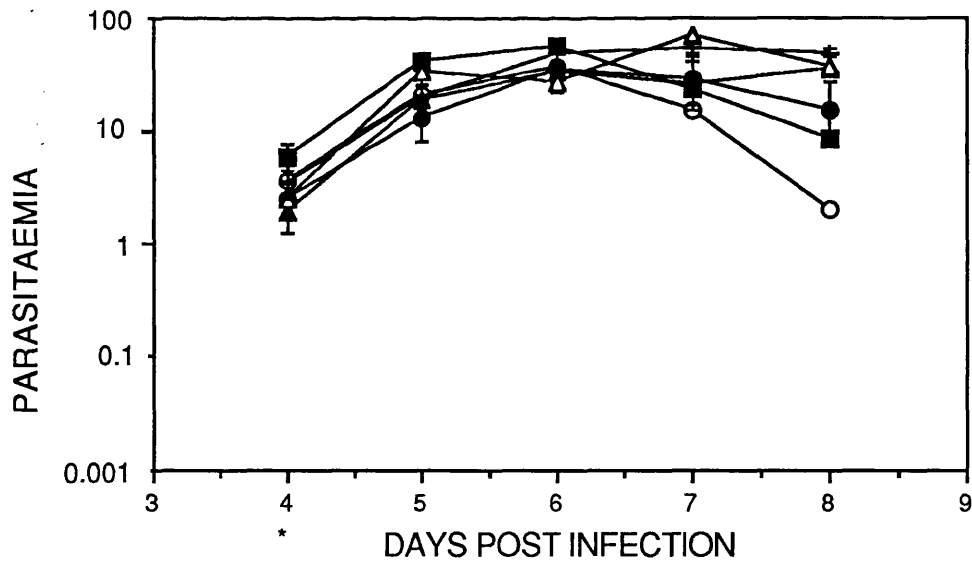
Figure 6.6b: Lethal *P.yoelii* (YM) infection *in vivo* and the effect of of the fraction from figure 6.6a.
10⁴ parasitised red blood cells i/v on day 0 and 0.5ml of fractionated serum i/p on days 0,2 and 4.
Symbols: —■—, Control infection; —○—, pooled fractions (equivalent to 1.5M NaCl ion exchange fraction); —●—, F1; —▲—, F2; —△—, F3; —+—, F4; *, days of sample injection.

Sample	Protein mg/ml
serum	
TNS	82.0
NRS	76.0
DE-52	
0.04M	0.1
0.08M	6.7
0.16M	47.0
1.50M	6.7
pool	56.6
S-200	
(DE52-0.08M)	NT
(DE52-0.16M)	
F1	0.23
F2	13.4
F3	16.0
F4	0.06
F5	0.02
pool	29.71
(DE52-1.50M)	
F1	1.02
F2	0.20
F3	0.94
F4	0.00
pool	2.16

Table 6.1: Protein concentrations in separated fractions of TNS as determined using the Bio-Rad protein assay Kit, with bovine serum albumin as the standard. NT; not tested.

SAMPLE	LPS (ng/ml)
TNS1	194
TNS2	180
TNS3	0
TNS4	323
F1 to F4	>340

Table 6.2: Concentration of Lipopolysaccharide (LPS) in tumour necrosis sera and fractions of tumour necrosis sera after ion-exchange chromatography. (F1 to F4 refer to the fractions in section 6.3). (F1 being 0.04M PBS and F4 being 1.50M NaCl). LPS was measured using a QCL-1000 assay kit (Whittaker Biotechnology).

← 116 KD

← 84　KD

← 58　KD

← 48.5 KD

← 36.6 KD

← 26.6 KD

← Front
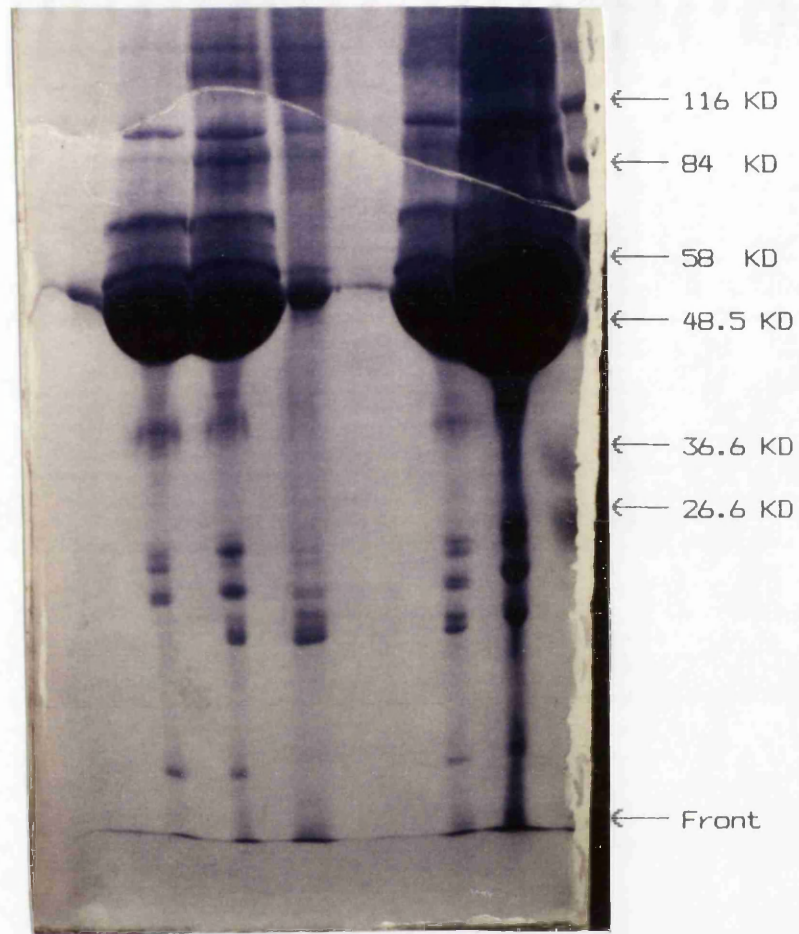
Plate 6.1: SDS-PAGE of 0.16M fraction of ion-exchange separated TNS
after S-200 molecular weight chromatography.
Lanes 6/7; 0.16M ion-exchanged fraction of TNS (1:10 dilution and
neat respectively)
Lanes 1-5; S-200 separated 0.16M fraction of TNS.

            1; fraction 5
            2; fraction 4
            3; fraction 3
            4; fraction 2
            5; fraction 1

Lane 8; Molecular weight markers.

6.5. How is TNS Acting *in vivo* ?

Because of the difficulties encountered in separating out the
activity in TNS, another approach was adopted. The effect of TNS was
clearly evident by day 4 and it was thought that TNS may be boosting
any non-specific antiparasitic effector mechanisms. One of which
could be an oxidative burst. One way to test this was to feed the
animals very high doses of antioxidants.

6.5.1. Effect of Vitamin C on TNS Activity *in vivo*.

Vitamin C was fed to the mice *ad libitum* in their drinking water
at 10mg/ml, following a loading dose of 20mg in PBS i/p, given 24
hours before LPY infection. TNS (0.5ml i/p) was injected on days 0,2
and 4. Water consumption was measured each day and the vitamin C
intake per mouse was calculated (figure 6.8b).

The control LPY infection increased as before and mice died from
day 7 onwards with 5/17 dead on day 7 and 13/17 dead by day 13. These
mice fell into two groups. Those whose parasitaemias reached nearly
100% or those which plateaued at approximately 50%, these then either
dropped or remained level for several days. This is in contrast to
the vitamin C treated mice whose parasitaemias rose continuously
until all the mice died by day 8. On the day before death all had
parasitaemias >75%, and 5/8 were >90%. The mean increase in
parasitaemia was slightly higher than the control but not
significantly so (Figure 6.7a). These mice consumed an average of
22.4mg of vitamin C per day.

The TNS treated mice fed vitamin C consumed 20.33 mg
vitamin C/mouse/day up to day 8 when consumption dropped to 14.22 mg
vitamin C/mouse/day, presumably because the mice were sick. This was
a general trend which occurred in all groups. However the mice fed
vitamin C never appeared to be as sick and immobile as the untreated
group of mice.

The effect of vitamin C on TNS was to partially block its parasiticidal activity (p<0.007) although there was still some activity when compared with the controls (p<0.0002 to day 7). Once TNS injections were stopped the non-vitamin C treated group's parasitaemia increased similar to a control infection, reaching 40% to day 14, whereas the TNS-vitamin C treated mice reached 40% parasitaemia on day 11.
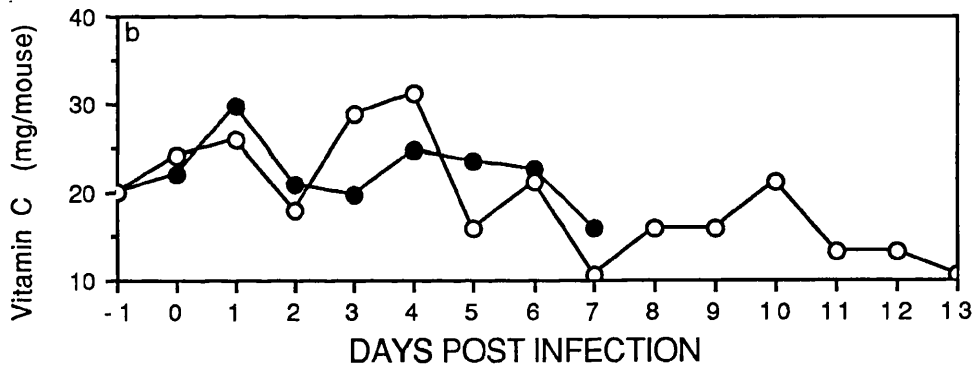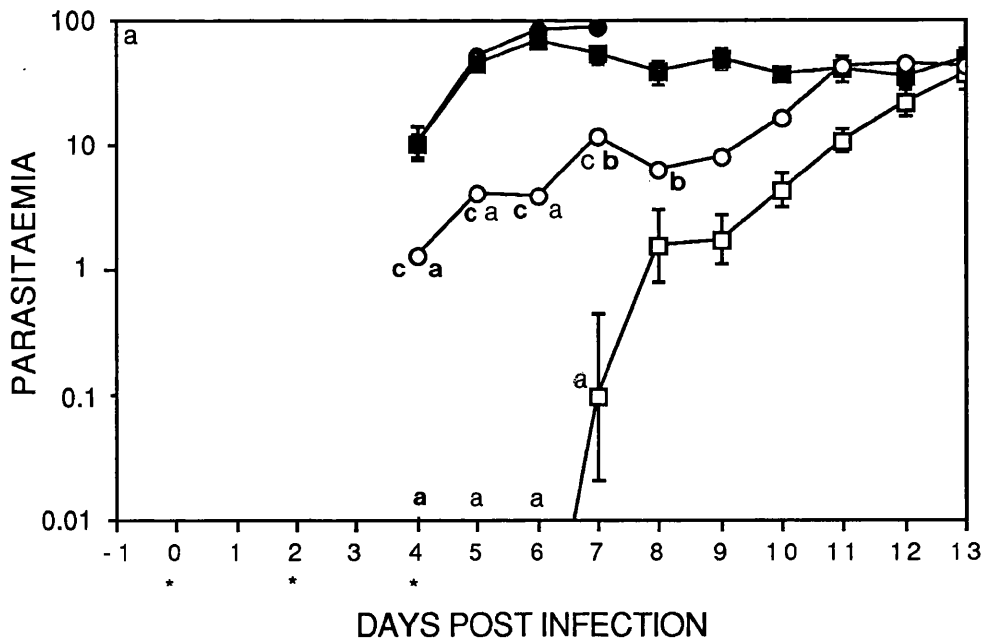
Figure 6.7: Inhibition of lethal *P.yoelii* (YM) infection *in vivo* by tumour necrosis serum and the effect of the antioxidant, Vitamin C.

10^4 parasitised red blood cells given i/v on day 0. 0.5ml TNS given i/p on days 0,2 and 4. Vitamin C fed *ad libitum* in the drinking water at 10mg/ml after an initial 20mg i/p 24 hours before infection.

Symbols: —■—, control infection; —□—, TNS i/p; —●—, control infection plus vitamin C; —○—, TNS i/p plus vitamin C. *, days of TNS injection; a, p<0.0002 compared with the control infection; b, 0.005<p<0.05 compared with the control infection; c, p<0.007 compared with TNS.

Vitamin E oil was fed to the mice *ad libitum* in their food at 2.5mg/g. TNS (0.5ml i/p) was injected on days 0,2 and 4 after infection with LPY. The food was weighed each day to determine the consumption of vitamin E/mouse/day (figure 6.8b).

The control group of mice and the vitamin E group did not have significantly different parasitaemias (Figure 6.8a). However unlike the vitamin C treated mice, the mortality of the vitamin E treated mice was not significantly different from the control mice, (3/6 by day 13 compared with 13/17 for the control group).

The intake of vitamin E remained at approximately 20 mg/mouse/day until day 6 when it fell to approximately 10 mg/mouse/day for the remainder of the assay. This change in eating corresponded to the parasitaemia reaching 50%. From this time until the mice recovered or died, they looked ruffled, sick and were passive for most of the time. The vitamin E fed mice were always far more active and less sick looking than the controls. This was, of course only a qualitative observation.

The effect of vitamin E on TNS activity was very similar to that of vitamin C, significantly reducing TNS activity (0.0001<p<0.05) to day 7. The parasitaemia of the vitamin E-TNS group reached 40% 3 days before the TNS group, after TNS injections were stopped (figure 6.9a). However the activity in the TNS-vitamin E group was still significant compared with the control infection (p<0.0002).
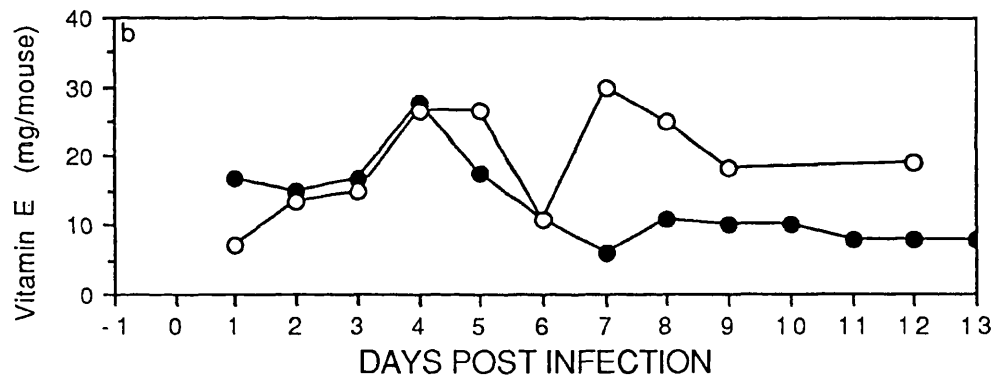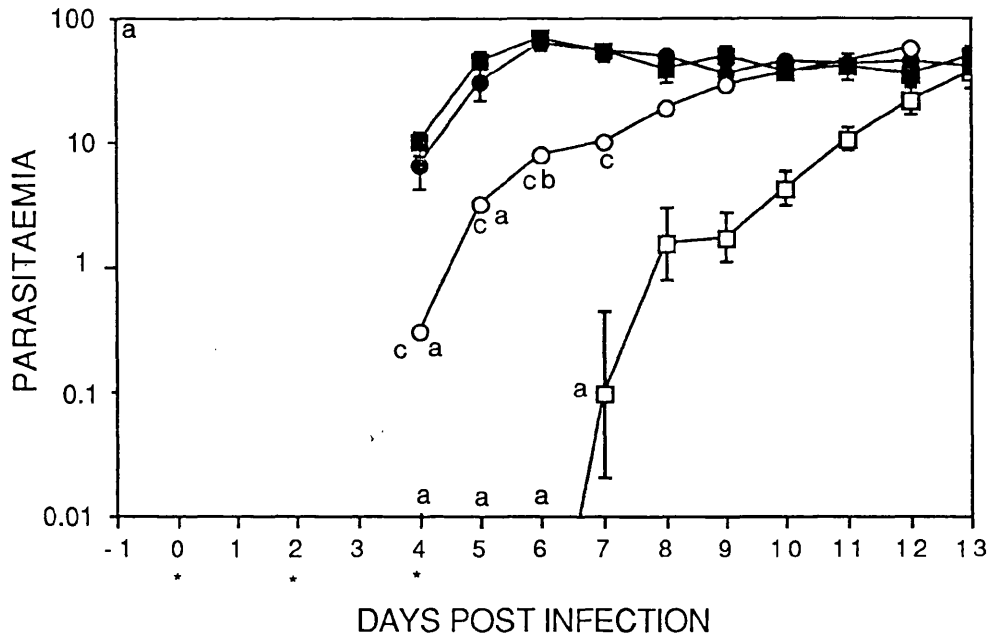
Figure 6.8: Inhibition of lethal *P.yoelii* (YM) infection *in vivo*
by tumour necrosis serum and the effect of the antioxidant,
Vitamin E.

10^4 parasitised red blood cells given   i/v on day 0. 0.5ml TNS
given i/p on days 0,2 and   4. Vitamin E fed *ad libitum* in the food
at 2·5 mg/g

Symbols: ─■─, control infection; ─□─, TNS i/p;
─●─, control infection plus vitamin E; ─○─, TNS i/p plus vitamin E;
*, days of TNS injection; a, p<0.0002 compared with the control
infection; b, p=0.0079 compared with the control infection; c,
0.0001<p<0.05 compared with TNS.

6.5.3. EFFECT OF VITAMIN C, VITAMIN E AND SUPEROXIDE DISMUTASE IN COMBINATION ON THE ACTIVITY OF TNS *IN VIVO*.

Mice were fed a combination of vitamin C and vitamin E as described above. One group also recieved injections of Superoxide Dismutase (SOD) i/p (2500 U/mouse/day) from day -1 to day 5.

TNS was injected (0.5ml i/p) on days 0,2 and 4.

The mice fed vitamin C and vitamin E had a reduced vitamin C intake compared with the previous experiment (=<10mg/mouse/day compared with 22mg/mouse/day). However the consumption of vitamin E was comparable to the previous experiment (figure 6.9b). This was because the food was made up with water and therefore the water consumption from the bottle dropped. This did not affect the final results.

The combination of vitamin C and vitamin E reduced the activity of TNS to the same extent as the vitamins alone, except that when TNS injections were stopped the increase in parasitaemia was faster than the vitamins alone as shown by the parasitaemia reaching 40% 4 to 5 days before the TNS group without vitamins, compared to 3 days in the vitamin C or vitamin E groups. This was also true for the group of mice fed vitamins C and E and SOD (figure 6.9a).

SOD alone did not alter TNS activity up to day 4, but after TNS injections were stopped, the parasitaemia increased much faster than in the vitamin treated mice. No deaths occurred in this group.

If these results are compared to the previous sections it can be seen that at the doses used, the individual vitamins were as effective as the combination. SOD may possibly be more effective alone, although not when used in conjunction with vitamins C and E.
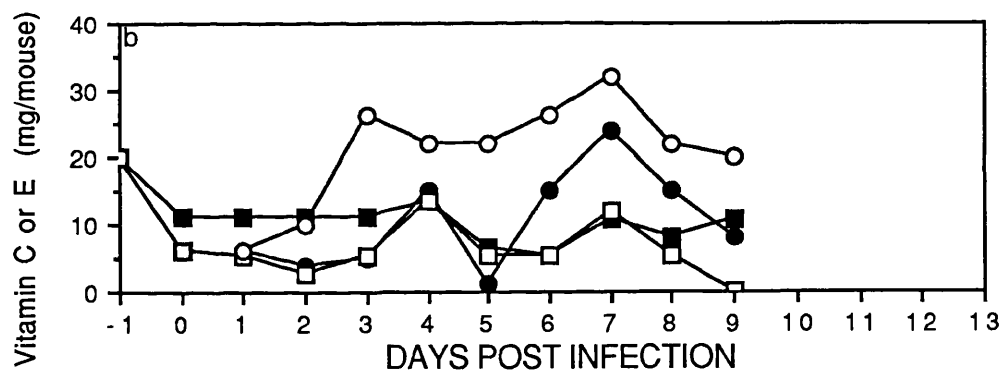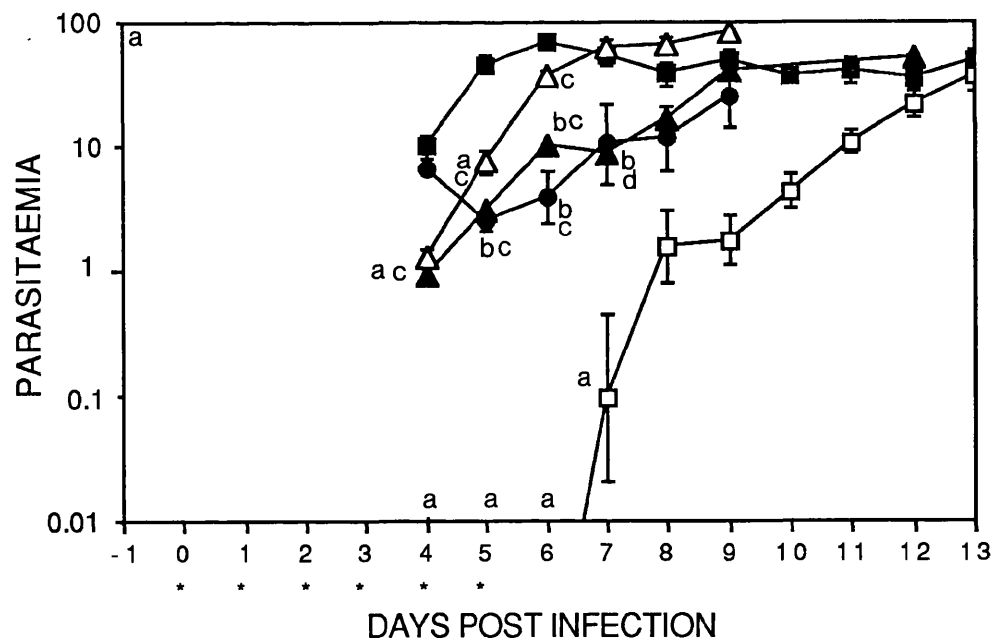
Figure 6.9: Inhibition of lethal *P.yoelii* (YM) infection *in vivo*
by tumour necrosis serum and the effect of the antioxidants,
Superoxide Dismutase alone; Vitamin C and Vitamin E; and Vitamin C,
Vitamin E and Superoxide Dismutase.
10^4 parasitised red blood cells given i/v on day 0. 0.5ml TNS given
i/p on days 0,2 and 4. Vitamin E fed *ad libitum* in the food at
2.5mg/g; Vitamin C fed *ad libitum* in the drinking water at 10mg/ml
after an initial 20mg dose i/p 24 hours beore infection; and
Superoxide Dismutase- 2500 U/0.5ml PBS/mouse/day from days 0 to 5
Symbols: Figure 6.9a; —■—, control infection; —□—, TNS i/p; —●—, TNS
i/p plus vitamin C and vitamin E; —▲—, TNS i/p plus vitamin C,E and
SOD; —△—, TNS i/p plus SOD; *, days of SOD injection i/p and TNS
injection (days 0,2 and 4 only); a, p<0.0002 compared with the
control infection; b, 0.0002<p<0.015 compared with the control
infection; c, p<0.0002 compared with TNS; d, 0.0002<p<0.035 compared
with TNS. Symbols: Figure 6.9b; TNS plus vitamin C (—□—) and vitamin
E (—○—); TNS plus vitamin C (—■—), vitamin E (—●—) and SOD.

6.6. EFFECT OF RECOMBINANT IFN-γ AND IL-1 ON LPY INFECTION.

Since the vitamin results suggested a role for oxygen metabolites *in vivo*, two cytokines which can stimulate an oxidative burst *in vivo* were tested. Although probably not present in TNS, their production would almost certainly be stimulated *in vivo* either by the TNS or by the infection. This would have to be the case for IFN-γ because rabbit IFN-γ would not be functional in a mouse.

Figure 6.10 shows that these cytokines were not active against LPY infection at the doses used (10 000 U IFN-γ i/p on days 0 and 2, 50 000 U IL-1 i/p on days 0 and 2). This seems to rule out these cytokines alone, though they might still act together with other factors. No polymorphonuclear cell infiltrate was seen.
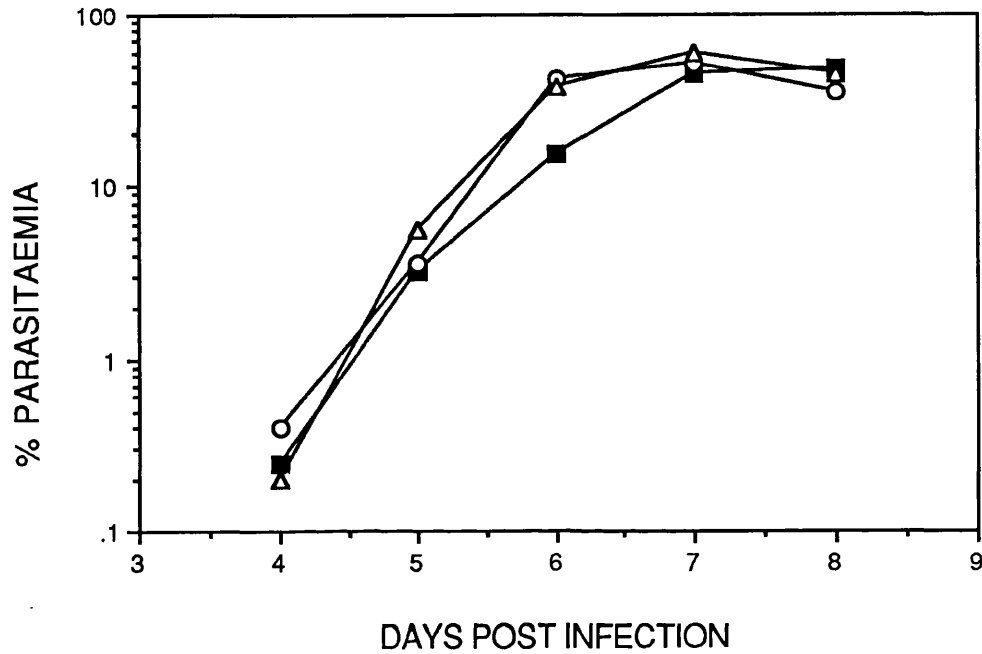
Figure 6.10: Effect of recombinant cytokines on the *in vivo* growth
of *P.yoelii* in Tuck No.1 mice.
10⁴ *P.yoelii* parasitised red blood cells injected i/v on day 0.
Recombinant cytokines injected i/p on days 0, 2 and 4.
Symbols: —■—, normal infection; —O—, 10,000 U recombinant murine
IFN-γ i/p; —△—, 50,000 U recombinant murine IL-1 i/p.

6.7. Effect of Antioxidants on Mouse Malaria Infections.

Vitamin C and Vitamin E were fed to mice as described previously. Each mouse consumed between 15 and 25 mg of vitamin C or E per day. Trolox was given as injections i/p at 20 mg/mouse/day. Nordihydroguariaretic acid was given at 10 mg/mouse/day i/p.

Three parasites were used to look at the effect of vitamin C and vitamin E on a primary infection. The effect of these on a LPY infection have already been described and are shown together in figure 6.11a. Vitamin E did not alter the course of the LPY infection, nor were the parasitaemias significantly different from the control. Vitamin C treatment although not significantly increasing the parasitaemia, caused all the mice to die by day 8 of infection.

Neither vitamin C nor vitamin E changed the course of a NLPY infection (figure 6.12). Parasite clearance still occurred at day 20. Vitamin C treatment did increase the parasitaemia between days 12 and 16 to about 55% compared with less than 50% for the control group. However both vitamins delayed the clearance of *P.chabaudi* by 24 to 48 hours, (figure 6.13a). The parasitaemias for each group up to the peaks were not significantly different from each other.

Vitamin E was also tested on a *P.chabaudi* infection in mice vaccinated with 10^7 formalin fixed *P.chabaudi* parasites 14 days earlier. The control parastaemia reached a peak of 10% on day 9, and then the parasites cleared by day 11 (figure 6.13b). Vitamin E altered this by increasing the peak parasitaemia to 30% and delaying the clearance by 24 to 48 hours.

In LPY vaccinated mice the control infection reached a peak at day 5 with a parasitaemia of 7%. The parasites were cleared by day 7. Four antioxidants were tested individually against a LPY infection in LPY-vaccinated mice. The data were pooled because the results were similar (Figure 6.11b). The antioxidants increased the peak parasitaemia to 12% compared with 6% and delayed parasite clearance by 24 hours. From day 6 to day 8 the antioxidant treated mice had a significantly greater parasitaemia compared with the control infection.
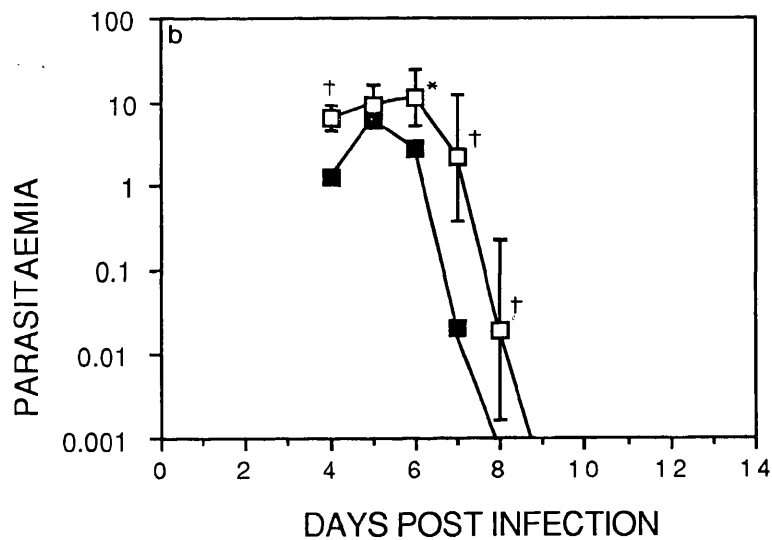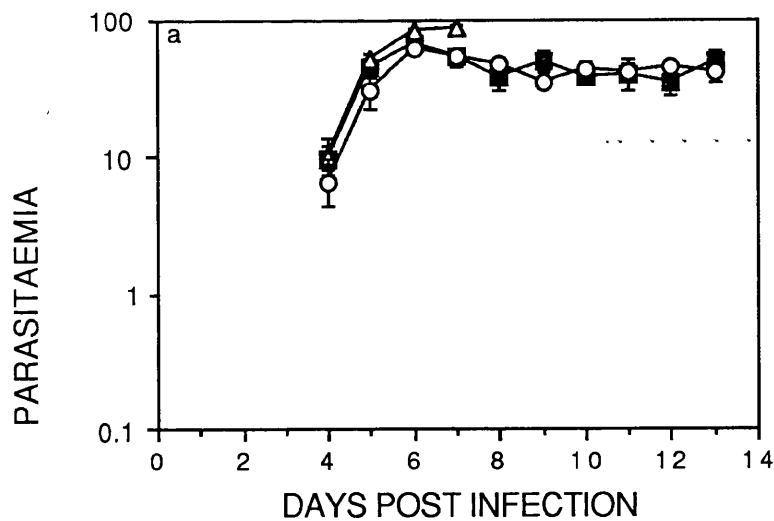
Figure 6.11:   Effect   of   antioxidants   on   the   course   of   a
lethal *P.yoelii* infection in mice.

Vitamin C was fed *ad libitum* in the water at 10mg/ml. Vitamin E was
fed *ad libitum* in the food at 2.5mg/g. Trolox; 20 mg i/p
per mouse/day. Nordihydroguariaretic acid; 10 mg i/p per mouse/day.
All were started 24 hours before infection with 10^4 parasitised red
blood cells   i/v.

a:Non-vaccinated Tuck No.1 mice; —■—, control infection; —O—, vitamin
E fed mice; —△—, vitamin C fed mice.

b: Vaccinated (C57black x   Balb/c)F1;   —■—,   control   infection;,
—□—, pooled data of all four antioxidants tested individually.

*, 0.03<p<0.0004; †, p<0.0004.
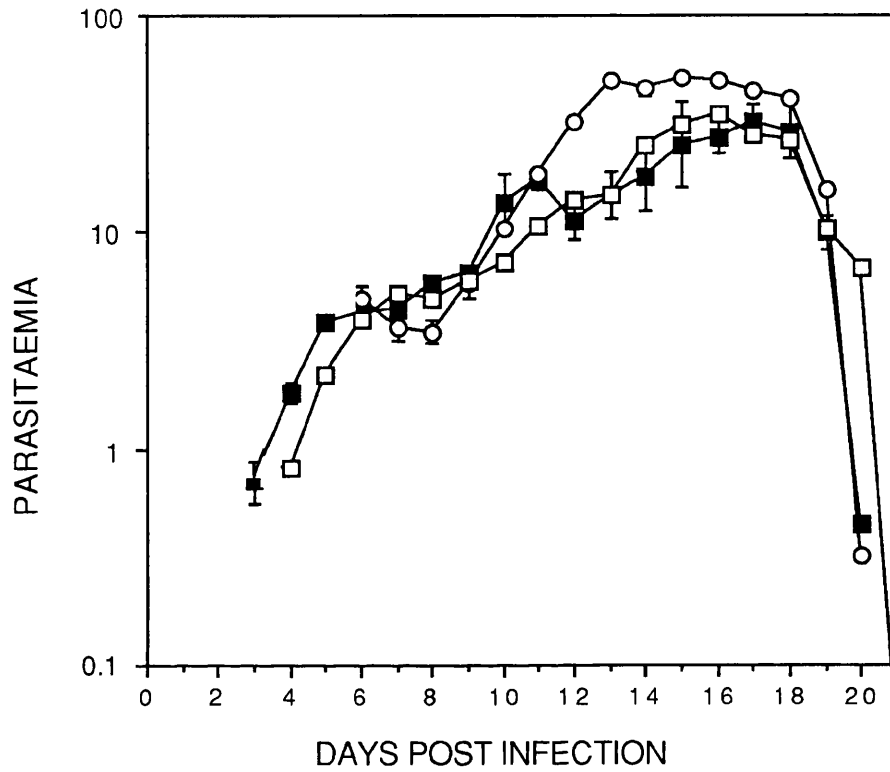
Figure 6.12: Effect of antioxidants on the course of a non-lethal
P.yoelii infection in mice. Vitamin C was fed *ad libitum* in the
water at 10mg/ml. Vitamin E was fed *ad libitum* in the food at
2.5mg/g. Both were started 24 hours before infection with 10^4
parasitised red blood cell i/v.
Non-vaccinated Tuck No.1 mice; ━■━, control infection; ━O━, vitamin C
fed mice; ━□━, vitamin E fed mice.
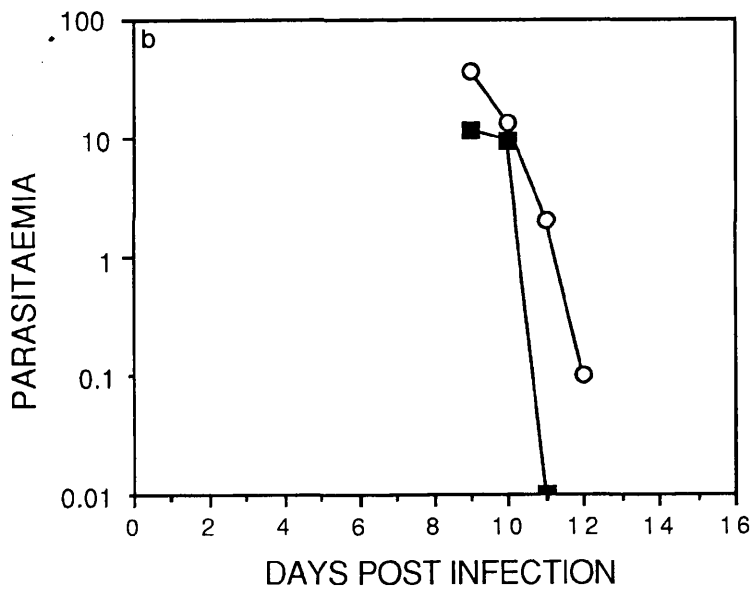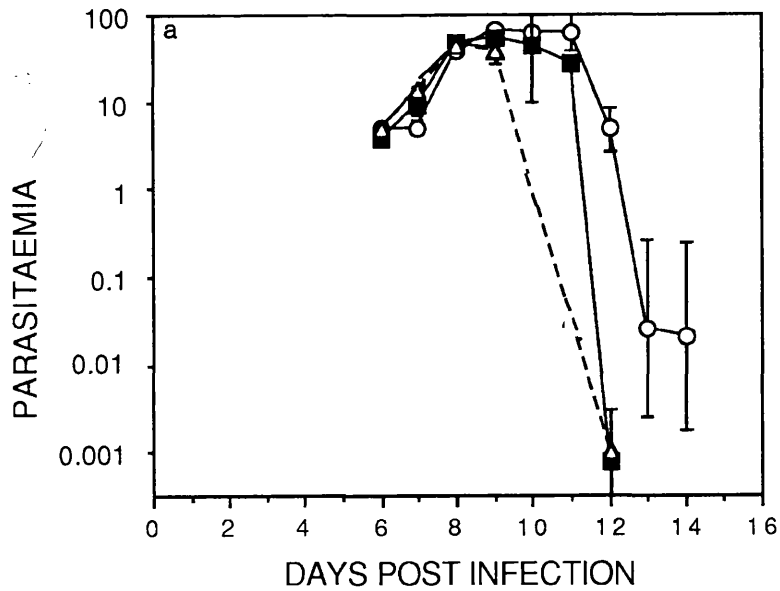
Figure 6.13: Effect of antioxidants on the course of a *P.chabaudi* infection in mice.
Vitamin C was fed *ad libitum* in the water at 10mg/ml. Vitamin E was fed *ad libitum* in the food at 2.5mg/g. Both were started 24 hours before infection with 10^4 parasitised red blood cell i/v.
a:Non-vaccinated (C57black x Balb/C)F1 mice; —■—, control infection; —O—, vitamin E fed mice; —△—, vitamin C fed mice.
b: Vaccinated (C57black x Balb/c)F1; —■—, control infection; —O—, vitamin E fed mice.

6.8. SUMMARY OF RESULTS.

1) TNS maintained LPY infection parasitaemias below 0.001%

2) TNF was not the main active factor in TNS.

3) 3 active fractions were separated by ion-exchange chromatography which maintained the LPY infection parasitaemias below 10%

4) Molecular weight chromatography lost the activity in TNS.

5) Vitamin C, vitamin E or SOD could almost totally reverse the antiparasitic effect of TNS.

6) Neither IL-1 nor γ-IFN affected the course of an LPY infection.

7) All antioxidants tested had an effect on an LPY infection in vaccinated mice.

Vitamin C and not vitamin E affected a primary infection of LPY.

Vitamin C and not vitamin E affected a primary infection of NLPY but not the time of recovery.

Vitamin E and not vitamin C affected a primary *P.chabaudi* infection by delaying recovery by 24-48 hours.

Vitamin E affected a *P.chabaudi* infection in vaccinated mice.

Antioxidants delayed parasite clearance in LPY infected ⁄ vaccinated mice

7. DISCUSSION II.

7.1. LIPIDS.

The *in vivo* effect of TNS on LPY has been known for some time (Taverne et al 1982). Up to now this activity has been attributed to two sorts of factors present in TNS; those able to kill the parasites directly and those acting indirectly. Following my results on the parasiticidal effect of TNS lipoproteins *in vitro*, I tried to repeat the effect *in vivo* using ultracentrifuge separated lipoproteins. These were administered both i/v and i/p. The results (figure 6.1) were identical and were therefore pooled. The lipoproteins from TNS had no significant effect on the LPY parasitaemia, although there was a tendency for the parasitaemia to be higher. However the non-lipid fraction was very active in maintaining a low parasitaemia, similar to the whole TNS. This is in complete contrast to the *in vitro* results, and suggests that the non-lipid fraction of TNS must be acting indirectly on the parasite. I cannot rule out the possibility that during an infection lipoproteins may play a parasiticidal role.

One possible reason for the lipoproteins having no effect *in vivo* is that when a mouse is injected i/v with 0.5ml of them, they would be diluted 1:5 by the blood. This dilution would have only 50% activity *in vitro*. In order to increase activity *in vivo*, the lipoproteins would have to be concentrated, but this proved impractical because the resulting solution was too viscous, and the lipids began to come out of solution. Experiments were done with artificial lipoproteins (section 4.7.2 and 4.7.3), but the initial results *in vitro* were not as promising as hoped, therefore they were not tested further.

7.2. TNF.

Because recombinant TNF had a slight but significant effect on the course of a LPY infection *in vivo* (Taverne et al 1987), the activity of TNS may partly be due to its TNF content, or some other molecules in TNS may only be active in the presence of, or

synergistic with, TNF. This was tested by using an anti-TNF
antiserum. This inhibited the TNF activity of TNS in a bioassay, but
had little effect on the *in vivo* activity of TNS on a LPY infection
(Figure 6.2).

Further experiments of this type should be done with more, or
different, antibodies, before dismissing the role of TNF
altogether.

Nevertheless these results do suggest that other molecules in TNS
are more important than TNF, especially when an occasional TNS (1:20)
has no *in vivo* activity, even though its TNF content was high
($>10^5$ U/ml).

Neither IL-1 nor γ-IFN alone induced a parasiticidal response. This
could be because the dose of cytokines used was too low, or that they
cannot act alone; certainly any IL-1 in TNS would contribute to the
activity of the serum by acting with other factors. γ-IFN on the
other hand because of its species specificity would have to be
induced within the animal before it could exert its effects.

7.3. SEPARATION OF TNS.

TNS was initially separated by ion-exchange chromatography into
four fractions. These were tested for *in vivo* activity against LPY.
The 0.04M PBS fraction had no activity whereas the other 3 fractions
had some activity, preventing the parasitaemia from rising above 10%.
When these fractions were pooled, the pool was as active as the
original TNS (figure 6.3). This suggests that the total activity of
TNS may be addition or synergism between at least 3 different
factors. Because of the method of separation it less likely for a
protein to be separated into more than one fraction. The lipids
eluted into the 0.16M PBS fraction and were not responsible for this
fraction's activity. TNF eluted in the 1.6M NaCl fraction, which is
in agreement with previously published results (Taverne et al 1984),
and it could be responsible for the activity of this fraction.

Further separation by molecular weight sieving lost any activity
that was present in the ion-exchange fractions (pooled fractions,
Figures 6.4b, 6.5b, 6.6b).

Even though no data could be obtained on the molecular weight of

the active factors, these results did show that foreign proteins were not responsible for the activity of rabbit TNS in mice on LPY, nor was it due to bacterial LPS picked up during the separation procedures, because fraction activity did not correlate with either protein concentration, or with LPS content.

7.4. ANTIOXIDANTS.

7.4.1. REVERSAL OF TNS ACTIVITY.

Three injections of 0.5ml TNS over the first four days of a LPY infection from an inoculum of 10^4 parasites was sufficient to cause a 3 log reduction in parasitaemia by day 4. This would be equivalent to the injection of 10^5 parasites rather than 10^8 (based on 5 x 10^9 RBC/ml and 2 ml blood). This timing is suggestive of a non-specific mechanism being responsible for the activity of TNS. because it is too early for a effective T or B cell response to have been mounted.

The hypothesis that TNS was triggering a oxidative burst was tested by feeding the mice very high doses of antioxidants. The daily doses of both vitamin C and vitamin E would be equivalent in humans to 5.6g per day. Although well tolerated this dose could cause some gastro-intestinal disturbances, but otherwise the vitamins are non-toxic (Reynolds 1989). Both vitamin C and vitamin E are readily absorbed from the intestines. Vitamin C passes directly into the plasma whilst vitamin E is taken up into the lymph and associates with the chylomicrons. These daily doses were based on published data (Godfrey 1957a,b, Eaton et al 1976).

The vitamins, singly, were able to almost completely reverse the parasiticidal activity of TNS. This reversal could not be improved upon by using both vitamins in the same animal. This suggests that the parasiticidal action of TNS is primarily via oxygen radicals and that the blocking capacity of both antioxidants is maximal. Under physiological conditions vitamin C synergises with vitamin E to neutralise oxygen radicals (Niki 1987). Therefore their effect together should be much greater than either one alone, unless the concentrations alone are in excess for the amount of ROI produced.

The parasitaemias in infected animals given antioxidants but not

TNS show that vitamins C and E were not inherently toxic to the parasite.

Superoxide Dismutase injections (2500U/mouse/day) from day -1 to 5 of infection, also almost totally reversed the parasiticidal activity of TNS. Its effects were possibly greater alone than when used in conjunction with vitamins C and E although not significant.

7.4.2. EFFECT OF ANTIOXIDANTS ON MALARIA PARASITES IN NON-VACCINATED AND VACCINATED MICE.

Three parasites were used: Lethal *P.yoelii*, Non-lethal *P.yoelii* and *P.chabaudi*. The *P.yoelii* parasites showed a a tendency to survive and grow better in vitamin C treated mice, as shown by increased parasitaemias. Thus the mice with LPY all died by day 8, compared to untreated mice, possibly due to anaemia as >90% of the erythrocytes were infected prior to death. Although an increased parasitaemia was seen in NLPY infection, the mice did not die. Indeed the parasites cleared at the same time as those in the untreated mice.

Vitamin E had no effect on *P.yoelii* infections, but it delayed the clearance of a *P.chabaudi* infection by 24 to 48 hours, which vitamin C did not. When antioxidants were tested on an LPY or *P.chabaudi* infection in vaccinated mice, they had similar effects. The peak parasitaemias were increased, and the clearance of the parasites was delayed by 24 to 48 hours.

These results suggest that ROI are important in vaccinated mice for controlling the growth of the parasite, but are not the most important mechanism for the clearance of the parasites. It is also implied that more oxygen radicals are released in vaccinated mice although the actual species of radicals may be different in vaccinated mice compared with non-vaccinated mice (see next section).

7.5. Role of ROI in Malaria Infections.

A primary infection does not appear to stimulate the release of ROI in quantities to inhibit parasite growth, because antioxidants have little effect. However they do seem to make the animals look better. While the animals in the control group are looking hunched, untidy and lethargic, the animals given vitamins look and act as normal, even though they have parasitaemias above 50%. This suggests that the oxygen radicals are damaging the host in the untreated groups of mice. This could be mediated through protein or lipid damage. The difference in effect of vitamin C and vitamin E on *P.yoelii* and *P.chabaudi* may reflect the species of radical induced by the two parasites. For example *P.yoelii* induce radicals which are active in an aqueous phase and therefore neutralised by vitamin C, whereas *P.chabaudi* induce radicals acting in a lipid phase which can be blocked with vitamin E.

Thus, even though the peak of macrophage activity as measured by L929 tumour cell cytoxicity (Taverne et al 1986) and ROI release (Dockerell et al 1986), occurs just before parasite clearance in *P.chabaudi* infection, ROI in the above infections are not essential for clearing the parasites.

When mice are vaccinated there is a large increase in macrophage activity (Taverne et al 1986) and ROI release (Dockrell et al 1986). This is reflected by the effect of antioxidants on LPY and *P.chabaudi* infections in vaccinated mice. Although parasite clearance still occurs, the results do show that ROI are involved in controlling the infection. Moreover both vitamin E and vitamin C have an effect. This could be due to the increased ROI production or possibly a change in the species of radicals produced.

However even in vaccinated mice the oxidative response is not as great as that in mice injected with TNS in which vitamins C and E significantly inhibit antiparasitic activity.

The effect of superoxide dismutase suggests that superoxide is an an important effector radical in the action of TNS against the parasite. Peroxide is produced from the reaction of superoxide and superoxide dismutase, therefore the peroxide must be disposed of quickly else it would be parasiticidal, as has been shown (Dockrell and Playfair 1984) unless the quantity of peroxide made in the above reaction is not parasiticidal.

Vitamin C can neutralise radicals released by effector cells into an aqueous environment, unless the effector cell is so close to the target cell so that the radicals are released directly onto the target cell's membrane. These radicals would be inhibited by vitamin E. However if a compartment is formed between the effector and target cells then there would initially be a limited antioxidant capacity before damage occurs. Direct release similar to this has been described (Wozencraft et al 1985).

Vitamin C can protect membrane proteins from becoming oxidised. Damage to these proteins would affect the exchange of nutrients between the plasma and the red cell, which would lead to parasite death by starvation. Ion channels in the membrane can be similarly affected. Parasite death could occur before red cell destruction and this may be one explanation of "crisis forms". Lipid peroxidation may result in red cell destruction before parasite death, unless the parasite is first killed by taking up the lipid peroxides into its own membrane. The two mechanisms above probably occur together and the extent of each would be determined by the ratio of lipid antioxidants to aqueous phase antioxidants. Whatever the exact mechanism of oxygen radical killing of LPY parasites after TNS injection, the results do show that it is the most important mechanism of action of TNS. The remaining activity after antioxidant treatment could still be due to ROI damage in situations where antioxidants cannot reach. For example close cell to cell contact or phagocytosis as described above. Alternatively it may reflect some quite different activity (see below).

7.6. MODE OF ACTION OF TNS.

As described above, TNS is primarily acting *in vivo* by inducing a ROI response, which kill the parasites. In each experiment with antioxidants there was still residual parasiticidal activity. This could be due to components of TNS acting directly on the parasite. On the other hand the activity may be due to ROI which cannot be blocked with antioxidants as described above. TNS may induce the release of other types of radicals, such as RNI. These may not be blocked by the antioxidants. RNI can attack sulphydryl groups and therefore damage proteins. Glutathione can neutralise their activity (Ignarro et al 1981).

TNS is more rapid but not more complete at inhibiting parasite growth than vaccination of the mice. The noted increase in blood leukocytes following TNS injections, may be a contributing factor to the increased ROI release. Alternatively TNS may supply all the necessary activating and growth promoting factors for ROI release, which would bypass the time required to synthesise and release them. TNS may also supply the initial trigger for macrophage activation, if not then the parasite certainly can (Wood and Clark 1984, Bate et al 1988, 1989).

7.7. IMPLICATIONS FOR FURTHER RESEARCH.

Given that the primary action of TNS *in vivo* was to stimulate an oxidative burst, it may be possible to devise an *in vitro* assay to measure the release of ROI from macrophages or other cell types after stimulation by TNS or separated fractions of TNS. This would have several advantages over the *in vivo* mouse assay.

a) Smaller volumes could be tested and therefore only small volumes of TNS would need to be separated (1 or 2 ml) compared with 6 to 10ml. This means shorter run times and sharper protein peaks.

b) Many more fractions could be collected and tested.

c) Shorter assay time.

d) Combinations of fractions could be tested.

e) The assay would be more sensitive than the parasite killing *in vivo*. The former makes a direct measurement of ROI release whereas the latter assay relies on sufficient radicals to kill the parasite.

An assay of this type might make it possible to identify which molecules are responsible for the activity of TNS and which cell types are stimulated. The assay could also be adapted to test for chemotactic factors.

Ultimately an artificial TNS could be developed with the effector molecules at optimum concentrations. The final test would be to see if it has activity *in vivo* against malaria parasites.

RNI could also be investigated in the above system. Unlike ROI, RNI are not as easy to inhibit *in vivo*. At present their synthesis can be competitively inhibited by using arginine analogues, but may themselves be parasiticidal.

7.8. SUMMARY.

1) TNS lipid peroxides are not antiparasitic *in vivo*, although lipid peroxides cannot be ruled out as an effector mechanism.

2) The *in vivo* parasiticidal activity of TNS is not due solely to TNF. Neither IL-1 nor Y-IFN alone can induce a parasiticidal response.

3) Separated TNS losses activity *in vivo*, suggesting the additive or synergistic nature of the effector molecules.

4) The activity of TNS can be almost totally reversed by antioxidants, showing that the primary effect of TNS *in vivo* is to induce the release of ROI.

5) Antioxidants allow greater parasite growth in vaccinated mice, and delay
by 24 to 48 hours, but do not prevent, clearance of the parasites.

8. DISCUSSION III.

I have demonstrated that the *in vivo* and *in vitro* parasiticidal activities of rabbit TNS are due to completely different components. *In vitro*, oxidised lipoproteins are alone responsible for the serum's parasiticidal activity, but *in vivo* they have no detectable effect on the course of a LPY infection. The non-lipid fraction is the active part of TNS *in vivo*. It was not possible to separate this activity into single active molecules because it could not be detected after separation regimens I used. However it does seem likely that there is more than one molecule responsible for the final activity of TNS, and that these act together additively or synergistically. This could be a reason why using individual cytokines had little (e.g. TNF) or no (e.g. IL-1, γ-IFN) effect. The large number of possible combinations of cytokines/TNS fractions to be tested prevented this being studied in the time available. The *in vitro* assay described in section 7.7 to test for ROI/RNI production from phagocytes after stimulation with TNS fractions would be better suited to the above experiment, because many combinations and dilutions could be tested simultaneously with the minimum requirement for material.

Two observations may account for the success of TNS *in vivo*: the leukocytopaenia which occurs by day 4 after beginning TNS treatment and infection. Also the almost total reversal of TNS activity *in vivo* with antioxidants. The apparent oxidative burst generated by TNS appears to be greater than that in either a normal primary infection or that produced in vaccinated-infected animals as demonstrated by the effects of antioxidants on the parasitaemias. The difference may be due to the fact that the oxidative burst occurs earlier and more suddenly in mice given TNS than in the natural infection, as shown by the parasitaemias on day 4 when TNS injected mice are compared with vaccinated mice. The generation of an oxidative burst as an effective antimalarial mechanism has been known for several years (Clark & Hunt 1983, Clark et al 1983) but the radicals may also be detrimental to the host animal. This would depend on several factors, including the nutritional status of the host and whether the effector cells have been primed in some way so that they release more radicals upon stimulation.

I have already described some of the ways in which ROI/RNI may be produced in a malaria infection. One observation may suggest that

they do indeed have an undesirable effect on the host. The mice fed antioxidants always appeared healthier than the control mice even though the parasitaemias were the same or higher. The antioxidants could be protecting host tissues from oxidant damage. Lipid peroxidation could be a major effect of oxidant attack. This does have implications for people with malaria.

8.1. DIET.

8.1.1. SATURATED FAT DIET.

A diet exclusive in saturated fats would eventually be harmful to the host. As well as the occurrence of tissue damage (table 2.1), this diet would also lead to a lowered inflammatory response. The essential fatty acid arachidonic acid is used by cells to make prostaglandins and leukotrienes. One effect of a deficiency in these two classes of molecules is a reduction in vascular permeability. This would reduce the number of leukocytes which could be recruited into the blood stream from the surrounding tissues (Ganong 1983). Therefore there would be a smaller oxidative burst in the blood, which combined with fewer circulating unsaturated fats would result in fewer lipid peroxides. However there would be always be a small amount of unsaturated fat because of host enzyme mechanisms (section 2.2.3). This situation may be advantageous to the parasite. Indeed in the short term a mixture high in saturated fats did not inhibit parasite growth. Long term effects could easily be studied by using *P. falciparum in vitro* and lipid depleted serum plus artificial saturated lipoproteins in the culture medium.

8.1.2. Unsaturated Fat Diet.

A diet high in unsaturated fats would prevent the host tissue damage described in table 2.1. Prostaglandin and leukotriene production would not be compromised as in a saturated fat diet, indeed it may be enhanced if there is a large reservoir of arachidonic acid. Also serum lipoproteins would be able to induce an increase in prostaglandin and leukotriene production (Kelley et al 1988). Therefore it would be easier for leukocytes to pass into the blood stream from the tissues, and the nett oxidative burst would be greater, when compared with that generated in animals on a saturated fat diet. The number of lipid peroxides generated would also be greater.

The effect of a high unsaturated fat diet on rodent malaria is quite dramatic (Godfrey 1957a). Compared with the control infection there is almost no parasite growth. This is probably not due to normal lipids as my results show (sections 4.7.3 and 4.7.4). The unsaturated fats are not parasiticidal until they are oxidised. In fact parasite growth is normal in animals on a high unsaturated fat diet if they are also given high doses of vitamin E or vitamin C.

8.1.3. Antioxidant Status.

Antioxidants have been shown to have parasite protective effects; firstly large doses of antioxidants reversed the parasiticidal effects of a high unsaturated fat diet and secondly, animals which are made vitamin E deficient, no longer support parasite growth. This is reversed by injecting antioxidants. These effects are summarised in table 8.1.

Under experimental conditions the above results suggest a possible application of diet to malaria therapy. However the desired manipulation of diets may not be very practical in malaria endemic areas, although the population of those countries do have in their favour a more predominantly vegetable diet than the western world, and this would favour the unsaturated rather than the saturated fats. On the other hand diets rich in antioxidants may be harmful because they could allow faster parasite growth though they might also

	Unsaturated Fats	Vitamin E Deficiency	Vitamin E	Vitamin C	TNS	Alloxan	Hydrogen Peroxide
P. chabaudi			Delays Recovery 24-48h	No effect	No effect		
P. chabaudi vaccinated			Peak Parasitae' 31% v's 6%				
P. vinckei						⇓ Clark and Hunt 1983 Cox 1983	
P. berghei	⇓ Godfrey 1957a	⇓ Godfrey 1957a Coatney and Greenberg 1961	Reverses unsat' fat inhibition Godfrey 1957a	Reverses unsat' fat inhibition Godfrey 1957a	Slight Reduction	⇓ Waki et al 1985	
LETHAL *P. yoelii*			No effect	All die by day 8	Keep parsitae' below 0.01%		⇓ Dockrell and Playfair 1983
P. yoelii vaccinated			Peak parasitae' 40% v's 6%	Peak parasitae' 35% v's 6%			
NON-LETHAL *P. yoelii*			No effect	Parasitae' reached 60 v's 45%	⇓ Taverne et al 1981		
Babesia	⇓ Godfrey 1957b	⇓ Godfrey 1957b	Reverses unsat fat inhibition Godfrey 1957b				
Avian malarias		⇓ Yarrington et al 1973					

Table 8.1: Summary of the effect of antioxidants or oxygen radical generating system on malaria parasites *in vivo*.
Published data is shown with a relevant reference. My data is also included.

protect the host from oxidative damage. From my data there is a tendency for antioxidants to increase parasite load.

However from table 8.1 several anomalies can be seen regarding the effects of antioxidants on parasite infections. *P.chabaudi* parasites live in mature red cells and therefore should be the most sensitive to oxygen radicals. But TNS does not have any effect on them and there is only a slight effect of vitamin E on the clearance of the parasites and none on their growth. This suggests that these parasites are already optimally protected against radical attack, and that the clearance of the parasites must therefore be due to some other mechanism (antibody for example).

P.berghei is in a similar position as regards TNS, suggesting that it too is protected against oxidant attack. Indeed antioxidants are required for parasite growth because it cannot grow in vitamin E deficient animals. It would be interesting to know the effects of high doses of antioxidants in normal infected animals.

I did look at the effects of vitamins C and E on a primary infection, and in vaccinated-infected animals. Only vitamin C affected the primary infection by hastening the death of the mice with LPY and causing a raised parasitaemia for NLPY. This could suggest that the parasites are amply protected against lipid attacking radicals but they lack antioxidant mechanisms for protecting against aqueous phase radicals. These radicals would attack the membrane transport proteins. Therefore *P.yoelii* appears to be not as well protected as *P.berghei* or *P.chabaudi*, and this may explain its sensitivity to TNS. In vaccinated animals either the quality of radicals is increased or there is a change in the pattern of radicals released, which is why all the antioxidants have an effect in vaccinated mice. It would be interesting to look at all the rodent malaria species for the pattern of ROI and RNI response before and after vaccination and after TNS injection.

8.1.4. STARVATION.

People during long term starvation show no symptoms of malaria neither are any parasites detectable in the blood (Murray et al 1975, Murray et al 1976). One might have expected the opposite because starvation leads to a decreased immune response, (Hayward 1977). However, starvation of the host may also lead to starvation of the parasite due to a lack of nutrients and also antioxidants. The former would slow parasite growth and the latter would make the parasite more susceptible to any oxidative response. This is supported by the fact that once the "starving" persons begin proper feeding, the malaria parasites reappear, as do the disease symptoms (Murray et al 1975, Murray et al 1976).

8.2. A UNIFIED SCHEME FOR NON-SPECIFIC PARASITICIDAL MECHANISMS.

Using published data and with the effects I have demonstrated by using TNS and antioxidants, I would like to propose the following scheme:

Parasites are phagocytosed and processed (Tosta and Wedderburn 1980, Celada et al 1983) and in doing so activate the reticuloendothelium system (Cox et al 1964, Kharazmi et al 1987). This will result in the release of ROI (Descamp-Latscha et al 1987) and cytokines (Wood and Clark 1984, Bate et al 1988, 1989). Many of the factors described in table 1.5 may also be released.

Parasites can also activate complement (Fogel et al 1966). The chemotactic molecules formed and those released from activated cells would attract leukoytes into the blood vessels. Such molecules would include prostaglandins and leukotrienes as described, plus IL-1 and TNF (Sayers et al 1988). TNS may contain several of these which act together to enhance the response.

Cytokines in TNS or released from cells after stimulation by parasites or TNS could activate the leukocytes. γ-IFN has been shown to make monocytes parasiticidal (Ockenhouse et al 1984a), but this was *in vitro* and γ-IFN alone does not induce a parasiticidal response *in vivo*. Neutrophils are parasiticidal after stimulation with

Zymosan or complement (Nnalue and Friedman 1988, Salmon et al 1986).
The parasiticidal activity is very likely to be oxygen radical
mediated. Factors in TNS may activate neutrophils, especially if IL-8
is present in TNS. From inhibitor studies two radicals involved in
neutrophil mediated parasiticidal activity are peroxide and
hypochloride (Dallegri et al 1983, Dallegri et al 1985).

From my antioxidant data I would suggest that in a primary
infection that the above response is not of major importance. But if
the animals are vaccinated or injected with TNS then the response is
increased, and in the case of TNS, speeded up. It is unfortunate that
in laboratory animals we cannot easily study chronic infections of
the human type. It is perhaps not surprising that BCG treated animals
cannot support parasite growth because the malaria parasites act like
LPS (although perhaps less potently) and as a result would be
endogenously generated TNS. The effect of antioxidants would be worth
investigating here.

The nearest situation to the above in humans would be tuberculosis.
Certainly serum from tuberculosis patients is parasiticidal (Geary et
al 1986). These patients had a active ongoing infection with acid
fast bacteria in their sputum. The presence of these may provide both
the priming and triggering necessary to produce the parasiticidal
response. Unlike *M.bovis* BCG which clearly does not. It is not
ethical to give people tuberculosis for a malaria infection, but a
BCG vaccination might be protective providing they have not already
be vaccinated with it.

Eosinophils may also be stimulated in the above scenario. Their
toxic cationic proteins have parasiticidal activity (Waters et al
1987). These proteins could puncture holes in the red cell membrane
in a similar way to complement (Young et al 1986). Eosinophils would
also enhance the oxidative response by releasing hypochlorous acid
made by the eosinophil peroxidase enzyme which uses hydrogen peroxide
as a substrate (Weiss et al 1986).

However the radicals are not totally beneficial and some of the bad
effects have already been discussed. TNF has also been implicated in
causing pathology in malaria. It may do this by causing an increase
in thrombospondin which in turn could increase the sequestration of
parasites (Roberts et al 1985, Rock et al 1988). TNF can also
increase the ROI release and other macrophage products (Clark et al
1989). TNF can encourage dyserythropoiesis and erythrophagocytosis
(Clark and Chaudri 1988). TNF has been found in the serum of people

with malaria (see Clark et al 1989 for review). But TNF does not appear to have any great parasiticidal activity *in vivo* as shown by the separated TNS and recombinant TNF experiments (Taverne et al 1986). Therefore it may be possible to use anti-TNF antibodies as therapy for severe malaria. Many of the activities of TNF can be mimicked by other cytokines (IL-1, γ-IFN, for example). Therefore a protective response could still occur but without any of the pathological consequences of TNF, described above. All the above mechanisms and some of those discussed earlier are shown in a summary diagram (figure 6.1) which tries to encompass the proven and postulated parasiticidal actions of TNS.

8.3. IMPLICATIONS.

I would like to suggest some possible approaches for treatment of malaria based on ideas generated from my work.

The basis of the approach is to generate oxygen and nitrogen radicals, the effectiveness of which will depend on the lipid status and antioxidant status of the person.

8.3.1. LIPID STATUS.

Increasing the proportion and concentration of unsaturated fats certainly decreases parasite growth. This could be mediated via lipid peroxidation and therefore generating an oxidative burst in the presence of unsaturated fats would increase the number of lipid peroxides and therefore their parasiticidal effect.

Experimentally, animals are fed for several weeks on high unsaturated fat diets to change the ratio of unsaturated to saturated fats in cell membranes. However, long term diets would not be practical and the next best situation would be to temporarily increase the serum lipid unsaturated fat content. This could be acheived by giving the person a meal high in unsaturated fats 2-3 hours prior to further treatment, by which time the unsaturated fats would be in the blood stream as chylomicrons.
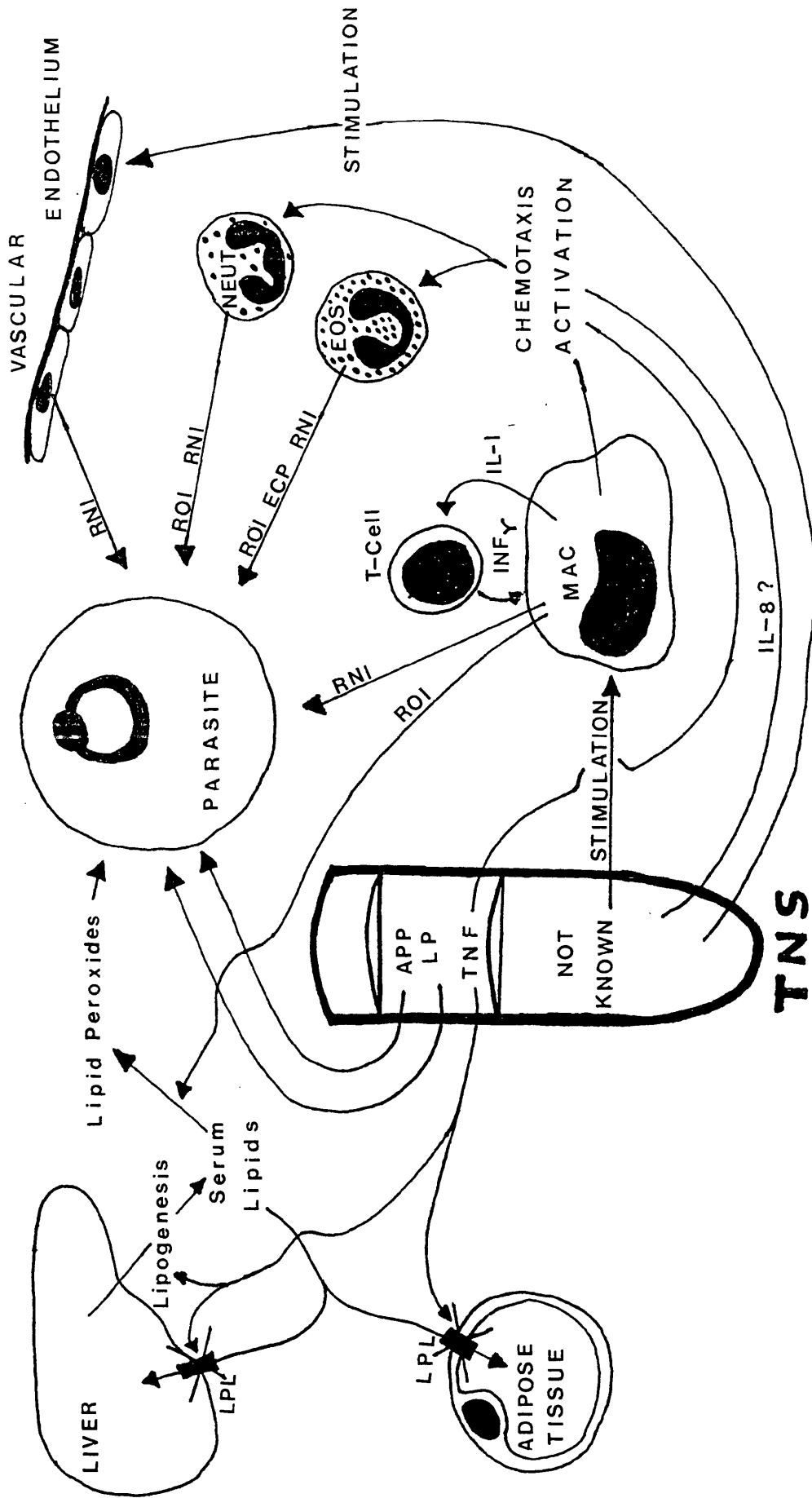
Figure 8.1: Schematic diagram of the parasiticidal actions of TNS.
(cells are not to scale).

Abbreviations:

TNS	Tumour necrosis serum
LPL	Lipoprotein lipsae enzyme
TNF	Tumour necrosis factor
APP	Acute phase protein
IL	Interleukin
IFN	Interferon
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
ECP	Eosinophil cationic protein
EOS	Eosinophil
NEUT	Neutrophil

Parasite survival will depend on a number of factors including
antioxidant capacity of the infected cells, age of red cell and the
parasite lipid requirements.

8.3.2. Antioxidant Status.

In general high antioxidant status (i.e. a high reduced status) is advantageous to the parasite. Therefore should the method of treatment be via oxygen radicals, their effect would be to shift the oxidant/reductant ratio towards the left and in favour of the host.

Experimentally this can be achieved by decreasing the hosts antioxidant pool by putting the animals on vitamin E free diets. However because this takes several weeks, it would not be very practical for humans. Another approach is to alter the host oxidant/antioxidant state *in vivo*. This has been done by converting reduced glutathione (GSH) to oxidised glutathione (GSSG) or by blocking the reduction of GSSG. The former reaction can be mediated by the drug Ebselen which acts like glutathione peroxidase (Müller et al 1984, Wendle et al 1984). Certainly this drug is parasiticidal *in vitro* against *P.falciparum*, but it has no effect on *P.berghei in vivo* (Hüther et al 1989). The latter effect was accounted for because the drug has a short *in vivo* half-life. However Ebselen has the advantage of low toxicity towards the animal (Mecurior and Combs 1986) unlike the drug Carmustine (BiCNU). This may not be suitable for long term use *in vivo* because of its many toxic side effects (BNF 1989, Reynolds 1989). One of its actions is to block glutathione reductase and therefore prevent the reduction of GSSG (Karplus et al 1988). Certainly *in vitro* this drug is antiparasitic (Zhang et al 1987). It would be interesting to test it *in vivo* on a malaria infection.

If the more oxidant state is achieved in the host, then the toxicity of oxygen radicals would be increased.

8.3.3. Generation of Oxygen Radicals.

Some currently-used antimalarial drugs can generate oxygen radicals. For example Artemesin (Quing hao su) (Krungkrai and Yuthavong 1987) and possibly Pyrimethamine (Kharazmi 1986) can do this.

TNS induces an oxidative burst *in vivo*, and if the active factor(s) could be isolated they may form the basis of a treatment. However, the antioxidant status of the host and parasite may be an

important factor in determining how susceptible the parasite is to oxygen radicals, so changing the host antioxidant status may be necessary. This could be tested using *P.chabaudi* and *P.berghei* in mice treated with Carmustine, and then injecting TNS. As an alternative to administering the active factors in TNS, it may be possible to induce their release *in vivo*, however it must be remembered that when TNS is made in the ordinary way the animal dies!

Further research could be carried out to look at the susceptibilities of other diseases to TNS. A parasite more sensitive to the toxic effects of TNS than *P.yoelii* may be found, thus enabling to test for separated TNS *in vivo*.

The ultimate aim of separating TNS would be to try and find a factor or factors which could perhaps form the basis of a treatment for malaria or some other disease by inducing an oxidative burst, which would be toxic to the parasite without causing irreversible damage to the host. This response would only be active whilst the factor is present and therefore would be more like a drug than a vaccine.

This idea of manipulating the body to release factors as a means of destroying parasitic cells is not new. The use of Y-IFN for priming and a streptococcal preparation for triggering, has been used with some success in cancer patients (Soma et al 1987, Tagaki et al 1987, Dobashi et al 1987). This treatment generates a novel TNF (TNF-S) which destroys the cancer cells unlike the TNF most people refer to (Mizuno et al 1987). As in the above case not all cancer patients had suitable cancers. The suggestion of a similar treatment but using TNS factors in malaria may only be suitable for certain categories of infected people, such as those people who are not going to remain in an endemic area, because after treatment they would be susceptible to reinfection, and, perhaps most of all, patients severely ill with drug-resistant strains of the parasite.

The use of oxygen radicals as a parasiticidal mechanism has the advantage over both chemotherapy and the activation of T or B cells (e.g by vaccines) that they may be less susceptible to parasite escape via genetic variation. It is still possible, of course, that mutation within the parasite may also enable it to evade oxidative damage, and oxidative mechanisms may not be so easy to evade. Certainly increasing red cell antioxidant capacity may be advantage to the parasite, but this can be made ineffective as I have already discussed.

Although the treatment of mice with whole TNS is crude, they do not appear to suffer any short or long term ill effects from the treatment and as my data shows it is a very effective means of preventing parasite growth. Therefore further research on TNS with the above ideas in mind may find a workable treatment whether for malaria or some other disease.

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10. APPENDIX.

10.1. COMPUTER PROGRAMS.

The following four programs were used to calculate exact p-valuse for Student's t-test.

Programs 1 and 2 must be run first to generate the semi-factorials in the equations (section 3.4.2.1). The data generated by these equations is then used by programs 3 and 4 to calculate the probability.

Equation 3 calculates 'p' from a given t value and degrees of freedom, while program 4 calculates 'p' from the means, standard deviations and sample sizes.

The programs were written on a Sinclair Spectrum +3. However the Basic should RUN on any computer. Apart from syntax the only change necessary, if any, would be to the file handling routines (i.e. SAVE and LOAD).

```
10 REM " calculate semi factorial for even DFs"
20 REM "i.e. 1*3*5.../2*4*6... etc"
30 REM "Kirk Allan Rockett 1989"
40 DIM s(100)
50 LET b=1: LET c=1
60 FOR a= 2 TO 56 STEP 2
70 LET b=b*(a-1)
80 LET c=c*a
90 LET s(a)=b/c
100 NEXT a
110 FOR a= 58 TO 100 STEP 2: LET s(a)=b/c: NEXT a
120 SAVE "tdata" DATA s()
```

```
10 REM "calculate semi factorials for odd DFs"
20 REM "i.e. 2*4*6.../3*5*7...  etc"
30 REM "Kirk Allan Rockett 1989"
40 DIM t(100)
50 LET b=1: LET c=1
60 FOR a= 2 TO 54 STEP 2
70 LET b=b*a
80 LET c=c*(a+1)
90 LET t(a)=b/c
100 NEXT a
110 FOR a= 56 TO 100 STEP 2: LET t(a)=b/c: NEXT a
120 SAVE "tdata1" DATA t()
```

```
10 REM "calculate P value from given t value and DF"
20 REM "Kirk Allan Rockett 1989"
30 DIM s(100): DIM t(100)
40 LOAD "tdata" DATA s()
50 LOAD "tdata1" DATA t()
60 LET sum =0
70 CLS
80 INPUT "t value ";t: PRINT "t value ";t
90 INPUT " df ";df: PRINT "Degrees of Freedom ";df
100 LET theta=FN a(t,df)
110 REM "calculate P value for odd df"
120 IF (INT (df/2))*2=df THEN GO TO 210
130 FOR a= 2 TO (df-3) STEP 2
140 LET sum= sum+(t(a)*((COS theta)^a))
150 NEXT a
160 LET sum =sum +1
170 LET sum=sum*(SIN theta) *(COS theta)*(2/PI)
180 LET ang =(theta * 2)/PI: LET sum=sum+ang
190 GO TO 270
200 REM "calculate P value for even DFs"
210 FOR a = 2 TO (df-2) STEP 2
220 LET sum =sum+ (s(a)* ( (COS theta)^a))
230 NEXT a
240 LET sum =sum+1
250 LET sum=sum*((SIN theta))
260 GO TO 270
270 PRINT "probability "; (1-sum)
280 REM "Equation for theta"
290 DEF FN a(a,b)= (ATN (a/SQR b))
```

```
10 REM "Calculate P values from means and SDs"
20 REM "Kirk Allan Rockett 1989"
30 CLS
40 DIM s(100): DIM t(100)
50 REM "load semi factorial data for equations"
60 LOAD "tdata" DATA s()
70 LOAD "tdata1" DATA t()
80 GO SUB 340
90 LET sum =0
100 LET df=n1+n2-2
110 PRINT "df:";df
120 LET theta=FN a(t,df)
130 REM "odd DF"
140 IF (INT (df/2))*2=df THEN GO TO 230
150 FOR a= 2 TO (df-3) STEP 2
160 LET sum= sum+(t(a)*((COS theta)^a))
170 NEXT a
180 LET sum =sum +1
190 LET sum=sum*(SIN theta) *(COS theta)*(2/PI)
200 LET ang =(theta * 2)/PI: LET sum=sum+ang
210 GO TO 290
220 REM "even DF"
230 FOR a = 2 TO (df-2) STEP 2
240 LET sum =sum+ (s(a)* ( (COS theta)^a))
250 NEXT a
260 LET sum =sum+1
270 LET sum=sum*((SIN theta))
280 GO TO 290
290 PRINT (1-sum)
300 REM " equation to calculate theta"
310 DEF FN a(a,b)= (ATN (a/SQR b))
320 GO TO 80
330 REM "input means etc"
340 INPUT "mean1,sd1,n1 ";mean1;" ";sd1;" ";n1
350 INPUT "mean2,sd2,n2 ";mean2;" ";sd2;" ";n2
360 REM " calculate population sd"
370 LET shat=SQR (((((n1-1)*(sd1*sd1))+((n2-1)*(sd2*sd2)))/(n1+n2-2)
)
380 REM "calculate t value"
390 LET t=(ABS (mean1-mean2))/  (shat*SQR ((1/n1)+(1/n2)))
400 PRINT "t:";t
410 RETURN
```

Killing of Blood-Stage *Plasmodium falciparum* by Lipid Peroxides from Tumor Necrosis Serum

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The multiplication of *Plasmodium falciparum* in culture, as measured by [³H]hypoxanthine incorporation, was inhibited in a dose-dependent manner by rabbit tumor necrosis serum. The regimen by which tumor necrosis serum is produced caused significant increases in the levels of triglycerides and lipid peroxides, with the latter being indicated by the level of malondialdehyde in the serum. When tumor necrosis serum was depleted of lipoproteins by aerosil (fumed silica), no parasiticidal activity remained, and when it was separated by ultracentrifugation, more than 70% of the parasiticidal activity was found in the lipoprotein fraction. This suggests that lipid peroxides may account for most of the parasiticidal activity in tumor necrosis serum but that a nonlipid toxic factor may also be present.

Tumor necrosis serum (TNS) is generated when animals are treated with *Mycobacterium bovis* BCG or *Propionibacterium acnes* (formerly *Corynebacterium parvum*) followed 14 days later with bacterial lipopolysaccharide (LPS) and then are bled 2 h later (4). This serum, which contains tumor necrosis factor and increased levels of numerous other molecules, has been shown to kill blood-stage malaria parasites in vitro (11) and in vivo (22); this effect in vitro is not due to tumor necrosis factor, as demonstrated by using the recombinant molecule (13, 23). However, the conditions under which this serum is made could implicate reactive oxygen intermediates (ROI). It has been shown in vitro that mitogenic stimulation of peritoneal macrophages 14 days after in vivo treatment with BCG gives an optimal hydrogen peroxide release (19) and that hydrogen peroxide is cytotoxic to blood-stage malaria parasites in vitro (6). However, hydrogen peroxide and other ROI have very short half-lives and are unlikely to be responsible for the in vitro effect of TNS; moreover, their effects would be blocked by antioxidants, which is not the case for the parasiticidal activity of TNS (10). However, lipid peroxides, which can be formed by reacting with ROI, are unaffected by antioxidants (18). Lipid peroxidation has the effect of stabilizing the reactive oxygen groups and thus creates a cytotoxic molecule with a much longer half-life. Also, many by-products of low molecular weight are formed; one is malondialdehyde (MDA) (17). This molecule can be used as an indicator of lipid peroxidation (2). Indeed, it has been shown that low-molecular-weight products are formed when *Plasmodium vinckei*-infected erythrocytes are exposed to oxidative stress (3) and that these products are toxic to the parasite when tested in vitro (5).

In this study, we have investigated the lipoprotein fraction of rabbit TNS to see whether lipid peroxidation occurs and whether these lipid peroxides are cytotoxic to *Plasmodium falciparum* in vitro.

MATERIALS AND METHODS

Culture of *P. falciparum*. The Ugandan strain of *P. falciparum* (Palo Alto) (7) was maintained in human O⁺ erythrocytes at a hematocrit of 5% in a standard culture system (25)

in RPMI 1640 medium containing 10% heat-inactivated human A⁺ serum, supplemented with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 0.2% NaHCO₃, and 40 U of streptomycin per ml (complete medium) in an atmosphere of 5% CO₂, 5% O₂, and 90% N₂. Cultures were synchronized by treatment with 5% sorbitol (16).

Rabbit sera. Normal rabbit serum was obtained from rabbits that had fasted overnight. TNS was produced as previously reported (22). Briefly, 5 × 10⁷ live *M. bovis* BCG organisms were injected intravenously. Two weeks later, after an overnight fast, the animals were given 80 μg of bacterial endotoxin (*Escherichia coli*; Glaxo Pharmaceuticals, Ltd., Greenford, United Kingdom) and 1.5 to 2 h later were bled out. The blood was allowed to clot overnight, and the serum was stored at −20°C before use.

Measurement of lipid peroxidation in serum. The following substances were added to 1 ml of fresh serum in a glass container: 5 μl of 0.3% butylated hydroxytoluene (to prevent peroxidation catalyzed by iron), 2 ml of solution containing 15% trichloroacetic acid, 0.375% thiobarbituric acid, and 0.25 N HCl. The container was then placed in boiling water for 15 min. The sample was cooled and centrifuged at 1,000 × *g* for 15 min, after which the optical density of the supernatant was measured at 535 nm, and the concentration of MDA was calculated by using an extinction coefficient of 1.56 × 10⁵ M⁻¹ cm⁻¹. The amount detected in phosphate-buffered saline (PBS) after the treatment was taken as the zero value in each assay (2).

Measurement of triglycerides. Triglycerides were measured by using an enzymatic method (kit 676519; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and an Encore chemical system (Baker Instruments Group, Allentown, Pa.).

Depletion of lipoproteins by aerosil. Aerosil (fumed silica; Sigma Chemical Co., St. Louis, Mo.) was used to deplete serum of lipoproteins (21). A 20-mg quantity of aerosil was added to 2 ml of serum (protein concentration, 7.5%; pH 7.2 to 7.8). After the serum was stirred in a water bath at 45°C for 4 h, the serum was cooled and centrifuged at 2,500 × *g* for 20 min. The supernatant was decanted and dialyzed against PBS–5 mM glutathione (reduced) (GR) before testing.
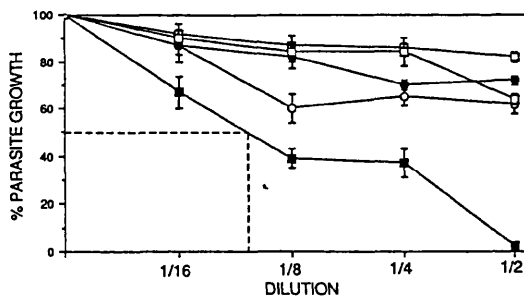
* Corresponding author.

FIG. 1. Inhibition of *P. falciparum* growth by serum samples from rabbits treated with BCG alone (●), LPS alone (○), BCG followed 14 days later by LPS (■), or serum samples from two normal rabbits (□). The data given are from a single experiment in which all sera were tested at the same time. The point used to compare parasiticidal activity in experiments is the dilution of serum required to inhibit parasite growth by 50% (– – –). Each datum point is the mean ± standard error of the mean for the sample tested in triplicate.

Separation of lipoproteins by ultracentrifugation. Serum dialyzed against PBS–5 mM GR was adjusted to a density of 1.21 g/ml with NaBr–5 mM GR (ρ = 1.51 g/ml) solution. A 1-ml quantity of NaBr–5 mM GR (1.21 g/ml) was layered on top of the serum preparation, which was then centrifuged for 44 h at 5°C in a 50 Ti rotor at 105,000 × *g*. After centrifugation was completed, the top 1-ml lipoprotein-containing fraction was removed from each sample. The serum protein pellet was then resuspended in the remaining volume. All samples were dialyzed extensively against PBS–5 mM GR and concentrated with polyethylene glycol (M_r, 20,000; BDH, Poole, England) as necessary to adjust volumes to the original serum volume.

Cytotoxicity assay. All samples for the cytotoxicity assay were extensively dialyzed against PBS–5 mM GR. Doubling dilutions of the test samples were made in PBS–5 mM GR in 50-μl volumes in 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark) in triplicate. Synchronized cultures of *P. falciparum* at the ring stage were adjusted to a parasitemia of between 0.5 and 1% at a hematocrit of 5% in complete medium, and 50-μl volumes were added to the test samples.

The plates were incubated at 37°C in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. At 24 h, tritiated hypoxanthine (0.4 μCi per well) was added to each well. At 48 h, the plates were harvested and incorporation was measured in a Tri-Carb 574 counter (Packard Instrument Co., Inc., Rockville, Md.).

Results were calculated as the mean percent inhibition of hypoxanthine uptake (± standard error of the mean) and compared with values for controls in which parasites were cultured in 50% PBS–5 mM GR in place of the test serum. From the dose-response curves produced, the concentration of samples that resulted in a 50% parasite growth inhibition was calculated. These concentrations were used to compare the killing of *P. falciparum* by the various serum samples.

RESULTS

Rabbits were injected either with BCG or LPS alone or with BCG and LPS. Only the combination of BCG followed by LPS triggered the production of significant parasiticidal activity (Fig. 1). Serum samples from rabbits given BCG

TABLE 1. Effects of BCG and LPS, alone and combined, in rabbits

Rabbit treatment	Serum level (mean ± SEM [n])		% Serum (mean ± SEM [n]) to inhibit parasite growth by 50%
	Triglycerides (mM)	MDA[a] (μM)	
Control	0.53 ± 0.06 (8)	2.43 ± 0.34 (12)	57.9 ± 5.53 (26)
LPS	1.82 (2)	3.31 (2)	75.0 ± 25.00 (3)
BCG	1.21 ± 0.09[b] (3)	2.36 ± 1.03 (3)	46.4 ± 17.90 (5)
TNS	1.55 ± 0.26[b] (7)	5.62 ± 0.29[b] (8)	22.5 ± 2.78[b] (22)

[a] MDA is an indicator of lipid peroxidation.
[b] $P < 0.01$ compared with control sample.

alone or LPS alone were unable to produce 50% parasite killing, even at a dilution of 1:2. The parasiticidal activity of each serum sample was measured here as the dilution resulting in a 50% inhibition of parasite growth, as measured by [³H]hypoxanthine incorporation.

In order to test whether the parasiticidal activity was related to the lipoproteins, the concentrations of triglycerides were measured in the sera. There was a significant increase in triglycerides ($P < 0.01$) following the administration of BCG or LPS or both (Table 1). This was not due to dietary factors as all animals were fasted overnight before they were bled. However, there was no direct relationship between the levels of triglycerides and parasite killing by serum.

MDA was measured next to give an indication of the oxidation state of the lipoproteins. TNS had the highest level of MDA ($P < 0.01$) and the level of MDA was also elevated in LPS serum (Table 1). No increase was seen in BCG serum. This suggests that lipid peroxidation, rather than simply a high concentration of lipid, may be involved in parasite killing. There was no correlation between the triglyceride concentration and the MDA concentration.

To test more directly for the involvement of lipoproteins, a method was required to deplete the serum of these molecules. Aerosil (fumed silica) almost exclusively removes lipoproteins (21). This treatment entirely depleted the small level of activity in normal rabbit serum and almost completely depleted the activity in TNS (Fig. 2). This suggests that the activity of normal rabbit serum is not due simply to the inability of a human parasite to thrive in the serum of a
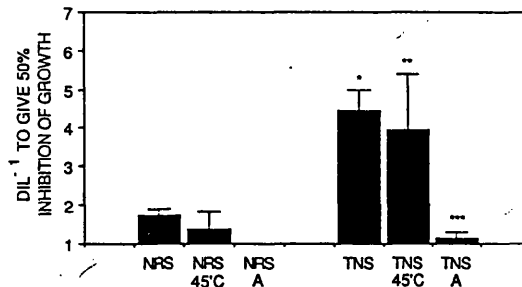
FIG. 2. Inhibition of *P. falciparum* growth by TNS and effect of depleting serum lipoproteins with aerosil. The values were calculated as in the legend to Fig. 1. Abbreviations: 45'C, Serum incubated for 4 h at 45°C; A, serum after treatment with aerosil at 45°C; Dil⁻¹, dilution⁻¹; NRS, normal rabbit serum. Each bar is the mean ± standard error of the mean for four to six experiments. Symbols: *, $P < 0.001$ compared with normal rabbit serum; **, not significant compared with TNS; ***, $P < 0.001$ compared with TNS.
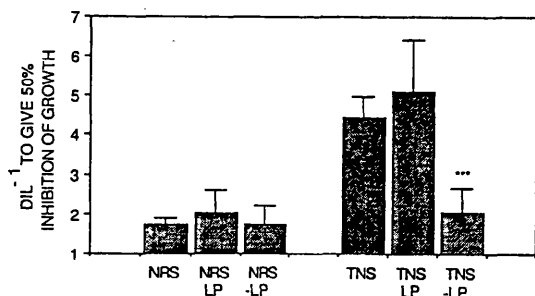
FIG. 3. Inhibition of *P. falciparum* growth by TNS and effect of preparative separation of lipoproteins by ultracentrifugation at 1.21 g/ml for 40 h at 105,000 × g. The values were calculated as described in the legend to Fig. 1. Abbreviations: LP, lipoprotein fraction of serum; −LP, serum depleted of lipoproteins; Dil^{-1}, dilution^{-1}; NRS, normal rabbit serum. Each bar is the mean ± standard error of the mean for six to nine experiments. Symbols: *, $P < 0.001$ compared with normal rabbit serum; **, $0.005 < P < 0.001$ compared with normal rabbit serum; ***, $0.02 < P < 0.01$ compared with TNS.

different species. This depletion was not due to the incubation of serum at 45°C for 4 h as shown in Fig. 2.

As a result of the nature of aerosil, it was not possible to recover the lipoproteins for testing. Instead, lipoproteins were preparatively separated by density gradient ultracentrifugation at a density of 1.21 g/ml. Figure 3 shows that the lipoproteins in TNS are toxic to the parasite ($P < 0.01$). The activity of the nonlipid fraction of TNS was reduced to a level not significantly different from that of normal rabbit serum. Thus, the depletion of lipids, while significantly reducing parasite killing, was not as effective as aerosil treatment, which removed essentially all activity. This may suggest the presence of two parasiticidal molecules, only one of which is lipoprotein in nature.

DISCUSSION

We have demonstrated that the administration of BCG followed by LPS leads to a rise in the concentration of serum triglycerides in rabbits and also to an increase in lipid peroxidation, as indicated by the thiobarbituric acid test. The elevated level of triglycerides is partly due to the BCG infection, as previously shown (24), but the LPS injection increases this level still further. These changes could be accounted for by several mechanisms. LPS can cause release of lipoproteins from various cell types. Also, this regimen causes the release of tumor necrosis factor, which in turn can block the action of lipoprotein lipase, thus blocking triglyceride clearance from the serum (15). LPS can also interfere with the activation of triglyceride clearance enzymes. It has been shown that the concentration of serum triglycerides can increase significantly within 2 h of LPS injection alone (14).

It is perhaps not surprising, then, that lipid peroxidation has occurred in TNS when the treatment is also optimal for macrophage activation (19). LPS alone can activate macrophages but not as well as treatment with BCG and LPS; therefore, some lipid peroxidation would be expected in the LPS serum, but it was not as high as in TNS (Table 1).

The results shown in Table 1 suggest that lipid peroxidation is not solely involved because the LPS serum should then have activity intermediate between that of TNS and

normal rabbit serum, which it clearly does not. However, the lipoprotein fraction of TNS was undoubtedly more toxic than that of normal rabbit serum (Fig. 3). If this effect were simply due to the triglyceride concentration, one would expect the BCG and LPS sera to be as toxic as TNS. There was no correlation between triglyceride concentration and the percent serum required to inhibit parasite growth by 50%.

If the aerosil results and the ultracentrifugation results are taken together, it is clear that there is another parasiticidal factor present which also binds to aerosil, suggesting a factor with high hydrophobicity. This molecule may also be present in normal rabbit serum but is either in a less toxic condition or at a lower concentration than in TNS. Preliminary data from chromatographic separations do support the idea of two cytotoxic fractions, one a lipoprotein fraction and a low-molecular-weight fraction (data not shown).

Lipid peroxides have been implicated in a number of diseases and have been shown to be cytotoxic to various cell types. They could kill cells in several ways. Since the parasites do not have a de novo triglyceride synthesis pathway (20), they must obtain all their lipid requirements from the host serum. If oxidized lipids are taken up by the parasite, they may have a direct cytotoxic effect on the parasite, for example, in cross-linking proteins. However, they are more likely to destabilize the cell membrane, in particular the erythrocyte membrane, which could then lead to parasite death without necessarily destroying the erythrocyte; this is quite possible as no significant hemolysis was seen in the cytotoxicity assays. It has been shown that in a malaria infection, the lipid content of the erythrocyte membrane can rise by up to 600% (1). Of the lipids, linoleic acid in particular increases. This is an unsaturated fatty acid which is readily oxidized. Therefore, linoleic peroxides are likely to be readily taken up by the parasitized erythrocytes. As well as causing membrane destabilization, the peroxides can initiate further oxidation in the presence of oxygen, especially in the presence of ferric ions, which act as a catalyst; both of these are available within the erythrocyte. The erythrocyte does have antioxidant pathways which would prevent this occurrence, but the potential oxidative capacity of TNS may be too much for the system. This threshold concept could explain why LPS serum is not parasiticidal, if the oxidative capacity of the LPS serum were within the antioxidant capacity of the erythrocyte.

If the erythrocyte were damaged enough, it would not support parasite growth. This process would be semiselective for the parasitized erythrocytes because parasitized erythrocytes would take up more lipid as mentioned above. There would be some effect on normal erythrocytes but not enough perhaps to hinder normal erythrocyte function, although there might be an effect on reinvasion by the parasites.

In summary, we have demonstrated that the direct parasiticidal effect of rabbit TNS can be attributed to an increase in lipid peroxides, with the probability that a second nonlipid parasiticidal molecule is also present. These molecules, then, must be added to the growing list of non-antibody-mediated anti-plasmodial molecules, which already includes eosinophil products (26), orosomucoid (9), polyamine oxidase (8), and crisis forming factor (12). We are currently investigating whether our nonlipid factor is related to one of these molecules.

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Inhibition of intraerythrocytic development of Plasmodium falciparum by protease inhibitors

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ABSTRACT

A group of inactivators of cysteinyl proteinases which function by covalent bond formation have been examined for their ability to inactivate plasmodia within red blood cells. The most effective of these caused inactivation of the parasite near 10^{-8}M concentration. The range of inhibitory action varied with peptide structure in a manner characteristic of affinity-labels for proteases suggesting that the target of inhibition was an unidentified protease, probably cysteinyl, but different from cathepsins B and L.

Plasmodial inhibition; antimalarials; protease inhibitors

INTRODUCTION

The study of plasmodial proteinases is currently under investigation in connection with various features of the life-cycle of the parasite (1-5) including erythrocyte invasion (1), intra-erythrocytic events such as hemoglobin digestion (2,4,5) and merozoite maturation (3). The use of specific inhibitors to obtain evidence for the physiological role of these proteinases and conceivably to lead to therapeutic applications is a logical extension of these studies. The success of this approach will depend on the specificity of the inhibitors since host cells employ similar proteinases for useful functions. Considerable progress has been made in the development of inactivators that function by affinity-labelling, using peptide sequences which satisfy the specificity of a target proteinase but are also capable of forming a covalent bond at the active center. Peptide derivatives that irreversibly inactivate serine and cysteinyl proteinases include peptidyl chloromethanes (6), fluoromethanes (7-13), diazomethyl ketones (14-18) and peptidyl methyl sulfonium salts (19,20). Variation in the peptidyl portion may provide selectivity of inactivation among closely related proteinases. This may be difficult to achieve within a common mechanistic family, but exploration of inhibitor structures has revealed useful enzyme differences, including topographical variations [17,18].

We found previously that certain peptidylfluoromethanes, and in particular, one containing a novel amino acid imidazolyl-norvaline, I_mNva, (12) are able to lyse the infectious but not

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the non-infectious forms of African and South American trypanosomes in vitro (Ashall, Angliker, and Shaw, manuscript in preparation). We subsequently examined a group of protease inhibitors of the peptidyl fluoromethane, diazomethane, and peptidylmethyl sulfonium salt classes for a possible effect on the intraerythrocytic development of the human malarial parasite, Plasmodium falciparum, with the results described below.

2. MATERIALS AND METHODS

Cultures of human A+ erythrocytes at a haematocrit of 5% in a standard culture system [21] were infected with P. falciparum strain NF54. Parasites were synchronized using 5% sorbitol and cultured for a further two hours. [22]. When parasites reached the ring stage, culture volumes were adjusted to a parasitaemia of 0.5 to 1% at a haematocrit of 5% then incubated with protease inhibitors at 37°C in an atmosphere of 5% CO_2/5% O_2/ 90% N_2. After 24 hours, tritiated hypoxanthine (4 μCi/ ml) was added to the cultures and after an additional 48.24 hours, erythrocytes were harvested and incorporation of radioactivity determined and used as an estimate of the rate of parasite multiplication [23].

3. RESULTS AND DISCUSSION

Nine synthetic proteinase inhibitors were examined, chiefly peptidyl fluoromethanes, but two other types were also included, a diazomethane and a pair of sulfonium salts. In the P_1 position [24] both neutral and positively charged amino acids were included since the specificity of proteolysis is often directed to this residue. Two of the inhibitors were particularly

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IC_{50} = inhibitory Concentration for 50% inhibition of Parasite growth.

effective in inhibiting development of intraerythrocytic P. falciparum (Table 1, top). The concentrations required to produce 50% inhibition of parasite division were similar to those of chloroquine, a widely used antimalarial drug (which for this strain has an IC_{50} of 1.28×10^{-8} M).

Morphology changes accompanying the inhibition of intraerythrocytic development of P. falciparum included enlargement of food vacuoles within the parasite (figure 1). It may be that the proteinase inhibitors produce these effects by inhibiting digestive proteases within parasite food vacuoles. Independent observations made with one of the less effective inhibitors of the present study, Cbz-Phe-AlaCH$_2$F, led to a similar interpretation of its action [5]. However, in that study it was concluded that the protease affected may be cathepsin-L like. In fact, such a possibility would suggest a lack of selective toxicity.

An important characteristic of affinity-labelling reagents such as those in Table 1 is the influence of peptide sequence. With an unknown protease, the variation in susceptibility to inhibitors of different sequences provides information about the specificity of the protease, analogous to examining a group of synthetic substrates. The results in Table 1 suggest that the most susceptible plasmodial protease is not cathepsin L-like, although they may belong to the same gene family, since cathepsin L is ten times more susceptible to an inhibitor containing the Cbz-Phe-Ala- sequence than one with Cbz-Leu-Tyr- [18], whereas in Table 1 the fluoromethane with the latter sequence is more than 50 times more effective, indicating quite a different binding

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site. This is promising with respect to developing inhibitors selective for an important plasmodial protease but without action on host cell function, thereby permitting treatment of human malarial infections in vivo. While it is not yet certain that the inhibition we describe is due to inactivation of a proteinase, it is very likely, since the fluoromethane group is unreactive and forms covalent bonds largely as a result of complex formation with a target enzyme [8]. The use of such reagents in radioactive form should permit the identification of the inactivated protease and characterization of its specificity in the usual manner.

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Table 1   Effects of protease inhibitors on intraerythrocytic

        development of <u>P. falciparum</u>.

Protease inhibitor	Concentration producing 50% inhibition of growth (nM)	Ref.
Cbz–Leu–TyrCH$_2$F	59	11
Cbz–Phe–ImNvaCH$_2$F	80	12
Cbz–Tyr–AlaCH$_2$F	1390	*
Cbz–Phe–AlaCH$_2$F	3380	7
Ala–Phe–LysCH$_2$F	4940	8
Cbz–Ala–PheCH$_2$F	15670	10
Cbz–Phe–AlaCHN$_2$	8790	14
Cbz–Phe–AlaCH$_2$S+(CH$_3$)$_2$	1950	19
Cbz–Phe–ArgCH$_2$S+(CH$_3$)$_2$	21080	20

    * In preparation

Figure 1: Appearence of *Plasmodium falciparum* trophozoites after 4
hours incubation in;
a: normal culture medium.
b: 0.05 µM protease inhibitor.
magnification x 1000. Stained with giemsa.