BIOCHEMICAL MECHANISMS OF CHLOROQUINE UPTAKE AND RESISTANCE IN MALARIA.

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by

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Dedicated to my parents,

Abel Oluedo

and

Elizabeth Diribe

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ABSTRACT

Mechanisms of (^{14}C) chloroquine accumulation and chloroquine resistance were studied in Plasmodium berghei (NK 65 and RC) in mouse erythrocytes in vitro.

The quantity of chloroquine taken up by erythrocytes infected with the chloroquine resistant RC strain was similar to that found for the sensitive NK 65 strain when percentage of erythrocytes parasitised was taken into account.

Chloroquine accumulation could be separated into saturable specific (high affinity), and an unsaturable non specific (low affinity) components. A specific receptor for chloroquine with a dissociation constant of 4.0×10^{-9} to 1.0×10^{-8} mol/l was found to be associated with uptake into erythrocytes infected with both NK 65 and RC, but was absent in uninfected erythrocytes or reticulocytes.

This receptor was 6 times more abundant in erythrocytes infected with NK 65 strain than in those infected with RC - where non-specific uptake accounted for up to 80% of the total chloroquine accumulation. It was tentatively concluded that non-specific uptake was mainly related to chloroquine binding by erythrocyte macromolecules; whilst specific uptake was related to receptors associated with the parasite itself.

Chloroquine uptake by NK 65 parasitised erythrocytes was highly dependent on glucose availability, which was not the case in uninfected erythrocytes. In parasitised erythrocytes, it seemed probable that only the specific component of uptake was dependent on D - glucose.

The ionophores Monensin and Nigericin inhibited chloroquine uptake non-competitively in high sodium and potassium buffer

respectively. This inhibition of uptake was achieved without significant changes in glycolytic rate. Partial alleviation by ouabain of the inhibitory effect of Monensin but not of Nigericin, suggested that the internal proton concentration of the erythrocyte compartment was involved in drug uptake. Valinomycin had much less effect indicating that membrane potential was a less important factor.

Mean internal pH values of NK 65 and RC infected erythrocytes were found to be about 0.2 units lower than uninfected erythrocytes and 0.55 units lower than the plasma.

It was concluded that chloroquine accumulation by P. berghei infected erythrocytes was dependent on several factors, but the basis of chloroquine resistance in RC strain was mainly a deficiency of specific receptor sites.

TABLE OF CONTENTS

CHAPTER I

CHAPTER 2

CHAPTER 3

CHAPTER 4

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

1. GENERAL INTRODUCTION

Malaria, one of the most important communicable diseases of man, is a principal health hazard in many countries. Presently, there are about 150 million cases of malaria annually. In Africa alone, more than one million children under the age of 14 die each year from malaria. It is a protozoan infection in which the causative agent, a member of the genus Plasmodium, undergoes a cyclical development both in the mammalian host and in the vector, the Anopheles mosquito. The genus Plasmodium is defined to include those parasites which reproduce sexually by sporogony in an anopheline mosquito, and asexually by schizogony in two cycles in the vertebrate host, one producing pigment in non-nucleated red cells and the other in parenchymal cells of the liver (World Health Organisation, 1963, Garnham, 1966).

There are more than 200 recognised species of malarial parasites, with a large number of birds, reptiles and mammals serving as hosts. Only four species of parasites are generally infectious to man, namely Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale and Plasmodium vivax.

The aetiology of malaria remained a mystery until the nineteenth century (Russell, 1955) Discovery of the malarial pigment by Lancisi in 1716, and subsequent appreciation of its association with the disease by Meckel in 1847 and Afanasiev in 1879, provided the foundation for Laveran's observation of the parasite in the blood (Garnham, 1966). Much of the proof of parasite growth and development within the insect is however due to Sir Ronald Ross, who first demonstrated

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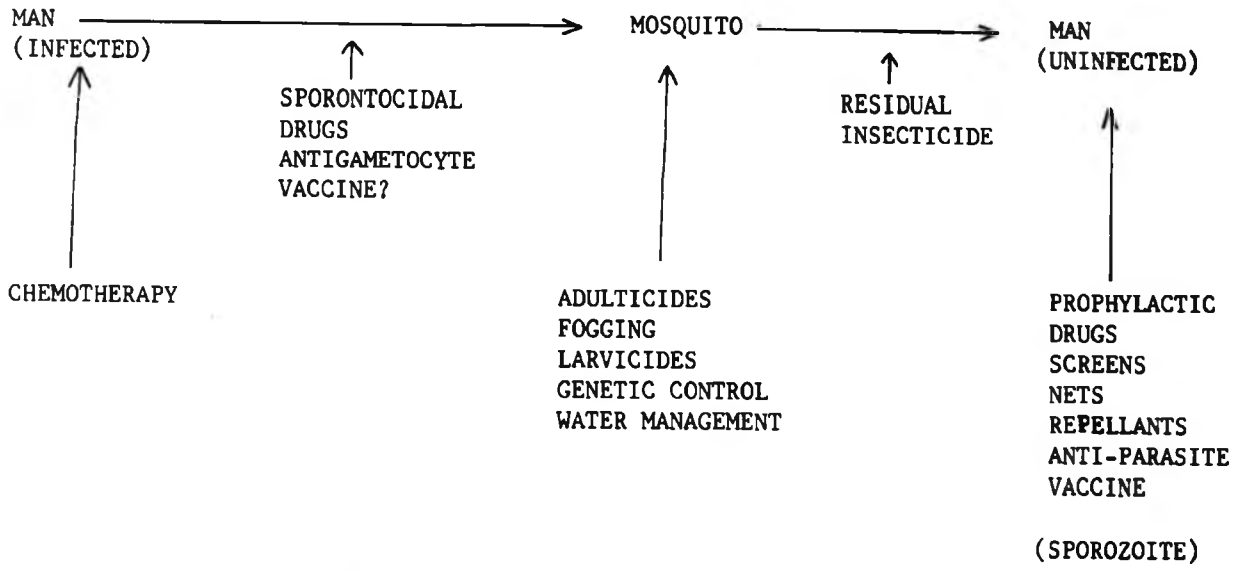
the presence of peculiar pigmented bodies in the gut wall of mosquitoes fed previously on malarious patients. Subsequently, Bignami and Grassi (1897-1900) demonstrated the whole life cycle in the mosquito (Garnham, 1966).

Following the important discoveries of the aetiologic agent and its mode of transmission, later researchers elucidated the life cycles of virtually all of the avian and many of the primate parasites. The knowledge gained from these studies led to the setting up of various programmes for both the rational treatment of malaria and the eradication of the disease.

Fig I. Illustrative points of attack in the Malaria transmission cycle



Adapted from HOWARD (1972).

1.2 REVIEW

1.2.1 HISTORICAL ACCOUNT OF CHEMOTHERAPY OF MALARIA

The complex development cycle of plasmodia indicated that control of malaria could be approached from the standpoint of curing the disease in man (or other mammalian host) or in the mosquito, or in eradicating the mosquito vector. Fig. I illustrates points of possible attack in the malaria transmission cycle.

Chemotherapy of malaria in man was initially aimed at treatment of florid infection but later prophylaxis against invasion of the body by plasmodia was also utilised. Treatment of malaria by clearing the bloodstream of plasmodia had long been known through administration of folk remedies such as quinine and febrifugine. However, the discovery of anti-malarial drugs resulted from the application to malaria parasites of the methods which had been used with trypanosomes (Schnitzer, 1963) Applying this method, the Bayer workers investigated methylene blue, went on to other ring systems until in 1924 they brought out the active compound Plasmoquine. It was however found to be toxic and inferior therapeutically to quinine so that by 1930, it had passed out of general use. Later, during the 1939-1945 war, it was reinvestigated in regard to its remarkable power of preventing relapses and became the starting point from which primaquine and similar compounds were derived.

In 1933, Mauss and Mietzch by attaching the active side chain of plasmoquine to another heterocyclic nucleus produced Atebrin (mepacrine, quinacrine) This antimalarial was neglected until 1942, when the Japanese captured Java and cut off supplies of quinine. With the loss of quinine, Atebrin proved an invaluable antimalarial in protecting troops; however, soon after

the war, it was supplanted by chloroquine which did not stain the skin yellow as mepacrine did.

Chloroquine which is now probably the most used drug for the suppression and treatment of human malaria also had a chequered history. This antimalarial drug and the 3-methyl derivative (sontoquine) were prepared about 1938 by the Bayer workers (Andersag et al; 1939). A small trial was made on a few general paralytic patients therapeutically inoculated with malaria, but the results suggested that its anti-malarial action was not sufficiently superior to that of Atebrin to encourage its commercial development. Later, in 1941, limited amounts of chloroquine and sontoquine were sent to Professor Sergent of the Institute Pasteur, Tunis, then under German domination. When the Anglo-American forces occupied Tunis in 1943, these drugs together with relevant information were handed over to the American Commander who then sent them to America for investigation. Chloroquine, after thorough laboratory and clinical trials was shown to be superior to mepacrine and has since then, become the mainstay of antimalarial chemotherapy.

Proguanil (Paludrine) was introduced by Curd et al in 1945 and pyrimethamine (Daraprim) by Hitchings et al in 1952. These compounds were selected by the new techniques of screening substances on Plasmodium gallinaceum in chicks and Plasmodium berghei in rodents. Since the introduction of these drugs, most other drugs used in antimalarial chemotherapy were generally not superior to those already discussed and also generally fell within the same classes. These classes will be discussed in detail later. Only recently did a new antimalarial drug which showed promise more than available drugs reach the stage of

clinical trials. This drug - mefloquine is being awaited with keen anticipation; and notes of caution arising from past experience. When pyrimethamine was introduced it was hailed as 'the greatest therapeutic triumph in malaria since the discovery of paludrine....' (Fairley, 1952) This analogy was unfortunate since pyrimethamine was to suffer the same fate as its predecessor. This fate - development of resistance to antimalarial drugs, continues to be a thorn in the flesh of malariologists and will continue to spur on more research to keep the malaria chemotherapy arsenal ahead of the development of resistance to "old" antimalarials.

1.2.2. VECTOR CONTROL AND MALARIA ERADICATION

Attempts at eradication of the mosquito vector followed the development of malaria chemotherapy. These attempts focused mainly on the drainage of the breeding grounds, use of insecticides and improvement in the ecological framework.

From 1936, the insecticide Pyrethrin was used in mass eradication of adult mosquitoes only to be displaced by a series of synthetic insecticides. Firstly by D.D.T. (dichlorodiphenyltrichloroethane) in 1939 and Gammexane in 1943. Since then, the use of these insecticides have presented problems, partly because of toxicity to humans and other animals and partly because the vector mosquitoes proved more adaptible than previously supposed.

It might be pertinent at this juncture to recall that painstaking research has led to the development of the fundamental understanding of the bases of the disease, establishment of the life cycles of the several plasmodia responsible for malaria. This has made

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possible definition of the problems involved in malaria, provision of protocol for tackling them; and not the least, raised the hope that some day, malaria will be a disease of the past. Such was this hope, that the World Health Organisation considered malaria eradication rather than control to be a realistic goal. Unfortunately, it did not fully reckon with the considerable range of biological adaptability which was to be so brazenly exhibited by both plasmodia-in developing resistance to anti-malarial drugs and by mosquitoes - in developing resistance to insecticides. The dulling of response of plasmodia to drugs became evident early in laboratory work with chlorguanide (Bishop and Birkett, 1947; Williamson and Lourie, 1947) and was soon followed by discouraging reports from widespread sections of the globe (Schnitzer, 1966). Indeed since 1961, there has been a rather uncomfortable realization that the battle for malaria might have taken a turn for the worse.

1.3. LIFE CYCLE OF MALARIA PARASITES AND DRUG INTERACTION

Malaria is caused by multiplication of haemosporidian protozoa of the family Plasmodiidae in the blood and other tissues of the host. More than 100 plasmodial species have been described, causing malaria in a wide range of vertebrates and exhibiting narrowly defined host specificity (Garnham 1966; Coatney, Collins, Warren and Contacos 1971). Infection is primarily initiated by the bite of a female anopheline mosquito which inoculates saliva containing sporozoites (a). The sporozoites are motile 11-12 µm long organisms and usually disappear from the circulation within 1 hour and initiate the tissue stage of primary exo-erythrocytic development (b-e). The exo-erythrocytic development occurs in liver parenchymal cells in

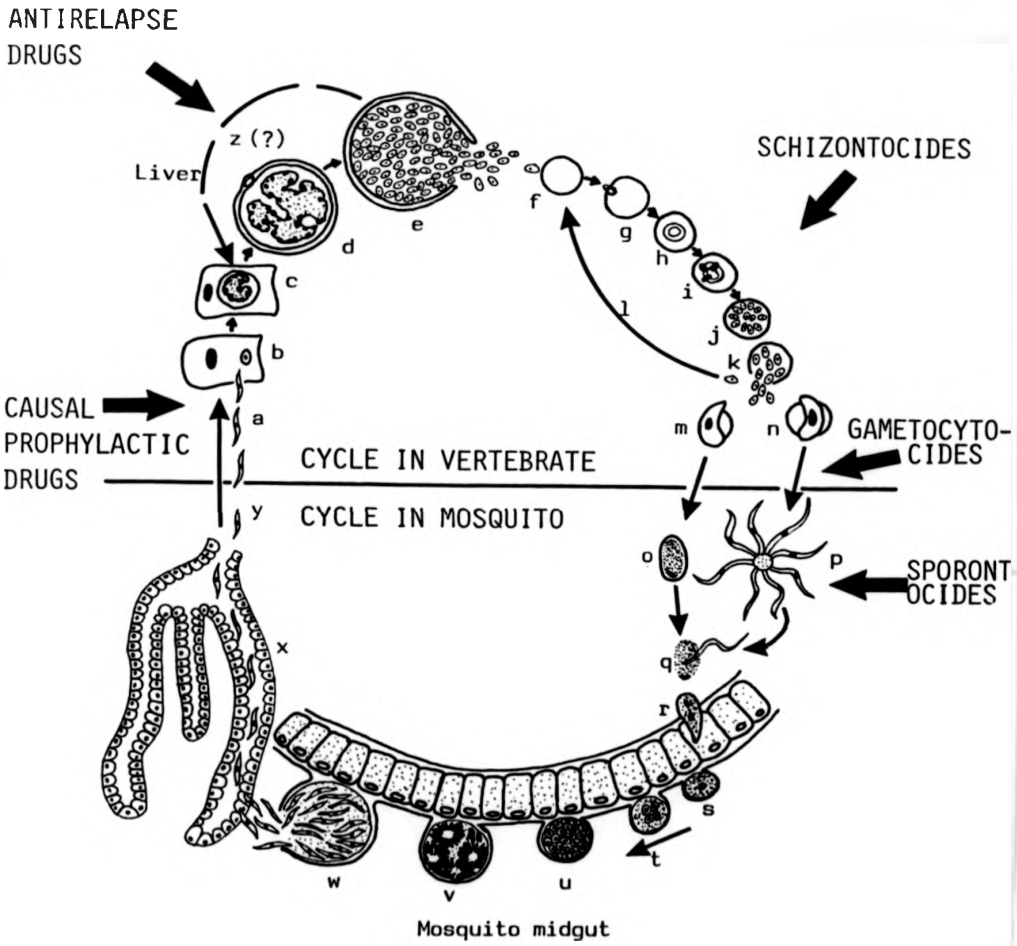

Fig. 2

Diagramatic representation of malaria parasite life cycle and drug interaction.

Adapted from Zaman, (1979) and Peters, (1970)

mammalian malarias but is considered to occur in mononuclear white cells close to the site of injection in avian malarias. Exoerythrocytic forms become multinucleate and within about 10 days they rupture the host cell to liberate between 100 to 30,000 exoerythrocytic merozoites (e), after repeated division by schizogony. The number of merozoites liberated depends on the species present and other factors. In avian malaria, these merozoites reinvade mesodermal tissues to initiate secondary exo-erythrocytic schizogony (z) the progeny of which invade either erythrocytes or further mesodermal tissues. In the majority of mammalian malarias on the other hand, primary exo-erythrocytic schizogony is followed exclusively by erythrocytic infection. Previously it was thought that Plasmodium vivax merozoites liberated from exoerythrocytic schizonts re-invaded fresh liver cells producing secondary exoerythrocytic stages and that entry of merozoites into the blood from secondary exoerythrocytic stages was the cause of relapses. It now appears that relapses are due to different rates of development of preerythrocytic schizonts resulting in 'early' and 'late' forms. The time of maturation of the primary exoerythrocytic stage apparently is determined by the genetic make up of the individual sporozoites which initiate their formation (Coatney, 1976, Krotoski et al 1980)

1.3.1 **DRUGS EFFECTIVE AGAINST EXO-ERYTHROCYTIC FORMS.**

Two major groups of antimalarial drugs are effective against the exo-erythrocytic stages. These are: (1) Causal Prophylactic drugs consisting of proguanil, pyrimethamine. (8 - aminoquinolines, e.g. primaquine

and pamaquine are also effective here but are not used because of toxicity)

(ii) Antirelapse drugs consisting of primaquine, quinocide and other 8 - aminoquinolines.

These drugs are useful in the radical cure of malaria. They are useful in prevention of mosquito infection by sterilising the gametocytes. However, pamaquine and primaquine are too toxic for prolonged use as causal prophylactics and are thus not used in practice for this purpose, but are generally reserved for use against persistent preerythrocytic forms of Plasmodium vivax and P.ovale.

Blood infection is initiated by merozoite invasion of red cells. This involves a sequence of random adherence to the erythrocyte, attachment of the apical prominence of the merozoite to a specific cell receptor and induction of endocytosis by the red cell (f-g) (Bannister et al 1975). Intracellular growth proceeds through stages of ring, trophozoite and schizont (h-j) and terminates with rupture of infected cells at 24-72h intervals, liberating 10-30 merozoites (k) which initiate a further cycle of development (1). The clinical signs and symptoms of malaria are associated in mammals exclusively with cyclical blood stage schizogony, but in birds exoerythrocytic schizogony causes significant additional pathology.

1.3.2. DRUGS EFFECTIVE AGAINST ERYTHROCYTIC FORMS

The main class of antimalarial drugs effective against erythrocytic forms is the schizontocides and can be classified into (a) Quick and potent

acting, consisting of quinine, mepacrine, chloroquine and amodiaquine. ("Blood schizonticides" - act only on blood stages) (b) Slower acting drugs consisting of proguanil, pyrimethamine, sulphonamides and sulphones. These also act on tissue forms. These drugs are effective against the growing intraerythrocytic stages of plasmodia. Another class of drugs, 8 - aminoquinolines such as primaquine and pamaquine are referred to as gametocytocides and are effective against blood gametocytes; causing destruction and sterilisation of gametocytes.

After a period of asexual multiplication of erythrocytic parasites, a proportion of newly invaded merozoites differentiate into male and female gametocytes (m & n), micro and macro gametocytes respectively. These mature without further cell division over a period of about 10 days. When ingested into the mosquito stomach, the microgametocyte under goes exflagellation (P) liberating 8 microgametes and fertilization occurs (q) The zygote (r) differentiates in the wall of the mosquito gut (s-v) and sporozoites finally accumulate in the insect's salivary glands (x) to complete the sexual cycle of development.

1.3.3. DRUGS EFFECTIVE AGAINST GAMETOCYTES AND OOCYTS IN THE MOSQUITO

These drugs are referred to as sporontocides and consist of proguanil, pyrimethamine, pamaquine and primaquine. They will prevent development of the sexual stages and the zygotes in the mosquito gut (Fig. 2 shows the interaction of groups of antimalarial drugs with the life cycle of malaria parasites. Letters in parenthesis in the text refer to letters in fig 2. Fig 3 is the summary

FIGURE 3. SCHEMATIC REPRESENTATION OF INTERACTION OF ANTIMALARIAL DRUGS WITH BLOOD, TISSUE & MOSQUITO FORMS OF MALARIA PARASITES.

DRUG	On Asexual forms in Blood. Blood Schizontocides		On tissue Schizont. Tissue Schizontocides			On Gametocytes			MAIN USES
			Preerythrocytic		Secondary Exoerythrocytic	In Blood gametocytocides		In Mosquito Sporontocides (1)	
	P.falciparum	Other Species	P. falciparum	Other Spp.		P. falciparum	Other Species		
Chloroquine Amodiaquine Mepacrine Quinine	+ +	+ +	-	-	-	-	+(2)	-(2)	Treatment of acute attack: radical cure of falciparum malaria. All except quinine are useful suppressives by action on blood schizonts
Proguanil Pyrimethamine	+	+	+	±	-	-	+	+	Causal prophylaxis infalciparum malaria. Suppression against other spp. Prevention of mosquito infection by sterilising gametocytes.
Primaquine Pamaquine	±	±	+(3)	+(3)	+	+	+	+	Radical cure of vivax and other relapsing malaria. Destruction and sterilisation of gametocytes.

(1) Sporontocides prevent development of the gametocyte after its ingestion by the mosquito.

(2) Although gametocytes of P.vivax and other species are removed from the blood rapidly, their development in the mosquito is not affected.

(3) Too toxic for prolonged use as causal prophylactics.

+ = active, - = not active.

of the drug interaction represented schematically). Insecticides interrupt the transmission cycle by killing the mosquito host.

1.4. PHYSIOLOGICAL ADAPTATION OF THE MALARIA PARASITE

In the mammalian host, malaria parasites generally elect to inhabit two types of cells.

(i) The exoerythrocytic schizonts inhabit the parenchymal cells of the liver.

(ii) The erythrocytic stages inhabit red blood cell or its precursors. Some species show a preference for younger cells e.g. P.vivax,others for older cells or cells of any age.

Previously, it was thought that P. falciparum preferred old cells but recent evidence suggests that P.falciparum also prefers younger cells in vivo and in vitro (Pasvol, et al 1980). Many strains of P.berghei preferentially invade immature cells,both polychromatophils and reticulocytes.

A third site of election unique to P.elongatum, a species of avian malaria parasites is the cells of the lymphoid-macrophage system with secondary exoerythrocytic schizogony occuring in erythrocyte stem cells of the haematopoietic tissues of bone marrow. This site of habitation is indeed a paradox of intraphagocytic parasitism (Peters, 1970), since the cells of the lymphoid-macrophage system are concerned with removal of parasites in defence of the host. Another analogous situation is the complete lack of cellular response to the exoerythrocytic parasites of mammalian malarias, up to the moment when they mature and erupt releasing their cryptozoites (merozoites) into the portal sinuses (Bray, 1957).

By virtue of its intracellular habitat, the malaria parasite has evolved a very complex interelation with its host cell. This interesting situation may possibly convey some degree of protection from extraneous toxic compounds (e.g. antimalarial drugs or antibodies) which must first penetrate the host cell membranes and contents before coming into contact with the parasite itself. On the contrary, the long and intimate interaction with the host has resulted, in some instances, in the parasite becoming more vulnerable because of its crucial dependence on the host for certain basic metabolites or possibly enzymes with which to utilize the metabolites. As a result of this dependence, the parasite has developed various metabolic deficiences. It has been suggested (Peters, 1970a) that the merozoite might have lost its rigid inner cell membrane inorder to gain better access to its host cell's contents, apart from being able to adopt amoeboid movements. Thus the parasite is able to take in portions of the host substance by phagotrophy as well as other more soluble substances by active membrane transport or free diffusion (Danforth, 1967, Jahn and Bovee, 1967).

One important feature of the erythrocytic stage of infection of malaria is the specialisation of the type of red blood cell infected by the various Plasmodium species. Plasmodium vinckei and P. chabaudi preferentially invade mature red cells whereas P.berghei and P.yoelii prefer reticulocytes. The human malaria, P.vivax infects predominately reticulocytes while P.malariae, P.ovale and P.falciparum appear to favour mature red cells. (but see Pasvol et al 1980).

The significance of the type of red cell infected lies in the difference in metabolic status of young and mature red cells since there are variations in levels of metabolites and various ions in both types of red blood cell, as shown in Fig. 4. However, in the erythrocyte, the importance of haemoglobin as a substrate cannot be overemphasised, but the position is less clear in the tissue phases. The parenchyma cells of the liver are rich in metabolites, and contain large quantities of glycogen, purines and B_{12} vitamins, as well as being a store for various substances transported by the blood and a factory for making nucleoproteins from amino acids and nucleotides.

As has been mentioned earlier, the intracellular habitat of the malaria parasite is a double-edged sword, protecting the parasite to some degree from toxic substances by obliging the toxic materials to first penetrate host red cell or liver cell membranes and contents before reaching the parasite itself, but rendering the parasite vulnerable because of its increasing dependence upon the host for certain basic nutrients or the enzymes with which to utilize them. Fig.5 shows some of the nutrients supplied to the parasite by the host cell and further underlines the crucial nature of the physiological dependence of the parasite on the host cell. It is pertinent at this point to mention the principal reactions which occur in plasmodial metabolism, all of which depend at one stage or other on the host cell.

1.4.1 UTILISATION OF GLUCOSE

Phosphorylation of glucose eventually provides energy required for the parasite's metabolism. The utilisation of glucose is predominantly via the glycolytic pathway

Fig 4 - Metabolic Characteristics of Red blood cells at 2 stages of development.

Metabolic changes	Reticulocyte*	Adult Cell
Glycolysis	+ + +	+ +
Oxygen Utilisation	+	+ +
Total - SH	+ + +	+ +
RNA present	+	0
Haem synthesis	+	0
Protein synthesis	+	0
Cytochrome and Electron transport system	+	0
Krebs Cycle	+	0
Electrolytes		
Potassium	+ + +	+ +
Sodium	+ + +	+ + (a)
Sodium	+	+ + (b)
Storage changes		
Breakdown of ATP	+	+ +

(a) Joyce, (1958)

(b) Bernstein, (1953)

*Symbols adapted from Harris J. W. (1963)

+ + +>+ +> +, 0 means virtually absent.

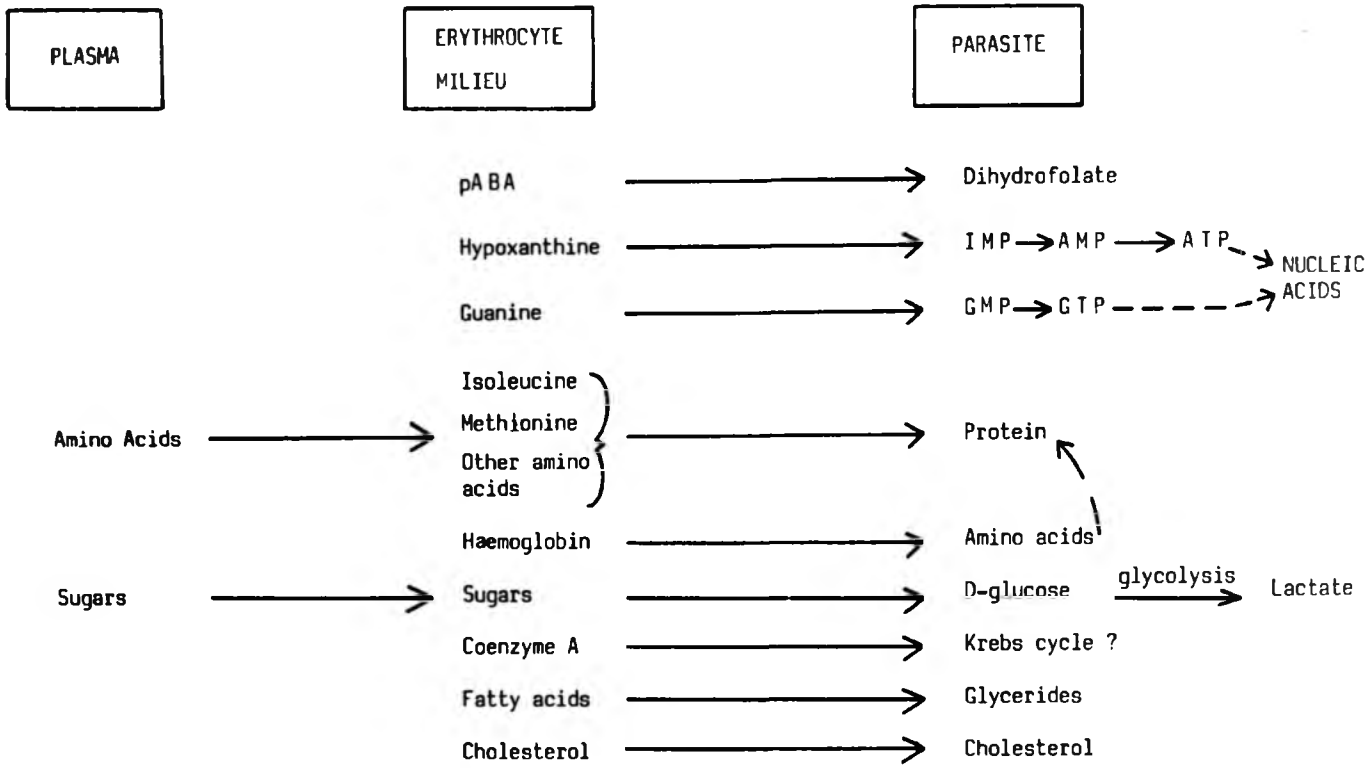

Fig. 5

Illustration of dependence of Parasite metabolism on supply of substrates from erythrocyte.

and very little glucose is utilised through the pentose phosphate pathway (Bowman et al 1961). Usually lactate is the end product of glycolysis in Plasmodium, and Fulton and Spooner (1956) have shown that the small amount of succinate found was probably produced by host cells. In general, all plasmodia break down glucose anaerobically through the classical Embden-Meyerhof pathway but the fate of the end product differs because of the lack of a Krebs cycle in many of them. Avian parasites can synthesise glutamate, aspartate and alanine through the Krebs cycle, but rodent parasites which lack the cycle (Howells 1970; Theakston, Fletcher and Maegraith, 1970) are more dependent upon CO_2- fixation for such synthesis. Free parasites of avian malaria can also form large amounts of acetate which is not further metabolised and it has been suggested that the basis for this shift in carbohydrate metabolism is probably due to the plasmodial requirement for preformed Coenzyme A from its host. (Sherman et al 1969).

1.4.2 UTILISATION OF HAEMOGLOBIN

Enzymatic break down of the globin portion of haemoglobin into amino acids and peptides, which are built up into parasite protein: It is apparent that malaria parasites hydrolyse globin, although an isolated plasmodial system capable of cleaving haemoglobin into haematin and globin is still to be demonstrated. In general, the amino acids produced in the highest concentration by parasitized erythrocytes are those found to be the most abundant in host haemoglobin but the parasite apparently produces many more amino acids than it can actually use (Cenedella, Rosen, Angel and Saxe, 1968) It is worth noting that other sources of amino acids obtainable by

malaria parasites include:

(a) Biosynthesis from other carbon sources; by fixing CO_2. The amino acids synthesised *via* this route include aspartic, glutamic acids and alanine (Sherman, 1977).

(b) Amino acid transport from host plasma across the surface of the host cell and the parasite.

1.4.3 SYNTHESIS OF LIPIDS

Growth of the malarial parasite, results in a marked rise in the lipid content of the infected erythrocyte, particularly in the phospholipid fraction. Notable increases occur particularly in phospholipids associated with the malaria parasite - phosphatidylinositol and polyglycerol phosphatides (Lawrence and Cenedella, 1969) *Plasmodium* is incapable of *denovo* biosynthesis of fatty acids and chol esterol and has to synthesize its glycerides and phosphoglycerides from host supplied fatty acids, nitrogenous bases, alcohols and coenzyme A. Cholesterol is similarly obtained from the host. Lipid metabolism of the parasite results in alterations in octadecenoic fatty acids and cholesterol composition of the erythrocyte membrane and leads to changes in permeability and increased fragility of the erythrocyte (Holz, 1977).

1.4.4. SYNTHESIS OF NUCLEIC ACIDS

Tetrahydrofolate - containing cofactors are required by both mammalian host cells and plasmodia for the synthesis of essential metabolites such as thymine. Folate metabolism in plasmodia differs from that of the host in starting from para aminobenzoic acid (PABA) and the parasite is unable to utilise exogenous preformed vitamins

folic and folinic acids, which man, for example can obtain from his diet.

It is now known, that malaria parasites make their own pyrimidines denovo, but the synthesis of purines is not performed denovo but they are obtained via metabolic salvage pathways. The salvage of preformed purines from the host can occur through the following pathways:

(1) Utilisation of adenine and adenosine to form adenosine monophosphate (AMP).
(2) Utilisation of inosine and hypoxanthine to form inosine monophosphate (IMP)
(3) Conversion of adenosine to adenine.

Fig 6 summarises the possible nucleotide pathways for the synthesis of nucleic acids in malarial parasites and emphasises that the salvage synthesis of purine nucleotides provides the precursor molecules for the biosynthesis of nucleic acids and the nucleotide coenzymes e.g. Guanosine 5' - triphosphate (GTP) which is the initial compound of the plasmodia - specific synthesis of dihydrofolate (Konigk, 1977)

The intraerythrocytic stages of plasmodia possess high rates of nucleic acid synthesis and protein synthesis and they require a vast supply of precursor molecules in amounts that will allow the successful growth and multiplication of the parasite within the cell. While the synthesis of dihydrofolate is an example of a parasite - specific sequence of reactions, the transformation of energy and the synthesis of nucleotides in the malaria parasite occur via metabolic routes very similar to those of their host cells. However, minor

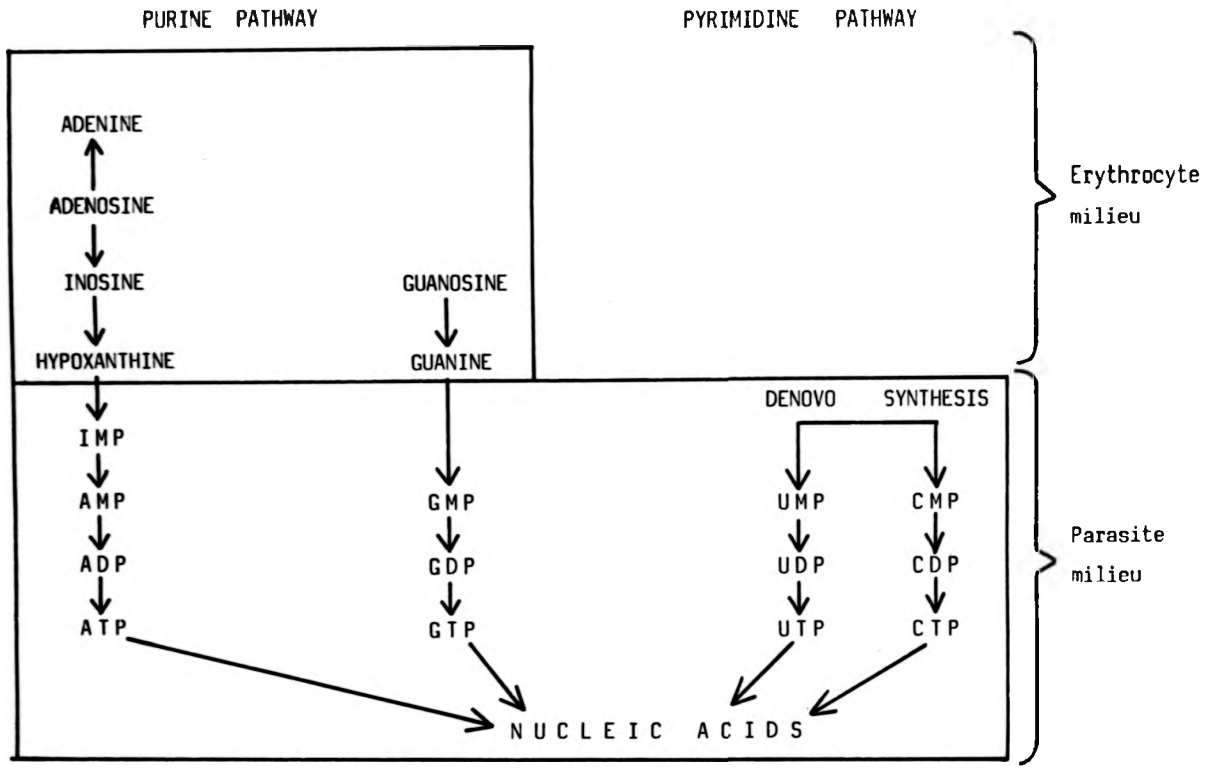

Fig. 6 Summary of possible nucleotide pathways for synthesis of nucleic acids in malaria parasites.

Adapted after Van Dyke, (1977)

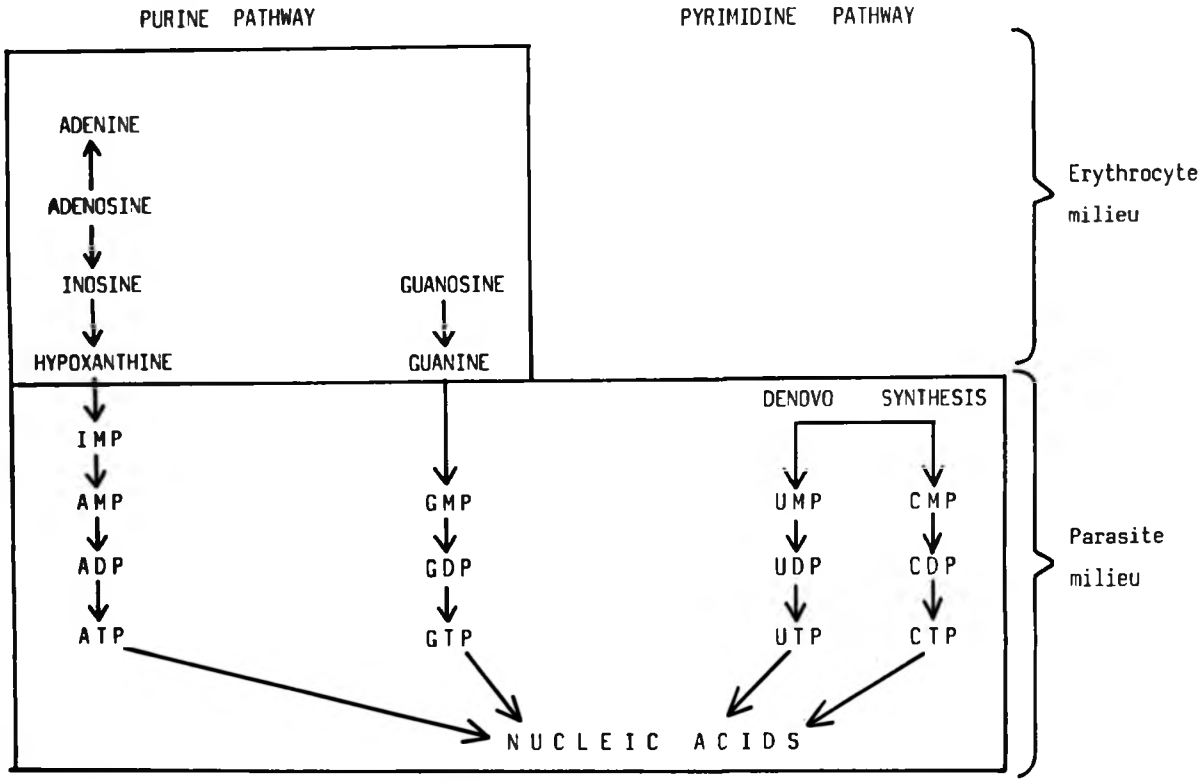

Fig. 6 Summary of possible nucleotide pathways for synthesis of nucleic acids in malaria parasites.

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differences may exist in the sequence and regulation of some reactions:- e.g. the synthesis of AMP from IMP has not been reported from human erythrocytes because of an apparent lack of adenylosuccinate synthetase and lyase, enzymes that have been observed in rabbit reticulocytes and in preparations of P.chabaudi (Konigk, 1977). Further differences between malaria parasites and host cells in the manner in which purines and pyrimidines are utilised have been reported (Van Dyke et al 1977).

1.5. CLASSES OF ANTIMALARIAL DRUGS AND THEIR MECHANISM OF ACTION.

Antimalarial drugs can be classified under three broad headings as follows:

(i) Blood schizontocides consisting of chloroquine, quinine, mepacrine, amodiaquine etc .

(ii) 8 - aminoquinolines, consisting mainly of primaquine and quinocide

(iii) Antimetabolites consisting of pyrimethamine, proguanil, sulphonamides and sulphones.

As will be seen later, the classes especially (i) and (iii) are not completely mutually exclusive, since for example, the antimetabolites proguanil, pyrimethamine and sulphonamides are also schizontocidal drugs though of limited action. (but they are not restricted to the blood stage).

1.5. BLOOD SCHIZONTOCIDES

The blood schizontocides chloroquine, quinine, and amodiaquine are quinoline derivatives; while mepacrine (also a blood schizontocide) is specifically an acridine derivative. These drugs are quick acting and are used therapeutically. They are effective on the growing intraerythrocytic stages of plasmodia,having a variable effect on developing gametocytes.

The selective toxicity of the blood schizontocides is based on the enormous concentrative ability of the intraerythrocytic malaria parasite for the drugs. This concentrative ability has been demonstrated for quinine and mepacrine (Bock, and Oesterlin see Warhurst 1980) and also more thoroughly for chloroquine (Macomber et al 1966, Polet and Barr 1969, Fitch 1969, 1970, 1973)

With a medium chloroquine concentration of 10 nmol/litre, infected erythrocytes can accumulate 600 times this concentration of radioloabelled drug. The uptake mechanism is saturable and has an apparent K_m of 10 nmol/litre (Fitch, 1969) The uptake of chloroquine into P.berghei is dependent on a supply of D-glucose and is inhibited by metabolic inhibitors (Fitch, et al 1974), similarly, uptake of chloroquine in P.falciparum is glucose dependent. However, uptake of amodiaquine was less glucose dependent, though glucose stimulated uptake of drug in the chloroquine resistant strain (Fitch et al 1974) The variation in the uptake of drug by resistant strains will be discussed later. It is Fitch's view that infected erythrocytes possess high affinity receptors for those drugs and that those receptors are absent in normal erythrocytes and reduced or absent in erythrocytes infected with drug resistant parasites. He further proposed that energy was required to make the receptors accessible to chloroquine and this accounts for the glucose requirement and explains the anomalous behaviour of amodiaquine which he proposes to be more accessible to receptors than chloroquine. An alternative explanation for the energy requirement has been suggested

(Warhurst and Thomas 1978). A two stage mechanism was suggested, the first stage of chloroquine uptake being binding to the high affinity site (non-energy dependent), the second stage was translocation of drug together with a proton into the parasite, under the influence of a proton gradient (energy dependent) .

The lysosome has also been postulated as a possible vehicle for the uptake of basic drugs in malaria

(Homewood et al 1972) The mechanism was initially proposed by Rollo, (1968) following partition profile studies indicating that the accumulation of basic antimalarials within the red cell was dependent upon differences between the pH of the plasma and the pH within the red cell. Rollo suggested that lactic acid production within the erythrocytic parasite led to a high hydrogen ion concentration enabling basic antimalarials to be concentrated. (This suggestion was based on the ionization characteristics of chloroquine. The ionization constants of chloroquine (pka_1 10.2, pka_2 8.1 - Irvin and Irvin 1947) indicate that at physiological pH, 18% of the drug is in the monoprotonated form which is soluble in lipid and hence capable of passing into the cell membrane. The drug would pass out readily from the membrane phase into a more acid cytoplasm with production of the doubly protonated form insoluble in lipid and incapable of passing back into the membrane. Chloroquine would thus cross the membrane into the cell provided that the pH inside the cell remained lower than that of the plasma).

Following the demonstration (Allison and Young 1964) that chloroquine concentrated in mammalian lysosomes, Homewood et al 1972, extended these observations to propose a physico-chemical basis for the accumulation of chloroquine within parasitised erythrocytes. These workers considered that a low pH within the parasite lysosomes would be effective in inducing accumulation of the drug within these organelles. The accumulation of chloroquine was followed by autophagic vacuole formation (Clumping) - enclosing the digestive vacuoles and their contents of haemozoin and haemoglobin. (Warhurst and Hockley, 1967)

The digestive vacuoles of malarial parasites (and other protozoa (Kudo 1966) are considered to be analogous to mammalian secondary lysosomes and to be at acid pH - an assumption supported by the localization of acid phosphatase within the pigment vesicle of P.berghei (Aikawa and Thompson 1971) and the presence of acid protease with a pH optimum of 4.0 in the parasite (Cook et al 1961). Warhurst (1980) has also observed the concentration of the basic dye neutral red from medium concentrations of 200 µmols/litre into digestive vacuoles, proving the acidic nature of these vacuoles. Concentration of the drug, it was argued would cause a rise in the pH of lysosome contents and this change might cause the clumping of the pigment vesicles. Evidence to support this concept was presented by the in vitro incubation of parasites in media made slightly alkaline with bicarbonate (HCO_3^-) It was observed that raising the extracellular pH of the parasites correspondingly increased the amount of clumping of the pigment. Also the protonation of chloroquine within the digestive vacuoles would deplete them of hydrogen ions and uptake of drug would then cease, unless more acid were secreted into the vacuoles or new acid containing vacuoles were formed, either case would require energy thus explaining the second energy dependent phase of chloroquine uptake described by Polet and Barr (1969). It is interesting to note that malaria infected rat erythrocytes have been reported to maintain a slightly higher average pH than the surrounding medium (Williams and Fanimo 1975), this observation would probably render Rollo's mechanism unlikely though not necessarily disproving the proposal of Homewood et al (1972). Indeed in mammalian cells the majority of evidence tends to support the intralysosomal

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concentration of chloroquine and similar drugs (De Duve et al 1974).

This mechanism for the intraparasitic concentration of chloroquine is also probably suggestive of one of the mechanisms of action of this group of antimalarial drugs. Depleting the food vacuole of acid would change its internal pH from that required for the most efficient action of digestive enzymes, digestion of haemoglobin would be considerably impaired, and the supply of amino acids to the parasite dramatically reduced. Because the mature mammalian erythrocyte is virtually impermeable to some amino acids (Winter and Christensen 1964) the normal rapid growth of the parasite would be halted. Hence chloroquine would act initially by raising the pH of malarial food vacuoles, thereby reducing the digestion of haemoglobin by the parasite and preventing its growth. Further support for this view arises from the observation that chloroquine in high concentrations inhibits certain mammalian proteolytic enzymes (Cowey and Whitehouse, 1966). However, Wibo and Poole, (1974) have observed that in mammalian cell free preparations, the proteolytic enzyme cathepsin B was 50% inhibited by 5 mmol/litre chloroquine, but cathepsins D, A and C, of which D would be most important in digestion of haemoglobin, were not affected. Despite these observations, the evidence is strong that lysosomal systems in malaria parasites are affected by blood schizontocides. The evidence includes chloroquine induced autophagic vacuole formation (pigment clumping) and the effect of prolonged quinine and mefloquine

treatment which causes cessation of haemozoin production and apparent dissolution of pigment (Peters, et al 1977)

Chloroquine at low concentrations (10^{-4}M) stabilizes erythrocyte membranes but at high concentration (10^{-3}M) labilizes them (Inglot and Wolma, 1968) Although the therapeutic concentration in plasma does not rise above 10^{-6}M (Berliner et al 1948) and the red cell membrane is therefore unaffected, it is quite possible that the concentration of chloroquine in malarial food vacuoles could rise sufficiently to labilize its membranes (Allison, 1968). Thus the formation of autophagic vacuoles could then be regarded as a defensive attempt by the cell to segregate the hydrolytic enzymes released into its cytoplasm. However, Warhurst and Williamson (1970), have shown that clumping appears before breakdown of RNA can be detected suggesting that lysis of cellular constituents follows rather than precedes autophagic vacuole formation. Moreover in the experiments of Homewood et al (1972), raising the pH to 8.0 did not cause haemolysis of the red cells although clumping occured.

Several groups of workers have demonstrated that chloroquine and quinine will, given several hours to become concentrated within the parasite; inhibit macromolecular synthesis such as that of DNA, RNA and protein. O'Brien and Hahn have offered a model to account for the antimalarial activity of chloroquine and its congeners (O'Brien and Hahn 1965) Some workers have also supported this model by showing that a high level of structure-activity correlation can be obtained with this model by comparing goodness of fit of regression lines obtained from calculated statistical quantities R^2,

F (overall) and explained variance (Bass et al 1971) However, there is no evidence that therapeutic levels of chloroquine affect macromolecular synthesis over the first hour (Homewood et al 1971, Warhurst, 1969), although this is the period during which the chloroquine induced autophagic vacuole is formed. The binding constant for chloroquine and DNA under physiological conditions of ionic strength is between 10^4- 10^5 l/mole and this is too low to explain concentrative uptake of the drug by the parasite. Moreover, the observation that mefloquine (WR 142, 490) which avidly binds to the high affinity uptake site (Fitch, 1972) will not interact with DNA in vitro (Davidson et al 1975, Peters, et al 1977) strongly negates the contention (Hahn, 1974) that DNA is the high affinity uptake site. Moreover chloroquine binds equally well to both mammalian and plasmodial DNA (Gutteridge et al 1972) Quinine and mepacrine like chloroquine intercalate into DNA (Hahn et al, 1966) and inhibit DNA and RNA synthesis. Mepacrine is a planar molecule with dimensions similar to those of the normal base pairs. It causes a local spreading of the distance between adjacent base pairs and a localised unwinding of the helix to accomodate this distortion. Given that high concentrations of these drugs are achieved in the parasite, their anti-DNA template activity must be highly important.

Chloroquine has also been reported to inhibit coenzymes (ubiquinones) Q8 and Q9 linked NADH -oxidase systems of P.lophurae at 0.1 mmol/litre (Skelton et al 1968) Ubiquinone production by P.knowlesi, P.cynomolgi, P.falciparum and P.loplaurae has been demonstrated (Skelton et al, 1970).

Evidence has also been presented for a CO_2 - fixing pathway in P.berghei (Siu, 1967) and it was demonstrated that chloroquine at a concentration of 0.2 mmol/l inhibited the action of parasite phosphoenolpyruvic carboxykinase by 50%. Other enzymes which have been shown to be inhibited by chloroquine (or other schizontocides) include deoxyribo-nuclease (Kurnick and Radcliff 1957), adenosine triphosphatase (Bagnall, 1957) cocarboxylase (Silverman, 1949) & diaphorase (Guggenheim and Shamir-Zernik 1953) The multiplicity of this enzyme inhibition probably indicates non-specific action which is difficult to correlate with the selective antimalarial effect of schizontocides. Hence, the relevance of the inhibition of these enzymes to the mode of action of the schizontocides in vivo is uncertain.

Chloroquine has been found to inhibit pinocytosis in cultured mouse macrophages. (Fedorko, et al, 1968) this observation could indicate effects on the feeding of plasmodia. Electron microscopic evidence (Rudzinska, 1960) showed that plasmodia feed on erythrocyte contents by pinocytosis, engulfing droplets of host cytoplasm into food vacuoles and thus producing extensive changes (shown up as Maurer's clefts in P.falciparum) The inhibition of pinocytosis coupled with the disruption of the digestive process, could lead to nutrient deprivation and parasite starvation.

1.5.2. 8 - AMINOQUINOLINES

The antimalarial action of 8 - aminoquinolines (primaquine, other 8 - aminoquinolines and napthoquinones) is predominantly on the exoerythrocytic schizonts with some effect on the gametocytes. Malaria parasites

posses mitochondria-like structures and are able to synthesise ubiquinone 8. The structure and function of these organelles are disrupted by the exposure of tissue stages of mammalian and avian plasmodia to 8 - aminoquinolines notably primaquine and to certain compounds of the napthoquinone series such as menoctone. The antimalarial effect of 8 - aminoquinolines is probably due to the quinoline 5, 6 diquinone active metabolites obtained by demethylation and oxidation (Tarlov et al 1962). The quinoline / quinone intermediates can act as intermediates in biological oxidation - reduction systems and are probably responsible for making erythrocytes susceptible to haemolysis especially in individuals with "G - 6 - PD" deficiency of the red blood cells. The quinoline / quinone intermediates, are active oxidants and may interfere with the NADH linked reductive processes in the tissue cells containing the schizonts, resulting in a lack of necessary substrates essential to the rapid development of the parasites.

It has recently been suggested (Gutteridge, et al 1979), that a link between ubiquinone and pyrimidine synthesis is the basis for the mode of action of primaquine and napthoquinones. The enzyme dihydroorotate dehydrogenase which is important in pyrimidine synthesis, is linked to the oxidation and reduction of ubiquinone. Hence the presence of active primaquine intermediates which are similar to the naturally occuring ubiquinone might be expected to interfere severely with synthesis of pyrimidines which is a crucial function in plasmodia. However, much work is needed to elucidate further the mode of action of 8 - aminoquinolines and naphthoquinones.

Unfortunately, primaquine is too toxic for use as a causal prophylactic (see fig 3) and its tendency to induce haemolysis in G6PD deficient individuals coupled with its low therapeutic index makes it an unsatisfactory drug.

1.5.3. THE ANTIMETABOLITES

An antimetabolite may be defined as a compound structurally related to a metabolite which prevents its further utilization by competing with it for an enzyme. The recognition that a compound, closely related to a metabolite, could prevent its participation in an essential biosynthetic pathway, arose from studies on the antibacterial effect of sulphanilamide and its reversal by the structurally related p-aminobenzoic acid (PABA). In malaria chemotherapy, there are two main groups of antimetabolites - antagonists of dihydrofolate (the metabolic product of proguanil (cycloguanil) and pyrimethamine) and competitors for PABA (sulphonamides and sulphones), both groups thus act at two main steps of the folate pathway in plasmodia (see fig 8)

Plasmodia like bacteria depend on p-aminobenzoic acid (pABA) together with pteridine and glutamic acid for the production of dihydrofolate. The enzyme dihydropteroate synthase (EC 2.5.1.15) catalyses the condensation of pABA with 6 - aminohydroxymethyl dihydroxy pteridine to form dihydropteroic acid. This enzyme is the site of action of sulphonamides which inhibit it competitively; by competing with pABA. Dihydropteroate synthase has

been found in P.chabaudi and P.berghei (FERONE, 1973) and has a K_m for pABA of 0.21 to 2.8 µmol/litre. Inhibition constants (K_i) for sulphadiazine, sulphathiazole and sulphanilamide are comparable to those obtained with the enzyme from bacterial sources (Brown, 1971) and satisfactorily confirm an enzymatic basis for the sulphonamide - pABA interrelationship observed in malaria in vivo and in vitro.

Dihydrofolate synthetase (EC 6.3.2.12) catalyses the reaction of dihydropteroate+L-glutamic acid + ATP to form dihydrofolate which is the next reaction in the sequence for the formation of dihydrofolate by analogy to bacteria. This enzyme has not been demonstrated in any malarial system. It has been speculated that P.berghei might utilise pABG (paraamino benzoyl glutamate) as an alternative substrate to form dihydrofolate directly (Ferone, 1977) .

Tetrahydrofolate dehydrogenase (EC 1.5.1.3) is the key enzyme in folate metabolism and converts dihydrofolate to tetrahydrofolate by reduction of the pteridine ring to the tetrahydro reduction state required for the reactions in which folate cofactors are utilised. These reactions include formation of methionine from homocysteine by donation of the S-methyl group, conversion of glycine to serine, generation of inosinate from purine precursors and synthesis of thymidylate from deoxyuridylate. Proguanil and pyrimethamine prevent the reduction of dihydrofolate to tetrahydrofolate by inhibiting tetrahydrofolate dehydrogenase. Proguanil is first metabolised to the active metabolite cycloguanil. The

affinity of these compounds (pyrimethamine and cycloguanil) for the plasmodial enzyme is much higher than for the mammalian enzyme and moreover these antagonists of dihydrofolate bind 10^4 - 10^5 times more strongly than dihydrofolate and so the inhibition is essentially an irreversible one (Bacq 1975).

The basis of the selective toxicity of sulphonamides to malaria parasites depends on the possession of a metabolic pathway to dihydrofolate distinct from the mammalian one, since mammals do not synthesise dihydrofolate via pABA but convert folic acid direct to dihydrofolate utilising folate reductase which is not present in plasmodia (Pratt, 1973) Mammals and plasmodia, convert dihydrofolate to tetrahydrofolate using tetrahydrofolate dehydrogenase. The basis of the selective toxicity of pyrimethamine and cycloguanil appears to be the much higher affinity of the plasmodial enzyme for these drugs (in the case of pyrimethamine up to 1000 times higher).

However, it is important to note that the fact that the antifolates and the sulphonamides are so effective in inhibiting plasmodial growth even in the liver which is rich in folate cofactors indicates that host folate cofactors are not available or cannot be used by the parasite. This phenomenon might be indicative of the inability of the parasites to take up preformed folates, as most micro organisms that synthesise folates de novo, are impermeable to exogenous folates (Hitchings and Burchall, 1965) probably due to a lack of a permease system (Albert, 1973). Fig 7 summarises the grouping of major antimalarial drugs and their probable mechanisms of action.

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FIGURE 7 - GROUPING OF MAJOR ANTIMALARIAL DRUGS AND ROBABLE MECHANISMS OF ACTION

Group	Chemical class.	Drug *	Synonym	Probable mechanisms of Action.
Schizontocides				
blood	4-amino quinoline	Chloroquine	Alvoclor Nivaquine Resochin Aralen etc.	After concentration Inhibition of digestive processes. Digestion of plasmodial cytoplasm due to cytolysosome formation. Binding to nucleic acid & inhibition of protein synthesis; other Biochemical effects including enzyme inhibition.
		Amodiaquine	Camoquin Flavoquine etc.	
	9-amino acridine	Mepacrine	Atebrin Atabrine Quinacrine etc.	After concentration Binding to DNA & RNA and inhibition of transcription, translation & protein synthesis.
	quinoline methanol	Quinine	Quininine	
tissue	8-amino quinolines	Primaquine	Neo-Quipenyl	Disruption of structure & function of malarial parasite mitochondrion-like organelles
Antimetabolites	Biguanide	Proguanil	Paludrine Chlorguanide etc	Inhibition of tetrahydrofolate dehydrogenase Competition with dihydrofolate.
	Diaminopy-rimidine	Pyrimethamine	Daraprim Malocide	Inhibition of tetrahydrofolate dehydrogenase Dihydrofolate antagonist.
	Sulphona-mides	Sulphadiazine etc.	Adiazine Co diazine Eustral etc	Antagonism of pABA Inhibition of dihydropteroate Synthetase
	Sulphones	Dapsone etc	Avlosulfone Damitone Udolac etc.	Antagonism of pABA Inhibition of dihydropteroate synthetase.

* International Nonproprietary Name

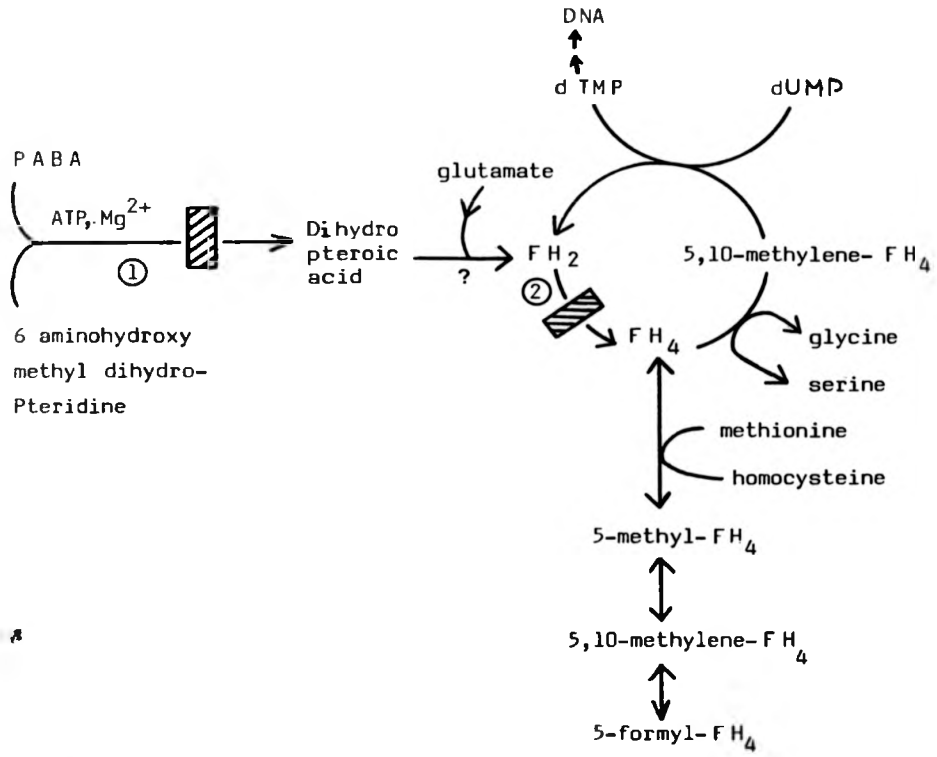

Fig. 8

Folate cofactor sythesis and utilisation in plasmodia.

Blocks show point of inhibition of sulphonamides and sulphones (1), pyrimethamine & cycloguanil (2).

Enzymes inhibited (1) Dihydropteroate synthetase

(2) Tetrahydrofolate dehydrogenase

(?) Dihydrofolate synthetase which catalyses this reaction has not been reported in malaria.

1.6. RESISTANCE TO ANTIMALARIAL DRUGS

Drug resistance in malaria has been defined as the 'ability of a parasite strain to survive and/or to multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the limits of tolerance of the patient' (W.H.O, 1965) This definition extends to all species of malarial parasite and all useful dosages of blood or tissue schizontocides, gametocytocides and sporontocides. However, drug resistance although occuring has never proved to be a serious obstacle to chemotherapy or prophylaxis of any of the relapsing malarias, i.e. in those infections due to P.vivax and P.ovale or of P.malariae. Hence, in practise the definition of resistance to antimalarial drugs is most commonly applied to the resistance of P.falciparum. Resistance to all antimalarial drugs can however be induced in experimental animals and in human volunteers under laboratory conditions.

Resistance of P.falciparum to antimalarials, ranges from a loss of effect demonstrable only by occasional recrudescence to a level of resistance at which the drug apparently has no effect on severe infections. The WHO Scientific Group on Chemotherapy of Malaria in 1967 proposed a grading system based on the response to normally recommended doses of chloroquine. The three grades of resistance RI, RII and RIII are as follows:

RI - Clearance of asexual parasitaemia as in sensitivity, followed by recrudescence.

Sensivitity is typified by clearance of asexual parasitaemia within 7 days of initiation of treatment, without subsequent recrudescence.

RII - Marked reduction of asexual parasitaemia, but no clearance.

RIII - No marked reduction of asexual parasitaemia.

The problem of drug resistant P.falciparum has become acute in the last decade with increasing reports of chloroquine and multi-resistant parasites. Usually resistance to chloroquine conveys resistance to mepacrine and quinine, as well as to other 4 - aminoquinolines in experimental animals and often in P.falciparum infections in man. There is little cross resistance between the antifolics and the 4 - aminoquinolines (Schnitzer, 1966; Peters, 1967), but resistance to pyrimethamine usually carries with it cross resistance to other tetrahydrofolate dehydrogenase inhibitors.

Chloroquine resistance in P.falciparum was first reported in Venezuela in 1960 by Maberti; but it was not until 1961 that the potentially catastrophic development attracted attention (Moore and Lanier, 1961) Since then, chloroquine resistance has been reported, again in Colombia (Comer, et al 1968), Bolivia and Paraguay (daSilva 1966, Rieckmann and Lopez-Antunano 1971) in Guyana (Giglioli et al 1967,) and in other areas of South America. Chloroquine resistance was suspected in Thailand in 1957 but was only confirmed in 1962 (Harinasuta et al 1962). A more recent study using Rieckmann's in vitro test demonstrates wide spread chloroquine resistance in central Thailand (Colwell et al 1972) and now probably extends to Laos, Sumatra and the Philippines. Until recently,

in sub-Sahara Africa, reports of P.falciparum resistant to chloroquine have remained open to challange, on the basis of W.H.O. criteria, of adequacy of chloroquine therapy or supervision of patient follow up (W.H.O. 1973) Bruce - Chwatt (1970) was unable to find convincing evidence of resistance to chloroquine in Africa upto 1970, since then additional reports (Dennis et al 1974, Omer, 1978, Fogh et al 1979, Moody 1979 and Eke 1979) have confirmed the existence of chloroquine resistant falciparum malaria in Africa.

Proguanil and pyrimethamine - resistant strains were readily obtained in human volunteers with cross-resistance between the drugs as early as 1949 (Adams and Seaton 1949; Burgess and Young 1959) but it was felt that the sporontocidal action of the drugs would limit the possibility of resistance spreading under natural conditions. Unfortunately this assumption has not been borne out by subsequent events.

Foci of proguanil resistance in Asia have been reported from India, Taiwan, West Pakistan, Malaya, Thailand, Cambodia, Vietnam and Java. Proguanil resistance also occurs in Africa with the foci in Senegal (Payet et al. 1966), Rhodesia (Lunn et al, 1964) Tanzania (Clyde 1967). It would appear that Proguanil resistance in P.falciparum often arises as a result of the parasite becoming insensitive to pyrimethamine though there is also considerable evidence that resistance also arises because of the use of the drug per se (WHO, 1965, Peters, 1970a) Pyrimethamine resistance is now widespread in much of East and West Africa, in Malaya, South America and the Far East. However, part of the resistance to anti-

metabolite drugs arises as part of a broader spectrum of drug resistance.

Sulphonamides and sulphones have been little used until recently for the prevention and treatment of malaria and even now, they tend to be used in combination with other drugs. Sulphonamides and sulphone drugs induce cross resistance to pyrimethamine in P. falciparum (Martin and Arnold 1968a, 1968b). Resistance to dapsone (a sulphone) develops rapidly, particularly in strains already resistant to pyrimethamine and this conveys cross resistance to sulphonamides (Verdrager et al 1969). Also, the possibility of induced resistance to sulphonamides and sulphones in various other pathogens exists, (Powell and Tigerrt 1968) this has occurred in Chad, where an epidemic has occurred of cerebrospinal meningitis caused by strains of Neisseria meningitidis which might have become resistant to sulphonamides as a result of malaria prophylaxis. (Sirol et al 1969).

Though resistance to antimalarial drugs by P. falciparum parasites, remains the most important type of resistance to antimalarials, it must be pointed out that, other strains of plasmodia may show relative insusceptibility. P.vivax is known to respond less quickly than P. falciparum.

to equivalent doses of quinine, while sulphonamides and sulphones have little if any action on P. vivax (WHO, 1973). Strains of P. vivax resistant to proguanil have been noted in West Malaysia while P. malariae resistant to proguanil has been reported in Indonesia and Taiwan. Insusceptibility to pyrimethamine has been shown by P. vivax in Kenya, Pakistan, and Venezuela (W.H.O, 1973) .

1.6.1 MECHANISM OF RESISTANCE TO CHLOROQUINE (AND RELATED DRUGS)

One of the early attempts to rationalise chloroquine resistance was that resistant plasmodia might have an abnormally high rate of production of ferrihaemic acid* (haemin) an intermediate in the synthesis of the malaria pigment (haemozoin). Chloroquine and quinine form complexes with haemin in vitro (Cohen et al, 1964, Schueler and Cantrell, 1964, Phifer et al 1966). This complex is less effective than the drugs per se when injected into mice infected with P. berghei. It was therefore suggested that the chloroquine - resistant plasmodia would more extensively complex with and inactivate the antimalarial by virtue of their excessive accumulation of haemin Though it has been suggested that strains of P. berghei resistant to chloroquine form less pigment probably because of excessive liberation of the soluble intermediate porphyrin - haemin (Peters, 1964, Peters et al 1965) resistant strains of P. falciparum contain normal levels of haemozoin. (McNamara et al 1967, Kellett et al 1968 and it is now clear that haemozoin formation ....

*ferriprotoporphyrin IX

in P.berghei is also related to the age of the red cell. Thus reduced pigment levels in chloroquine resistant P.berghei are associated with its presence in immature red cells and not necessarily to drug resistance as such (Howells et al; 1968a, b) Moreover it has also been demonstrated that one of the most virulent strains of P.berghei (NS line) which shows an innate resistance to chloroquine produces abundant haemozoin in mature erythrocytes. Hence reduced pigment formation does not appear to be causally associated with chloroquine resistance (Ladda and Sprinz, 1969). Similarly, these points appear to argue against the contention that interference with the formation of pigment from haemoglobin during digestion in the parasite was the basis of resistance (Peters et al, 1965) especially, since certain very virulent strains of P.berghei produce large amounts of haemozoin and presumably the intermediate haemin but are still resistant to chloroquine (Peters, 1970b)

It has also been postulated that a general impairment of the parasitised erythrocyte's concentrating mechanism for chloroquine could lead to the development of resistance (Macomber et al 1966) and that this impairment accounts for the cross resistance to mepacrine and quinine which have been shown like chloroquine to be concentrated in lysosomes (Allison and Young, 1964). Indirect evidence for a role of parasite lysosomes in the antimalarial action of chloroquine and related drugs was provided by experiments that demonstrated synergism between cortisone (Cantrell and Kendrick, 1963) or promethazine (Warhurst, 1965a) and chloroquine.

Both cortisone and promethazine like chloroquine have been shown to stabilise lysosome membranes (Weissman, 1965). (However, although several attempts have been made to demonstrate lysosomes in malaria parasites by classical methods other than cytochemistry, (Aikawa and Thompson 1971) no success has yet resulted) Rollo (1968) has also suggested that some unspecified change in the general metabolism of the resistant parasite might raise their overall pH and that this could lead to a reduced accumulation of chloroquine. This suggestion has been further elaborated by Homewood.

Chloroquine resistant erythrocytic parasites do concentrate less chloroquine than sensitive ones (Macomber et al. 1966, Fitch, 1969, 1970) and some apparently degrade less haemoglobin. These observations have been thought of (Homewood et al 1972) as being due to a reduction of the acidity of food vacuoles of resistant parasites. Lack of acid would reduce the uptake of chloroquine and also prevent effective haemoglobin digestion by enzymes requiring acid pH for maximum activity. The inability of the resistant parasite to digest sufficient haemoglobin would necessitate an alternative source of amino acids possibly by transamination of citric acid cycle intermediates (Howells, et al 1970) Howells had earlier proposed that a switch to the breakdown of glucose via the citric acid cycle in the resistant parasite would confer resistance by enabling the parasites to synthesise amino acids by the transamination of citric cycle intermediates. The presence of a demonstrable succinic dehydrogenase activity in the parasite using the blue

tetrazolium technique at the light microscope level presented preliminary evidence to support this theory. Unfortunately, there has been no further evidence to support the presence of a citric acid cycle in chloroquine resistant P. berghei, and there have been doubts raised regarding the specificity of localisation of tetrazolium salts following staining (Pearse and Hess, 1961) and attempts made at both electrophoretic separation and the fine structural localisation of succinic dehydrogenase were unsuccessful (Howells et al, 1972). It is important to mention that avian malarias utilize the citric acid cycle, synthesise amino acids by transamination, produce haemozoin and are sensitive to chloroquine!

Evidence from uptake studies in erythrocytes suggest that chloroquine and quinine are selectively toxic to plasmodia because of their preferential accumulation in parasitised erythrocytes (Macomber et al, 1966; Polet & Barr, 1968) Thus mouse erythrocytes infected with chloroquine sensitive P.berghei showed over a 600: 1 (cells : medium) concentration gradient when exposed to 10^{-8} moles/l chloroquine at 22^{o}C and physiological pH; while similar cells infected with chloroquine resistant strains showed only 100 : 1 gradients, controls containing no parasites had gradients of only 14 ; 1 (Fitch, 1969). The erythrocytes containing chloroquine sensitive P.berghei had high-affinity receptors for the drug which were absent in normal erythrocytes and reduced in number or absent in erythrocytes infected with chloroquine resistant parasites. In subsequent work, Fitch showed that a similar drug concentrating mechanism with a K_m of 67 nmol/l was present in owl monkey erythrocytes infected with a relatively chloroquine sensitive P.falciparum

(Camp) and deficient in a chloroquine - resistant (Monterey) strain (Fitch, 1970) Uptake of chloroquine into P. berghei depends on a supply of glucose and is prevented by metabolic inhibitors (Fitch, et al; 1974a), similarly in P. falciparum uptake of chloroquine is glucose dependent but in the chloroquine resistant strain glucose does not stimulate uptake. From these and other experiments - (Fitch, et al; 1974b), Fitch has concluded that chloroquine resistance is specifically associated with a reduction in the number or efficiency of the high affinity binding sites. The high affinity binding site has been suggested to be identical to haemin (ferriprotoporphyrin IX; Chou, et al, 1980)

The very diversity of actions of chloroquine and related drugs upon plasmodial metabolism makes it likely that the parasite must have to adopt more than one method to protect itself from chloroquine attack. Episomal transfer of resistance (which has so far been excluded in plasmodia - Peters, 1980) usually expresses itself in a reduction in permeability of the organism to drugs (Watanabe, 1963) and deficiencies in the binding of chloroquine, be it to iron - porphyrin or to other substrates may indeed be only one of the possible changes to be implicated in the development of chloroquine resistance.

1.6.2. MECHANISM OF RESISTANCE TO PRIMAQUINE

Primaquine resistance in P.berghei may involve the ability of the parasite to increase its respiratory capacity by biogenesis of mitochondria, in the same way that the erythrocytic forms of sensitive malaria parasites resist the oxidative actions of primaquine as compared with the tissue forms (Howells et al, 1968b, 1970) It has been speculated that resistance to chloroquine may also express itself in a similar way, because erythrocytes infected with chloroquine resistant P.berghei take up twice as much oxygen as do normal parasites (Paleck et al 1967, Cho and Aviado, 1968) While this may simply reflect on increase in the oxygen carrying capacity of the host haemoglobin, it is possible that it is associated with the marked increases in mitochondrial cytochrome oxidase activity observed in chloroquine-and quinacrine-resistant strains of parasite (Howells et al, 1969) Though the poor activity of 8 - aminoquinolines like primaquine if used alone against erythrocytic forms of plasmodia is a clear incentive to emergence of resistant strains, they are unlikely to appear in the field because the drugs are usually administered together with a quick acting schizontocide and also because of their pronounced gametocytocidal effects which prohibit transmission to the mosquito.

1.6.3 MECHANISM OF RESISTANCE TO ANTIMETABOLITES

Metabolic adaptation plays an important role in the development of drug resistance in protozoa just as it does in bacteria. The enzymes involved in this adaptation may be quite new or simply derepressed. Pyrimethamine, cycloguanil and other inhibitors of tetrahydrofolate dehydrogenase interact with the parasite enzyme *in vitro* in a highly selective, stoichiometric manner. This specific binding is the basis of their antimalarial action (Ferone, *et al* 1969) Rodent parasites such as *P. berghei* (Diggens, *et al* 1970; Ferone, 1970) and *P.vinckei* (Ferone, *et al* 1970) and *P. knowlesi* (Gutteridge and Trigg, 1971) produce a mutant tetrahydrofolate dehydrogenase that is different from the normal parasite enzyme. The mutant enzyme has a larger specific activity than the normal enzyme (17.4 nmole/min/mg protein as compared to 4.8 for normal *P.vinckei* tetrahydrofolate dehydrogenase (Pinder, 1973)) This increase in specific activity is due to the increased number of catalytic sites on the protein chain effectively increasing the enzyme levels of the parasite. Also, there is a corresponding decrease in the affinity of the mutant enzyme for both dihydrofolate and the drugs. The two factors combine to increase the parasite's ability to synthesise tetrahydrofolate even in the presence of previously inhibiting concentrations of pyrimethamine and cycloguanil. The decrease in affinity for dihydrofolate accounts for the observation that a pyrimethamine resistant strain of *P.berghei* had an increased requirement for pABA (Jacobs, 1964; Vary, 1970) Resistance to pyrimethamine does not confer a similar resistance to sulphonamides and sulphones and there may be a certain degree of hypersensitivity to sulpha drugs

due to the reduction of affinity of the enzyme for dihydrofolate.

Sulphonamide resistance has been speculated to arise from the ability of the parasite to commence folate synthesis at a stage beyond pABA or utilisation of pABG (paraaminobenzoylglutamic acid) in place of pABA.

It is clear that there are various possible mechanisms for the emergence of drug resistant strains in plasmodia. These include the selection of resistant mutant strains, superinfection by environmental strains of a resistant nature, the transfer of genetic information from resistant to sensitive strains and the ability to induce adaptive enzymes. A unique mechanism for the transfer of pyrimethamine resistance in rodent malaria has been proposed by Yoeli et al (1969). The mechanism was termed "Synpholia" and involved 'nonsexual' transfer of pyrimethamine resistance from pyrimethamine resistant P.vinckei to P.berghei while the two parasites lived together in the same host. Peters, (1970a) has critised the experimental procedure in the original experiment and has been unable to repeat the original experiments in his laboratory. However, Ferone et al (1970) have shown that the dihydrofolate reductase of pyrimethamine resistant P. berghei produced by "synpholia" in the experiment reported by Yoeli et al, (1969) had characteristics intermediate between the dihydrofolate reductase of sensitive P. berghei and of the pyrimethamine resistant P. vinckei.

1.7. MECHANISMS OF UPTAKE OF DRUGS ACROSS MEMBRANES

There are fundamentally two broad mechanisms whereby drug pass across biological membranes. These are

(1) Passive transfer consisting of
   (a) Simple diffusion
   (b) Filtration (hydrodynamic flow)

(2) Specialised transport consisting of
   (a) Carrier mediated transport with components of
      - Active transport
      - Facilitated diffusion
      - Exchange diffusion
   (b) Facilitated diffusion not associated with membrane carriers.
   (c) Pinocytosis

In simple diffusion, the rate of transfer of drug is directly proportional to the concentration gradient across the membrane. The speed of penetration of drug is also determined by the lipid to water partition coefficient. The cardiac glycosides afford clinically interesting examples of the effect of partition coefficients on permeability. Digitoxin, the most readily accumulated glycoside in clinical use, is the most lipophilic.

Partly ionised drugs may distribute unequally because of a Donnan type of ionic distribution or because of differences in hydrogen ion concentration on the two sides of the membrane. Donnan distribution occurs whenever the solution on one side contains some ions that cannot cross to the other side. A difference in pH

on the two sides of membrane affects the distribution of a partly ionised drug because of preferential permeability of membranes to the lipidsoluble unionised forms of compounds. Accordingly, the concentration of the solute (ionised plus unionised) on each side of the membrane is a function of the pH of the two fluids and the dissociation constant of the solute. According to the Henderson - Hasselbach equation;

$$pH = pk_a + \log\frac{[base]}{[acid]} \quad \ldots\ldots\ (1)$$

or $$\log\frac{[base]}{[acid]} = pH - pK_a \quad \ldots\ldots\ (2)$$

or by approximation

$$\log\frac{Cm}{C_1} = pK_a - pH \text{ for acids} \ldots\ (3)$$

$$\log\frac{C_1}{Cm} = pK_a - pH \text{ for bases} \ldots\ (4)$$

where Cm = total drug concentration

C_1 = concentration of drug in ionized state

pKa = negative logarithm of the acidic dissociation constant of the acid or base.

Similarly, for a weak acid

$$\frac{C_1}{C_2} = \frac{1 + 10^{(pH_1 - pK_a)}}{1 + 10^{(pH_2 - pK_a)}} \quad \ldots\ldots\ (5)$$

and for a weak base

$$\frac{C_1}{C_2} = \frac{1 + 10^{(pK_a - pH_1)}}{1 + 10^{(pK_a - pH_2)}} \quad \ldots\ldots\ (6)$$

on the two sides of membrane affects the distribution of a partly ionised drug because of preferential permeability of membranes to the lipidsoluble unionised forms of compounds. Accordingly, the concentration of the solute (ionised plus unionised) on each side of the membrane is a function of the pH of the two fluids and the dissociation constant of the solute. According to the Henderson - Hasselbach equation;

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where Cm = total drug concentration

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and for a weak base

$$\frac{C_1}{C_2} = \frac{1 + 10^{(pK_a - pH_1)}}{1 + 10^{(pK_a - pH_2)}} \quad \ldots\ldots\ (6)$$

where C_1 and C_2 are the concentrations of drug in the two fluids. (Schanker, 1962)

Equations (2) - (6) emphasize once again the importance of pH in the distribution of the concentration of certain drugs in different fluid compartments of an organism.

Filtration occurs as a result of hydrostatic or osmotic pressure difference across a membrane, the flow of the water and its solutes is passive in nature. For example, water that filters across the glomerular membrane is accompanied by all of the solutes of plasma except the large protein molecules.

Carrier transport offers a tentative explanation of the peculiar permeability of cell membranes to certain lipid insoluble solutes. Active transport has the following characteristics:

(a) Drugs move across the membrane against a concentration gradient or against an electrochemical gradient if an ion.

(b) The transport mechanism becomes saturated when the concentration of drug is raised high enough.

(c) The process shows specificity for a particular type of chemical structure.

(d) If two substances are transported by the same mechanism, one will competitively inhibit the transport of the other.

(e) The transport mechanism is inhibited non-competitively by substances which interfere with cell metabolism.

Facilitated diffusion describes carrier transport in which the substrate does not move against a concentration gradient. For example D-glucose readily penetrates the human red cell attaining the same concentration inside the cell as that on the outside by a process that shows specificity, saturability and sensitivity to certain metabolic inhibitors (Lefevre, 1961)

Facilitated diffusion not associated with membrane carriers has been proposed to explain the accelerated diffusion of certain lipid-insoluble substances across membranes. Stein and Danielli proposed that penetration might occur through hydrogen bonding structures (polar pores) extending through the thickness of the lipid membrane. Polar substances would penetrate rapidly if their stereochemical characteristics corresponded closely to those of the hydrogen-bonding pore. In addition there would be competition for transfer through the pores and the pores would be saturable (Stein and Danielli, 1956).

During exchange diffusion, a carrier is thought to transport the substrate from one surface of the membrane to the opposite surface where it releases the substrate, picks up another molecule of substrate and transports it to the original surface. Exchange diffusion may occur in any carrier - mediated transport system that is near saturation. (Wilbrant and Rosenberg, 1961).

Cells growing in tissue culture take up small droplets of the external medium by an engulfing or sucking in process known as pinocytosis. There is electronmicroscopic evidence suggesting that pinocytosis occurs in certain mammalian cells (Holter, 1959). Very little is known about the physiological significance of pinocytosis, but the process is too slow to account for the rapid cellular uptake of natural substances and some drugs, though it could account for uptake of small amounts of proteins and other macromolecules. (Christensen et al, 1958).

It must be pointed out that the mechanisms whereby a drug secures passage across membranes may not necessarily belong to one class only. There might be cases of interaction of two or more mechanisms further complicating the picture. Moreover, the way in which energy is coupled to drug transport is poorly understood, progress being hindered both by the present lack of information about its molecular basis and by the complexity of plausible kinetic models of energy coupling in transport.

1.8. RODENT MALARIA AS A MODEL FOR STUDY OF HUMAN MALARIA.

The most important single factor for the massive utilisation of P.berghei in malaria research since 1964 was the development of resistance to chloroquine by human malaria parasites. Experimental chloroquine resistance in P.berghei was produced by Ramakrishnan et al, (1957), Hawking and Gammage (1962), Peters, (1964). Since then, there has been a massive search for antimalarials active against chloroquine resistant strains using the P.berghei - mouse model. There is no doubt that it would not have been possible to launch the search for antimalarials without this model being available (Peters, 1980) and in almost all instances, a drug effective in the human malaria has been proved also effective in rodent malaria. After the discovery of Plasmodium berghei by Vincke and Lips in 1948 in the blood of the tree rat (Grammomys surdaster) in the Belgian Congo, and its successful passage into laboratory mice, resistance to virtually all antimalarial drugs has been induced and laboratory investigations have allowed examination of the factors governing emergence of resistance and of the fundamental biochemical mechanisms underlying the problem.

Recently it has been pointed out that the moderately chloroquine - resistant NS line of P.berghei in secondary screening of antimalarials was a good model for chloroquine resistant P.falciparum, while the highly resistant R C strain of P.berghei is valuable for its predictive value as to whether or not a parasite that is already chloroquine - resistant can become resistant

to another drug (Peters, 1980) For example mefloquine the new antimalarial drug presently undergoing clinical trials is effective against P.berghei NS and multiple - resistant P.falciparum, but has less activity against P.berghei RC, thus proving that it could be relatively easy to develop strains resistant to mefloquine from P.berghei NS and also forecasting the probability of further development of resistance of P.falciparum to this drug.

It is significant that a rough estimation of potency index based on activity of antimalarial drugs against P.berghei is valid for human forms of malaria (Aviado, 1969) except for a few anomalies like menoctone which was a complete failure in man probably because of inadequate absorption from the human intestinal tract; and also RC12 which could not " fulfil" in man its promise as a tissue schizontocide (Peters, 1980) Evidence from ^{14}C - chloroquine uptake studies shows that a similar mechanism of uptake, and resistance to chloroquine might be operating in P.berghei and P.falciparum (Fitch 1969, 1970) Both models have shown similar kinetic parameters and substrate utilisation (Fitch, 1970, Fitch et al, 1974a, b) Moreover, the tetrahydrofolate dehydrogenase inhibitors (pyrimethamine, cycloguanil) that have proved to be causally prophylactic in mouse are also active in man. Indeed, the mechanism of resistance to these antimetabolites was largely worked out in rodent malaria (Ferone, 1970).

1.9 AIMS OF THE RESEARCH PROJECT

Plasmodium falciparum malaria resistant to chloroquine and in some cases to quinine is now prevalent in South America, and South East Asia and constitutes a renewed major threat to world health. In Africa, though the situation is still confusing, there is no doubt that the continent might be the next citadel to fall! In approaching the subject of development of drug resistance by plasmodia, a fundamental area of investigation must lie in assessing the biochemical mechanisms which give rise to the phenomenon. Though many advances have been made in the study of morphological changes (Peters 1964, 1965a,b,c; Jacobs and Warren; 1967) and pathological changes (Sadavongvivad and Aviado, 1969) much is yet to be done from the biochemical standpoint if the basis of resistance is to be understood and overtaken.

The present work was undertaken in order to obtain a better understanding of the biochemical mechanisms governing the accumulation of chloroquine. In order to attack chloroquine resistant malaria, it is important to understand the basis of selective toxicity, mechanism of resistance and mode of action of drugs such as mefloquine which are capable of treating the resistant strains. In this way, it should be possible to design new approaches to rational therapy (for example see Warhurst and Thomas, 1975) and to counteract both resistance to chloroquine and to its replacements should it arise.

CHAPTER 2

2. MATERIALS AND METHODS

2.1. CHEMICALS AND DRUGS

Chloroquine (Ring - 3 - ^{14}C) diphosphate (specific activity 30.0 mCi/mmol) was obtained from New England Nuclear, Boston, Mass. USA.

Inulin (^{14}C) carboxylic acid (CFA 399) (Specific activity 12.6 mCi/mmol), L - (1-^{14}C) glucose (Specific activity 61.4 mCi/mmol), n-(1-^{14}C) hexadecane (specific activity 0.526 mCi/mg) were obtained from the Radiochemical Centre, Limited, Amersham, England.

The ionophores - monensin sodium and nigericin sodium were obtained from Eli Lilly and Co. Indianapolis, USA.

Chloroquine (7-chloro - 4 (4-diethylamino-1-methyl-butylamino) quinoline) diphosphate salt, ouabain (strophanthin G), 5 - hydroxytryptamine (5HT), L-epinephrine, 2 - deoxy - D - glucose, Valinomycin, N-ethyl maleimide, Reserpine (R.0875) (3,4,5-trimethoxybenzoyl methyl reserpate), were obtained from Sigma Chemical Co.

Foetal Calf Serum was supplied by Wellcome Reagents Limited, Beckenham, England.

4 - chloromecuribenzoic acid and other reagents were obtained from Hopkin and Williams, Chadwell, Heath, Essex, England.

2.2 INCUBATION MEDIA

Malaria usually alters erythrocyte cation transport in all erythrocytes (Dunn, 1969b) Red blood cell sodium concentration is usually elevated and cellular potassium decreased in proportion to the elevation of sodium. To assess the probable effects of the altered cation metabolism, the cation content of the media used in experiments was varied. The following systems were tested:

(i) Standard medium I (SBI) consisting of NaCl - 60 mmol/litre, Mg SO_4 - 2.0 mmol/litre, KCl - 5.0 mmol/litre, Na_2HPO_4 - 50 mmol/litre, NaH_2PO_4 - 5.0 mmol/litre, D-glucose - 5.0 mmol/litre.

(ii) Standard medium II (SB2) consisting of NaCl 25 mmol/litre, Mg SO_4 1.2 mmol/litre, Na_2HPO_4 50 mmol/litre. This medium was modified from that used by Fitch (1969) by reducing the glucose concentration from 86 mmol/litre, (later reported to be inhibitory, Fitch, 1975) to 5.0 mmol/litre.

(iii) High Potassium buffer (HKB) consisting of KCl - 65 mmol/litre, Mg SO_4 2.0 mmol/litre K_2HPO_4 - 50 mmol/litre, $NaH_2PO_4.2H_2O$ - 5.0 mmol/litre, D-glucose - 5.0 mmol/litre.

Incubation media SBI and HKB were adjusted to pH 7.4 with 0.IN HCl and average osmolarity was 286 mOsm/kg when measured at room temperature in a Digimatic Osmometer, Model 3D II (Advanced Instruments inc. Mass. USA). Standard medium II (SB2) after adjustment to pH 7.4 with 0.IN HCl had an osmolarity of 185 mOsm/Kg.

2.3 PARASITE LINE

2.3.1. Plasmodium berghei berghei NK 65. The N.K 65 strain was orginally isolated by Professor M. Yoeli in January 1964 in the forest gallery of the River Kisanga near Lumbumbashi (Yoeli and Most, 1965) and was maintained by cyclical transmission in his laboratory. This strain was later sent to Dr.D. C. Warhurst at the National Institute for Medical Research from where it was sent to Dr. D. Walliker at the Institute of Animal Genetics and designated Edinburgh stabilate No. 277. The strain was passaged into mice and sent to London School of Hygiene and Tropical Medicine, Winches farm station, where blood from the infected mice was cryopreserved in liquid nitrogen and this strain was now designated LUMP 1150. This strain has now been passaged continously in mice for about three years, without intervening mosquito passage.

2.3.2 PLASMODIUM BERGHEI BERGHEI RC STRAIN

This RC strain was obtained from the Department of Medical Parasitology of the Liverpool School of Tropical Medicine and is a line of a strain originally developed by Professor W. Peters. Strain N(K173) isolated from the same area as NK 65 but in 1948 (Vincke and Lips 1948) was made resistant to chloroquine by treating mice infected with Plasmodium berghei Vincke and Lips 1948, with increasing doses of chloroquine parenterally. The resistance of this RC strain is unstable in the absence of drug pressure. On receipt from the University of Liverpool, the stabilate was passaged into T.O mice and after one subpassage was tested for chloroquine

sensitivity (5.0mg/kg base, i.p. x 5 days) Thereafter, the strain was kept under drug pressure to ensure the retention of resistance. Drug pressure was maintained by intraperitoneal administration of chloroquine at a dose of 10 mg/kg wt. of mice twice weekly (I.P). However before utilisation of infected T. O. mouse red cells for experiments, the infection was passaged at least once without concomitant drug pressure.

2.4. ROUTINE PASSAGE TECHNIQUES

2.4.1. Blood Passage

Parasite strains were maintained in 18 - 22g male Theiler's Original (T.O.) albino mice, which were free from infection with Eperythrozoon coccoides. The mice were kept at about 25°C, fed on Dixon's diet - 86, (Dixon and Sons Limited, Crane Mead, Mills) and supplied with water ad libitum.

The parasitaemia (percentage of erythrocytes infected) of mice was assesed by examination of their blood films. Prior to a blood passage, the tips of the tails of donor mice were snipped with a pair of sterilised scissors, a drop of blood was removed and thin blood smears were prepared. The smears were labelled, fixed in absolute methanol and stained for 45 minutes in a 10% solution of Giemsa's stain (10ml Giemsa's stain (B.D.H); diluted with 90 ml phosphate buffered water pH 7.2 - The phosphate buffered water was prepared by dissolving in 1 litre distilled water 3.0g of anhydrous Na_2 HPO4

and 1.2g of anhydrous KH_2 PO4, the concentration of phosphate in the medium was thus 0.03 mol/litre). At the end of the staining, the excess stain was washed off carefully in running water and the slides air dried. Stained blood smears were examined under the 100 x oil immersion objective and 10 x eyepiece of a Zeiss standard microscope and the percentage of parasitised cells estimated by examination of 500 erythrocytes. Random fields from appropriate parts of the blood films were examined.

The donor mouse at a suitable parasitaemia was anaesthetised with chloroform or CO_2 and blood collected by cardiac puncture using a heparinised syringe (approximately 10 units of heparin per 0.5 ml blood). To pass on the infection, normal mice were each given an intraperitoneal injection of 0.2 ml of parasitised blood; suitably diluted in ice cold foetal calf serum/sterile saline (1:1) Occasionally, the intravenous route of injection was utilised via the tail veins which were previously enlarged by warming the mice under an infra-red heater for 10 minutes. The degree of dilution utilised in passaging was based on the following rationale:

Assuming there are 8 million (8.0×10^6) red blood cells per mm^3 in healthy mouse, then there are about 8.0×10^9 red cells in 1.0ml and therefore 1.6×10^9 in a standard inoculum of 0.2ml.

Hence the formula:

$\frac{X}{100} \times 1.6 \times 10^9$; gives the total number of parasitised cells in 0.2ml blood obtained from a donor mouse with parasitaemia of X%. For example, if the mice had a parasitaemia of 10%, 0.2ml of its blood will have:

$$\frac{X}{100} \times 1.6 \times 10^9 \text{ parasites}$$

$$= 1.6 \times 10^8 \text{ parasites.}$$

The above rationale is based on the assumptions that the number of red blood cells/mm^3 is the same in each mouse and that each infected red blood cell contains one parasite. These assumptions are not always correct since multiple infection of red blood cells can occur with P.berghei (Garnham, 1966) Hence the calculated number of parasites is only an approximation.

2.4.2 INDUCTION OF RETICULOCYTOSIS IN MOUSE INVIVO.
Phenylhydrazine - HCl (PH), a haemolysis inducing drug was used to induce reticulocytosis in T. O. mice. This drug was dissolved in deionised water just before use and injected subcutaneously into the experimental mouse in doses of 60mg/kg for three days. The rise in reticulocytes was followed by light microscope examination of New Methylene blue-stained slides of blood from phenylhydrazine HCl treated donor mice. Equal volumes of blood and New methylene blue stain (1.0g/100ml 3% citrate

saline) were drawn into a haematocrit tube, mixed and placed in an incubator at 37°C for 15 min. and then smears made without fixing. Reticulocytosis was expressed as percent of total red blood cell population examined which stained with new methylene blue.

2.5. PURIFICATION OF INFECTED RED BLOOD CELLS.

Red blood cells from groups of 5 mice were pooled and "purified" as follows:

The pooled heparinised blood (4-6ml) was added to an equal volume of standard medium 1, supplemented with heparin and kept in ice. The blood cells were collected by centrifugation at 700g for 5 mins. Subsequently, the blood cells were washed twice with 5 volumes of standard medium 1. After washing the cells the white blood cells were removed, using an adaptation of the cellulose powder CF 11 procedure (Williams and Richards, 1973, Baggaley and Atkinson, 1972).

1.0 ml of packed blood cells previously washed and kept in ice was made up to 6ml with standard medium. The suspension of red blood cells was carefully layered on an plastic column of dry CF 11 cellulose powder (Whatman Limited, Maidstone, Kent). The plastic column was made from a 20 ml plastic syringe, with a piece of cotton wool covering the needle end. The column was packed to a depth of 6 cm with 6-7g Whatman CF11. The red blood cells were then eluted with 10ml of ice cold standard medium 1 applied under slight pressure from a 20ml syringe, the needle of which was inserted through a tightly fitting rubber bung at the top of the column. The eluted red blood cell suspension (not more than 10ml) was then centrifuged at 700g for 5 min. and the resultant pellet suspended in standard medium 1 to a ratio of 1:14 (V:V) and kept in ice.

FIGURE 9

PROTOCOL FOR CHLOROQUINE UPTAKE STUDIES IN MOUSE ERYTHROCYTES INFECTED WITH PLASMODIUM BERGHEI.

Heparinised blood from donor mice in equal volume of ice cold standard medium, I.

↓ centrifuge 700g x 5 min.

supernatant ← | ↓

packed rbcs

↓ suspend in 5 vols medium and wash twice

suspend washed packed rbcs in 5 vol standard medium and pass through dry cfll Cellulose column, eluting with more medium.

↓

rbcs, free from wbcs and platelets

↓ wash and suspend pellet in standard medium 1:14.

1 ml rbc suspension mixed with 2ml medium containing radioactive chloroquine and 1 ml medium containing other additions if needed, incubated at 37°C for varying periods of time in weighed sterilin plastic centrifuge tubes and then centrifuged to separate the cells from the medium.

SUPERNATANT	PELLET
[contains unabsorbed chloroquine]	chloroquine in parasite-erythrocyte complex and in water space (inulin space)
↓	pellet lysed after weighing.
3ml added to 0.3mg cold chloroquine in 1ml water - 1 ml 5N NaOH added, extraction for 20 min into 2.5 ml heptane. 1ml extract read in 10 ml toluene scintillator.	(1ml distilled water added) and 0.1 mg cold chloroquine. Then 1ml 2N NaOH, and extract into 2.5 ml heptane. 1 ml extract read in 10 ml toluene scintillator.
↓	↓
Calculation of 14C chloroquine concentration in supernatant.	Calculation of 14C chloroquine concentration in pellet. Efficiency of counting measured by channels ratio method. Using the inulin space, concentration of 14C chloroquine per Kg rbc calculated.

FIGURE 9

PROTOCOL FOR CHLOROQUINE UPTAKE STUDIES IN MOUSE ERYTHROCYTES INFECTED WITH PLASMODIUM BERGHEI.

Heparinised blood from donor mice in equal volume of ice cold standard medium, I.

↓ centrifuge 700g x 5 min.

supernatant ← | ↓

packed rbcs

↓ suspend in 5 vols medium and wash twice

suspend washed packed rbcs in 5 vol standard medium and pass through dry cfll Cellulose column, eluting with more medium.

↓

rbcs, free from wbcs and platelets

↓ wash and suspend pellet in standard medium 1:14.

1 ml rbc suspension mixed with 2ml medium containing radioactive chloroquine and 1 ml medium containing other additions if needed, incubated at 37°C for varying periods of time in weighed sterilin plastic centrifuge tubes and then centrifuged to separate the cells from the medium.

↓

SUPERNATANT ← | → PELLET

[contains unabsorbed chloroquine]

↓

3ml added to 0.3mg cold chloroquine in 1ml water - 1 ml 5N NaOH added, extraction for 20 min into 2.5 ml heptane. 1ml extract read in 10 ml toluene scintillator.

↓

Calculation of 14C chloroquine concentration in supernatant.

chloroquine in parasite-erythrocyte complex and in water space (inulin space)

pellet lysed after weighing.

(1ml distilled water added) and 0.1 mg cold chloroquine. Then 1ml 2N NaOH, and extract into 2.5 ml heptone. 1 ml extract read in 10 ml toluene scintillator.

↓

Calculation of 14C chloroquine concentration in pellet. Efficiency of counting measured by channels ratio method. Using the inulin space, concentration of 14C chloroquine per Kg rbc calculated.

2.6. DETERMINATION OF PACKED CELL VOLUME OF MOUSE BLOOD

The sample of whole mouse blood was thoroughly mixed and sucked into a heparinised capillary pipette to within 2.0 cm of the top by means of a teat and adapter. The top end of the capillary pipette was sealed in a microburner and allowed to cool. The capillary pipette was then centrifuged at 10,000 r.p.m. in a Hawksley micro haematocrit centrifuge for 15 min. (in preliminary experiments, the packed cells were measured at intervals until a constant volume of packed cells had been obtained, the average time required to obtain a constant packed volume was found to be 15 min.) The height of the blood column (A) and that of the packed cells (B) was measured. The packed cell volume (PCV) was calculated as the fraction of packed cells, $\frac{B}{A}$.

2.7. ANALYSIS OF SIZE DISTRIBUTION OF BLOOD CELLS

Blood samples to be analysed were suitably diluted, usually utilising the "two step dilution" method. 40µl of whole blood was mixed with 20 ml of standard buffer 1 giving a dilution of 1:500. Then 200µl of the above suspension was added to an additional 20ml of standard medium kept at room temperature to minimize changes in red blood cell size. The resistivity of the standard medium was usually between 10-20 kilo ohms across the aperture used giving an acceptable signal to noise ratio (Brechner 1962). The standard medium was previously cleaned by passing it through a millipore filter (size 0.22 µm), and light microscope examination did not detect any swelling or shrinkage of the cells by standard medium 1. The final diluted suspensions (1:50,000) were then analysed on a coulter counter (Model B) with an automatic particle size distribution analyser (Model J). The Coulter counter was set at the matching switch of L64H with an aperture opening of 100 microns. However amplitude and aperture current gain settings were adjusted to yield 'normal' particle pulses on the oscilloscope. The settings were calibrated by use of latex P.V.P. particle standards of mean diameter 15.0 µm. The mean volume of the major cell component was determined and a normal curve, obtained, from the histogram produced by the plotter, by joining the midpoints of the peak of each histogram plot. Any changes in plotter settings are indicated in the appropriate graphs.

2.8. ASSESMENT OF HAEMOLYSIS OF INCUBATED ERYTHROCYTES

In order to avoid errors introduced by binding of chloroquine to exogenous haemoglobin, the extent of haemolysis in the incubated erythrocytes was evaluated routinely as follows:

A "100%" haemolysed solution of erythrocytes was prepared by lysing 0.1ml of packed purified red blood cells in 50µl of distilled water and made up to 4.0ml with isotonic saline (154 mmol/litre). "0%" haemolysed solution was prepared by diluting 0.1ml of packed purified red blood cells to 4.0 ml with isotonic saline. Both solutions were then centrifuged at 700g for 5 min. and the optical density of the supernatants obtained were found by measuring at 600 nm in a UNICAM SP 600 spectrophotometer.

By using varying quantities of the supernatants from the 100% and 0% haemolysed solutions, optical density readings were obtained for 20%, 40%, 60% and 80% haemolysis and a calibration curve for the instrument prepared (Fig 10) The degree of haemolysis (if any) after each experiment was then checked by comparing the optical density reading of the supernatant, with the calibration curve at the same wavelength. During experiments, plasticware and siliconised glassware only were used to minimise haemolysis. The same precaution was adopted during collection and purification of blood.

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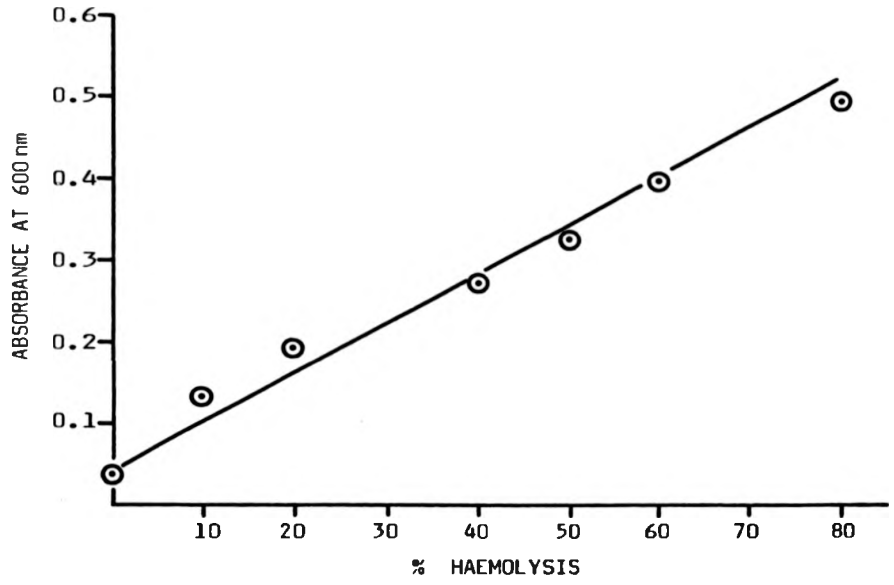

Fig. 10

Haemolysis calibration curve.

This curve was utilised to check degree of haemolysis (if any) after each experiment. Haemolysis below 2 x 0% haemolysis was accepted as insignificant. Results obtained from experiments with haemolysis above 2 x 0% haemolysis* are indicated in relevant tables.

*2 x control (0% haemolysis)

2.9 DETECTION AND MEASUREMENT OF RADIOACTIVITY

A Packard TriCarb Liquid Scintillation Spectrometer (Model 3003 - Packard Instruments Limited, Caversham, Berkshire) was used for the detection and measurement of radioactivity in the radiolabelled drugs and substrates utilised in various experiments in the project. The selected window settings of the scintillation spectrometer are shown in Fig 11.

In order to correct for optical, colour and chemical quenching, the counting efficiency of the scintillation spectrometer was determined using the sample channels ratio method. The method involved the preparation of a quench correction curve. The procedure adopted was essentially as follows:

A sample was prepared in heptane containing a known amount of standard carbon-14 labelled hexadecane (P disintegrations per min) and was counted in toluene scintillator (Packard) in two channels (Q counts per min in the green (medium energy ^{14}C) channel (2) and R counts per min. in the red (low energy ^{3}H channel (1) The counting efficiency in channel 2 was then calculated (Q/P x 100%). The sample channels ratio is the ratio of counts recorded in channel 2 and channel 1 (i.e. R/Q). Increasing amounts (0.1 ml increments) of chloroform (quenching agent) were then added to the sample and counting repeated. This procedure was repeated four times and the counting efficiency in channel 2, and the

channels ratio were calculated after each increment of the quenching agent. A Calibration curve (Fig 11) was then prepared which was used when determining the counting efficiency of the scintillation spectrometer with various experimental samples. The channels ratio for each sample was determined and the counting efficiency calculated using the slope and intercept values for the regression of % of efficiency of counting against channels ratio. The calibration curve applied to all samples containing the same isotope.

It was found that counting efficiency for samples extracted into n-heptane and counted in toluene scintillator was generally high. (75 - 85%). Percentage recovery of radioactivity was also generally high and averaged over 90%. Recovery of radioactivity was evaluated by comparing radioactivity added (from specific activity converted to dpm) to radioactivity obtained by summation of total dpm obtained in pellet and dpm left in supernatant.

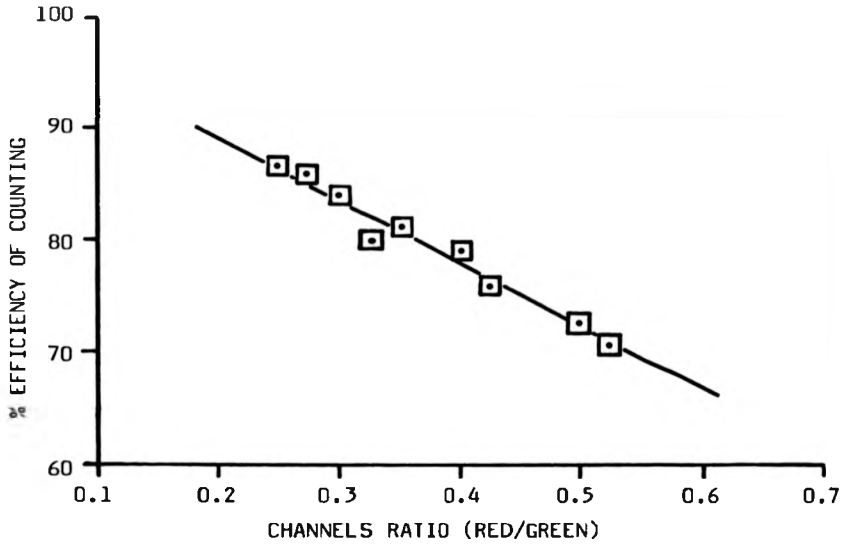

Fig. 11

Channels ratio quench correction curve using n - (1-^{14}C) Hexadecane standard.

The slope of the regression line (correlation coefficient 0.98) and the interception on the y axis were utilised in the programme for calculating total disintegrations per min /g erythrocytes (Appendix 1).
The liquid scintillation spectrometer settings are as follows:

CHANNELS	1	2	3
BACKGROUND SUBTRACTION	NIL	NIL	NIL
GAIN	5,1 (100%)	5,0 (10%)	1,0 (100%)
WINDOW	A-B	C- ∞	E-∞
	A-B	C-D	E-F
	0-10	0-10	0-10
	50- 2	50-0	50-0

2.9.1 ESTIMATION OF TRAPPED FLUID VOLUME (INULIN SPACE)

Washed and "purified" T. O. mouse red blood cells (1.0ml) were suspended in 3.0 ml of SB2, pH 7.4 containing Inulin (^{14}C) carboxylic acid (which does not enter erythrocytes) at 100,000 d.p.m/ml. After incubation for 45 min at 37°C, red cells were separated by centrifugation at 700g for 5 min, the supernatant was aspirated, and the wet pellet weighed. The amount of radioactivity in the trapped fluid volume of the red blood cells was measured as follows:

After weighing the pellet, 2.0ml of 5% (W/V) trichloroacetic acid (TCA) was added to the pellet and left overnight. After stirring and centrifugation at 700g for 5 min, the radioactivity in the TCA extract was determined by adding 1.0ml of the TCA extract to 10ml of Xylene based Emusifier scintillator (Special M1-96) and counting radioactivity as described earlier. The proportion of wet red blood cell weight contributed by water in the "inulin space" was determined by measuring the total radioactivity (dpm) of the extract and calculating what this represented in terms of the volume of extracellular water (100,000 dpm/ml). This was expressed as a proportion of the weight of the pellet and was then used to correct both the "wet weight" of red blood cell pellets and the total radioactivity taken up by the red blood cells (see appendix 1). The mean value was found to be 0.15 (inulin space = 15%).

2.9.2. DETERMINATION OF (^{14}C) CHLOROQUINE UPTAKE BY INFECTED AND UNINFECTED T..O. MOUSE ERYTHROCYTES

1.0ml of a 1:14 (packed red cell: incubation medium, v/v) dilution of purified red blood cells was added to 2.0ml of radioactive chloroquine in buffer equilibrated to 37°C. To bring the incubation medium to a total volume of 4.0ml, 1.0 ml of standard medium pH 7.4, was also added. (Usually, the same buffer system, SBI or SB2 or HKB was used in preparing the dilution of the red blood cells and during incubation,) On various occasions, the 1.0 ml of standard medium also contained compounds under study.

The incubation mixtures in Sterilin plastic centrifuge tubes, were left at 37°C on a roller tube apparatus. Red blood cells were separated after 45 mins by centrifugation at 700g for 5 min. at room temperature. In other instances, incubation time was varied.

Immediately after centrifugation, the supernatant medium was aspirated from the pellet and the wet pellet weighed. All calculations of the amounts of radioactive chloroquine taken up by erythrocytes are based on wet weight corrected for inulin space. In order to determine chloroquine uptake, the amounts of radioactivity in the supernatant medium and red blood cell pellets were measured according to the following method based on Fitch, 1969:

Approximately 0.10g pellet was lysed with 1.0ml water containing 0.10mg non-radioactive chloroquine. To the lysate was then added 1.0ml of 2M NaOH. 2.5ml of n-heptane containing 1.5% (V/V) isoamyl alcohol was added and the mixture shaken vigorously on a "Whirlimixer" for 1 minute. 1.0 ml of the heptane extract was added to 10ml of Packard toluene scintillator (containing 0.1g POPOP (1,4-bis (2-(5-phenyloxazolyl) benzene and 5.0g PPO (2,5-diphenyloxazole) per litre) and the radioactivity read.

After addition of 1.0ml water containing 0.3mg non-radioactive chloroquine to 3.0ml of supernatant medium, the mixture was made alkaline by addition of 1.0ml of 5M NaOH. The mixture was extracted into 2.5ml heptane containing 1.5% isoamyl alcohol as before and radioactivity similarly read. The absolute concentration (μmol/kg wet weight erythrocytes) of the radioactive chloroquine in pellet and in the supernatant medium (μmol/litre) was computed using the programmes in Appendices I, II and III using a Texas TI Programmable 57, calculator. (See also Fig. 9)

In prelimary experiments, it was observed that n-heptane containing 1.5% isoamyl alcohol could extract over 95% of radioactive chloroquine present in the incubation medium. Binding of radioactive chloroquine to the walls of the sterilin plastic centrifuge tubes and glass tubes used during the experiments was negligible.

2.10 EFFECTS OF SUBSTRATES AND DRUGS ON UPTAKE OF (^{14}C) CHLOROQUINE

2.10.1 PREPARATION OF CHEMICALS

D-glucose and glucose analogues (2- deoxy- D-glucose, 3 - O - methyl - D - glucose) were prepared directly in the appropriate buffers and serial dilutions made when required. All stock solutions of D-glucose and analogues were prepared fresh. Concentrations of all substrates used are indicated in appropriate places in the text.

Ionophores (Monensin, Nigericin, Valinomycin) and ouabain were dissolved first in a very small volume of absolute ethanol, usually less than 0.5 ml and stored refrigerated at - 20°C. Working concentrations were made up immediately before use with appropriate buffer. Finally, the final concentration of ethanol in the incubation medium was less than 1% (V/V). This concentration of ethanol was shown (see results) to have no effect on (^{14}C) chloroquine uptake.

Sulphydryl reactive agents, N-ethyl-maleimide (NEM) and parachloromercuribenzoic acid (pCMB) were dissolved in SB1. In the case of p-chloromercuribenzoic acid, a few drops of 5N NaOH was added to the stock solution to aid dissolution. Any variation from pH 7.4 was adjusted accordingly with 0.1M acid or alkali. N-ethyl maleimide was always prepared fresh to avoid slow hydrolysis to N-ethylmaleamate.

Reserpine, 5-hydroxytryptamine and L-epinephrine were first dissolved in a small volume of glacial acetic acid (less than 0.1ml) and then made up to correct concentration by dilution with appropriate buffer. 1.0M NaOH was utilised to correct variations in pH to 7.4.

2.10.2 DOSE RESPONSE

1.0ml of a suitably diluted suspension of infected or uninfected erythrocytes were incubated for 45 minutes at 37°C in 3.0ml of medium containing (^{14}C) chloroquine at a concentration*of 50 nmol/litre and a range of concentrations of the compounds under investigation. The dose range was usually 0.125 - 40 mmol/litre for D-glucose and analogues and 1.0×10^{-10} - 2.0×10^{-6} mol/litre for the ionophores - nigericin and monensin. The incubations were usually in duplicate and controls which did not contain either D-glucose and analogues or ionophores were included. At the end of the incubation period, the tubes were centrifuged at 700g for 5min and the supernatant and pellet assayed for radioactivity as indicated previously. From the results of the experiments, log dose response curves were prepared.

* final

2.10.3 TIME COURSE OF EFFECT OF IONOPHORES

The time course of action of the ionophores was also investigated as follows:

After purifying the erythrocytes, 1.0ml of a suitably diluted suspension was preincubated with 3.0 ml of medium containing 0.5µmol/litre* of appropriate ionophore at room temperature. For experiments with valinomycin and nigericin, the medium (SBI) also contained an additional 40 mmol/litre KCl. After the preincubation for varying periods of time (5 min - 60 min), the tubes were centrifuged at 700g for 2 min and the pellet was washed once in SBI and resuspended in the incubation medium. The resuspended erythrocytes were then incubated as usual (in an incubation medium containing (^{14}C) chloroquine at a concentration of 50 nmol/litre) for 45 min at 37^{o}C. At the end of this time, the red blood cells were separated and drug uptake determined radiochemically as previously described. The time course of the effect of the ionophores was determined by plotting drug uptake against time of preincubation.

*final concentration

2.11 KINETICS OF (^{14}C) CHLOROQUINE UPTAKE BY UNINFECTED AND INFECTED T.O. MOUSE ERYTHROCYTES

2.11.1 TIME COURSE OF DRUG UPTAKE

The time course of (^{14}C) chloroquine uptake was investigated over concentration ranges where the uptake of drug varied linearly with drug concentration in the medium, giving a first order kinetics. Using a (^{14}C) chloroquine concentration within this range, the time course of drug uptake was determined as follows:

1.0 ml of "purified" erythrocyte suspension was added to 12 siliconised stoppered glass test tubes containing 3.0ml of incubation medium (SBI) with 50nmol/litre* (^{14}C) chloroquine. These tubes were then incubated at 37°C in a roller tube apparatus for varying periods of time ranging from 5 minutes to 60 minutes. At the end of each incubation period, the tubes (in duplicate) were centrifuged at 700g for 5 min. The supernatant was aspirated and the pellet was weighed. The amount of radioactive drug in the pellet and supernatant was then determined as previously stated. This experiment was performed for P.berghei - infected and uninfected T.O mouse erythrocytes.

* final concentration

2.11.2 AFFINITY OF UPTAKE SYSTEM FOR CHLOROQUINE.
Purified T.O. mouse erythrocyte suspensions infected with P.berghei NK65, RC strain, and uninfected, were divided into 1.0ml aliquots and incubated with a series of concentrations of (^{14}C) chloroquine ($2.5x10^{-7}$-$9.75x10^{-10}$ mol/litre) After 45 mins incubation at 37°C in a roller tube apparatus, the erythrocytes were collected by centrifugation at 700 x g for 5 min and the amount of (^{14}C) chloroquine concentration in the pellet and supernatant were determined.

Double reciprocal graphs of these data were used to determine the dissociation constants of the chloroquine drug 'receptor' complex. In another series of similar experiments, the purified erythrocytes were incubated with the same series of concentrations of (^{14}C) chloroquine with the addition of a constant concentration of nonradioactive inhibitor test drug. The inhibitor test drugs examined were quinine, Monensin and L-epinephrine. Double reciprocal graphs of the data obtained from these experiments were also used to evaluate the type of inhibition caused by the drugs and to estimate the inhibition constant (K_i) for the drug if the inhibition was competitive.

2.11.3 SPECIFICITY OF UPTAKE OF (^{14}C) CHLOROQUINE

The erythrocyte - parasite system is a very complex system containing various components and "barrier" systems. One result of this complexity, is that there could be nonspecific uptake components in the system which would be non-saturable at therapeutic chloroquine concentrations and might be distinguishable from the saturable "specific" uptake sites of high affinity. Experiments were therefore devised to distinguish between "specific" and "non specific" uptake.

This distinction has been made by parallel incubations over the same range of radioactive drug (5.0×10^{-7} - 3.9×10^{-10} mol/litre) in the presence and absence of an excess of nonradioactive drug (1.25×10^{-5} mol/litre). This excess only minimally occupies the "nonspecific" uptake system and therefore does not block "nonspecific uptake". On the otherhand, the specific uptake system is overwhelmingly filled by the excess nonradioactive drug and a reduced amount of radioactive drug is taken up via this system. When the nonspecific uptake is subtracted from the total uptake, a specific uptake value is found.

Hence, by parallel incubations of (^{14}C) chloroquine alone (Total uptake) and in the presence of excess non radioactive chloroquine (nonspecific uptake) variations or similarities in the "specificity" of uptake of (^{14}C) chloroquine into uninfected T. O. mouse erythrocytes, P.berghei NK 65 and P.berghei RC strain were compared.

2.12 MEASUREMENT OF PERMEABILITY OF ERYTHROCYTES TO (^{14}C) L-GLUCOSE

1.0 ml of a 1:14 dilution (packed purified red blood cells: SBI) of red blood cells was added to 2.0 ml of L - (1-^{14}C) glucose in standard medium 1 at pH 7.4. The incubating medium was made up to 4.0 ml by addition of an extra 1.0 ml of medium with or without a test compound.

After incubation at 37^{o}C for 45 min and centrifuging at 700 x g for 5 min. the supernatant was removed and the pellet weighed. Any L-(1-^{14}C) glucose taken up into the pellet was extracted into 5% (W/V) trichloroacetic acid. The radioactivity was determined by adding 1.0 ml of the T.C.A extract to 10.0ml of xylene based emulsifier scintillator (special M1-96) and radioactivity was counted.

The amount of L - (1-^{14}C) glucose left in the supernatant was similarly determined. Absolute concentrations of the radioactive L-glucose were then computed using the programmes in Appendices 1, 11 and 111.

This experiment was done using various concentrations of (^{14}C) L-glucose (5.0×10^{-7}-3.9×10^{-10} mol/litre) for both uninfected and P.berghei infected T. O. mouse erythrocytes.

2.13 MEASUREMENT OF D-GLUCOSE UTILISATION AND L(+) LACTATE PRODUCTION.

1.0ml of a 1 in 10 dilution of purified P.berghei infected T. O mouse erythrocytes was incubated at 37°C in 4.0ml of standard medium (SBI) containing a range of concentrations of D-glucose (0.5-5.0 mmol/l) 1.0 ml of sample was removed from the incubation medium at room temperature at time zero and therefter 1.0 ml of sample was revoved at 15 minutes intervals until thirty minutes had elapsed. Immediately after removal of aliquots from the incubation medium, they were deproteinised with 0.1 ml of 6 M perchloric acid and kept chilled in ice. After spinning the chilled aliquot in a centrifuge at 4°C for 10 min at 700g., 0.5 ml of each supernatant was transferred to a clean sterilin tube and neutralised with 0.091 ml of $KHCO_3$ (3 mol/l). This neutralised sample was used for assay for both D-glucose and L(+) lactate in the sample. This experiment was done for both P.berghei NK 65 - and RC strain - infected T. O. mouse erythrocytes. The effect of the ionophore Monensin on utilisation of D-glucose and production of L(+) lactate was investigated by running parallel experiments containing the ionophore.

2.13.1 ASSAY OF D - GLUCOSE

The method used for the assay of D-glucose is a modification of the method of Bergmeyer (1974). The principle is based on the formation of NADPH as measured by the change in extinction at 340nm, the NADPH formed is proportional to the amount of glucose present as shown in equations below:

D-glucose + ATP $\xrightarrow{\text{Hexokinase}}$ G-6-P+ATP.

G-6-P+ $NADP^+$ $\xrightarrow{\text{G6P-DH}}$ 6-Phosphogluconate + NADPH $+H^+$

0.1ml of standards and neutralised deproteinised samples were made up in 1.0 ml cuvettes containing 0.82ml of 0.3M triethanolamine (TEA) with 3 mmol/l $MgSO_4$ (5.6g TEA and 740mg $MgSO_4.7H_2O$ dissolved in 7.0ml H_2O, and adjusted to pH 7.5 with 1M NaOH, then made up to 100 ml) and 0.06 ml of ATP/NADP (455mg ATP.Na_2.$3H_2O$ and 50mg $NADP^+$. Na_2 made up in 5.0ml of TEA buffer). After measuring the absorbance of this mixture at 340nm in an SP600 spectrophotometer, 0.02 ml of a mixture of G6P-DH and hexokinase (1mg/ml in 3.2 mol/l $(NH_4)_2SO_4$) was added to the 1.0 ml cuvettes containing the mixtures and the increase in absorbance followed at 340nm to completion (5-15 min) The increase in absorbance was calculated. The amount of D-glucose present was calculated with reference to a calibration run with a D-glucose standard.

2.13.2 ASSAY OF L(+) - LACTATE

L-(+)-Lactate was assayed using a modification of the method of Gutman and Wanefield (Bergmeyer 1974). The assay is based on the production of NADH by the oxidation of L-(+) - lactate to pyruvate by NAD in the enzymatic reaction catalysed by lactate dehydrogenase (LDH)

$$\text{L(+) Lactate} + NAD^+ \underset{}{\overset{LDH}{\rightleftharpoons}} \text{Pyruvate} + NADH + H^+$$

The formation of NADH was measured in an SP 600 spectrophotometer by the increase in extinction at 340nm. The equilibrium of the reaction lies in favour of lactate and NAD. However pyruvate is trapped by reaction with hydrazine as the hydrazone while the protons are removed by an alkaline reaction medium to yield:

$$\text{L-(+) - lactate} + NAD^+ + \text{Hydrazine} \xrightarrow[\text{PH 9.0}]{LDH} \text{Pyruvate}$$

$$\text{hydrazone} + NADH + H^+$$

0.8ml of neutralised deproteinised samples were added to 3.0ml cuvettes containing 2.0 ml hydrazine/glycine buffer (0.4 mol/l hydrazine; 0.5 mol/l glycine pH 9.0) and 0.2ml NAD^+ (40 mmol/l) After measuring the absorbance of this mixture at 340nm, 0.02 ml of 5mg/ml LDH was added to the reaction mixture and stirred. The reaction was allowed to go to completion in a water bath at 37°C. After 30 min. the increase in extinction at 340nm was measured, and the change in optical density used to calculate the amount of L-(+)-lactate in the sample; according to the method of Bergmeyer (1974).

2.14 DETERMINATION OF H^+ EFFLUX INDUCED BY IONOPHORES.

The measurement of pH changes induced by the ionophores Monensin and Nigericin was carried out in a cylindrical Sterilin plastic cell of 5.0ml capacity with a combination glass micro electrode containing a silver-silver chloride reference cell (W.G Pye Limited, Cambridge) The cylindrical cell was mounted on an electrically driven horizontal turntable with a constant speed of 24 rev. per min. The pH meter (model 290) was connected to a potentiometric flatbed recorder (RE 541.20 - Smith Industries Limited, London) via its external outlet which had a variable resistor inserted. The potentiometric flatbed recorder was calibrated to give a sensitivity of 1 mm per 0.01 pH change between the pH range of 6.0 to 8.0. At this amplification, the background noise level corresponded to less than 2mm on the chart. Control experiments with various solutions used, indicated a constant response time by the flatbed recorder, over the whole recording width. The medium used in the experiments had a constant volume of 4.0ml. Generally, the procedure was as follows:

4.0ml of the medium (sterile saline 154 mM, or KCl 154 mM or K^+ supplemented isotonic saline, details in relevant result text) was pipetted into the cylindrical cell and allowed to equilibrate. The pH meter was then turned on to "read" and the variation in the meter response followed over a duration of 10 min. 1.0 ml of the medium was gradually pipetted off and 1.0 ml of "purified" red blood cells in the same medium was added and the resultant change in pH followed over the next 10 minutes. To test the effect of various compounds, 10 µl of a solution of the

compound was added and the pH change induced followed over 10 min with constant stirring by the horizontal mixer. At the end of each experiment, the glass micro electrode was wiped clean with paper tissue soaked in deionised water and the micro electrode temporarily stored in a concentrated solution of KCL (3 mol/l). The linearity of the calibrated scale on the flatbed recorder was checked in between experiments by measuring the pH and corresponding pen positions on the scale when the microelectrode was transferred into solutions of pH standards 6.0, 7.0 and 8.0 respectively.

2.15 MEASUREMENT OF INTERNAL pH (pHi) OF RED BLOOD CELLS

A modification of the method of Deane and Smith (1957) was used in measuring the intracellular pH of red blood cells:

Blood from groups of 10 T. O. mice was collected in 5ml glass haematology bottles pretreated with heparin and stored in ice. Prior to collecting blood, the mice were anesthetised with chloroform only. After thoroughly mixing the blood in the haematology bottles, the pH of the whole blood at room temperature (25°C) was recorded and the blood centrifuged at 700g for 3 min.

The plasma obtained after centrifugation was allowed to to attain room temperature (25°C) and the pH measured. The buffy coat on top of the packed red blood cells was carefully removed and the top half of the packed cells was transferred to another glass haematology bottle. For P.berghei - infected mice the top fraction removed was usually about one third the volume of packed red blood cells containing parasites in various developmental stages. Aliquots of the top fractions were then pooled to provide a sizable portion. 0.3ml of Verislube silicone oil was carefully layered on top of the sample and then gradually mixed with the packed red blood cells.

The mixture was then centrifuged at 700g for 3 min after which, the Verislube silicone oil layer together with the trapped plasma was gently aspirated off. The packed cells were then frozen rapidly in liquid nitrogen and then thawed. The cycle of freezing and thawing was repeated twice. When the lysed red blood cells had attained room temperature, the pH of the resulting liquid was then

measured using the micro glass electrode. The pH value thus obtained is the average intracellular pH (pHi) of the red blood cells.

CHAPTER 3

3. RESULTS

3.1 VARIATION OF PACKED CELL VOLUME.

The packed cell volume (PCV) of normal (uninfected) T.O. mouse red blood cells having 2.5 - 5.5 % reticulocytes was found to be 0.30 ± 0.03. For phenylhydrazine treated T.O. mice, reticulocytes increased to 65% ± 5.0 and the packed cell volume was 0.201 ± 0.001. From Coulter counter readings, the average concentration of red blood cells in normal mice was 5.54×10^{12} per litre whole blood while that of reticulocyte enhanced blood was 3.28×10^{12} per litre whole blood. Packed cell volume varied with the degree of parasitaemia for both *P. berghei* NK 65 and RC strain infected erythrocytes.

At about 50% parasitaemia, the packed cell volume of *P. berghei* NK 65 infected erythrocytes was 0.23 ± 0.09 with reticulocytosis of 16% ± 4.0, while *P. berghei* RC strain infected blood at a parasitaemia of 30% had a packed cell volume of 0.172 ± 0.04 and reticulocytosis of 55% ± 5.0. The concentration of purified red blood cells in infected T.O. mouse blood was 1.05×10^{11} per litre (at the dilution (1:14) of the standard erythrocyte suspension used in experiments) for NK 65 and 8.0×10^{10} per litre for RC strain.

3.2 SIZE DISTRIBUTION OF RED BLOOD CELLS.

Results obtained from the automatic particle size distribution analyser, show that P. berghei NK 65 and RC strains infect different populations of red cells. After calibration of the analyser with P.D.V.B latex particles, the mean cell volume and volume distribution of uninfected erythrocytes was obtained. The mean cell volume of uninfected T.O. mouse erythrocytes was 46.0 μm^3 and that of reticulocyte enhanced blood was 65.0 μm^3.

After infection with P. berghei, there was a slight reduction in the mean cell volume, and at about 50% parasitaemia, the mean volume of T.O. mouse red cells was 40.0 μm^3 . However with P. berghei RC, there was a marked increase in the mean cell volume to 63.0 μm^3; towards peak parasitaemia. Figs 12 and 13 are the coulter counter calibration curve and size distribution graphs respectively. The mean cell volume of erythrocytes infected with P. berghei RC was evidently the same as the mean cell volume obtained for reticulocyte-enhanced mouse red blood.

TABLE 1 VARIATION OF HAEMATOLOGICAL DATA OF T.O. MOUSE BLOOD

Strain and Stage of Infection	Population (x10 ^{12}/Litre)	% RETS.	Mean Cell Volume (mm^3)	PCV
Normal (a)	5.54 ± 0.01	4 ± 1.5	46.0	0.30 ± 0.03
Normal +PH(a)	3.28 ± 0.06	65 ± 5.0	65.0	0.201 ± 0.001
NK 65 (b) 50%	0.105 ± 0.001	16 ± 4.0	40	0.23 ± 0.01
RC strain (b) 30%	0.08 ± 0.002	55 ± 5.0	63.0	0.172 ± 0.04

(a) Population determined for whole blood.

(b) Population determined for purified blood. (i.e. 1:14 dilution)

Results given as mean ± S.D for 3 experiments.

PH is phenylhydrazine hydrochloride.

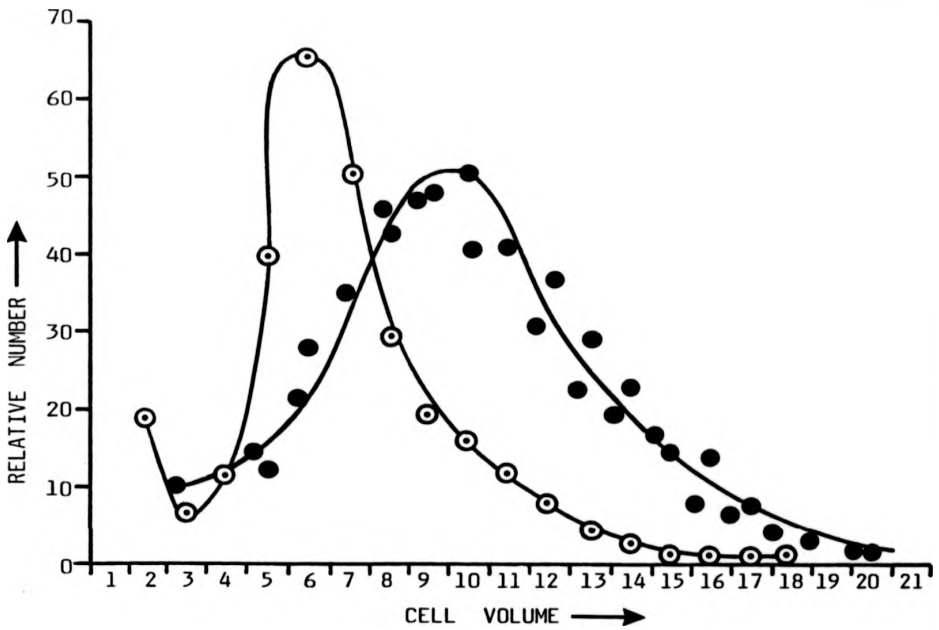

Fig. 12

Coulter counter calibration curve

●—● P.D.V.B. Latex calibration standard at Coulter counter aperture current of 2.0 and amplification of 0.06. Mean volume of PDVB latex particles is 1767 μm^3 ; with a spread of 358.22 - 3140.0 μm^3.

⊙—⊙ Size distribution of unifected T.O. mouse erythrocytes at coulter counter aperture current setting of 2.0 and amplification of 2.0 . 1 unit volume is equivalent to 7.0 cubic microns and mean volume of unifected T.O. mouse erythrocytes is approximately 46.0 μm^3(a).

(a) The unit volume can be obtained from the P.D.V.B. latex calibration standard curve only after correction for any differences in aperture current and amplification. This "correction" together with any variations in the venier scale of automatic particle size distribution analyser generates the calibration factor which was used to derive the unit volume of the red blood cell from that of the P.D.V.B. latex standard.

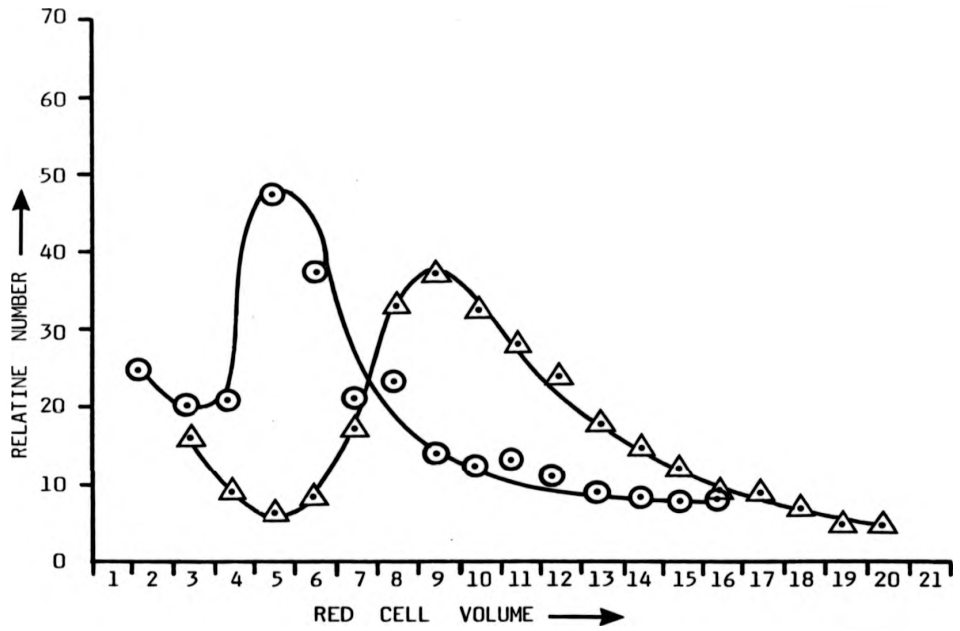

Fig. 13

Size distribution graph.

⊙ P.berghei NK 65 infected T.O. mouse red cells

△ P.berghei RC strain infected T.O. mouse red cells

One unit volume of red cell is equivalent to 7.0 cubic microns. Mean cell volume for P.berghei NK 65 infected red cells is approximately 40 cubic microns and for P.berghei RC strain infected red cells is 63.0 cubic microns.

% parasitaemia is about 40% for both strains.

Coulter Counter aperture current setting = 2.0

Amplification setting = 2.0

3.3 DEVELOPMENT OF MALARIA INFECTION IN MICE INFECTED WITH P. BERGHEI.

After inoculation of 10^6 parasitised red cells intraperitoneally, the P. berghei NK 65 infection developed more rapidly than P. berghei RC. Fig 14 shows the course of parasitaemia for both strains. Linear regression analysis of the curves show that the pre-2% period (Warhurst and Folwell, 1968) for P. berghei NK 65 was 2.4 days while that of P. berghei RC strain was 5.4 days. The maximum parasitaemia obtainable was 80.1% $\pm$ 5.0 for P. berghei NK 65 by the 7th day after infection, and 37% $\pm$ 2.5 for RC strain by the 14th day. This yields a growth index of 11.4 for NK 65 strain and 2.6 for RC strain (Growth index is obtained here by dividing maximum parasitaemia by time in days required to attain it.)

While P. berghei NK 65 did not show any preference for polychromatophiles, P. berghei RC strain, invaded mainly immature polychromatophilic cells. P. berghei NK 65 infection usually kills the mice by the 7th day while RC strain usually took 14 days or more.

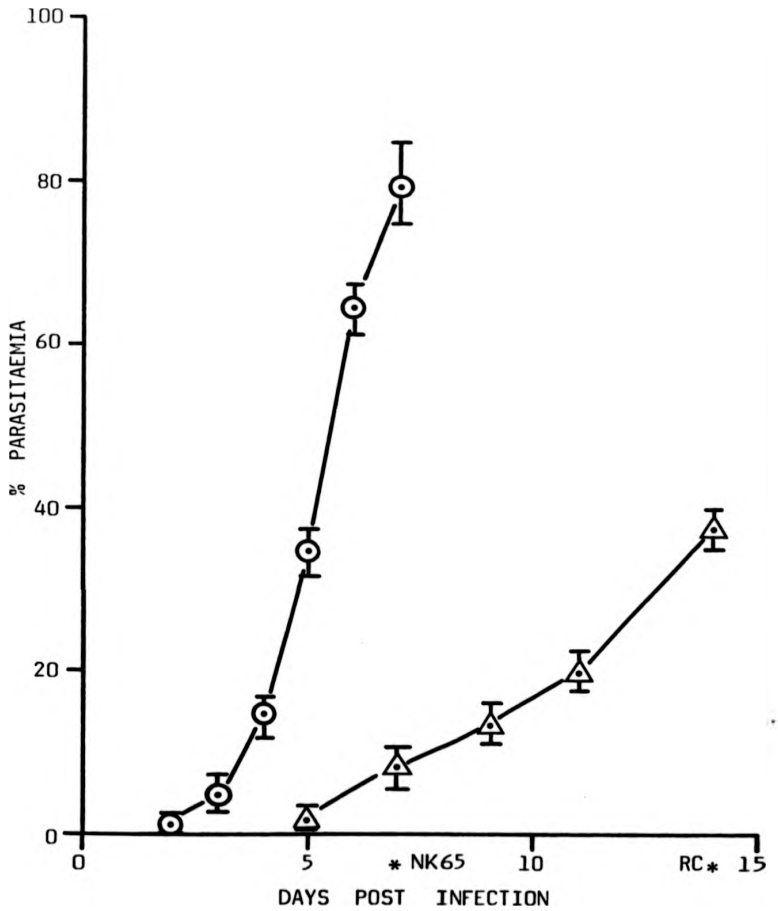

Fig.14

Profile of infection of P.berghei NK 65 ⊙ and P.berghei RC strain △, in T.O. mice.

Inoculum containing 1.0×10^6 infected red cells from donor mouse was inoculated intraperitoneally on day 0 . Average weight of mice is 17.5g.

Points are mean ± S.D. of four experiments, with 5 mice per experiment. Asterisks indicate usual time of death of infected mice.

3.4 TIME COURSE OF (^{14}C) CHLOROQUINE UPTAKE.

The curve obtained for the time course of the uptake of chloroquine by P. berghei NK 65 infected erythrocytes is a rectangular hyperbola (Fig 15) and suggests that the uptake is of first order kinetics. The initial rate of uptake was rapid and could not be determined accurately. However, uptake of drug appeared to attain a steady state at 40 - 45 minutes.

Since the uptake was first order, it can be described by the equation :

$$\frac{d(x)}{dt} = k' (a-x)$$

where a is the initial concentration of drug. The characteristics of this system is that for each time interval (within the linear uptake period) a constant fraction x is taken up (Voicu and Olinescu, 1977). From the slopes of the linear portion of the graphs, the infected erythrocytes accumulated drug at a rate 10 times the uninfected. K' is rate constant and t is time elapsed.

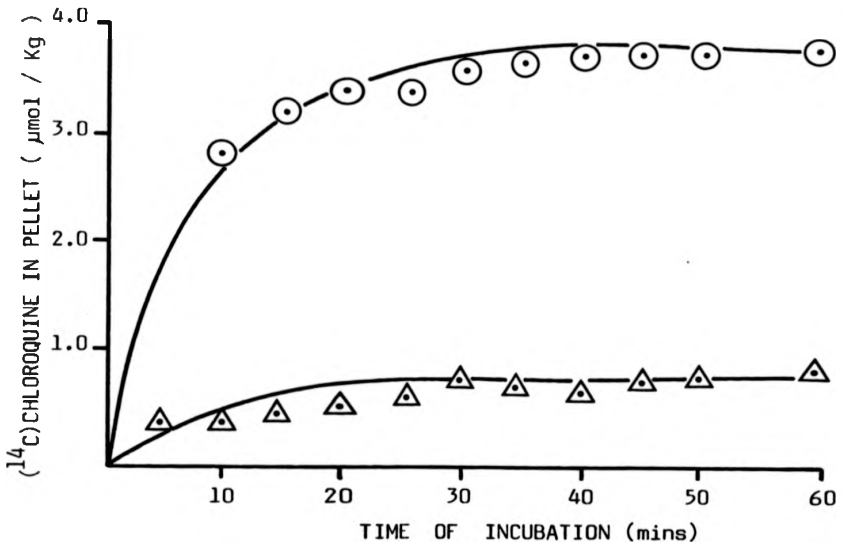

Fig. 15

Time course of uptake of (^{14}C)chloroquine by T.O.mouse erythrocytes.

⊙ T.O. mouse infected with P.berghei NK 65

△ Uninfected T.O. mouse erythrocytes.

Washed erythrocytes were incubated in 50 nmol/litre chloroquine in SB II at 37°C for varying periods of time the initial pH of incubating medium was 7.4. Points on curve are mean of three experiments. Five minutes was the earliest possible time a determination could be made using this technique.

3.5 RELATIONSHIP OF PARASITAEMIA TO DRUG UPTAKE.

The degree of parasitaemia was found to be directly proportional to drug uptake in both *P. berghei* NK 65 and RC strain infected T.O. mouse erythrocytes. Fig 16 shows the correlation of (^{14}C) chloroquine uptake with parasitaemia in *P. berghei* NK 65 infected T.O. mouse erythrocytes. Uptake of (^{14}C) chloroquine by *P. berghei* RC strain infected T.O. mouse erythrocytes was also found to fit into linear regression line drawn for *P. berghei* NK 65 at the appropriate parasitaemia.

The trend equation for the regression line was: $y = (0.06x + 1.1)$ which is in the form of the straight line equation $y = mx + c$, where x is the percentage parasitaemia and y is drug uptake in μmol/kg. The initial (^{14}C) chloroquine concentration in the medium was 5.0×10^{-8} mol/litre. When the data obtained for (^{14}C) chloroquine uptake at different parasitaemias of *P. berghei* RC infected erythrocytes was included with those of *P. berghei* NK 65, the points were found to fit a line with a correlation coefficient of 0.998 (with $P < 0.001$) The trend line equation was $y = (0.06 + 1.2)$ Fig 17 shows the regression for *P. berghei* NK and RC strains.

It is interesting to observe that the intercept on the y-axis (0% parasitaemia) corresponds, to the uptake of (^{14}C) chloroquine by uninfected erythrocytes in SBI. (see Table 2).

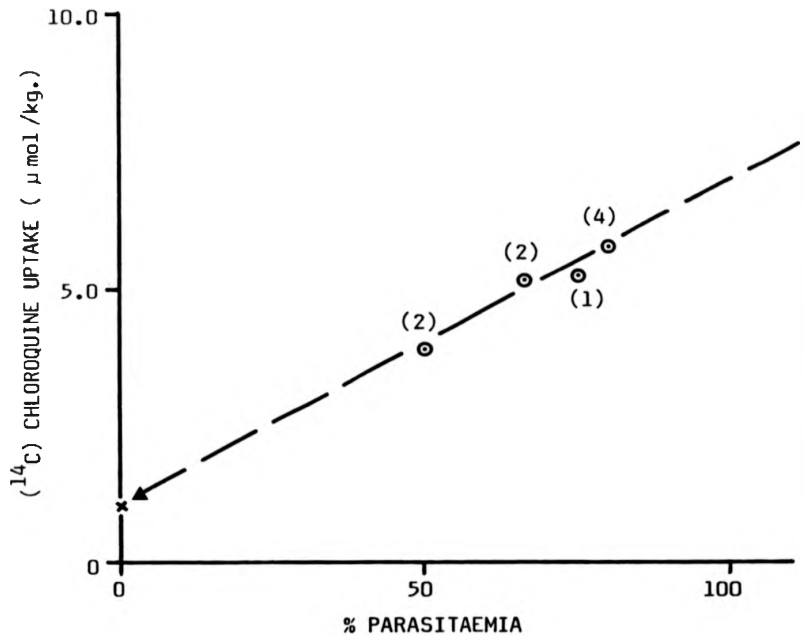

Fig. 16

Correlation of (^{14}C) chloroquine uptake (in P.berghei NK 65) with parasitaemia.

Numbers in parenthsis are no. of experiments.

Dashed line is regression line with $P < 0.001$.

All experimentswere carried out in SB1 containing 5.0×10^{-8} mol/l (^{14}C) chloroquine at 37 °C.

The trend line equation is : $y = 0.06x + 1.1$

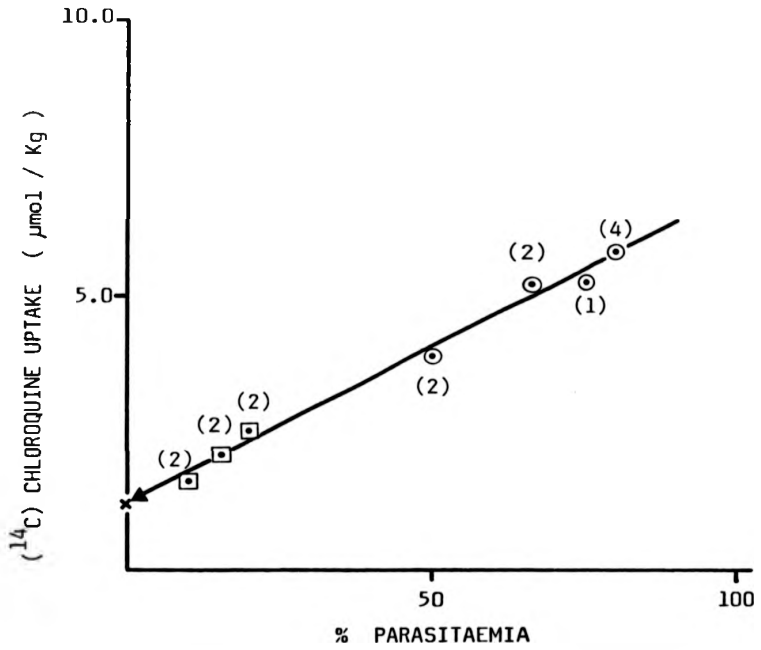

Fig. 17

Correlation of (^{14}C) chloroquine uptake in P.berghei NK 65 ⊙ , and P.berghei RC ⊡ , with parasitaemia.

Numbers in parenthesis are no. of experiments.

Dashed line is regression line with $P < 0.001$.

The trend line equation is $y = \{ 0.06x + 1.2 \}$

Correlation coefficient of points is 0.998.

3.6 EFFECTS OF VARIATION OF IONIC COMPOSITION OF INCUBATION MEDIUM ON UPTAKE OF (^{14}C) CHLOROQUINE

3.6.1 UNINFECTED ERYTHROCYTES

High K^+ buffer (HKB) at pH 7.4 depressed uptake of drug by uninfected erythrocytes. After incubation for 45 minutes at 37°C, the uptake of drug was 0.62 ± 0.01 µmol/kg from a medium concentration of 5.0×10^{-8} mol/litre, (^{14}C) chloroquine. This is an inhibition of 48.3%, when compared to an uptake of 1.2 ± 0.05 µmol/kg using standard medium (SBI) The decrease in drug uptake in high K^+ medium was statistically significant ($p < 0.05$, student t test).

There was also a similar reduction in drug uptake ratio. Here, the drug uptake ratio is the ratio of the distribution of drug between the erythrocyte and the medium at equilibrium. The concentration of drug taken up by the erythrocyte (V_s) = F_s(Se), where F_s represents uptake ratio and Se is the equilibrium extra cellular drug concentration in the medium (in µmol/litre). V_s is in µmol/kg. The equation $V_s = F_s$ (Se) is adapted from Whitfield and Schworer (1978) who used F_s to represent the "carrier" coefficient for uptake of D-glucose into avian erythrocytes. Applying the above equation,

we obtain an uptake ratio of 13.2 when the incubation medium is high K^+ buffer (HKB) and 27.9, when standard medium 1 (SBI) is the incubation medium. There is thus a two fold variation in the activity of the uptake system as indicated by the uptake ratio (F_s) (see Tables 2-3).

3.6.2 P. BERGHEI NK 65 INFECTED ERYTHROCYTES

High K^+ buffer did not cause any marked inhibition of (^{14}C) chloroquine uptake by erythrocytes infected with P. berghei NK 65. Uptake of (^{14}C) chloroquine from high K^+ incubation medium containing 5.0×10^{-8} mol/litre (^{14}C) chloroquine was 3.91 ± 0.04 µmol/kg, while the uptake in standard medium 1 was 3.99 ± 0.06 µmol/kg. The drug uptake ratio was 5171.9 in the presence of high K^+ medium and 5377.4 in standard medium 1; indicating that the uptake system was not adversely affected by high K^+ in the incubating medium. (see also Tables 2 - 3).

3.6.3 P. BERGHEI RC STRAIN INFECTED ERYTHROCYTES.

High K^+ buffer (HKB) inhibited the uptake of (^{14}C) chloroquine by P. berghei RC infected erythrocytes. Uptake of (^{14}C) chloroquine was 3.09 $\pm$ 0.10 µmol/kg in high K^+ medium compared to 3.89 $\pm$ 0.10 µmol/kg obtained in standard medium 1. This represents an inhibition of 20.6%.

The uptake ratio also decreased from 1088.8 in the presence of standard medium to 543.1 in the high K^+ medium. This is a two fold decrease.

It is interesting to note that though RC strain infected erythrocytes accumulated almost as much drug as P. berghei NK 65 infected erythrocytes (especially in SBI, see Table 2a) the comparative drug uptake ratios (ie uptake ratio in HKB divided by uptake ratio in SBI) was similar for both uninfected and RC strain infected erythrocytes (see Table 3).

TABLE 2a ; COMPARISON OF (^{14}C) CHLOROQUINE UPTAKE IN 2 MEDIA.

STRAIN	High K^+ medium HKB	High Na^+ medium SBI
URBC	0.62 ± 0.01	1.2 ± 0.05
P. berghei NK 65	3.91 ± 0.04	3.99 ± 0.06
P. berghei RC	3.09 ± 0.02	3.80 ± 0.1

URBC = uninfected erythrocytes.

Results (in µmol/kg) are given as mean ± S.D (3 experiments)

Parasitaemia of *P. berghei* NK 65 approximately 50%; *P. berghei* RC, 40%.

TABLE 2b; MEDIUM CONCENTRATION OF (^{14}C) CHLOROQUINE AFTER 45 MINS. INCUBATION AT 37°C.

STRAIN	Medium concentration of chloroquine (µmol/litre)	
	High K^+ medium	High Na^+ medium
URBC	4.69×10^{-2}	4.3×10^{-2}
P.berghei NK 65	7.56×10^{-4}	7.42×10^{-4}
P.berghei RC	5.69×10^{-3}	3.49×10^{-3}

TABLE 3; VARIATION IN DRUG UPTAKE RATIOS.

STRAIN	F_s HKB	F_s SBI	Fc= $\frac{F_s \text{ in HKB}}{F_s \text{ in SBI}}$
URBC	13.2	27.9	0.47
P.berghei NK 65	5171.9	5377.4	0.96
P.berghei RC	543.1	1088.8	0.50

3.7 EFFECT OF D-GLUCOSE ON UPTAKE OF (^{14}C) CHLOROQUINE

3.7.1 UNINFECTED ERYTHROCYTES

Between the concentration range 0.1 - 400 mmol/litre, D-glucose showed little effect on the uptake of (^{14}C) chloroquine by uninfected erythrocytes. At a (^{14}C) chloroquine concentration of 5.0×10^{-8} mol/litre, in SBI, the uptake of (^{14}C) chloroquine by uninfected T.O. mouse erythrocytes was 1.05 ± 0.162 µmol/kg (n = 7). In the absence of D-glucose in the incubating medium, T.O. mouse erythrocytes could take up 0.90 µmol/kg, (^{14}C) chloroquine. The drug uptake ratio (erythrocytes : medium) remained virtually unchanged in the presence or absence of D-glucose. In the incubating medium (SBI) alone, the uptake ratio was 32.53 ± 4.1, while in the presence of D-glucose in the medium, the uptake ratio was 38.50 ± 4.1. (see Table 4)

3.7.2 P. BERGHEI NK 65 INFECTED ERYTHROCYTES.

In the absence of D-glucose, P. berghei NK 65 infected erythrocytes can accumulate chloroquine up to 2.37 $\pm$ 0.3 µmol/kg at an initial medium (^{14}C) chloroquine concentration of 5.0×10^{-8} mol/litre. With D-glucose in the medium (SBI), there is stimulation of (^{14}C) chloroquine uptake. This activation of drug uptake is linear with the D-glucose concentration range of 0.125 - 2.0 mmol/litre. The increased uptake plateaus between 2.0 - 20 mmol/litre and then declines steadily at D-glucose concentrations greater than 40 mmol/litre. Fig 18a shows the log-dose relationship of D-glucose and (^{14}C) chloroquine uptake. At maximum stimulation, an activation of up to 65.82 % over the total uptake in the absence of D-glucose in the incubating medium (SBI) is obtained. The peak uptake (3.93 $\pm$ 0.3 µmol/kg) represents an uptake ratio of 801.7 $\pm$ 37.7 compared to an uptake ratio of 56.30 $\pm$ 5.2 obtained in the absence of D-glucose. (see table 5)

3.7.3 P. BERGHEI RC STRAIN INFECTED ERYTHROCYTES.

Presence of D-glucose in the incubating medium (SBI) showed a small but consistent effect on the uptake of (^{14}C) chloroquine into P. berghei RC infected erythrocytes. In the absence of D-glucose in the incubating medium, uptake of radiolabelled drug amounted to 2.92 $\pm$ 0.13 µmol/kg. With 5.0 mmol/litre D-glucose in the medium, uptake amounted to 3.48 $\pm$ 0.28 µmol/kg. However, the uptake ratios showed a wide variation of 165.90 $\pm$ 24.9 for uptake in the absence of D-glucose and 749.87 $\pm$ 65.96 for uptake in the presence of 5.0 mmol/litre D-glucose. The maximal effect of D-glucose (5.0 mmol/litre) produced an activation of uptake of (^{14}C) chloroquine of 19.2 % (see Table 6).

3.8 EFFECT OF 2-DEOXY-D-GLUCOSE ON UPTAKE OF (^{14}C) CHLOROQUINE.

3.8.1 UNINFECTED T.O. MOUSE ERYTHROCYTES.

2-deoxy-D-glucose showed virtually no inhibition of (^{14}C) chloroquine uptake in this model. The uptake in the presence of 5.0 mmol/litre 2-deoxy-D-glucose amounted to 0.88 $\pm$ 0.05 µmol/kg compared to 1.05 $\pm$ 0.162 µmol/kg total uptake with 5.0 mmol/litre D-glucose alone in SBI. Also there was little change in the drug uptake ratios. An uptake ratio of 38.30 $\pm$ 4.1 was obtained in the presence of 5.0 mmol/litre D-glucose alone in the medium and 32.62 $\pm$ 4.0 in the presence of 5.0 mmol/litre D-glucose and 5.0 mmol/litre, 2-deoxy-D-glucose. Table 4 summarises the effect of D-glucose and 2-deoxy-D-glucose on uptake of radiolabelled chloroquine by uninfected erythrocytes.

TABLES 4,5, AND 6. LEGEND.

(a) Concentration of (^{14}C) chloroquine after 45 minutes incubation at 37°C in standard buffer.

(b) Uptake ratio is ratio of (^{14}C) chloroquine concentration in cells (μmol/kg) to free (^{14}C) chloroquine (μmol/l) left in incubating medium after 45 min. incubation at 37°C.

(c) % Parasitaemia of P. berghei NK infected erythrocytes is 46.4 $\pm$ 10.0.

(d) % Parasitaemia of P. berghei RC infected erythrocytes is 30.96 $\pm$ 7.5

Results given as mean $\pm$ S.D. of 4 experiments.

TABLE 4. THE EFFECT OF D-GLUCOSE AND 2-DEOXY-D-GLUCOSE ON THE UPTAKE OF (^{14}C) CHLOROQUINE BY UNINFECTED ERYTHROCYTES.

Additions	Uptake (a) µmol/kg	Uptake Ratio (b)
Incubation medium (SBI) only	0.90 ± 0.06	32.53 ± 4.0
+ 5.0M D-glucose	1.05 ± 0.162	38.30 ± 4.1
+ 5.0 mM D-glucose + 5.0 mM 2-Deoxy-D-glucose	0.88 ± 0.05	32.62 ± 4.0

TABLE 5. P. BERGHEI NK 65 (c) INFECTED ERYTHROCYTES.

Additions	Uptake µmol/kg	Uptake Ratio
Incubation medium only	2.37 ± 0.3	56.30 ± 5.21
+ 5.0 mM D-glucose	3.93 ± 0.3	801.7 ± 37.7
+ 5.0 mM D-glucose + 5.0 mM 2-deoxy-D-glucose	1.47 ± 0.02	33.78 ± 4.3

TABLE 7 - LEGEND

(e) Total drug uptake in the presence of 5.0 mmol/l D-glucose in incubating medium (SBI)

(f) Uptake ratio for (^{14}C) chloroquine uptake in incubating medium alone regarded as having an index of 1.0.

Results calculated from "means" only.
URBC is uninfected T.O. mouse red blood cells.

TABLE 6. P. BERGHEI RC[(d)] INFECTED ERYTHROCYTES.

Additions	Uptake µmol/kg	Uptake Ratio
Incubation medium only	2.92 ± 0.13	165.90 ± 24.9
+ 5.0 mM D-glucose	3.48 ± 0.28	749.87 ± 65.96
+ 5.0 mM D-glucose + 5.0 mM 2-deoxy-D-glucose	3.04 ± 0.05	366.5 ± 25.7

TABLE 7 . SUMMARY OF INHIBITION PROFILE AND VARIATION OF UPTAKE RATIO OF (^{14}C) CHLOROQUINE BY T.O. MOUSE ERYTHROCYTES.

	Percentile Inhibition of mean of maximal drug uptake[(e)]		Change in uptake Index (f)	
STRAIN	+ 5.0 mmol/l 2-Deoxy-D-glucose	Incubation medium only	+ 5.0 mmol/l D-glucose	+ 5.0mmol/l 2-Deoxy-D-glucose
URBC	16.2	14.29	1.17	1.002
NK 65	62.60	39.7	14.24	0.6
RC strain	12.64	16.9	4.52	2.2

3.8.2 P. BERGHEI NK 65 INFECTED T.O. MOUSE ERYTHROCYTES.

The activation of (^{14}C) chloroquine uptake induced by D-glucose (2.0 - 20 mmol/litre) was consistently inhibited by 2-deoxy-D-glucose. Within the 2-deoxy-D-glucose concentration range of 2.0 - 20 mmol/litre, the inhibition of (^{14}C) chloroquine uptake was 62.75 % $\pm$ 2.33. However, when compared to the uptake in the absence of D-glucose, the resultant inhibition was 39.57% $\pm$ 3.78.

The drug uptake ratio was also reduced significantly, from 801.7 $\pm$ 37.7 in the presence of D-glucose (5.0 mmol/l) to 33.78 $\pm$ 4.3 in the presence of 5 mmol/l, 2-deoxy-D-glucose and 5 mmol/l, D-glucose. Fig 18b shows the inhibitory effect of deoxy-D-glucose on uptake of radiolabelled chloroquine by P. berghei NK 65 - infected erythrocytes. Table 5 also shows the effect of D-glucose and 2-deoxy-D-glucose on uptake of (^{14}C) chloroquine by this model.

3.8.3 P. BERGHEI RC STRAIN INFECTED T.O. MOUSE ERYTHROCYTES.

Between 2.0 - 20 mmol/l, 2-deoxy-D-glucose, there was fairly uniform inhibition of (^{14}C) chloroquine uptake of 18.8% $\pm$ 4.15, in the presence of 5.0 mmol/l, D-glucose in the incubating medium. When compared to uptake of (^{14}C) chloroquine in the absence of D-glucose in the incubating medium (SBI), the inhibition amounted to only 1.2% $\pm$ 0.20. However, very high concentrations (100 - 200 mmol/l) of 2-deoxy-D-glucose, showed inhibition of 27.0 - 32.8% at incubating medium (SBI) D-glucose concentration of 5 mmol/l.

At 2-deoxy-D-glucose concentration of 5.0 mmol/l, the total uptake of (^{14}C) chloroquine was 3.04 $\pm$ 0.05 µmol/kg and corresponded to an uptake ratio of 366.6 $\pm$ 25.7, compared to an uptake ratio of 165.90 $\pm$ 24.9 for uptake of radiolabelled drug in the absence of D-glucose and 749.87 $\pm$ 65.96 for uptake in the presence of 5.0 mmol/l D-glucose alone, in the incubating medium. Table 6, shows the effect of D-glucose and 2-deoxy-D-glucose on uptake of (^{14}C) chloroquine by *P. berghei* RC strain infected T.O. mouse erythrocytes.

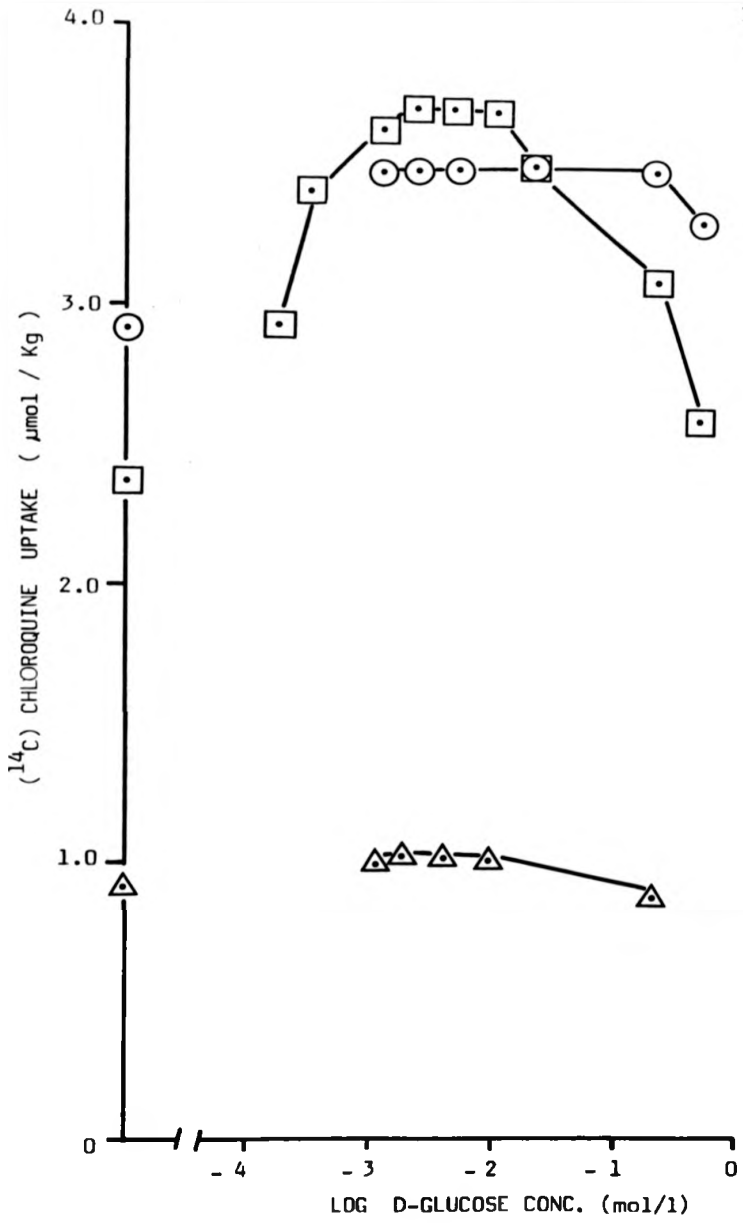

Fig. 18a
Effect of D-glucose on (^{14}C) chloroquine uptake

⊡ Uptake into P.berghei NK 65 infected T.O.mouse erythrocytes. Average parasitaemia = 45%

⊙ Uptake into P.berghei RC strain infected T.O. mouse erythrocytes. Average parasitaemia = 38%

△ Uptake into uninfected T.O. mouse erythrocytes

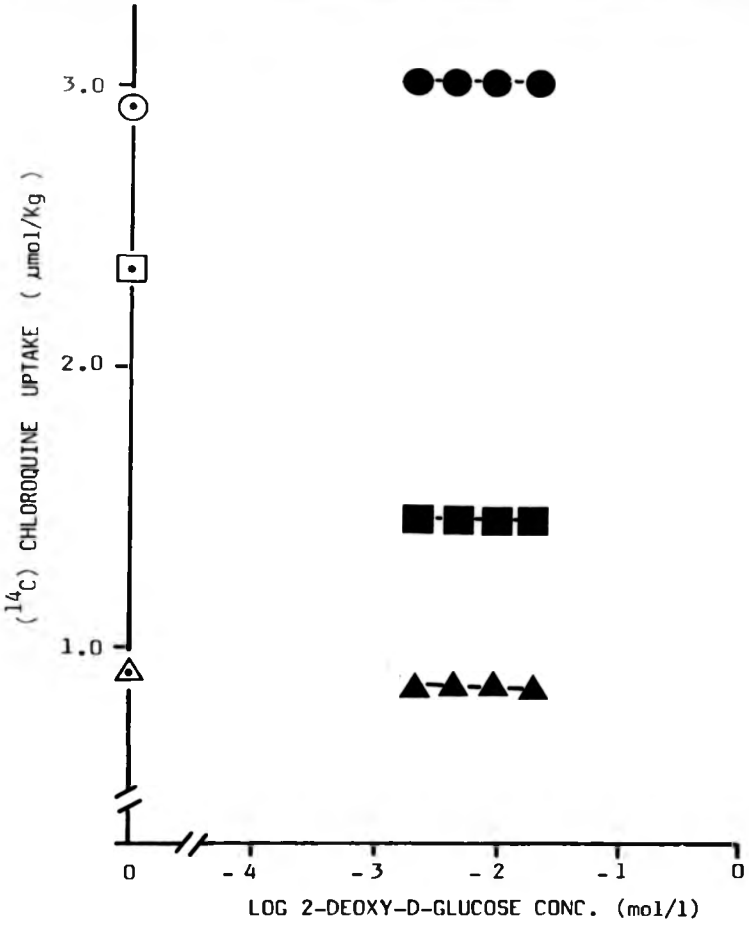

Fig. 18b

Effect of 2-Deoxy-D-glucose on uptake of (^{14}C) chloroquine.

■ Uptake into P.berghei NK 65 infected T.O. mouse erythrocytes. % parasitaemia = 45

● Uptake into P.berghei RC strain infected T.O. mouse erythrocytes. % parasitaemia = 38

▲ Uptake into uninfected T.O. mouse erythrocytes.

Incubating medium in addition to various concentrations of 2-Deoxy-D-glucose contained 5.0 mmol/l D-glucose.

Points on y axis at zero Deoxy-D-glucose concentrations represent uptake in the absence of D-glucose.

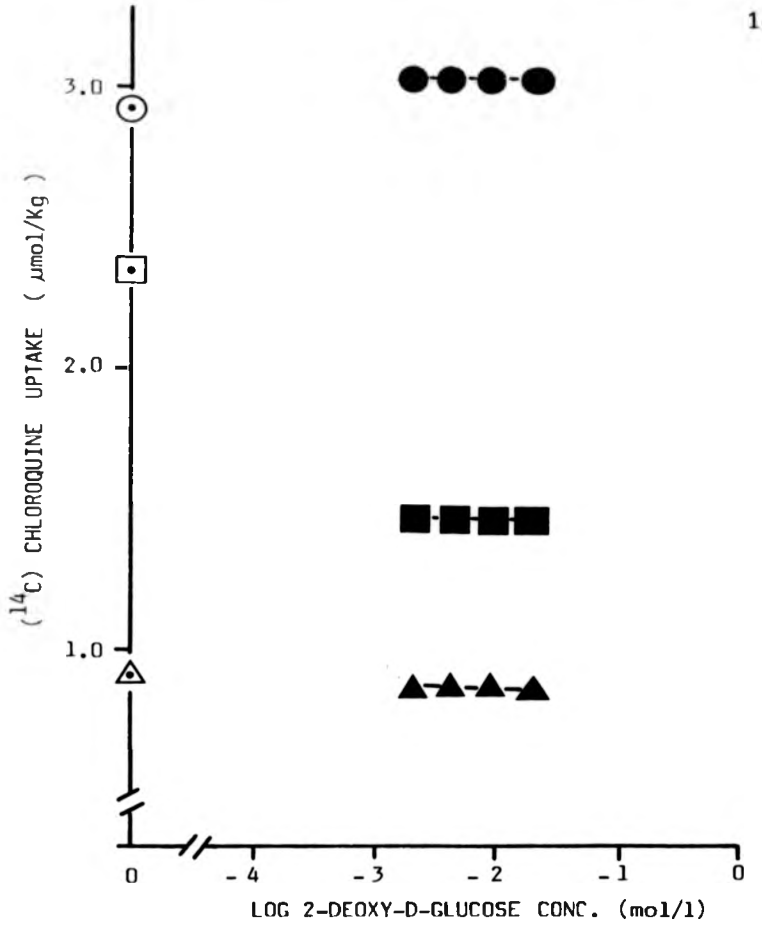

Fig. 18b

Effect of 2-Deoxy-D-glucose on uptake of (^{14}C) chloroquine.

■ Uptake into P.berghei NK 65 infected T.O. mouse erythrocytes. % parasitaemia = 45

● Uptake into P.berghei RC strain infected T.O. mouse erythrocytes. % parasitaemia = 38

▲ Uptake into uninfected T.O. mouse erythrocytes.

Incubating medium in addition to various concentrations of 2-Deoxy-D-glucose contained 5.0 mmol/l D-glucose.

Points on y axis at zero Deoxy-D-glucose concentrations represent uptake in the absence of D-glucose.

3.9 EFFECT OF 3-O-METHYL-D-GLUCOSE ON UPTAKE OF (^{14}C) CHLOROQUINE.

This analogue of D-glucose was tested within the concentration range of 2.5 - 400 mmol/l. There was no significant effect on uptake of (^{14}C) chloroquine by *P. berghei* NK 65, *P. berghei* RC and uninfected erythrocytes; in the absence of and in the presence of 5 mmol/l D-glucose.

3.10 EFFECT OF L-GLUCOSE ON UPTAKE OF (^{14}C) CHLOROQUINE.

Between the concentration range of 2.5 - 400 mmol/l, L-glucose did not show any significant effect on the uptake of (^{14}C) chloroquine by *P. berghei* NK 65, *P. berghei* RC and uninfected T.O. mouse erythrocytes; both in the presence and absence of D-glucose (5 mmol/l).

3.11 EFFECT OF IONOPHORES ON (^{14}C) CHLOROQUINE UPTAKE.

3.11.1 MONENSIN AND NIGERICIN

These carboxylic polyether antibiotics (structures in appendix) exchange protons across membranes for Na^+ and K^+ respectively (Pressman. 1976). These "ionophores" showed significant inhibition of (^{14}C) chloroquine uptake by both infected and uninfected T.O. mouse erythrocytes. The inhibition was both dose and time dependent.

3.11.2 DOSE RESPONSE ANALYSIS

Between the concentration range of 1.0×10^{-10} - 2.0×10^{-6} mol/l, both Monensin and Nigericin showed graded and dose dependent inhibition of drug uptake into *P. berghei* - infected and uninfected erythrocytes. In the high Na^+ incubation medium (SBI) the ID_{50} (concentration causing 50% inhibition of maximal chloroquine uptake) for monensin was $2.5 - 5.0 \times 10^{-7}$ mol/l This ID_{50} was increased to 1.0×10^{-6} mol/l when the incubation medium was SB2 and contained a lower Na^+ concentration. In the presence of high K^+ (eg In HKB) 5.0×10^{-7} mol/l monensin, caused only 25.41% $\pm$ 4.6 inhibition of (^{14}C) chloroquine uptake by erythrocytes infected with *P. berghei* NK 65.

In the presence of high K^+ concentration in the incubation medium (HKB or SB2 supplemented with 40 mmol/l KCl) Nigericin caused significant inhibition of drug uptake into both *P. berghei* infected and uninfected T.O. mouse erythrocytes. The ID_{50} for Nigericin was $1.0 - 2.0 \times 10^{-7}$ mol/l for *P. berghei* NK 65 infected erythrocytes and $0.6 - 2.0 \times 10^{-7}$ mol/l for uninfected erythrocytes. In high Na^+ incubating medium alone, 2.0×10^{-7} mol/l Nigericin could only elicit 12% $\pm$ 2.6 inhibition of drug uptake by erythrocytes infected with *P. berghei* NK 65.

However, at concentrations greater than 3.0×10^{-6} mol/l both Monensin and Nigericin caused partial lysis of the incubated T.O. mouse erythrocytes and all such results were discarded. Figs 19 and 20 are log dose response curves for Monensin, while Figs 21 and 22 are log dose response curves for Nigericin. Fig 33 shows the effect of Monensin on (^{14}C) chloroquine uptake at varying medium (^{14}C) chloroquine concentration.

The inhibition of drug uptake by Monensin was also found to be noncompetitive. Fig 23 shows the inhibition of chloroquine uptake by monensin in erythrocytes infected with P. berghei NK 65. The inhibition is noncompetitive since Umax (maximum uptake at infinite medium chloroquine concentration) is changed from 50 to 7.46 µmol/kg.

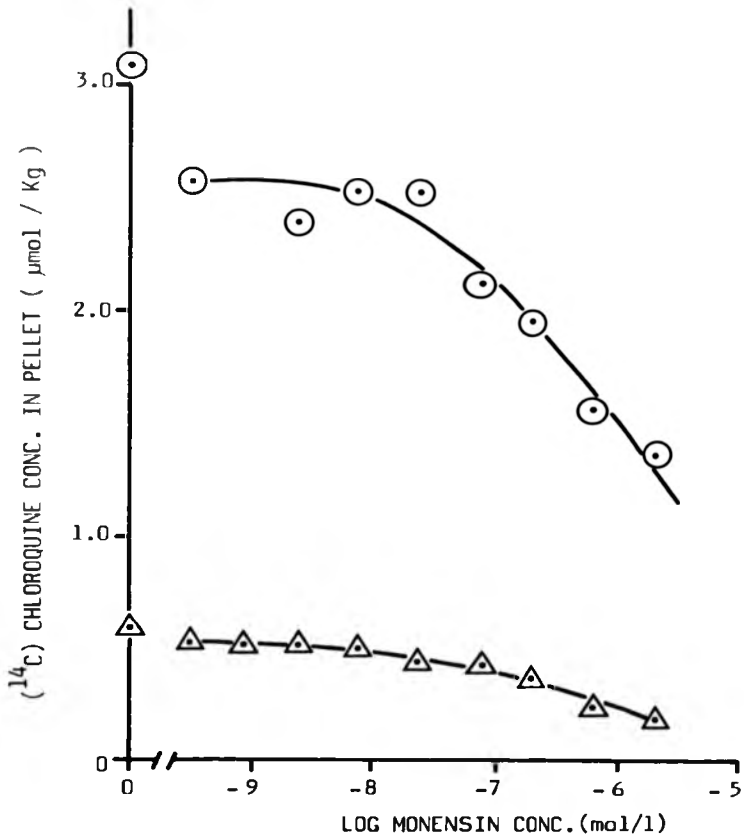

Fig. 19

Log - Dose response curve of Monensin

⊙ Uptake of (^{14}C) chloroquine by P.berghei NK 65 infected T.O. mouse erythrocytes.

△ Uptake of (^{14}C) chloroquine by uninfected T.O. mouse erythrocytes.

Infected and uninfected T.O. mouse erythrocytes were incubated in SB II medium (reduced Na^+) at 37°C for 45min. Initial concentration of radio labelled chloroquine in medium was 5.0×10^{-8} mol/l and initial pH of incubating medium was 7.4 .

Points on the curves are means of three experiments. Points on ordinate indicate value of drug uptake in absence of monensin.

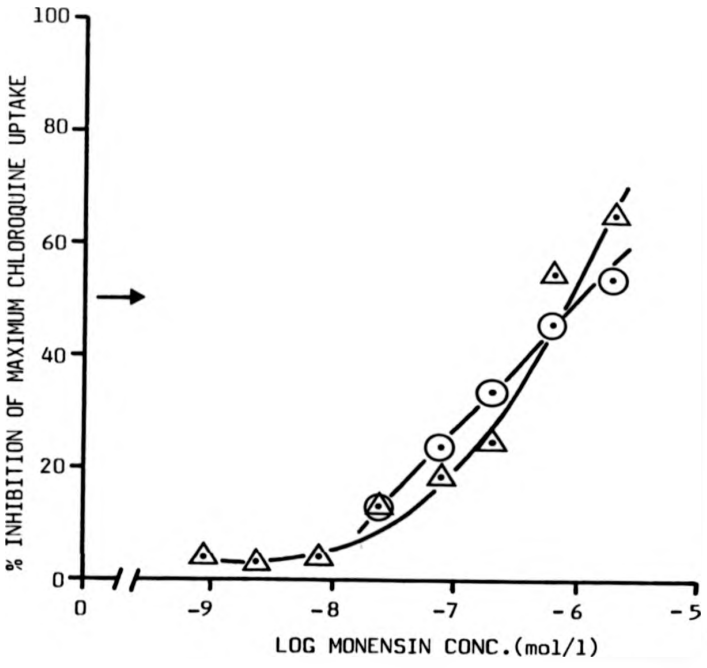

Fig. 20

Log - Dose response curve of Monensin

⊙ Percentage of maximum uptake of (^{14}C) chloroquine by P.berghei NK 65 infected T.O. mouse erythrocytes.

△ Percentage of maximum uptake of (^{14}C) chloroquine by uninfected T.O. mouse erythrocytes.

Arrow indicates $I.D_{50}$ and is approximately 1.0×10^{-6} mol/l for both uninfected mouse erythrocytes and P.berghei NK 65 infected T.O. mouse erythrocytes.
Incubating medium is SBII. (reduced Na^+)
ID_{50} of Monensin for P.berghei NK 65 infected T.O.mouse erythrocytes in SB 1 (high Na^+) is $2.5 - 5.0 \times 10^{-7}$ mol/l.
ID_{50} is regarded as concentration of ionophone required to inhibit uptake of radiolabelled drug by 50%

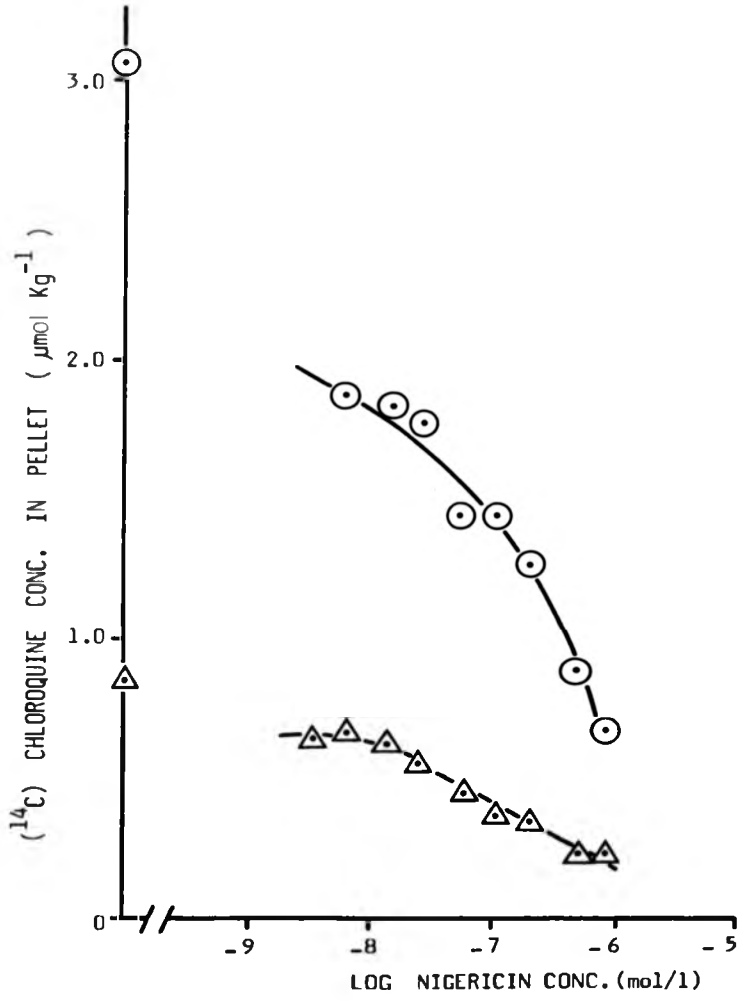

Fig. 21

Log - Dose response curve of Nigericin

⊙ Uptake of (^{14}C) chloroquine by P.berghei NK 65 infected T.O. mouse erythrocytes.

△ Uptake of (^{14}C) chloroquine by uninfected T.O. mouse erythrocytes.

Incubation medium was SBII supplemented with 40 mmol/l. KCl
Points on the curves are means of three experiments.
(Total K^+ conc. = 45 mmol/l).
Points on ordinate indicate value of drug uptake in absence of nigericin.

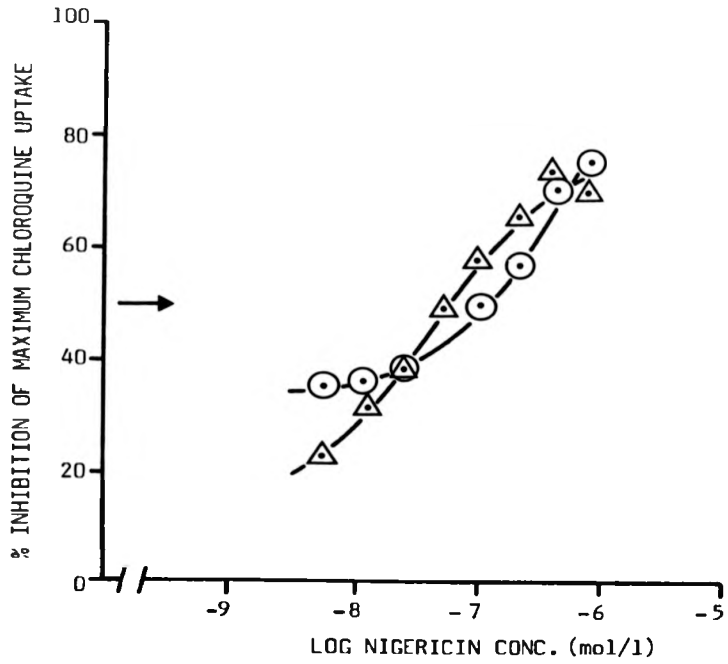

Fig. 22

Log - Dose response curve of Nigericin

⊙ Percentage of maximum uptake of (^{14}C) chloroquine by P.berghei NK 65 infected T.O.mouse erythrocytes.

△ Percentage of maximum uptake of (^{14}C) chloroquine by uninfected T.O. mouse erythrocytes.

Arrow indicates ID_{50} and is 1.0×10^{-7} mol/l for P.berghei NK 65 infected T.O. mouse erthyrocytes.

Incubating medium is SBII supplemented with 40 mmol/l KCl. (Total K^+ concentration = 45 mmol/l).

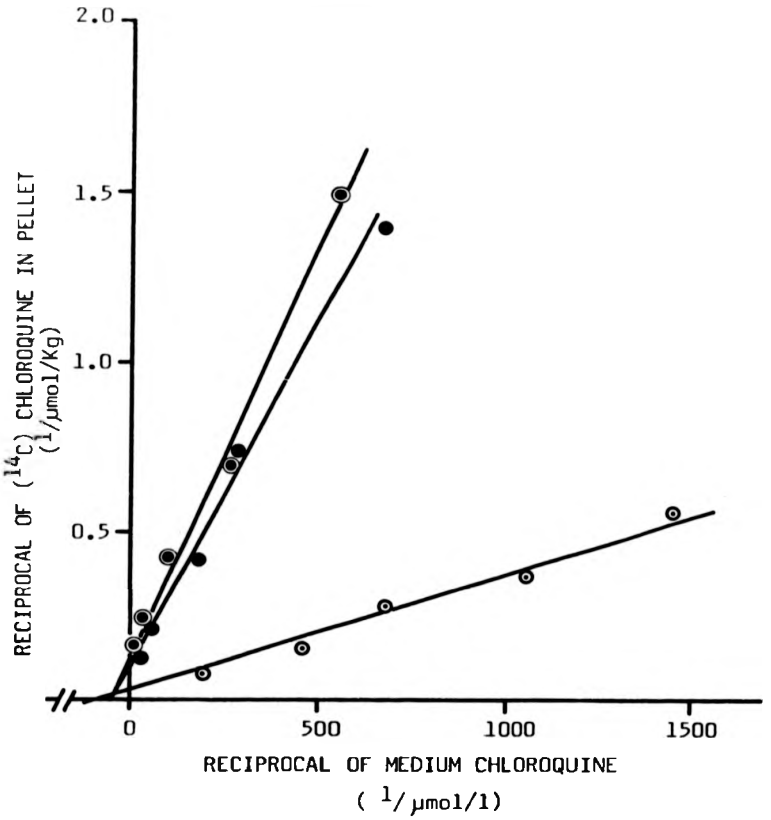

Fig. 23

Double reciprocal plot of uptake of radioactive chloroquine by P.berghei NK 65 infected T.O. mouse erythrocytes in the presence of monensin.

- ⊙ Control, No Monensin in incubation medium.
- ● 5.0×10^{-8} mol/l Monensin in incubation medium.
- ◉ 2.5×10^{-7} mol/l Monensin in incubation medium.

Incubation was for 45 min at 37°C in SBII

Lines are calculated regression lines.

Abscissa is reciprocal of medium chloroquine at equilibrium.

3.12 TIME COURSE OF INHIBITION OF (^{14}C) CHLOROQUINE UPTAKE BY IONOPHORES.

3.12.1 MONENSIN AND NIGERICIN

Inhibition of (^{14}C) chloroquine uptake by monensin and nigericin is proportional to the time of pre-incubation of T.O. mouse erythrocytes in the ionophore. The relationship of inhibition to time pre-incubated in 5.0×10^{-7} mol/l Monensin is relatively linear for the first 15 min. of preincubation and attains equilibrium by 45 minutes.

Fig 24 illustrates the effect of preincubation of *P. berghei* NK 65 infected mouse erythrocytes over 60 minutes in 5.0×10^{-7} mol/l Monensin in SB2. Nigericin in K^+ supplemented buffer showed a similar profile.

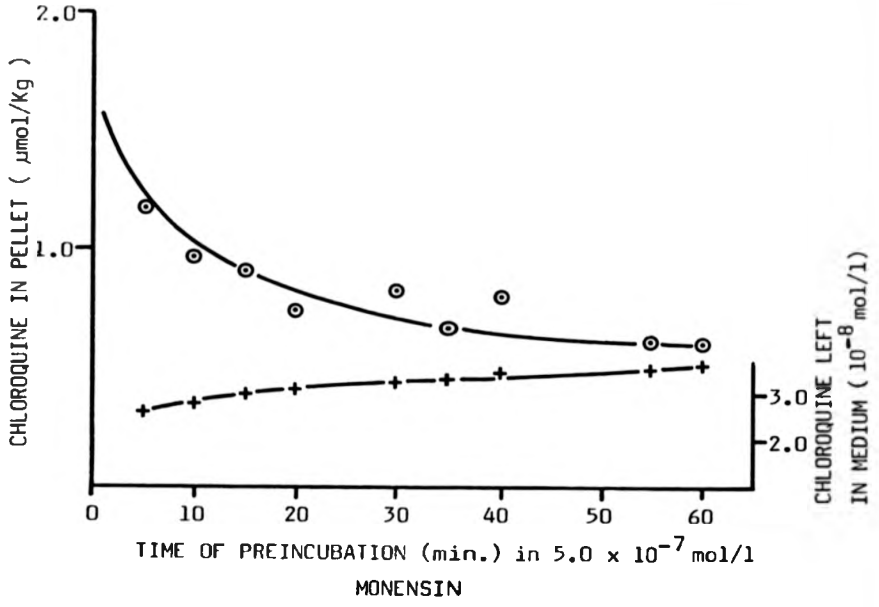

Fig. 24

Effect of preincubation of mouse erythrocytes in 5 x 10^{-7}mol/l Monensin.

⊙ Uptake of (^{14}C) chloroquine by P.berghei NK 65 infected T.O. mouse erythrocytes.

\+ (^{14}C) chloroquine concentration left in incubation medium at the end of 45 mins incubation at 37 ^{o}C . Initial concentration of (^{14}C) chloroquine in incubation medium was 5.0 x 10^{-8}mol/l.

Inhibition of (^{14}C) chloroquine uptake into P.berghei RC infected and uninfected T.O. mouse erythrocytes showed the same profile. A similar result was obtained for Nigericin in the appropriate buffer.

3.12.2 VALINOMYCIN

This cyclic dodecadepsipeptide antibiotic catalyses electrogenic transfer of K^+ across membranes (Pressman, 1976). Over the concentration range of 1.0×10^{-10} - 1.0×10^{-6} mol/l, valinomycin in high Na^+ medium (SB2) showed no effect on uptake of (^{14}C) chloroquine. In the presence of SB1 supplemented with 40 mmol/l KCl, valinomycin (2.0×10^{-7} mol/l) showed only 8% inhibition of uptake of (^{14}C) chloroquine by both infected and uninfected T.O. mouse erythrocytes.

3.13.1 EFFECT OF OUABAIN ON UPTAKE OF (^{14}C) CHLOROQUINE.

The Na^+/K^+ ATPase inhibitor ouabain was tested for its effects on uptake of drug. In the concentration range of 5.0×10^{-4} - 5.0×10^{-5} mol/l, Ouabain inhibited uptake of radiolabelled chloroquine in uninfected, *P. berghei* NK 65 and RC strain infected T.O. mouse erythrocytes. Maximum inhibition of drug uptake was obtained at ouabain concentrations of 1.0×10^{-4} mol/l and above. This inhibition for uninfected T.O. mouse erythrocytes was 0.27 ± 0.04 µmol/kg and represented a percentage inhibition of 25.48 ± 3.51. For *P. berghei* NK 65 infected erythrocytes, inhibition was 0.75 ± 0.1 µmol/kg equivalent to $23.3 \pm 5.0\%$, and 1.01 ± 0.19 µmol/kg equivalent to $28.25 \pm 5.5\%$ for *P.berghei* RC infected T.O. mouse erythrocytes (see tables 8, 9 and 10).
It is thus evident that the percentage inhibition of (^{14}C) chloroquine uptake caused by ouabain (1.0×10^{-4} mol/l) was similar in all three models, though the absolute reduction in drug uptake was in the order RC > NK 65 > URBC. Ethanol at concentrations up to 1% did not show any significant inhibition of drug uptake.

On combination of 1.0×10^{-4} mol/l ouabain with 2.0×10^{-7} mol/l Monensin, ouabain partially alleviated the

inhibitory effect of Monensin in SB1 incubating medium. For uninfected T.O. mouse erythrocytes, inhibition caused by 2.0×10^{-7} mol/l Monensin was reduced from 0.51 ± 0.06 µmol/kg to 0.30 ± 0.03 µmol/kg; a reduction of 19.4%. This percentage reduction was statistically significant $P < 0.05$, t-test. There was also similar reduction of inhibition of (^{14}C) chloroquine uptake caused by Monensin in P. berghei NK 65, but a lesser and more variable effect in RC strain infected erythrocytes. For P. berghei NK 65, the reduction was from 1.74 ± 0.06 µmol/kg to 1.1 ± 0.14 µmol/kg; a reduction of 18.2% which was statistically significant, $P = 0.05$, t-test. For P. berghei RC strain infected erythrocytes, there was a slight reduction in the inhibitory effect of Monensin by ouabain, from 1.47 ± 0.3 µmol/kg to 1.19 ± 0.03 umol/kg. However, this reduction was not statistically significant ($P > 0.05$ student t test with DF = 6).

Ouabain (1.0×10^{-4} mol/l) had no alleviating effect on inhibition of (^{14}C) chloroquine uptake caused by Nigericin (2.0×10^{-7} mol/l) in all three systems. For P. berghei NK 65 infected erythrocytes, there was an enhanced depression of uptake, indicating an additive effect, from 42.0 ± 2.0 % inhibition to 48.35 ± 1.35 (see table 9). For RC strain infected cells and for uninfected erythrocytes there was very little difference from the effect of Nigericin alone (see table 8 and 10).

3.13.2 EFFECT OF BIOGENIC AMINES ON UPTAKE OF (^{14}C) CHLOROQUINE.

L-epinephrine and 5-hydroxytryptamine (5HT, serotonin) were the biogenic amines tested. Both showed some inhibition of (^{14}C) chloroquine uptake in SB1 containing 5.0×10^{-8} mol/l (^{14}C) chloroquine, in all three systems. At 2.0×10^{-7} mol/l, L-epinephrine inhibited drug uptake by 47.39 % $\pm$ 3.46 (0.52 $\pm$ 0.06 µmol/kg) in uninfected mouse erythrocytes whilst 5HT (2.0×10^{-7} mol/l) caused a similar inhibition of 48.02% $\pm$ 5.51 (0.53 $\pm$ 0.06 µmol/kg). For P. berghei NK 65 infected T.O. mouse erythrocytes, the inhibition of drug uptake by L-epinephrine was 13.3% $\pm$ 1.87 (0.54 $\pm$ 0.07 µmol/kg) whilst 5HT inhibited drug uptake by 16.6% $\pm$ 3.3 (0.63 $\pm$ 0.05 µmol/kg).

For P. berghei RC strain infected erythrocytes, the inhibition for L-epinephrine and 5HT was 16.6 $\pm$ 3.7 (0.60 $\pm$ 0.13 µmol/kg) and 25.2% $\pm$ 7.9 (0.91 $\pm$ 0.3 µmol/Kg) respectively. It is thus evident that both L-epinephrine and 5HT each caused a similar percentage inhibition and about the same absolute amount of drug uptake was inhibited in all three models by L-epinephrine and 5HT.
(0.55 $\pm$ 0.03 µmol/kg inhibition by L-epinephrine, mean $\pm$ S.D. for all three models).
0.69 $\pm$ 0.16 µmol/kg inhibition by 5HT, mean $\pm$ S.D. for all three models).
Tables 8, 9 and 10 also summarises the effect of L-epinephrine and 5HT on uptake of (^{14}C) chloroquine by the three models.

The inhibition of drug uptake by L-epinephrine was found to be competitive. Fig 25 is the double reciprocal plot of uptake of chloroquine by P. berghei NK 65 infected erythrocytes in the absence and presence of L-epinephrine. The inhibition is competitive since Umax (maximum uptake at infinite chloroquine concentration) is unchanged but the dissociation constant is raised The inhibition constant K_i is calculated from Gaddum's equation:

$$DR-1 = \frac{(i)}{K_i}$$

where DR (Dose ratio) is

$\frac{K_m}{K_m}$ for chloroquine in presence of inhibitor in absence of inhibitor, and (i) is the molar concentration of inhibitor (Paton, 1970). The inhibition constant K_i for inhibition by L-epinephrine was calculated to be $1.0 - 2.0 \times 10^{-7}$ mol/l.

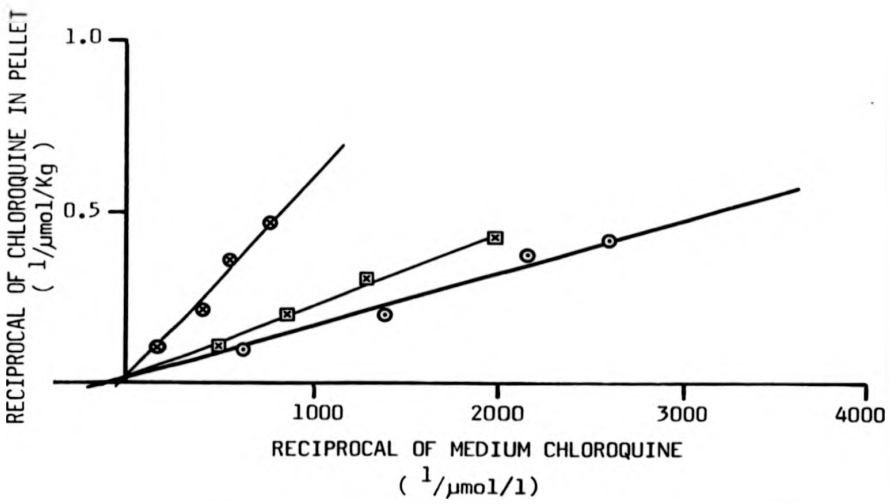

Fig. 25

Effect of non radioactive L-Epinephrine on uptake of (^{14}C) chloroquine by P.berghei NK 65 infected T.O. mouse erythrocytes.

- ● 2.0×10^{-7} mol/l Epinephrine
- ⊠ 5.0×10^{-8} mol/l Epinephrine
- ⊙ Control (NK 65)

L-Epinephrine was dissolved in minimal amount of glacial acetic acid and then diluted appropriately with high Na^+ buffer (SB 1).

Incubation was in SB 1 at 37 °C for 45 min.

3.13.3 EFFECTS OF SULPHYDRYL REACTIVE AGENTS ON UPTAKE OF (^{14}C) CHLOROQUINE.

The -SH blocking agents parachloromecuribenzoate (pCMB) and N-ethylmaleimide (NEM) were tested within the concentration range 1.0×10^{-5} mol/l to 1.0×10^{-3} mol/l in SBI containing 5.0×10^{-8} mol/l (^{14}C) chloroquine. Below 10^{-4} mol/l, both pCMB and NEM showed no effect on the uptake of radiolabelled drug. But at 10^{-3}M, both compounds inhibited the uptake of (^{14}C) chloroquine into uninfected and infected erythrocytes.

At 10^{-3}M, pCMB reduced uptake of radioactive chloroquine into *P. berghei* NK 65 infected erythrocytes by 38.75% $\pm$ 3.05, however this reduction was accompanied by haemolysis far greater than twice the control. At the same concentration NEM caused a 67.2% $\pm$ 1.1 reduction in uptake of radioactive chloroquine in this model. These percentage decreases correspond to decreases in drug concentrations of 1.60 $\pm$ 0.13 and 2.75 $\pm$ 0.05 µmol/kg respectively.

For *P. berghei* RC strain infected erythrocytes, pCMB (10^{-3} mol/l) caused an inhibition of 61.8% $\pm$ 6.0 (2.23 $\pm$ 0.22 µmol/kg) while NEM caused an inhibition of 73.4% $\pm$ 7.9 (2.65 $\pm$ 0.29 µmol/kg).

There was lysis in both cases with pCMB showing greater lytic effect. This lysis not withstanding, there was similarity of inhibition by both pCMB and NEM in this model. (see tables, 8, 9 and 10).

TABLE 8. EFFECTS OF VARIOUS AGENTS ON UPTAKE OF (^{14}C) CHLOROQUINE BY UNINFECTED ERYTHROCYTES.

Addition to medium	^{14}C CQ uptake µmol/kg	Inhibition by agent µmol/kg(b)	Inhibition by agent % (c)
Control	1.09 ± 0.06	-	-
Monensin 2.0×10^{-7} mol/l	0.58 ± 0.07	0.51	47.45 ± 7.07
Nigericin(a) 2.0×10^{-7} mol/l	0.52 ± 0.06	0.57	49.66 ± 8.62
Ouabain 1.0×10^{-4} mol/l	0.82 ± 0.03	0.27	25.48 ± 3.51
L-Epinephrine 2.0×10^{-7} mol/l	0.57 ± 0.05	0.52	47.39 ± 3.46
5HT 2.0×10^{-7} mol/l	0.56 ± 0.04	0.53	48.02 ± 5.5
Ouabain/Monensin	0.79 ± 0.07	0.30	28.01 ± 3.5
Ouabain/Nigericin	0.56 ± 0.02	0.53	48.6 ± 6.9
Ethanol 1% v/v	1.03 ± 0.03	0.06	5.5 ± 0.21
Valinomycin (a)	1.00 ± 0.07	0.09	8.25 ± 0.4

(a) 40.0 mM KCl added to SB1.
Values are means ± S.D of four experiments.

(b) Values given for comparison only.

(c) Actual % inhibition in individual experiments summed up to give mean ± S.D.

TABLE 9. EFFECT OF VARIOUS AGENTS ON UPTAKE OF (^{14}C) CHLOROQUINE BY P. BERGHEI NK 65 INFECTED ERYTHROCYTES (d).

Addition to medium	^{14}C CQ uptake µmol/kg	Inhibition by Agent µmol/kg(b)	Inhibition by Agent %(c)
Control	4.1 ± 0.17	-	-
Monensin 2.0×10^{-7} mol/l	2.36 ± 0.12	1.74	42.5 ± 1.5
Nigericin(a) 2.0×10^{-7} mol/l	2.43 ± 0.17	1.67	42.0 ± 2.0
Ouabain 1.0×10^{-4} mol/l	3.35 ± 0.13	0.75	23.3 ± 5.0
L-Epinephrine 2.0×10^{-7} mol/l	3.56 ± 0.10	0.54	13.13 ± 1.87
5HT 2.0×10^{-7} mol/l	3.47 ± 0.11	0.63	16.6 ± 1.4
Ouabain/Monensin	3.0 ± 0.1	1.1	24.3 ± 3.3
Ouabain/Nigericin	2.06 ± 0.1	2.04	48.35 ± 1.35
* PCMB 10^{-3} mol/l	2.50 ± 0.18	1.63	38.75 ± 3.05
* NEM 10^{-3} mol/l	1.35 ± 0.04	2.75	67.2 ± 1.1
Reserpine	4.05 ± 0.1	-	-
Ethanol 1%	4.03 ± 0.1	-	-
Valinomycin(a)	4.10 ± 0.2	-	-

(d) Average parasitaemia was 55%

* Haemolysis greater than 2x "control" haemolysis.

TABLE 10. EFFECTS OF VARIOUS AGENTS ON UPTAKE OF (^{14}C) CHLOROQUINE BY P. BERGHEI RC-INFECTED ERYTHROCYTES[e]

Addition to medium	^{14}C CQ uptake µmol/kg	Inhibition by Agent µmol/kg[b]	% [c]
Control	3.61 ± 0.23	-	-
Monensin 2.0×10^{-7} mol/l	2.14 ± 0.40	1.47	40.97 ± 10.4
Nigericin[a] 2.0×10^{-7} mol/l	2.01 ± 0.25	1.61	44.6 ± 6.8
Oubain 1.0×10^{-4} mol/l	2.60 ± 0.20	1.01	28.3 ± 5.5
L-Epinephrine 2.0×10^{-7} mol/l	3.01 ± 0.20	0.60	16.6 ± 3.7
5HT 2.0×10^{-7} mol/l	2.7 ± 0.30	0.91	25.2 ± 7.9
Oubain/Monensin	2.42 ± 0.40	1.19	32.7 ± 9.2
Oubain/Nigericin	1.98 ± 0.2	1.63	45.3 ± 5.0
* PCMB 10^{-3} mol/l	1.38 ± 0.2	2.23	61.8 ± 6.0
* NEM 10^{-3} mol/l	0.96 ± 0.04	2.65	73.4 ± 7.9
Reserpine	3.26 ± 0.3	-	-
Valinomycin[a]	3.52 ± 0.1	-	-

(e) Parasitaemia was maximal after 14 days of inoculation

* Haemolysis greater than 2 x "control" haemolysis.

3.14 KINETICS OF (^{14}C) CHLOROQUINE UPTAKE

3.14.1 AFFINITY OF UPTAKE SYSTEM FOR CHLOROQUINE

When reciprocals of the 45 min. (^{14}C) chloroquine uptake values were plotted against reciprocals of the equilibrium concentrations of radiolabelled chloroquine in the incubating medium, straight lines were obtained for uninfected, P.berghei NK 65 and RC strain infected T. O. mouse erythrocytes. This suggests that the amount of radiolabelled chloroquine in the erythrocytes at equilibrium in the presence of increasing external concentrations of chloroquine follows the Langmuir absorption isotherm:

$$\frac{1}{C_{in}} = K\frac{1}{C_{ex}} + \frac{1}{U_{max}}$$

where C_{ex} is the external concentration of chloroquine, C_{in} the amount of drug taken up by the erythrocyte and Umax is the maximal amount taken up by the erythrocyte or erythrocyte/parasite complex at saturating concentrations of chloroquine. (Capacity of the cells).

The dissociation constant K_d was obtained from the intercept on the abscissa and was found to be between 1.0 - 2.0×10^{-8} mol/l for P.berghei NK 65 and 2.7×10^{-8} mol/l for P.berghei RC infected T. O. mouse erythrocytes. The uptake maximum (Umax) was found to be the same for both P.berghei RC strain and uninfected T. O. mouse erythrocytes - approximately 20 µmol/kg, while Umax for P.berghei NK 65 infected T. O. mouse erythrocytes was 53.5 µmol/kg.

The plot for uninfected T. O. mouse erythrocytes almost passed through the intersection of the ordinate and abscissa, but the K_d obtained from the linear regression was 1.33×10^{-6}

mol/l. Fig 26 shows the double reciprocal plots of uptake of (^{14}C) chloroquine in the 3 cell systems used.

Fig 27, illustrates the inhibition of (^{14}C) chloroquine uptake by quinine and non radioloabelled chloroquine. The capacity of the cells (Umax) remainsunchanged in the presence of the inhibitors, unlike the effect of monensin (Fig. 23). The inhibition constant K_i for inhibition by quinine was calculated to be 2.2×10^{-6} mol/l from Gaddum's equation. K_i for "cold" chloroquine was calculated to be 7.7×10^{-7} mol/l. The competitive inhibition showed that quinine probably shared the same uptake system as chloroquine.

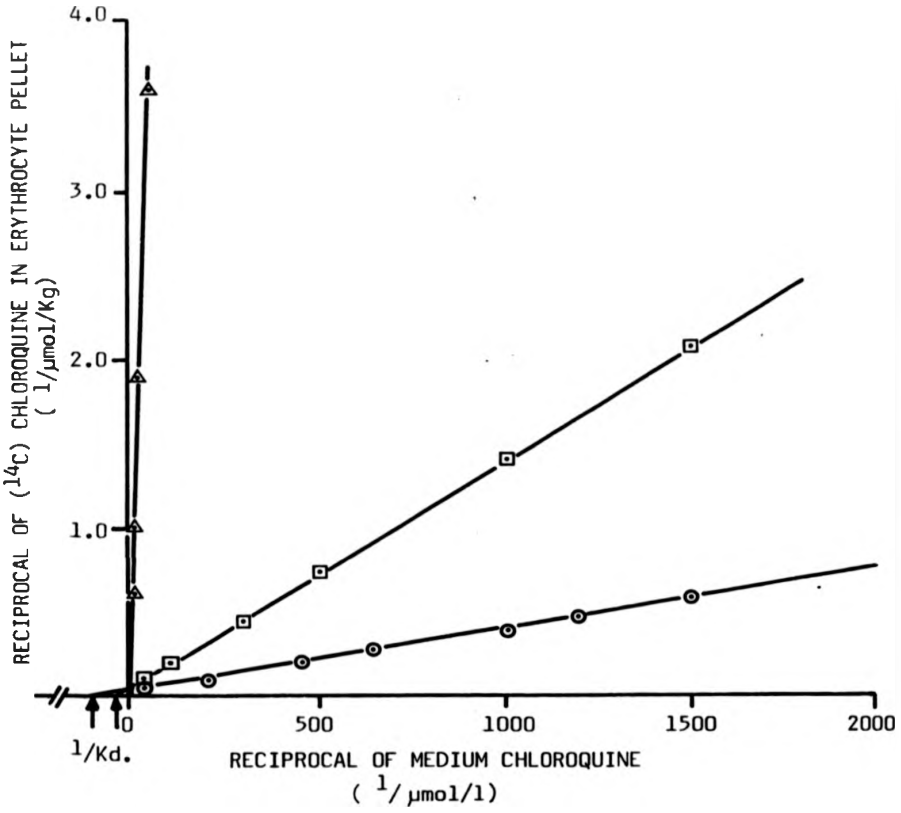

Fig. 26

Double reciprocal plots of uptake of radioactive chloroquine by T.O. mouse erythrocytes as a function of medium concentration of chloroquine at equilibrium.

- ⊙ Uptake by P.berghei NK 65 infected T.O. mouse erythrocytes.
- ⊡ Uptake by P.berghei RC strain infected T.O. mouse erythrocytes.
- ▲ Uptake by uninfected T.O. mouse erythrocytes.

All incubation was in SBIIfor 45 min.at 37°C .
Initial pH of incubating medium was 7.4.

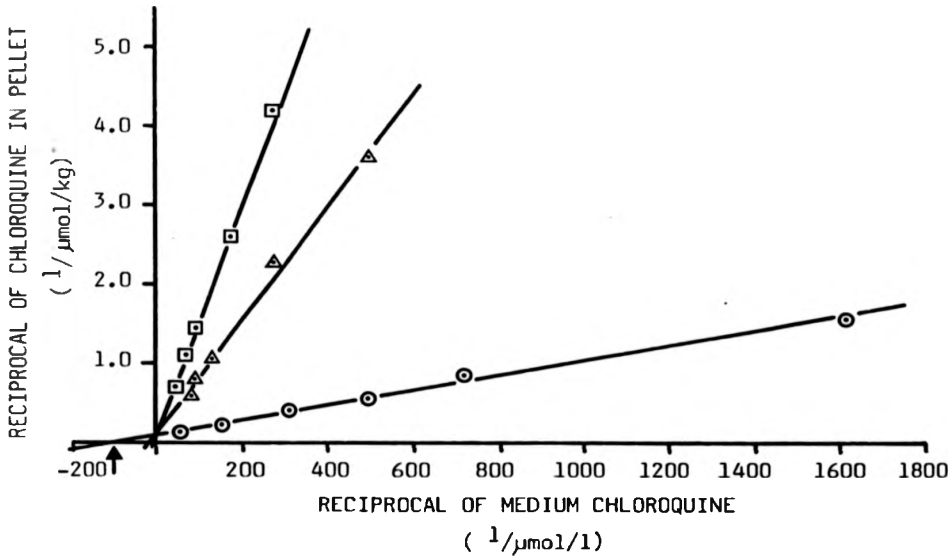

Fig.27

Effect of non radioactive quinine and chloroquine on uptake of radio labelled chloroquine by P.berghei NK 65 infected T.O. mouse erythrocytes.

- ⊙ Uptake by infected erythrocytes in absence of any other drug.
- ▲ Uptake by infected erythrocytes in presence of 10^{-5}mol/l non radio labelled quinine.
- ⊡ Uptake by infected erythrocytes in presence of 10^{-6}mol/l non radio labelled chloroquine.

Incubation was in SBII at 37 °C for 45 min.

3.14.2 SPECIFICITY OF UPTAKE OF (^{14}C) CHLOROQUINE

Over the same range of concentrations of radioactive chloroquine (5.0×10^{-7} - 4.0×10^{-10} mol/l) *P.berghei* NK 65 infected T. O. mouse erythrocytes took up 55.82% ± 2.3 of the drug "specifically" as compared to 23.87 ± 2.42 for *P.berghei* RC strain. Uninfected T. O. mouse erythrocytes showed a "specific uptake" of 40.24% ± 6.18 compared to 45.4% ± 3.0 for reticulocyte enhanced T. O. mouse blood. Tables 11 & 12 show the variation in specific uptake of (^{14}C) chloroquine at varying initial drug concentrations in the medium (SBI).

Nonspecific uptake in chloroquine resistant *P.berghei* RC strain is almost twice that of chloroquine susceptible *P.berghei* NK 65. Figs 28, 29 and 30 are plots of uptake of drug against equilibrium free drug concentration (drug concentration left in medium, at the end of the experiment) for *P.berghei* NK 65, RC strain and uninfected erythrocytes respectively. Fig 31 shows specific uptake for the three models. Fig 32 is the "Scatchard" transformation (Scatchard, 1949) of specific uptake plots (Fig 31) from the slopes of the Scatchard plots, the dissociation constant (K_d) values for specific uptake are 1.0×10^{-8} mol/l for the NK 65 strain and 4.0×10^{-9} mol/l for the RC strain. The intercept on the X axis shows that there is a sixfold difference in the total specific uptake of radiolabelled chloroquine, 3.4×10^{-5} mol/kg and 5.7×10^{-6} mol/kg for *P.berghei* NK 65 and RC strains. This intercept (n) also represents the total receptor sites available for uptake. (Goldstein *et al* 1968, Klotz & Hunston, 1971).

TABLE 11. UPTAKE OF (^{14}C) CHLOROQUINE BY RETICULOCYTE ENHANCED RED BLOOD CELLS.

Initial (^{14}C) CQ concentration in medium (mol/l)	Uptake of (^{14}C) chloroquine (μmol/kg)	Uptake Ratio	Non-Specific uptake. (μmol/kg)	Specific Uptake. (μmol/kg)
5.0×10^{-8}	1.3 ± 0.06	43.2	0.71 ± 0.09	0.59

Reticulocytosis was 60 %
Incubation was at 37°C for 45 min. in SBI
with 5.0 mmol/l D-glucose.
% specific uptake = 45.4 ± 3.0

TABLE 12. VARIATION OF "SPECIFIC" UPTAKE OF (^{14}C) CHLOROQUINE.

Initial (^{14}C) CQ concentration in medium (mol/l)	"Specific" uptake (µmol/kg)		
	NK 65	RC Strain	U R B C
1.25×10^{-7}	6.62 ± 0.15	2.89 ± 0.12	0.70 ± 0.05
6.25×10^{-8}	3.23 ± 0.15	1.88 ± 0.33	0.56 ± 0.10
3.125×10^{-8}	1.76 ± 0.19	0.79 ± 0.09	0.26 ± 0.02
1.56×10^{-8}	0.90 ± 0.04	0.36 ± 0.11	0.16 ± 0.02
7.8×10^{-9}	0.44 ± 0.03	0.16 ± 0.0	0.09 ± 0.03
3.9×10^{-9}	0.17 ± 0.02	0.07 ± 0.02	0.02 ± 0.0

Values for specific uptake obtained by subtracting "non specific" uptake from total uptake.

% of specific uptake is 55.82 ± 2.3 for NK 65, 23.87 ± 2.42 for RC strain and 40.24 + 6.18 for uninfected T.O. mouse erythrocytes (URBC).

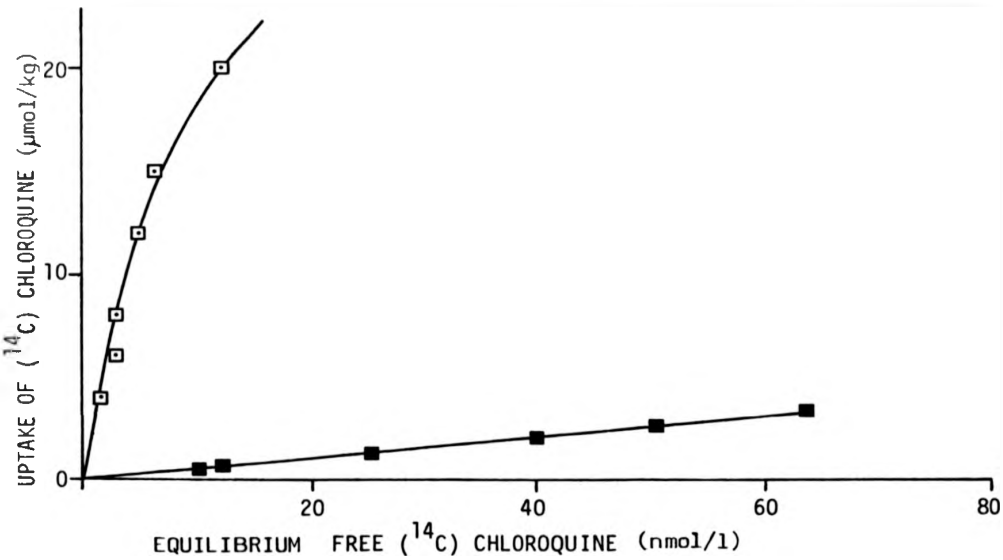

FIG.28

Uptake of (^{14}C) chloroquine over a range of free drug concentrations in P.berghei NK 65 infected T.O. mouse erythrocytes

- ⊡ Total (^{14}C) chloroquine uptake
- ■ "non specific" (^{14}C) chloroquine uptake

% Parasitaemia 59.25 ± 14.46 (n = 8)

n = no. of mice/experiment ; points are mean of values from three experiments in SB 1 .

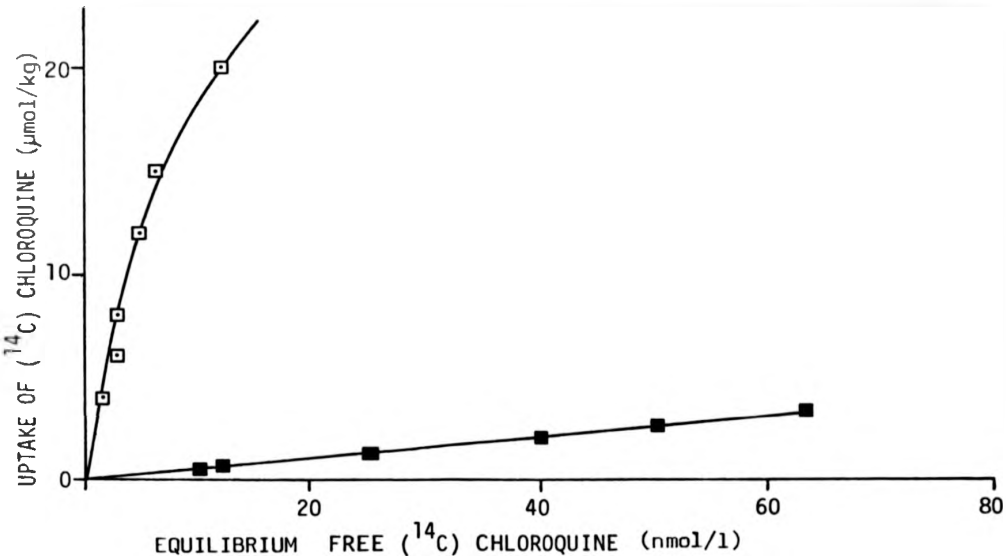

FIG.28

Uptake of (^{14}C) chloroquine over a range of free drug concentrations in P.berghei NK 65 infected T.O. mouse erythrocytes

⊡ Total (^{14}C) chloroquine uptake

■ "non specific" (^{14}C) chloroquine uptake

% Parasitaemia 59.25 ± 14.46 (n = 8)

n = no. of mice/experiment ; points are mean of values from three experiments in SB 1 .

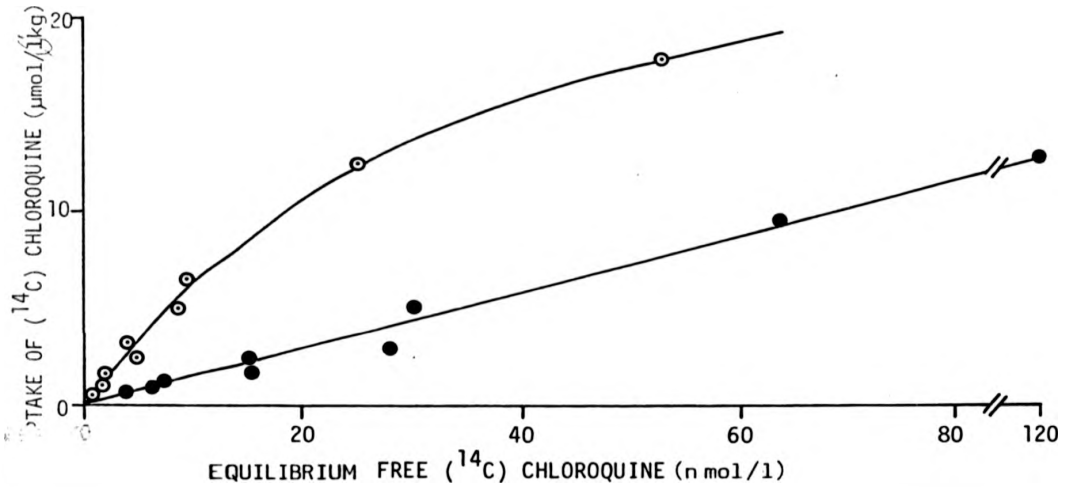

FIG. 29

Uptake of (^{14}C) chloroquine over a range of free drug concentrations in P.berghei RC strain infected T.O. mouse erythrocytes.

⊙ Total (^{14}C) chloroquine uptake

● "non specific" (^{14}C) chloroquine uptake

Average % parasitaemia 31.5% . Points are mean of values from three experiments in SBl.

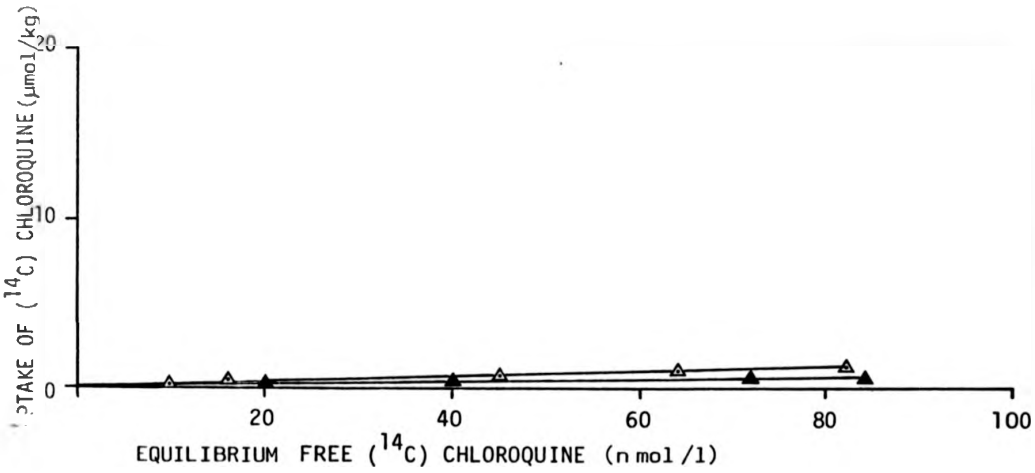

FIG. 30

Uptake of (^{14}C) chloroquine over a range of free drug concentrations in uninfected T.O. mouse erythrocytes.

△ Total (^{14}C) chloroquine uptake

▲ "non specific" (^{14}C) chloroquine uptake

Points are mean of values from three experiments in SB1.

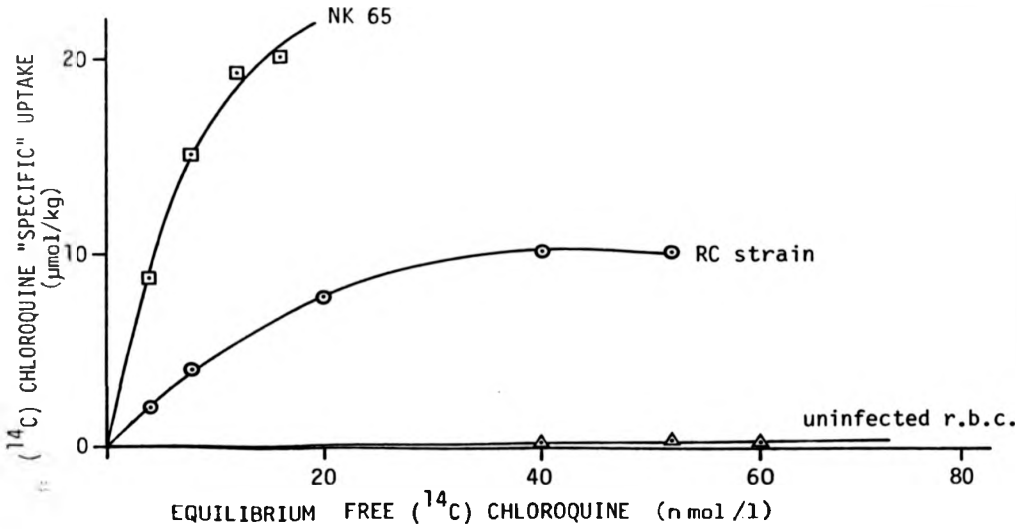

FIG. 31

"Specific"uptake of (^{14}C) chloroquine over a range of free drug concentrations in Plasmodium berghei NK 65 ⊡ , RC strain ⊙ and uninfected T.O. mouse erythrocytes ▲

"Specific" uptake is generated by subtracting non specific uptake from total uptake

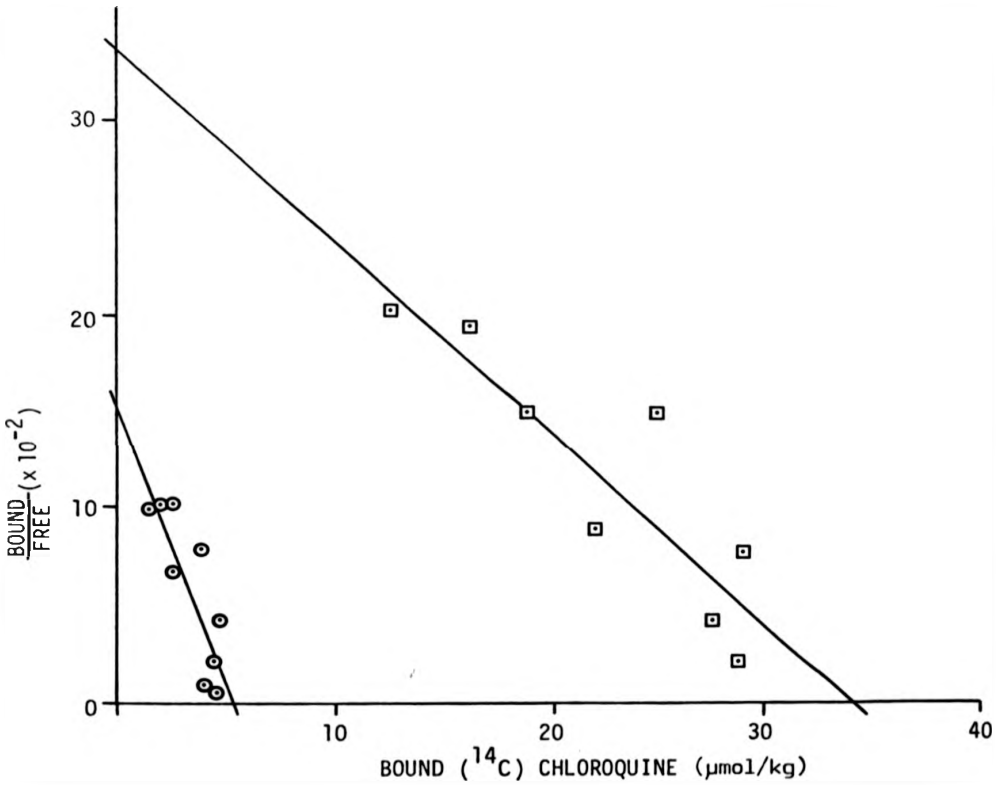

FIG. 32

Transformation of data shown in Fig.31 to a "Scatchard" plot

Intercept on Y axis = nk

Intercept on X axis = n, where k is equivalent to affinity constant

⊡ P.berghei NK 65

⊙ P.berghei RC strain

Both lines are Regression lines with $p < 0.01$, comparison of linear regression coefficients by student t test yielded no significant difference

3.15 ENTRY OF (^{14}C) - L - GLUCOSE INTO T. O. MOUSE ERYTHROCYTES

Between the initial (^{14}C) - L - glucose medium concentration range of 4.0×10^{-9} - 5.0×10^{-7} mol/l, there was no detectable uptake of L - glucose from the medium (SBI) into uninfected T. O. mouse erythrocytes. There was however uptake of radiolobelled L-glucose into both P.berghei NK 65 and P.berghei RC strain infected erythrocytes. The cells: medium ratio was between 0.38 and 0.9, hence there was no concentration of L-glucose by the erythrocyte parasite complex.

At an initial medium concentration of 5.0×10^{-7} mol/l (^{14}C) - L-glucose, only 0.181 µmol/kg L-glucose was taken up and at 4.0×10^{-9} mol/l (^{14}C) - L - glucose, only 0.003 µmol/kg L-glucose was taken up by the infected T. O. mouse erythrocytes. The ionophores Monensin, Nigericin and Valinomycin did not affect uptake of L-glucose into infected T. O. mouse erythrocytes. Similarly, ouabain (1.0×10^{-4} mol/l), L-epinephrine and 5- hydroxytryptamine (2.0×10^{-7} mol/l) did not alter uptake of L-glucose.

Fig 33 is a semilog plot of the effect of Monensin on the uptake of chloroquine and L-glucose by P.berghei NK 65 - infected erythrocytes.

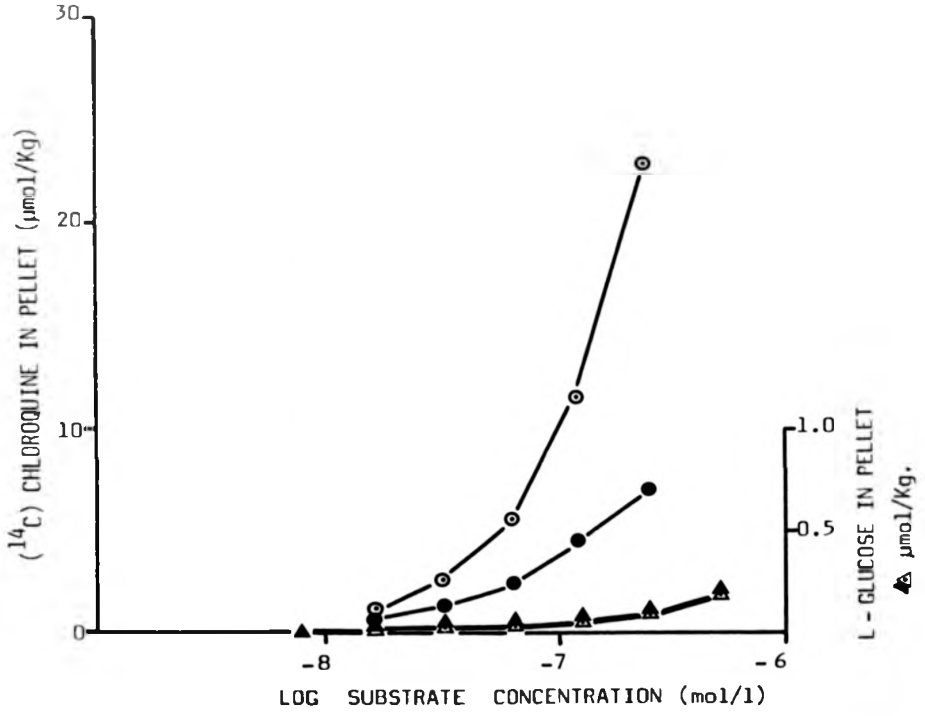

Fig.33

Effect of Monensin on the uptake of (^{14}C) chloroquine and (^{14}C) L-glucose by P.berghei NK 65 infected T.O. mouse erythrocytes.

- ▲ Uptake of L-glucose in absence of Monensin
- ▲ Uptake of L-glucose in the presence of 5.0×10^{-7} mol/l Monensin
- ⊙ Uptake of (^{14}C) chloroquine in absence of Monensin
- ● Uptake of (^{14}C) chloroquine in presence of 5.0×10^{-7} mol/l Monensin

Average parasitaemia was 53%

3.16 UTILISATION OF D-GLUCOSE AND PRODUCTION OF L(+) LACTATE BY T. O. MOUSE ERYTHROCYTES.

Unfortunately the assay system used for determination of D-glucose was not sufficiently sensitive in the concentration range utilised for the experiments (0.5 - 5.0 µmol/ml). Results obtained from experiments to determine glucose utilisation varied widely and the reason for the inconsistency could not be detected. However, the lactate assay was sensitive and was thus used as an indirect measure of glucose utilisation.

Fig 34 shows the similarity of production of L-(+)-lactate in both P.berghei NK 65 and RC strain infected T. O. mouse erythrocytes. L-(+)-lactate production was linearly related to initial glucose concentration in both strains from 0.5 to 2.0 µmol/ml. At low D-glucose concentration (0.5 µmol/ml), up to 85.7% of D.glucose utilised, could be accounted for by L-(+)lactate production while at high D-glucose concentration (5.0µmol/ml) only about 40% D-glucose utilised could be accounted for by L(+) lactate production. (2 molecules of lactate production by glycolysis of 1 molecule of glucose - see Homewood, 1978). However, variations in these results were quite wide as mentioned above.

Monensin (2.0×10^{-7} mol/l) had only a small effect on L(+) Lactate production. Thus for P.berghei NK65 infected erythrocytes, the maximum L(+) lactate produced after 30 minutes incubation in the presence of 5.0 µmol/ml D-glucose was 0.79 ± 0.03 µmol/ml and for P.berghei RC strain infected T. O. Mouse erythrocytes 0.81 ± 0.04

µmol/ml. In the presence of Monensin, L-(+)-lactate produced was 0.76 ± 0.02 and 0.67 ± 0.04 µmol/ml for P.berghei NK 65 and RC strain infected T.O. mouse erythrocytes respectively.

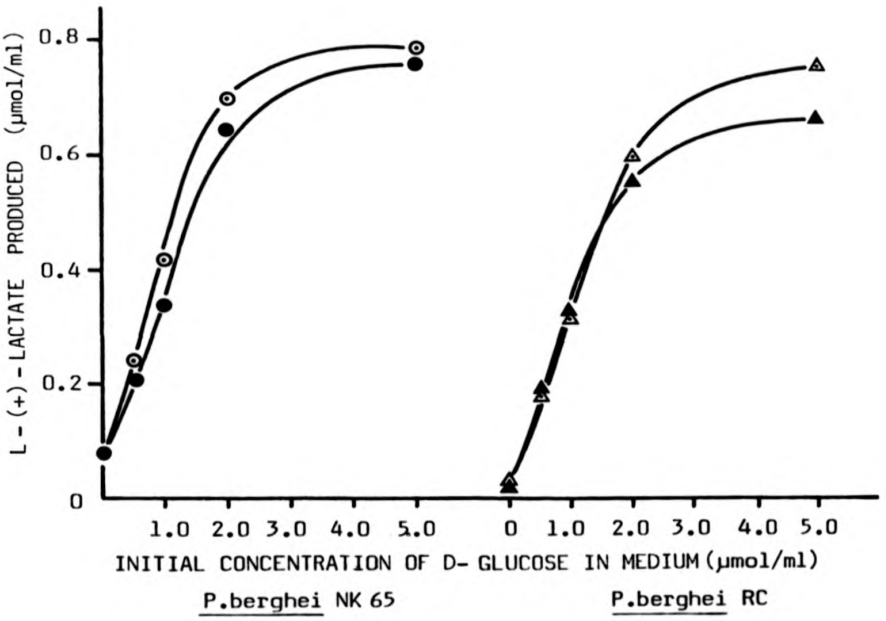

Fig.34

Production of L (+) lactate in P.berghei NK 65 ⊙ and RC strain ▲ , infected T.O. mouse erythrocytes.

- ● Production of L (+) lactate by P.berghei NK 65 in the presence of 2.0×10^{-7} mol/l Monensin in incubating medium (SB1).
- ▲ Production of L (+) lactate by P.berghei RC in the presence of 2.0×10^{-7} mol/l Monensin.

3.17 PROTON EFFLUX INDUCED BY IONOPHORES.

Results obtained showed that there was no detectable efflux of H^+ from uninfected erythrocytes in the absence of D-glucose in the medium; on the otherhand, there was a steady but slow efflux of H^+ from both P.berghei NK 65 and RC strain infected erythrocytes. On addition of 20 mmol/1 D-glucose (final concentration), both infected and uninfected erythrocytes showed a marked efflux of H^+. The glucose stimulated efflux of H^+ was in the order P.berghei NK 65>RC strain>Uninfected T. O. mouse erythrocytes. Computation of initial rates of H^+ efflux showed that NK 65 strain had a relative H^+ efflux of 3.0 by the 1st minute of addition of 20 mmol/1 D-glucose while RC strain had a relative H^+ efflux of 2.0 in the 1st minute and uninfected T. O. mouse erythrocytes 1.0. (Relative H^+ efflux here was computed by calculating the slope of the curve produced on the printout per minute, calculated and expressed as ΔpH x 50 per minute, since 100 mm on the printout was equivalent to 2 pH units i.e. 1 unit on the ordinate (relative H^+ efflux) is equivalent to 0.02 pH units).

After about 5 minutes, the D-glucose induced H^+ efflux levelled at 1.0 for both NK 65 and RC strains and zero for uninfected T. O. mouse erythrocytes.

On addition of 5.0×10^{-7} mol/1 Monensin, there was a rapid efflux of H^+, peaking at a value of 14.0 for NK 65 infected erythrocytes (14.0 ± 2.0) and 13.0 for RC strain infected and uninfected erythrocytes (13.0 ± 1.5)

A similar effect was observed when Nigericin was used in 154 mM KCl solution.

Fig 35 is a copy of typical results obtained from the potentiometric flatbed recorder.

Fig 36 is a graphical transformation of the recording.

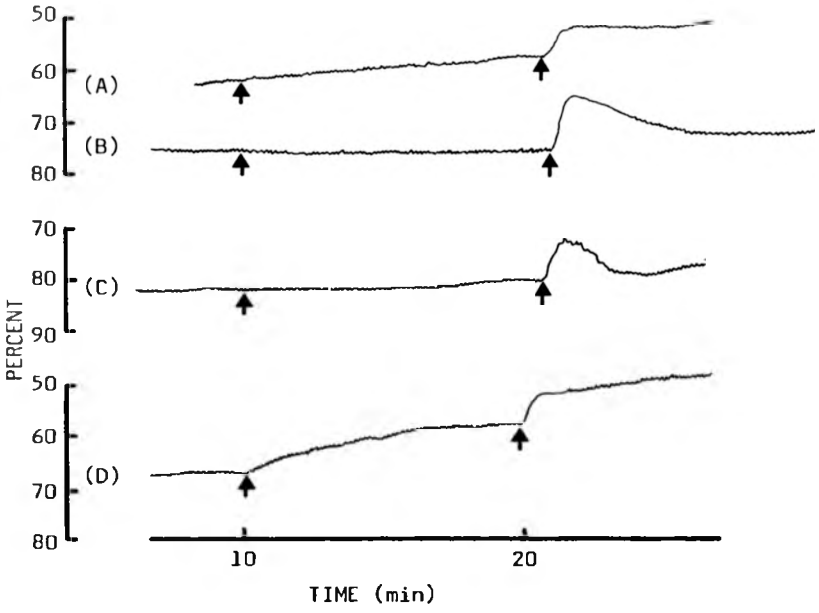

Fig. 35

Effect of ionophores on H^+ efflux recorded by potentiometric flat bed recorder.

pH changes recorded as % after calibration between pH range 6 - 8 equivalent to 0 - 100. (ie. 100 = 2pH units, 1 = 0.02 pH units)

Erythrocytes were equilibrated in appropriate medium for 10 min. before the addition of 10 µl D-glucose 20 mmol/l (first arrow). Ionophores were added 10 minutes later (second arrow).

(A) Effect of 10 µl Monensin (5.0×10^{-7} mol/l) on H^+ efflux by P.berghei RC strain infected T.O. mouse erythrocytes in 154 mmol/l NaCl

(B) Effect of 10 µl Monensin (5.0×10^{-7} mol/l) on H^+ efflux by uninfected T.O. mouse erythrocytes (URBC) in 154 mmol/l Na Cl.

(C) Effect of 10 µl Nigericin (5.0×10^{-7} mol/l) on H^+ efflux by unifected T.O. mouse erythrocytes in 143 mmol/l kCl and 12 mmol/l Na Cl.

(D) Effect of 10 µl Monensin (5.0×10^{-7} mol/l) on H^+ efflux by P.berghei NK65 infected T.O. mouse erythrocytes in 154 mmol/l Na Cl.

Concentrations given are final concentrations.

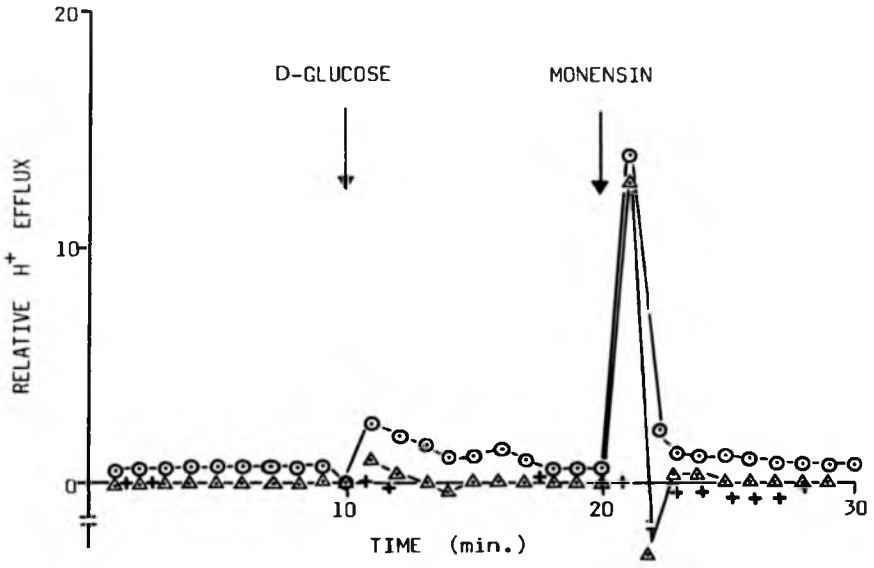

Fig. 36

Effect of Monensin on rate of H^+ efflux from T.O. mouse erythrocytes.

- ⊙ Efflux from P.berghei NK 65 infected T.O. mouse erythrocytes.
- △ Efflux from uninfected T.O. mouse erythrocytes.
- \+ Control, no erythrocytes present in medium.

The relative H^+ efflux is generated from the slope of the curve produced on the print out (fig.35)with units Δ pH x 50/min.

D.Glucose (20 mmol/l) and Monensin in (5.0×10^{-7}mol/l)were added at the times indicated by arrows.

3.18 INTERNAL pH (pHi) of T. O. MOUSE RED BLOOD CELLS

Table 13 summarises the variation of the plasma and internal pH of T. O. mouse red blood cells. The result shows that the intracellular pH is appreciably lower than the extracellular pH. The mean plasma pH obtained was 7.50 for uninfected T. O. mouse. 7.45, for P.berghei NK 65 infected T. O. mouse and 7.41, for P.berghei RC infected T. O. mouse. (In preliminary experiments, it was also found that the mean plasma pH could go down to as low as 6.90 if CO_2 was used as the anaesthetic)

The intracellular pH values obtained were 7.10, 6.9 and 6.84 for uninfected, P.berghei NK 65 and RC infected T. O. mouse red blood cells respectively. The average intracellular pH (pHi) was thus in the order URBC>NK 65> RC strain. Though the average intracellular pH of the chloroquine sensitive, P.berghei NK 65, infected T. O. mouse red blood cell is slightly higher than the resistant one (P.berghei RC), both values were about 0.2 pH units lower than that for uninfected erythrocytes (0.16 ± 0.01) and 0.55 pH units lower than the plasma (0.56 ± 0.01).

Table 13. VARIATION OF EXTERNAL AND INTERNAL pH OF T. O. MOUSE RED BLOOD CELLS

Strain	Mean* plasma pH.	Mean Intracellular pH (pHi)	Δ pH	Calculated ΔH^+ at pH 7.4(mol/l)	Ratio of (H^+) (URBC =1.0)
URBC	7.50	7.10	-0.4	$3.2x10^{-8}$	1.0
NK 65	7.45	6.90	-0.55	$8.62x10^{-8}$	2.2
RC	7.41	6.84	-0.57	$10.47X10^{-8}$	2.7

(H^+) calculated from equation $pH = -\log (H^+)$

* Mean of 3 experiments involving 10 mice per strain per experiment, with similar S.D $\pm$ 0.1 for plasma pH and 0.05 for intracellular pH.

Average % Parasitaemia of NK 65 infected red blood cells was 60%.

Average % Parasitaemia of RC infected red blood cells was 40%.

CHAPTER 4

4. DISCUSSION

4.1 CHARACTERISTICS OF INFECTIONS OF P. BERGHEI IN T.O. MOUSE AND RELATIONSHIP TO CHLOROQUINE UPTAKE.

Results obtained from this project confirm that distinct characteristics are exhibited by infections of *P. berghei* NK 65 and RC strains in T.O. mice. Changes in the packed cell volume (PCV) showed that there is a greater degree of anaemia in mice infected with *P. berghei* RC strain than by NK 65 strain. Table 1 shows that while NK 65 infected T.O. mouse blood had a PCV of 0.23 ± 0.01, the RC strain infected T.O. mouse blood had a PCV of 0.172 ± 0.04; even though the parasitaemia was less. These results confirm part of the work of Palecek *et al* (1967). Similarly, variation of the mean cell volume of the infected blood cells confirmed the association of the chloroquine-resistant RC strain with obligatory development in immature large red cells. As shown in Table 1, the mean cell volume of *P. berghei* RC strain infected cells, corresponded to that of phenylhydrazine-induced reticulocytes (63.0 μm^3 and 65.0 μm^3 respectively). *P. berghei* NK 65 infected blood cells, however, had about the same mean volume as those of the

uninfected normal T.O. mouse ($40\,\mu m^3$ to $46.0\,\mu m^3$).

The rate of development of parasitaemia, showed that P. berghei RC strain infection was less severe than that of P. berghei NK 65. After inoculation of one million parasitised cells intraperitoneally, the NK 65 strain attains a parasitaemia of 2% in about 2.4 days while the RC strain takes about 5.4 days. Moreover, within 7 days of infection, a fulminating infection of NK 65 strain results, with parasitaemia exceeding 80%. This is in contrast to the gradual increase of parasitaemia of the RC strain, which attains maximal parasitaemia of 40% by the 14th day. After this period, destruction of red cells is so pronounced that the estimated degree of parasitaemia becomes unreliable, though the mouse usually dies within the third week.

The accumulation of chloroquine in vitro was found to be directly proportional to the degree of parasitaemia. At the same parasitaemia, the chloroquine resistant strain took up the same amount of drug as the sensitive strain. Total uptake of (^{14}C) chloroquine by P. berghei RC strain infected erythrocytes, was found to fit the linear regression line drawn for uptake of radiolabelled drug by P. berghei NK 65 infected erythrocytes. The

similarity in the total uptake of chloroquine (after correction for degree of parasitaemia) is interesting and appears to agree with the observation of Warhurst (1965b) that "Resistance to chloroquine and quinacrine in P. berghei does not appear to be related to changes in drug uptake by the cell....."

Previously, variations in the morphology of chloroquine-resistant P. berghei had been reported. Peters (1965d) showed that there were up to nine food vacuoles in the chloroquine resistant RC strain as compared to a single food vacuole in P. berghei NK 65. Moreover, there was proliferation of multilamellate whorls of membranes. Rudzinska et al (1965) tried to attribute a metabolic function to both the multilamellate whorls of membrane and the increased food vacuoles, but there has been little evidence to support this suggested mitochondrion-like function. Howells et al (1968a) have also shown the lack of typical malaria pigment grains of P. berghei in the chloroquine resistant RC strain but correlated this observation with the growth of the parasites in immature host cells.

Though there are differences in the character-

istics of infection and morphology between the drug sensitive and resistant strains, the results obtained show that there is similarity of total uptake of radioactive chloroquine *in vitro*. Analysis of this total uptake into specific (high affinity) and nonspecific (low affinity) components, shows that there are differences in the specificity of uptake of drug *in vitro* between the sensitive and resistant strain. This difference in specificity of uptake will be discussed more fully later.

4.2 TIME COURSE OF UPTAKE OF (^{14}C) CHLOROQUINE.

The time course of uptake of radiolabelled chloroquine by infected and uninfected erythrocytes (Fig 15) is similar. It is evident that there is an initial rapid uptake followed by a slow increase to a saturation value in 40 - 45 minutes. It is probable that at the saturation value, a state of equilibrium exists between the drug left in the medium and the drug taken up by the erythrocytes.

From the slopes of the linear portion of the graphs, it is also evident that P. berghei infected erythrocytes accumulate radiolabelled chloroquine at an initial rate 10 times that of uninfected erythrocytes; even though saturation appeared to be attained uniformly. Because of the uniformity in attainment of equilibrium, it was decided to adopt 45 minutes as the standard time for incubation during experiments.

However, it is important to note that the attainment of equilibrium will be affected by a number of parameters including the final concentration of drug in the incubating medium, the temperature of incubation, the density of erythrocytes in the incubation medium etc. In this project, it was ensured that conditions of the experiments were

kept constant and varied only when necessary. It is possible that variation in these parameters could have lead to other investigators obtaining higher equilibrium times. For example, Polet and Barr (1969), using an incubation medium of Hanks' salt solution with 10% dialysed human serum found that after 3 hours, the amount of tritiated chloroquine remained about constant. Fitch, (1969) using an incubation medium consisting of the following (mmol/l) : NaCl, 25, KCl 4.8, $MgSO_4$, 1.2; glucose 80; and Na_2HPO_4, 50; at 22°C, found a saturation time of about 60 minutes.

4.3 INFLUENCE OF IONIC COMPOSITION OF INCUBATION MEDIUM ON UPTAKE OF CHLOROQUINE IN VITRO.

Tables 2 and 3 showed that the variation of Na^+ and K^+ composition of the incubating medium had little effect on the uptake of radiolabelled drug by *P. berghei* NK 65 but appreciably affected drug uptake by both uninfected and *P.berghei* RC infected T.O. mouse erythrocytes. This effect was reflected in a reduction of the "carrier coefficient" of the drug uptake system in uninfected and *P. berghei* RC strain infected erythrocytes. The significance of this differential effect, might lie in variations in the drug pools within the various compartments of the erythrocyte-parasite complex. It is interesting to compare this effect with the observation that high concentration of K^+ releases norepinephrine bound by rat brain tissue fractions (Baldessarini and Marcella, 1971). Though the relationship of this observation to the inhibitory effect of high K^+ concentration on uptake of (^{14}C) chloroquine in erythrocytes *in vitro* is not quite clear now, it would appear that ionic interactions play a major role in the availability of free (unbound) chloroquine in the erythrocyte cytoplasm. (see for example Phifer *et al*, 1966; Kramer and Matusik, 1971 and Yuthavong 1980).

It is known that changes in external concentrations of K^+ affect the functioning of the enzyme Na^+/K^+ ATPase and this requirement for K^+ has to be fulfilled from the exterior of the membrane (Whittam, 1962). Moreover, experiments with Ouabain (Na^+/K^+ ATPase inhibitor - Skou, 1960) showed that in the presence of this compound (1.0×10^{-4} mol/l) uptake of radiolabelled drug into both uninfected and infected T.O. mouse erythrocytes was partially inhibited. The possible involvement of Na^+/K^+ ATPase in the uptake of (^{14}C) chloroquine is not quite clear, but it would appear that proper functioning of the enzyme is necessary for adequate regulation of the metabolism of the red cell. Since inhibitors of metabolism also inhibit chloroquine uptake, (Polet and Barr, 1969, Fitch, et al 1979) alteration of the metabolic status of the red cell by inhibition of Na^+/K^+ ATPase would affect drug uptake. It is important to note however, that ouabain does not completely arrest glycolysis, indeed the inhibition of ion transport in red cells by cardiac glycosides has been shown to have an ATP sparing effect (Davis, 1973) Moreover, the observation by Fitch and coworkers (Fitch et al 1974c) that there was an inverse relationship between the amounts of ATP in infected erythrocytes and ability to accumulate

chloroquine from dilute solutions, further highlights the probability that the linkage of ATP to drug uptake might not be a direct one, but maybe *via* some other metabolism dependent factor which is partially vulnerable to ouabain.

The absolute depression of uptake due to ouabain is greater in *P. berghei* infected T.O. mouse erythrocytes (1.01 and 0.75 µmol/kg for RC strain and NK 65 respectively as against 0.27 µmol/kg for uninfected T.O. mouse) and it might be that active Na^+/K^+ ATPase favours uptake of(^{14}C) chloroquine into erythrocytes and from the erythrocyte cytoplasm into the parasite. However, Warhurst and Thomas (1978) found no effect of ouabain on chloroquine induced pigment clumping (CIPC); this indicates that any effects of ouabain on drug uptake are related to the erythrocyte compartment.

It might be important at this stage, to make some comments on the uptake ratios obtained in some experiments, especially in Tables 2 and 3. see also tables 4, 5 and 6. Since the calculated uptake of drug is dependent on the percentage of erythrocytes parasitised (See Figs 16 and 17), the uptake ratio will thus vary and will increase with increasing

parasitaemia, since less drug will be left in the medium. This observation might account for the rather high uptake ratios obtained in Tables 2 and 3.

4.4 ROLE OF D-GLUCOSE IN UPTAKE OF (^{14}C) CHLOROQUINE

(^{14}C) Chloroquine uptake is enhanced by D-glucose in both P. berghei NK 65 and RC strain infected red blood cells but D-glucose does not enhance uptake in uninfected T.O. mouse erythrocytes. It has been suggested by Fitch and coworkers, that the role of D-glucose was to provide energy to unmask receptors. The unmasking of receptors involved the digestion of haemoglobin (proton dependent) releasing the high affinity receptor considered to be ferriprotophorphyrin IX (haemin) (Chou et al 1980). Another role of energy was put forward by Warhurst and Thomas (1978) and involved the translocation of drug, together with a proton, into the parasite, under the influence of a proton-gradient maintained by a membrane located energy-dependent, proton pump.

It is thus generally agreed that the crucial role of D-glucose utilisation was the provision of protons or generation of proton gradients which in some way modulated drug uptake. However, as shown by the production of L-(+)-lactate (Fig 34), both P. berghei NK 65 and RC strain infected erythrocytes utilise D-glucose via the glycolytic cycle

to the same extent, but D-glucose does not stimulate chloroquine uptake in P. berghei RC strain infected erythrocytes as much as in P. berghei NK 65 infected erythrocytes. It would thus appear that D-glucose stimulates only the component of overall drug uptake, which is reduced in P. berghei RC infected erythrocytes. Moreover, the marked inhibition shown by high concentrations of D-glucose in P. berghei NK 65 (Fig 18a) and by 2-deoxy-D-glucose (Fig 18b), emphasises the tighter linkage of energy to drug uptake in the chloroquine sensitive P. berghei NK 65 strain than in the drug resistant RC strain.

It is known that in bacteria, uptake of substrates via membrane carriers can be facilitated by a proton gradient (low H^+ concentration inside, higher concentration outside) maintained by membrane proton pumps (Harold, 1977). Examples include symport of sugars and amino acids with one or more protons, symport of anionic metabolites and the extrusion of sodium and calcium by antiport with protons (Simoni and Postma, 1975) Symport refers to metabolite transport coupled to that of another ion in the same direction, while antiport is in the opposite direction. Also in the eukaryote fungus Neurospora uptake of glucose is

by symport with protons (Slayman and Slayman, 1974). If uptake of D-glucose into plasmodia is controlled by protons, then the inhibition of chloroquine uptake *in vitro* by high concentrations of D-glucose can be explained by the excessive inflow of protons into the parasite brought about by the inward passage of glucose and protons in symport. However, 3-O-methyl-D-glucose which utilises the same transport system as D-glucose (Teh, 1975, Regan and Morgan, 1964) does not appreciably inhibit uptake of radiolabelled chloroquine suggesting that this may not be the mechanism of inhibition by high concentrations of D-glucose.

It is however possible that as high concentrations of D-glucose enter the cell, glucose is rapidly phosphorylated thereby causing a precipitous drop in cellular ATP which would otherwise have been utilised (by ATPase) to generate the protons necessary for drug uptake. This indeed might be the case, since O-methyl-D-glucose is not known to be phosphorylated appreciably (Schneider and Wiley, 1971, Scarborough, 1970) and does not inhibit chloroquine uptake. Also, deoxy-D-glucose which inhibits drug uptake, uses a tremendous amount of ATP for its own phosphorylation (Detwiler, 1971) and does not replace the lost ATP, since

deoxy-D-glucose does not completely go through the glycolytic sequence as deoxyglucose phosphate but inhibits the phosphohexose isomerase reaction, which is the next reaction in glycolysis after the phosphorylation of glucose.

However, since an appreciable amount of chloroquine is taken up by both uninfected and infected erythrocytes in the absence of D-glucose in the incubating medium, in vitro, it is most probable, that only "specific uptake" by the parasite (see later) is activated by additional D-glucose in the incubating medium. This contention is further supported by the equivalence of % specific uptake to % activation of drug uptake by D-glucose in P. berghei NK 65 (55.82% specific uptake and 65%activation), and (23.87% specific uptake compared to 19% activation) in P. berghei RC infected erythrocytes. (Tables 5,6 and 11).

4.5 UPTAKE OF L-GLUCOSE BY T.O. MOUSE ERYTHROCYTES

Uninfected erythrocytes do not accumulate (^{14}C) L-glucose but on infection with P. berghei an appreciable permeability to (^{14}C) L-glucose is found with uptake being proportional to medium L-glucose concentrations (Fig 33). This confirms previous findings on the alteration of the permeability of erythrocytes on being infected with P. berghei (Homewood and Neame, 1974, Neame, et al, 1975) and with Babesia rodhaini (Homewood et al, 1975).

The uptake of L-glucose by P. berghei infected T.O. mouse erythrocytes was not concentrative and was not affected by Monensin (Fig 33), Nigericin, Valinomycin, Epinephrine or 5HT. These results indicate that the altered membrane permeability that has given rise to the uptake of L-glucose by infected erythrocytes, is not responsible for increased chloroquine uptake.

4.6 VARIATIONS IN INTRACELLULAR pH OF T.O. MOUSE RED BLOOD CELLS.

The H^+ concentration of a cell plays an important role in the uptake of chloroquine into that cell. Rollo (1968b) had speculated that the average intracellular pH of the aqueous compartment in a parasitised erythrocyte is below that of the exterior fluid and that basic drugs such as chloroquine are concentrated by passive diffusion into these acidic regions and bound there by components of the cell matrix.

Results from experiments to determine the intracellular pH of uninfected and P. berghei infected erythrocytes, show that the average intracellular pH values of NK 65 and RC strain infected erythrocytes are 0.2-0.26 units lower than uninfected erythrocytes and 0.55-0.57 units lower than the plasma. (Table 13). The intracellular pH of uninfected T.O. mouse erythrocytes is 0.4 pH units less than the pH of plasma. These results show that there is a pH gradient, in favour of the red cell interior (ie red cell interior acidic). These results appear to be in conflict with the work of Williams and Fanimo (1974) using rat blood infected with P. berghei who found that "The

intracellular pH of both the parasitised populations (chloroquine sensitive and resistant strains), however is somewhat greater than that of the uninfected cells." The reason for this discrepancy, may lie with the methods used in assessing intracellular pH. Williams and Fanimo used the distribution of the weak acid 5, 5-dimethyloxazolidine-2, 4-dione (DMO) after equilibration in vitro with 5% CO_2 in air. During preliminary experiments, it was found that merely anaesthetising the mice in CO_2 leads to a decrease in both the plasma pH and the intracellular pH. Because of this, CO_2 was not used in these experiments to determine intracellular pH using micro glass electrodes.

Sanslone and Muntwyler (1964) using a method similar to that of Williams and Fanimo, working with Wistar rats, found an extracellular pH of 7.4 and an intracellular pH of 7.16 for uninfected normal Wistar rat. This result thus shows that a pH gradient of at least - 0.24 exists in Wistar rat red blood cells.

However, Battaglia and Behrman (1965) have critised the use of silicon oil during determination of intracellular pH, as pH might be slightly higher due to loss of CO_2 through the oil; though valid, this criticism would tend to lend weight to

the result obtained here that a pH gradient of about - 0.4 exists between the plasma and intracellular pH; since loss of CO_2 from the red cell cytoplasm would have led to higher intracellular pH. However, it would appear reasonable that loss of CO_2 from the plasma would correct for corresponding loss of CO_2 from the red cell. However, if the loss of CO_2 from the plasma far outweighs the loss from the red cell, then the plasma pH obtained could be higher than the pH <u>in vivo</u>.

These constraints not withstanding, the method of determining intracellular pH by microglasselectrode after freezing and thawing, is a well established method (Waddel and Bates, 1969) and the adaption here is suited for the comparison of pH between two different red cell populations.

However, the calculated ΔH^+ at 7.4 (Table 12) shows that the ratio of ΔH^+, is 1:2:2.7 (URBC : NK 65 : RC strain), it is important to note that calculation of H^+ concentration by taking the reciprocal of the antilog of the pH value; only gives a comparative amount of "relative acidity" and "makes no statement about absolute hydrogen ion concentration" (Waddell and Bates, 1969).

Though the results obtained here also differ from those of Williams and Fanimo in showing that a pH gradient exists between the infected red cell interior and exterior, we are in agreement that the mean intracellular pH values of erythrocytes parasitised with either sensitive or resistant strains of P. berghei are very similar.

4.7 THE ROLE OF PROTON GRADIENTS IN UPTAKE OF CHLOROQUINE.

4 aminoquinolines, have been demonstrated to have the greatest basicity among the various mono-amino derivatives of quinoline. The enhanced basicity (attraction for protons) was attributed to the stabilization of the cation formed on acceptance of protons. (Irvin and Irvin, 1947). At physiological pH, monoprotonated and diprotonated forms will predominate. Indeed calculation of the percentage of chloroquine ionized using the pK_a and pH values (8.06, 10.16 at pH 7.0) show that the three states exist in the following proportions.

$$\underset{0.03\%}{CQ} \rightleftharpoons \underset{16.6\%}{CQH^+} \rightleftharpoons \underset{83.37\%}{CQ2H^+}$$

The diprotonated form is not permeable through membranes and hence only the unprotonated and monoprotonated forms can cross membranes by virtue of the partition coefficient (oleyl-alcohol/water) of 9 at pH 7.0 (Warhurst and Mallory, 1973). Having passed through the membrane, these species can traverse back into the exterior unless they are further protonated. Hence H^+ in the cytoplasm may play a predominant

role in "trapping" chloroquine within the red cell cytoplasm. Note also, that proteins and other macromolecules (eg nucleic acids) will also "trap" various ionised species especially the diprotonated and dipolar forms which can interact with charges on macromolecules.

However, calculation of ΔH^+ (Table 12) generated at pH 7.4 show that the total internal excess concentration of protons available (as reflected by pH) is only in the range 0.3 - 1.0×10^{-7} mol/l; which is not sufficient to account for total uptake into infected erythrocytes. Moreover, the distribution ratios obtained using equation (6), show that only uptake ratios of 2.31 ± 0.14, 3.04 ± 0.1 and 3.5 ± 0.2 can be obtained for uninfected, P. berghei NK 65 and RC infected T.O. mouse erythrocytes respectively.

These ratios do not correspond to the observed distribution ratio for these three models. However, since increase in the pH of red cell cytoplasm (using ionophores) leads to dose dependent decrease in drug uptake into erythrocytes, there is no doubt that at least a proportion of drug uptake is intimately linked to the H^+ status

of the cell. For *P. berghei* - infected T.O. mouse erythrocytes, extra H^+ needed to support higher uptake of chloroquine might possibly be generated by a proton pump, either by sequestration of H^+ *per se* (say into lysosomes), or by efflux of metabolic endproducts in symport with protons - an example of which is the generation of an electrochemical proton gradient by lactate efflux in membrane vesicles of *Escherichia coli* (Brink and Konings, 1980).

It is clear that proton motive force can be used by cells to drive energy consuming processes such as secondary solute transport, ATP synthesis (Mitchell, 1966) or motility (flagellar motion - Manson *et al* 1977). Whether malaria parasites utilise proton gradients in these ways is presently not clear and requires further studies.

4.8 IONOPHORES AND (^{14}C) CHLOROQUINE UPTAKE.

The carboxylic polyether ionophores, Monensin and Nigericin are capable of transporting monovalent ions across cell membranes down their concentration gradients. Monensin is 10 times more selective for Na^+ than K^+, while Nigericin is 45 times more selective for K^+ than Na^+; charge differences on either side of the membrane are not affected as H^+ is exchanged for Na^+ or K^+ respectively (Pressman, 1976).

Monensin and Nigericin inhibit (^{14}C) chloroquine uptake, 50% inhibition being shown in medium concentrations of 1.0×10^{-7} - 1.0×10^{-6} mol/l depending on the external Na^+ concentration for Monensin and external K^+ concentration for Nigericin. (Figs 19-22). Complete inhibition was not seen at non-lytic concentrations. Measurements of relative proton efflux (Figs 35 and 36) confirm that Monensin (in high Na^+ medium) and Nigericin (in high K^+ medium) cause a marked efflux of H^+ from uninfected and *P. berghei* infected T.O. mouse erythrocytes. This depletion of internal H^+ may be related to their inhibitory effect on drug uptake.

The effect of these ionophores is specific. If the relevant mediating ion is changed, the inhibition of the ionophore is dramatically reduced. For example if Na^+ is replaced by K^+ in the incubating medium, the inhibition of drug uptake caused by 5.0×10^{-7} mol/l Monensin drops from over 50% to 25%. Similarly, if K^+ is replaced by Na^+, the inhibition of drug uptake caused by 2.0×10^{-7} mol/l Nigericin is reduced from 50% to 12%.

Inhibition of chloroquine uptake by these ionophores is noncompetitive (Fig 25) and hence these agents are unlikely to be linking to chloroquine binding sites. Furthermore, the Na^+/K^+ ATPase inhibitor ouabain alleviates the inhibitory effect of Monensin in both uninfected and infected T.O. mouse erythrocytes in SBI, while increasing the inhibitory effect of Nigericin in P. berghei NK 65 infected erythrocytes in K^+ enhanced buffer.

This differential effect of ouabain, is dependent on the ionic composition of the incubating medium. In high Na^+ buffer (SBI), ouabain alleviates the inhibitory effect of Monensin probably by causing the intraerythrocytic sodium

concentration to be increased (as a result of Na^+/K^+ ATPase inactivation) thus reducing the amount of intracellular H^+ exchanged for sodium from the medium. In high K^+ buffer, ouabain slightly increases the inhibitory effect of Nigericin in P. berghei NK 65 infected erythrocytes, but has no effect on the inhibition by Nigericin in uninfected or P. berghei RC infected erythrocytes. The slight potentiation of the inhibition of Nigericin in P. berghei NK 65 is as expected, since Nigericin is specific for K^+ and accumulation of Na^+ due to ouabain inhibition would not be expected appreciably to reverse the effect of Nigericin. The situation with RC strain is more complicated, especially since high external K^+ per se inhibits (^{14}C) chloroquine uptake in RC strain infected and uninfected erythrocytes.

These results show that in the presence of sodium in the external medium, Monensin would raise intraerythrocytic pH and inhibit drug uptake, while Nigericin in the presence of K^+ in the medium will have a similar effect. It thus appears that chloroquine accumulation in T.O. mouse erythrocytes is partially dependent on an electrochemical proton gradient ($\Delta\tilde{\mu}H^+$) across

the red cell membrane (internal acidic). Indeed results from measurement of intracellular pH of red blood cells show that such a proton gradient exists.

The electrochemical proton gradient consists of an electrical parameter, the membrane potential ($\Delta\psi$) and a chemical parameter, the pH difference across the red cell membrane (ΔpH)

$$\Delta\tilde{\mu}H^{+} = \Delta\psi - Z\Delta pH$$

where $Z = 2.3 \frac{RT}{F}$, F is the Faraday, T is temperature in degrees Kelvin, R is the gas constant. (Mitchell, 1966).

However, from results of experiments using the electrogenic ionophore valinomycin (Tables 8, 9 and 10) it would appear that Δ pH is the major determinant of chloroquine uptake by uninfected T.O. mouse erythrocytes, not $\Delta\psi$.

It is likely that a second electrochemical proton gradient exists across the limiting membranes of the intracellular P. berghei parasites (probably with internal parasite pH greater than erythrocyte cytoplasm pH). If this is true, then

a membrane potential (internal - ve) could regulate the uptake of drug from the erythrocyte into the parasite. Whether the parasite membrane potential plays a significant role in the uptake of chloroquine from the red cell cytoplasm into the parasite is not clear and requires further study. However, the diminished inhibition of Monensin in the presence of ouabain (Tables 8, 9 and 10) could be interpreted to be due to relief of the effect of Monensin on membrane potential, since Monensin has no effect on membrane potential in the presence of ouabain (Lichtshtein, et al 1979). If this is true, then, the results obtained from the interaction of ionophores with (^{14}C) chloroquine uptake suggest that chloroquine accumulation into P. berghei infected T.O. mouse erythrocytes can occur in the presence of either a transmembrane proton gradient, or a transmembrane potential gradient, and that maximal uptake is achieved when both components of the proton motive force are present.

It would appear that the inhibitory effect of high K^+ concentration on uptake of (^{14}C) chloroquine further suggests a role for a transmembrane potential gradient (internal - ve),

since excess K^+ in the medium would tend to reduce the transmembrane potential gradient by slowing down outward leakage of K^+. However, a proper interpretation of the role of a transmembrane potential gradient is hindered by the multicompartmentation of the erythrocyte/parasite complex. For example, it is probable that part of the uptake of drug is regulated by the lysosome - a highly acidic organelle. Similarly, it is probable that part of the effect of electroneutral ionophores (monensin, nigericin) can be manifested on the lysosomes by depletion of hydrogen ions within the lysosomes. Especially since excess hydrogen ions in the lysosomes can trap chloroquine within that organelle by protonation of the monocation species into the non permeable dication species. Further work on the distribution of chloroquine within various organelles is needed in order to confirm the role of the lysosome as the major reservoir for chloroquine taken up by the parasite; since chloroquine is already known as a lysosomotropic drug (De Duve et al 1974).

4.9 INTERACTION OF BIOGENIC AMINES WITH (^{14}C) CHLOROQUINE UPTAKE IN VITRO.

The biogenic amines L-epinephrine and 5-hydroxy tryptamine, showed similar inhibition of chloroquine uptake in uninfected and P. berghei infected T.O. mouse erythrocytes. It is interesting that the inhibition due to these biogenic amines at a medium concentration of 200 nmol/l was 0.62 $\pm$ 0.10 µmol/kg for all three models. The implication of the similarity in inhibition is probably that the same component of drug uptake is being affected. Since the erythrocyte compartment is the only compartment common to all three systems, it seems logical, to propose that the effect of L-epinephrine and 5HT is purely on the erythrocyte compartment. Further evidence supporting this proposal is from the observation that L-epinephrine had no inhibitory effect on chloroquine induced pigment clumping (Warhurst and Thomas, 1975) a phenomenon which occurs within the parasite. Recently, Warhurst (personal communication) has also observed that 5HT did not inhibit chloroquine induced pigment clumping.

Analysis of the kinetic data obtained from double reciprocal plots of epinephrine inhibition

of(^{14}C) chloroquine uptake (Fig 25) show that the inhibition caused by L-epinephrine is competitive and has a K_i value of 1.0 - 2.0 x 10^{-7} mol/l. This K_i value is similar to the K_i value of 3.8 x 10^{-7} mol/l found by Warhurst and Thomas (1975), for the competitive inhibition of chloroquine induced pigment clumping by the lipophilic β-blocker dichloroisoproterenol. The competitive nature of the inhibition of drug uptake by epinephrine, might suggest that L-epinephrine is probably accumulated into erythrocytes *via* the same mechanism as chloroquine.

There is further evidence in other systems of relationship between chloroquine and biogenic amines. It has been known for some time that the mechanism of supersensitivity to catecholamines following chloroquine administration is *via* liberation of catecholamines from storage tissues by chloroquine (Pandya, *et al* 1968). It is also known that basic drugs accumulate in 5HT storage organelles of rabbit blood platelets (da Prada & Pletcher 1975) Indeed while several of the basic compounds which release 5HT from platelets are localised in the membrane (reserpine, chlopromazine), others (tyramine, chloroquine and mepacrine) are

specifically accumulated in the interior of the organelles. (Pletscher and da Prada, 1975).

Recently, it has been shown that accumulation of epinephrine by chromaffin granule membrane vesicles was *via* a transmembrane pH gradient (interior acid) driven by ATP and inhibited by the proton ionophores carbonylcyanide trifluoromethoxy phenylhydrazone and nigericin but not by valinomycin. (Schuldiner *et al*, 1978). Similarly, it has been shown that 5HT is accumulated by resealed bovine chromaffin granule "ghosts", if a pH gradient (acid inside) is imposed across their membranes; by preincubation of the granules at low pH. (Phillips, 1978). These findings strongly suggest that biogenic amines (L-epinephrine and 5HT) are transported across membranes in response to a pH gradient and may inhibit chloroquine uptake by competition for this system.

The probability that L-epinephrine could also influence chloroquine uptake in erythrocytes by modulating the glycolytic rate, can, however not be ruled out. This possibility is supported by the finding that epinephrine influences the degradation of cyclic AMP in chicken erythrocytes. (Gorin and Dickbuck, 1979).

4.10 EFFECT OF SULPHYDRYL AGENTS ON DRUG UPTAKE.

The thiol reactive agents PCMB and NEM did not show appreciable inhibition of drug uptake below 10^{-3} mol/l. In all models, 10^{-3} mol/l PCMB and NEM caused inhibition of (^{14}C) chloroquine uptake; coupled with lysis of the red blood cells. This inhibition of (^{14}C) chloroquine uptake at high concentration can be logically attributed to lysis of red cells and parasites, leading to binding of chloroquine to released proteins and nucleic acids in the medium.

It has been shown that high NEM and PCMB concentrations cause cation gradients across the red cell membrane to be disrupted with a loss of cellular K^+, a gain of Na^+ and water (Jacobs and Jandl, 1962). This influx of water caused by the unopposed osmotic pressure of haemoglobin and other macromolecules leads to spherocytosis and swelling of the red cells, which result eventually in the leakage of haemoglobin (haemolysis).

At present, no useful deductions can be made from the differential inhibition (ie 38.75% and 67.2%) by PCMB and NEM respectively, on (^{14}C) chloroquine uptake in *P. berghei* NK 65 infected

erythrocytes (see Table 9). It is only probable that differential lysis gave rise to this effect; but even this contention is further complicated by the observation that at 10^{-4} mol/l, both NEM and PCMB show a similar inhibition (16%) of (^{14}C) chloroquine uptake in both P. berghei NK 65 and RC infected T.O. mouse erythrocytes.

4.11 PHARMACOKINETICS OF UPTAKE OF (^{14}C) CHLOROQUINE.

Results obtained from experiments to establish the specificity of uptake of drug into infected and uninfected T.O. mouse erythrocytes showed that specific uptake (high affinity) is predominant only in the chloroquine sensitive P. berghei NK 65 infected T.O. mouse erythrocytes. This specific uptake accounted for about 60% of total uptake into this model, while specific uptake accounted for less than 30% of total uptake into the chloroquine resistant P. berghei RC strain infected erythrocytes. Thus nonspecific (low affinity) uptake is predominant in the chloroquine resistant strain.

The capacity of the erythrocytes determined from the intercept on the Y axis of the double reciprocal plots (Umax) and calculated by linear regression analysis showed that the capacity of uninfected erythrocytes and P. berghei RC infected erythrocytes were similar (20 μmol/kg) while the capacity of erythrocytes infected with P. berghei NK 65 was 53.5 μmol/kg.

The dissociation constant (K_d) of

$1.0 - 2.0 \times 10^{-8}$ mol/l (associated with drug-receptor interaction during uptake into infected erythrocytes) here can also be compared with the apparent dissociation constant of 5.0×10^{-8} mol/l found by Fitch et al (1974c). Similarly, the results here showed the inhibition constants (K_i) of quinine and chloroquine to be 2.2×10^{-6} mol/l and 7.7×10^{-7} mol/l respectively, while Fitch (1972) found apparent K_i of 2.0×10^{-6} mol/l and 5.0×10^{-7} mol/l for quinine and chloroquine. Though these results are similar, it is important to point out that Fitch did not routinely remove white cells from red blood cell preparations, a purification step which he reports had no effect on the outcome of his experiments (Fitch et al 1974c). However, the importance of including this purification step has been strongly advocated. (see Homewood, 1978). Moreover, Fitch carried out his experiments at 25°C while the experiments here were carried out at 37°C; any changes in the temperature of the experiment are indicated in the relevant texts.

Experiments with reticulocyte enriched blood cells, showed that at $5.0 - 6.0 \times 10^{-8}$ mol/l (^{14}C) chloroquine in the incubating medium, the "specific"

uptake was 0.59 µmol/kg (45.4%) similar to the "specific" uptake by mature uninfected rbcs .0.56 µmol/Kg (40.26%) (see Tables 11 and 12). Fitch et al 1975 have also shown that uninfected polychromatophils accumulate chloroquine but not at a high affinity. The results here confirm that accumulation of chloroquine by uninfected erythrocytes is a nonspecific low affinity process (K_d = 1.33 x 10^{-6} mol/l) and a dissociation constant for the specific receptor-drug interaction (representing the high affinity process) could not be determined by the Scatchard plot (Fig 32).

The dissociation constant of the drug-specific receptor interaction is about the same for both strains 1.0 x 10^{-8} mol/l (NK 65) and 4.0 x 10^{-9} mol/l (RC strain) (both values are not significantly different P> 0.5, student t test). The specific receptor, is therefore present and similar in both sensitive and resistant strains. However, when the number of receptor sites (n) is compared, there are 6 times as many in NK 65 as in RC strain. (Fig 32). This observation is important as it shows a unique difference in the kinetics of uptake of chloroquine between the drug sensitive and resistant strain. Chou et al (1980) have

proposed ferriprotoporphyrin IX (haemin) as the chloroquine receptor of malaria parasites. In equilibrium dialysis experiments, the chloroquine/ ferriprotophorphyrin IX complex was found to have a dissociation constant of 3.5×10^{-9} mol/l which is similar to the dissociation constant for chloroquine obtained here for the specific receptor.

The similarity of the dissociation constant for the specific receptor and that obtained for ferriprotoporphyrin IX by equilibrium dialysis, suggests that this specific receptor is probably identical to ferriprotoporphyrin IX.

From Fig 31, it will be seen that there is virtually no specific uptake into the uninfected T.O. mouse erythrocytes and the dissociation constant for the specific receptor-drug interaction could not be obtained. The absence of specific uptake into uninfected erythrocytes suggest that specific uptake is into the parasite. Probably, nonspecific uptake represents the proportion of drug taken up by the red cell and bound to macromolecules (nucleic acids, proteins etc) in the red cell cytoplasm. Since, the nonspecific uptake is far more in RC strain than in any other

model (compare for example Figs 28 and 29), sequestration of chloroquine due to nonspecific binding is probably one of the means by which drug resistant P. berghei RC strain in immature red cells (which have a higher nucleoprotein concentration - see Fig 4) evades the effect of chloroquine.

4.12 MODEL FOR CHLOROQUINE UPTAKE AND RESISTANCE

Fig 37 summarises the proposed model for chloroquine uptake and resistance in *P. berghei* infected erythrocytes.

The model proposes that chloroquine uptake can be segregated into specific (high affinity) and nonspecific (low affinity) uptake. Specific uptake is apparently into the parasite while nonspecific uptake represents bound drug in erythrocyte cytoplasm and membrane. (Total uptake = specific uptake + nonspecific uptake). In the erythrocyte cytoplasm, nonspecific binding to proteins and nucleic acids determines the amount of "free" drug available for specific uptake by parasite.

A specific receptor (probably ferriprotoporphyrin IX) is responsible for mediating specific uptake into the parasite and perhaps also acts in conjunction with chloroquine in eliciting parasiticidal effect, since haemin (ferriprotoporphyrin IX) is known to cause damage to membranes (Tappel and Zalkin, 1959). It is further proposed that negatively charged groups on the receptor bind

(ferriprotoporphyrin IX) is produced inside a parasite organelle, eg in lysosomes. In this case, uptake of monoprotonated chloroquine from the erythrocyte milieu into the parasite milieu becomes very crucial and if a transmembrane potential regulates the uptake of this monocation, then generation of the membrane potential will be a crucial step in the uptake of chloroquine. However, it is still possible that a receptor produced within one of the parasite's organelles can regulate uptake of drug from the erythrocyte cytoplasm into the parasite.

The model also proposes two possible mechanisms of resistance to chloroquine. One mechanism involves the red cell as a site of loss of drug by sequestration due to nonspecific binding of chloroquine to macromolecules. This site of loss of drug is more pronounced in the reticulocyte, probably because of its high ribonucleoprotein content (see Fig 4). It is also possible that increased production of haemin binding protein normally incorporated into haemozoin (Yamada and Sherman, 1979) may be responsible for non specific binding in the chloroquine-resistant strain. Indeed, increase in haemin-binding protein could effectively reduce the number of free ferriprotoporphyrin IX

with the positively charged chloroquine mono or dications and the drug-receptor complex permeates into the parasite. If the chloroquine cations dissociate from the receptor inside the parasite, then in regions of high hydrogen ion concentration (eg lysosomes), the diprotonated forms will remain charged and will not be able to pass back into the erythrocyte cytoplasm, whilst the monoprotonated forms will become diprotonated and also cannot pass back through the membrane.

The model speculates that the role of protons includes generation of appropriately charged chloroquine cations, receptors and perhaps a parasite membrane potential, which could be influential in attracting the receptor drug complex and/or the monoprotonated drug. A role is further envisaged for a proton pump. At present, it is not known whether the proton pump probably operates by efflux of (H^+) *per se* or by efflux of other metabolic products eg lactate. (Brink and Konings, 1980).

It is important to mention that the above mechanism for the uptake of chloroquine is a simplification of an inherently complex system. For example, it is probable that the receptor

(ferriprotoporphyrin IX) is produced inside a parasite organelle, eg in lysosomes. In this case, uptake of monoprotonated chloroquine from the erythrocyte milieu into the parasite milieu becomes very crucial and if a transmembrane potential regulates the uptake of this monocation, then generation of the membrane potential will be a crucial step in the uptake of chloroquine. However, it is still possible that a receptor produced within one of the parasite's organelles can regulate uptake of drug from the erythrocyte cytoplasm into the parasite.

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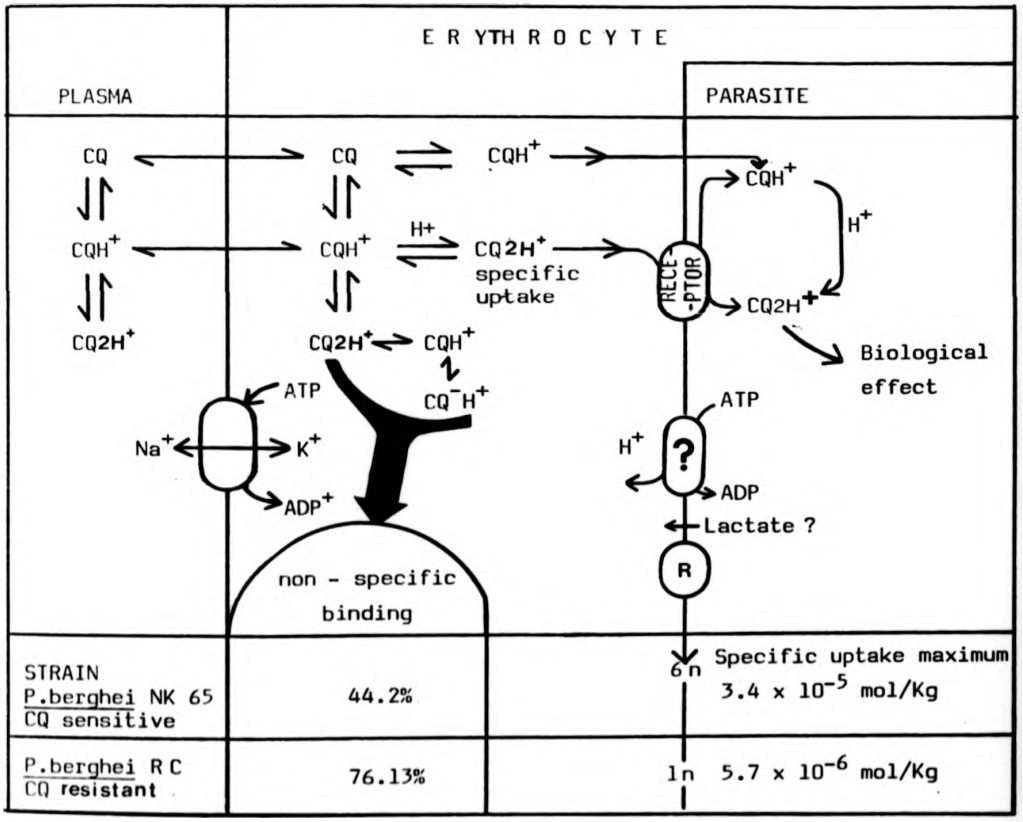

Fig. 37

Schematic model for chloroquine uptake into P.berghei infected erythrocyte.

molecules available for binding chloroquine (Warhurst 1980 - WHO report).

The second mechanism of resistance in P. berghei RC infected T.O. mouse erythrocytes, is the decrease in the number of drug receptors available. There are six times more specific drug receptors in the erythrocytes infected with the chloroquine sensitive strain than in the drug resistant strain (See Fig 32). Accordingly, the drug sensitive strain can accumulate chloroquine "specifically" up to a maximum of 3.4×10^{-5} mol/kg while the resistant strain can accumulate a maximum of 5.7×10^{-6} mol/kg. It is interesting to compare the result for the chloroquine sensitive strain obtained here with that of Fitch et al (1975) who obtained a value of 26 ± 2.4 µmol/kg for maximal accumulation of chloroquine by the "saturable" process; estimated from double reciprocal graphs. Unfortunately no value was given in that same work for the chloroquine resistant strain of P. berghei. However, the dissociation constant (K_d) for the interaction of drug with the specific receptor 4.0×10^{-9} - 1.0×10^{-8} mol/l is identical to the reciprocal of the apparent intrinsic association constant (K) of the

saturable class of binding sites : 0.4 to $1.6 \times 10^{8}\ M^{-1}$ obtained by Fitch (1969). The higher maximum specific uptake obtained here is not surprising, since at 37°C this glucose dependent process will be more efficient than at 22°C - the temperature at which Fitch did his experiments.

That the drug receptor is associated with ferriprotoporphyrin IX is supported by the following observations :

(i) The similarity of dissociation constant for specific receptor-chloroquine interaction (1.0×10^{-8} - 4.0×10^{-9} mol/l) found here and that found in equilibrium dialysis experiments (3.9×10^{-9} mol/l) for ferriprotophorphyrin - chloroquine interaction (Chou et al 1980).

(ii) Erythrocytes infected with chloroquine-susceptible P. berghei, degrade haemoglobin in normocytes and accumulate malaria pigment which contains ferriprotoporphyrin IX, whereas in reticulocytes, it produces little pigment. In addition, when chloroquine resistant lines of P. berghei revert to chloroquine sensitivity, they also revert to the production of malaria pigment.

Thus, the two lesions contributing to the development of resistance to chloroquine, (increased non specific binding and decrease in drug receptor sites leading to decrease in specific uptake) are uniquely combined in the infection of reticulocytes by P. berghei RC strain and can explain the linkage of chloroquine resistance in P. berghei to infection of immature erythrocytes.

This model for chloroquine uptake and resistance accounts for most of the known characteristics of chloroquine uptake and resistance in P. berghei and lends itself to experimental verification - (see later). It will be interesting to see how this model compares with P. falciparum infection in man. Of course more research work has to be undertaken in order to fully elucidate the model. There is need to find out more about the mechanisms whereby malaria parasites couple their metabolism with uptake of metabolites and perhaps drugs, because a better understanding of the system could provide further chemotherapeutic targets. Also the sequence of events leading to the generation of chloroquine receptors in the erythrocyte parasite complex needs to be studied.

Further studies of this model should include subcellular fractionation of the erythrocyte parasite complex with a view to :

(i) Investigating the distribution of drug into various compartments - erythrocyte milieu components and parasite organelles.

(ii) Assessing the nature of binding of drug to the subcellular fractions.

Already, some work in this direction shows that useful results can be obtained from this approach. For example Kramer and Matusik (1971) found that by differential lysis of mouse erythrocytes parasitised with chloroquine sensitive Plasmodium berghei, they could differentiate the regions responsible for the saturable chloroquine binding process. They found that the highest affinity sites (affinity constant = $10^8 M^{-1}$) appeared to be associated with the parasite membranes, whereas those with affinity constants of about $10^5 M^{-1}$ were associated with the cytoplasmic fraction of the parasite.

It is hoped that a more rigorous examination

of distribution of chloroquine into subcellular fractions will probably show the location of the chloroquine receptor and perhaps the predominant target of chloroquine action.

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ABBREVIATIONS

AMP	-	Adenosine 5'-monophosphate.
ADP	-	Adenosine 5'-diphosphate.
ATP	-	Adenosine 5'-triphosphate.
CMP	-	Cytidine 5'-monophosphate.
CDP	-	Cytidine 5'- diphosphate.
CTP	-	Cytidine 5'- triphosphate.
CIPC	-	Chloroquine induced Pigment clumping.
CQ	-	Chloroquine.
CQH^+	-	Monoprotonated chloroquine.
$CQ2H^+$	-	Diprotonated chloroquine.
DDT	-	Dichlorodiphenyltrichloroethane.
DPM	-	Disintegrations per minute.
dTMP	-	Deoxythymidine 5' - monophosphate.
dUMP	-	Deoxyuridine 5' - monophosphate.
DNA	-	Deoxyribonucleic acid.
FH_2	-	Dihydrofolic acid.
FH_4	-	Tetrahydrafolic acid.
G-6-P	-	Glucose - 6 - phosphate.
G6P-DH	-	Glucose - 6 - phosphate dehydrogenase.
GMP	-	Guanosine 5' - monophosphate.
GDP	-	Guanosine 5' - diphosphate.
GTP	-	Guanosine 5' - triphosphate.

ABBREVIATIONS

AMP	-	Adenosine 5'-monophosphate.
ADP	-	Adenosine 5'-diphosphate.
ATP	-	Adenosine 5'-triphosphate.
CMP	-	Cytidine 5'-monophosphate.
CDP	-	Cytidine 5'- diphosphate.
CTP	-	Cytidine 5'- triphosphate.
CIPC	-	Chloroquine induced Pigment clumping.
CQ	-	Chloroquine.
CQH^+	-	Monoprotonated chloroquine.
$CQ2H^+$	-	Diprotonated chloroquine.
DDT	-	Dichlorodiphenyltrichloroethane.
DPM	-	Disintegrations per minute.
dTMP	-	Deoxythymidine 5' - monophosphate.
dUMP	-	Deoxyuridine 5' - monophosphate.
DNA	-	Deoxyribonucleic acid.
FH_2	-	Dihydrofolic acid.
FH_4	-	Tetrahydrafolic acid.
G-6-P	-	Glucose - 6 - phosphate.
G6P-DH	-	Glucose - 6 - phosphate dehydrogenase.
GMP	-	Guanosine 5' - monophosphate.
GDP	-	Guanosine 5' - diphosphate.
GTP	-	Guanosine 5' - triphosphate.

HKB	-	High Potassium buffer.
5HT	-	5 - Hydroxytryptamine.
IMP	-	Inosine 5' - monophosphate.
I.P	-	Intraperitoneal.
NAD	-	Nicotinamide adenine dinucleotide.
NADH	-	Reduced NAD.
NADP	-	Nicotinamide adenine dinucleotide phosphate.
NADPH	-	Reduced NADP.
NEM	-	N-ethyl maleimide.
pABA	-	para amino benzoic acid.
pAGA	-	para amino benzoyl glutamic acid.
PH	-	Phenylhydrazine hydrochloride.
PCMB	-	para chloromercuri benzoic acid.
PCV	-	Packed cell volume.
RNA	-	Ribonucleic acid.
SB1	-	Standard buffer 1.
SB2	-	Standard buffer 2.
UMP	-	Uridine 5' - monophosphate.
UDP	-	Uridine 5' - diphosphate.
UTP	-	Uridine 5' - triphosphate.
URBC	-	Uninfected red blood cells.

TEXAS T1-57 PROGRAMMES

I

PROGRAMME TITLE : Calculation of total disintegrations per minute from uncorrected radioactivity counts. (Texas T 1-57).

Programme :

KEY	STEP	COMMENTS
LRN	00	
-	01	
RCL 1	02	
=	03	
STO O	04	
R/S	05	Channel 1 (red) background correction.
-	06	
RCL 7	07	
=	08	
STO 6	09	
R/S	10	Channel 2 (green) background correction.
RCL O	11	
÷	12	
RCL 6	13	
x	14	
58.4 *	15-18	
+/-	19	

KEY	STEP	COMMENTS
=	20	
+	21	
101.67 *	22-27	* slope and intercept from quenching correction curve.
=	28	
÷	29	
100	30-32	
=	33	
STO 2	34	
R/S	35	Relative efficiency of counting.
RCL 6	36	
÷	37	
RCL 3	38	
x	39	
RCL 4	40	
÷	41	
RCL 5	42	
÷	43	
RCL 2	44	
=	45	
R/S	46	Total Dpm / Extract.
LRN	47	
RST	48	

(USER INSTRUCTIONS.)

STORE CHANNEL 1 BKG COUNTS IN MEMORY BANK 1.
STORE CHANNEL 2 BKG COUNTS IN MEMORY BANK 7.
STORE COUNTING TIME IN MINUTES IN MEMORY BANK 3.
STORE TOTAL VOLUME OF HEPTANE EXTRACT IN MEMORY BANK 4.
STORE VOLUME OF EXTRACT ADDED TO EACH COUNTING VIAL IN MEMORY BANK 5.

To operate,

Key in counts from channel 1 : R/S gives background counts correction, Key in counts from channel 2; R/S gives background correction, R/S gives relative efficiency and R/S gives total DPM in extract.
RST (return to start) after each run.

II

PROGRAMME TITLE : Calculation of D.p.m per gramme erythrocyte.

Programme :

KEY	STEP	COMMENTS
LRN	00	
STO 0	01	
R/S	02	Weight of erythrocyte pellet.
STO 1	03	
R/S	04	Dpm/ml supernatant.
÷	05	
RCL 0	06	
=	07	
STO 2	08	
CLR	09	
.15 *	10-12	* Calculated average "Inulin space".
x	13	
RCL 1	14	
=	15	
+/-	16	
+	17	
RCL 2	18	
=	19	
÷	20	

KEY	STEP	COMMENTS
.85	21-23	
=	24	
R/S	25	Dpm/g erythrocyte.
LRN	26	
RST	27	

To operate,

Key in weight of pellet R/S, then key in DPM/ml supernatant R/S, key in DPM in pellet; R/S gives DPM/gm erythrocytes.

III

PROGRAMME TITLE : Calculation of drug concentration in μmol/kg wet weight erythrocyte.

Programme :

KEY	STEP	COMMENTS
LRN	00	
EE	01	
3	02	
÷	03	
RCL 0	04	
=	05	
÷	06	
22.2	07-10	
=	11	
÷	12	
1	13	
EE	14	
5	15	
=	16	
R/S	17	Concentration in μmol/kg or μmol/l.
LRN	18	
RST	19	Return to start.

STORE SPECIFIC ACTIVITY OF RADIOLABELLED COMPOUND (μCi/μmole) IN MEMORY BANK 0.

To operate,

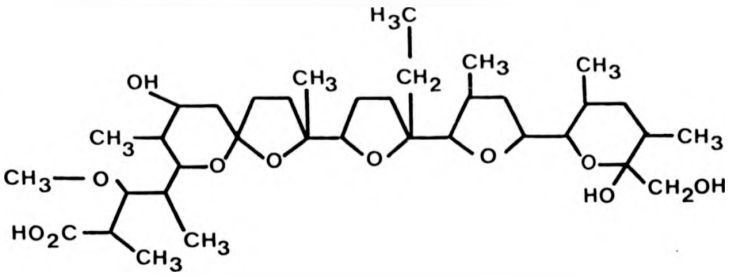
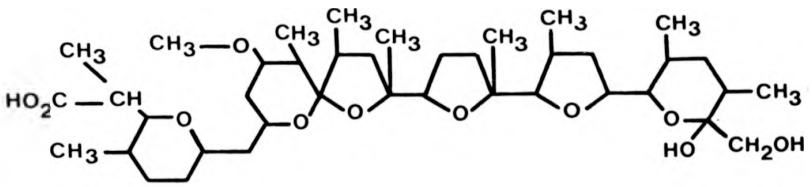
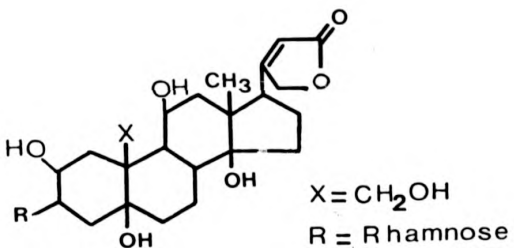
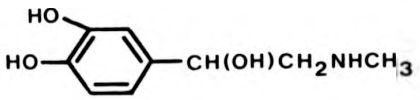
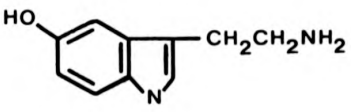
Key in DPM/g or DPM/ml; RS gives concentration of radiolabelled drug in μmol/kg wet weight erythrocyte.

* Programmes supplied by D.C. Warhurst.

To operate,

Key in DPM/g or DPM/ml; RS gives concentration of radiolabelled drug in μmol/kg wet weight erythrocyte.

* Programmes supplied by D.C. Warhurst.

COMPOUND	STRUCTURE
MONENSIN	
NIGERICIN	
OUABAIN	 X = CH_2OH; R = Rhamnose
EPINEPHRINE	
5-HT	
CHLOROQUINE