IN VITRO STUDIES ON THE EFFECT OF CHLOROQUINE PHOSPHATE ON THE METABOLISM OF THE RAT RED BLOOD CELLS

BY

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D E C L A R A T I O N

THE EXPERIMENTAL WORK DESCRIBED IN THIS THESIS WAS CARRIED OUT BY ME AT THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF GHANA, LEGON UNDER THE JOINT SUPERVISION OF PROFESSOR G.S. ASANTE, DR. CHERYL LOVELACE AND DR. PETER LARWAY.

I CERTIFY THAT THIS WORK HAS NOT PREVIOUSLY BEEN ACCEPTED FOR ANY DEGREE AND IS NOT BEING CONCURRENTLY SUBMITTED IN CANDIDATURE FOR ANY OTHER DEGREE.

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V I T A

The author was born on 29th January, 1947 at Asamankase, Eastern Region, Ghana. He attended the Ghana Secondary School, Koforidua and the West Africa Secondary School, Accra respectively.

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LIST OF ABBREVIATIONS

ADP	:	Adenosine 5' diphosphate
ATP	:	Adenosine 5' - triphosphate
DHAP	:	dihydroxyacetone phosphate
2,3-DPGA	:	2,3'-diphosphoglyceric acid
2,3-DPGM	:	2,3' - diphosphoglycerate mutase
DTNB	:	5,5'-dithiobis - (2 nitrobenzoic acid)
EDTA	:	Ethylenediaminetetra-acetate
FDP	:	Fructose 1,6-diphosphate
F-6-P	:	Fructose - 6 - phosphate
G3PD	:	glyceraldehyde-3-phosphate dehydrogenase
G-3-P	:	glyceraldehyde-3-phosphate
G-6-P	:	glucose - 6 - phosphate
G6PD	:	glucose - 6 - phosphate dehydrogenase
GPX	:	gluthathione peroxidase
GSH	:	reduced gluthathione
GSSG	:	oxidized gluthathione
Hb	:	reduced hemoglobin
Hk	:	Hexokinase
LDH	:	Lactate dehydrogenase
Met Hb	:	met hemoglobin
NAD^+	:	Nicothinamide-adenine dinucleotide
NADH	:	Nicotinamide-adenine dinucleotide (reduced)

$NADP^+$	:	Nicotinamide-adenine dinucleotide phosphate
NADPH	:	Nicotinamide-adenine dinucleotide phosphate (reduced)
Pi	:	Inorganic Phosphate
PCV	:	Packed Cell Volume or Hematocrit
PEP	:	Phosphoenol Pyruvate
PFK	:	Phosphofructo-Kinase
PK	:	Pyruvate Kinase
1,3-PGA	:	1, 3-Phosphoglyceric Acid
2-PGA	:	2-Phosphoglyceric Acid
3-PGA	:	3-Phosphoglyceric Acid
6PGD	:	6-Phosphogluconate Dehydrognase
TA	:	Transaldolase
TK	:	Transketolase
TI	:	Trioseisomerase

SUMMARY

Evidence is presented to show that *in vitro*, (1) High levels of chloroquine phosphate can induce hemolysis in the rat red blood cells; and (2) chloroquine-induced hemolysis is characterised by a fall in GSH levels, unless glucose is present in very high concentrations.

Chloroquine phosphate is a member of the 4-aminoquinoline series of drugs used for treating acute malaria due to infection by *Plasmodium vivax, Plasmodium falciparum, Plasmodium malariae* and *Plasmodium ovale*.

In vitro studies were made on red blood cells incubated with chloroquine phosphate to investigate a possible chloroquine-induced hemolysis in rat red blood cells.

A wide range of chloroquine phosphate concentrations were tested. After 4 hours incubation of the mixture at 37°C; 6.3×10^{-4}M caused only 5% hemolysis in the rat red blood cells; whereas 2.5×10^{-2}M chloroquine caused 66.5% hemolysis. Complete hemolysis was however observed when 4.2×10^{-2}M chloroquine phosphate was used in the incubation system.

Either 3.78×10^{-1}M glucose or 2.0×10^{-2}M ATP protected the red blood cells from hemolysis induced by 2.5×10^{-2}M chloroquine phosphate. Hemolysis induced by chloroquine phosphate was found to be characterised by (a) a fall in GSH level, and (b) an increase in the osmotic fragility of the rat red blood cells. These characteristics are similar to primaquine-induced hemolysis in [illegible] red blood cells. The possibility is discussed that based upon osmotic fragility studies, the site of hemolytic action of chloroquine phosphate could be directly on the red cell membrane where the drug might interfere with sulfhydryl groups.

EFFECT OF CHLOROQUINE PHOSPHATE ON THE METABOLISM OF THE RAT RED BLOOD CELLS

INTRODUCTION OF LITERATURE REVIEW

The main function of the mature mammalian red blood cell is the transport of oxygen from the lungs to all cells of the animal and the returning of the CO_2 resulting from internal respiration of foods. The oxygen so transported is used by living cells of the body for the prolific synthesis of ATP, through aerobic oxidation of foods. The ATP produced serves as a source of energy for numerous transactions in living cells such as:

(1) muscle contraction.

(2) synthesis and breakdown of biologically important compounds such as proteins,

and (3) transport of substances across cell membranes.

The mature circulating mammalian red blood cell has no nucleus or ribosomes, no complete Kreb cycle or cytochrome oxidase system, and requires glucose for survival.

Biochemically, the red blood cell can be considered as three interacting units (Carson and Frischer, 1966).

(1) The cell membrane, characterised not only by its glycolipid, glycoprotein and lipoprotein structure but also by its enzymes, e.g. adenosintriphosphatase (ATPase).

2) The hemoglobin which transports oxygen to other cells of the body.

3) The remaining intracellular soluble elements especially the enzymes, coenzymes and substrates for glucose metabolism (Carson and Fricher, 1966).

Respiration of the red blood cell in vitro, is almost negligible compared to most nucleated cells, with 90% of glucose metabolised by glycolysis through the Embden-Meyerhoff pathway and 10% via hexose monophosphate shunt (Murphy, 1960a). Utilising the energy derived from glucose metabolism through these pathways, the red blood cell can maintain itself for approximately 120 days in vivo, during which time it supplies oxygen to all cells of the body (Harris et al, 1970). The red blood cell may be subjected to numerous, intense and unremitting physical and chemical stresses such as the toxic products ensuing from drugs and the degradative effects of oxidative substances. The end results of all these factors is probably the slow depletion of enzymes and confactors which cannot be replaced by the cell (Yunis, and Yasmineh, 1969). The red blood cell eventually undergoes hemolysis. Hemolysis in this context therefore refers to a reduction in the survival time of the red blood cell in the circulation, and could imply rupture of red blood cell thus releasing its contents. The mechanism by which the survival of the red blood cell is impaired in the various hemolytic disorders

encompass most of the fundamental threats to life, including injury, allergy, fatigue and ageing (Jandl, 1968).

Among the many causes of hemolysis is that induced by drugs and their metabolites. Hemolysis due to the employment of primaquine, an 8-aminoquinoline antimalarial agent has well been documented by Dern et al (1954). The primary biochemical defect responsible for primaquine induced hemolysis has been recognised as a functional impairment of the pentose Shunt created by an intrinsic deficiency in the enzyme glucose-6-phosphate dehydrogenase (G6PD) (Carson et al. 1956).

The significance of drug induced hemolysis in primaquine-sensitive individuals lies in the fact that, many drugs commonly used in the clinical practice of medicine can also cause hemolysis when ingested by individuals lacking G6PD. These drugs include the latest of the antimalarial drugs, dapsone; which has been reported to induce hemolysis in normal cells [illegible] (WHO, 1967).

Malaria is among the leading causes of morbidity all over the world - ranking 6th at the Korle Bu teaching hospital, Ghana, in 1968; with a record of 372 admissions over a period of 12 months (Pobee 1972). Economically, malaria appears the world's most expensive disease not only in terms of the treatment required for it, but also in terms of the loss of productivity of individuals who are incapacitated by it. This fact is supported by a report from a central African hospital that hospital admissions of

malaria ranged from 5 to 15% of total admissions, and that out of 1,873 autopsies, malaria appeared to have been the cause of death in 12.3% of infants and 11.2 to 13.6% of older children (Janssen et al. 1966).

The seriousness of the malaria problem is clearly demonstrated in the statistical revelation of the World Health Organisation (WHO) that approximately 10% of the almost 250 million people who suffer clinical attacks of malaria annually, die of the disease (Williams, 1959).

Raitt (1970) points out that, the awareness that malaria kills more people and hence interferes more with agriculture and food production than any other disease, engendered the establishment of the WHO in 1948 to give priority to malaria attacks and its global eradication.

Now malaria is an eradicable disease in most areas - the sequel of advances in its treatment and in the control of mosquito vectors. However, in Ghana, antimalarial drugs remain the bulwark against this debilitating disease. Among the most widely used antimalarial agents are: quinacrine, chloroquanide, amodiaquine, primaquine and its congeners, and chloroquine.

Richards (1970) has reported the potency of synergistic use of sulfones and sulfonamides with pyrimethamine in malaria therapy, especially in case where certain strains of P. falciparum have developed resistance to chloroquine.

present complex problems.

Malaria in man is characterised by successive fevers, chills and sweat, anaemia and splenic enlargement. It is caused by the presence in the red blood cell of 4 closely related species of protozoal organisms belonging to the genus Plasmodium. These are the P. falciparum, P. vivax, P. malariae, and P. ovale. In Rats malaria is caused by the presence of P. berghei in their red blood cells.

P.falciparum and P. vivax are reported to account for 95% malaria cases all over the world, whilst P. ovale is particularly found in Africa (Pampama E., 1963). P. falciparum or malignant malaria frequently results in fatalities unless a suitable drug is promptly administered after the initial manifestation of the disease.

The life cycle of the parasite in the mammalian host begins when the sporozoites, which are mainly in the salivary gland of the anopheles mosquito, areinjected into the blood stream as an infected mosquito feeds. The final stages of the life cycle occurs in the red blood cells. The rationale behind the continued use of therapeutic agents in eradication programmes lies in the fact that, the life cycle of plasmodium in man is represented by a number of stages which vary in their degree of susceptibility to chemotherapeutic treatment - depending upon species and drug in question. In this respect chloroquine, a 4-aminoquinoline antimalarial has proved highly effective against the asexual erthrocytic forms of P. vivax and P. falciparum and gametocytes of P. vivax - hence it is the drug

of choice for treating acute malaria. Chloroquine has thus become a household name in West Africa, and appears under such trade names as Resochin, Avloclor, Aralen, Malarex, etc. (for the phosphate) and Nivaquine for the sulfate.

From the time of its synthesis by Adersag in the laboratory of the Bayer Company in Elberfield in 1934, chloroquine has faced a precarious existence. The history of its early fate, its rejection on grounds of toxicity and its subsequent revival have been admirably described by Coatney (1963).

During the Second World War, practically all the world's regular supply of quinine was denied to the Allies following the Japanese invasion of Pearl Harbour, Honolulu, Hawaii, in 1941. The need for alternative antimalarial drugs to meet the exigencies of that time resulted in the screeing of about 16,000 drugs for antimalarial activity. Atabrine and primaquine proved promising successors of quinine. The realization that atabrine imparts stains to the skin and eyes; causes gastro-intestinal irritations, and its failure to demonstrate curative properties against tissues stages of plasmodia precipitated its later rejection (Coatney 1963).

The usefulness to which primaquine could be put has since its discovery been bedevilled by primaquine's capability to induce hemolysis in G6PD deficient individuals (Faulkner et al. 1968).

Chloroquine which had then been rejected on false alert of toxicity was retested and found not only to be less toxic than atabrine, but also more potent than quinine, Chloroquine then,

having recovered with flying colors, had a clear run from 1946 onwards and was indeed the cornerstone of surveillance operation of the newly formulated eradication campaigns. After 15 years of service, the threat of chloroquine resistance was monitored by Thompson (1966) and hovered on all horizons.

Now it is realized that the problem is apparently more than drug resistance. To the clinical biochemist, the most serious obstacle to the continued use of chloroquine lies in accumulated data which indicate that chloroquine is not quite the harmless drug that it was long believed to be.

Carson et al, (1966) observed mild hemolysis but no anaemia in G6PD deficient male negroes on daily chloroquine dosage of about 300 mg. Bell and Davidson (1965) reported that cholineesterase activity in human red cell is inhibited by chloroquine, and Inglot and Wolna (1968) have also shown that high concentrations of chloroquine (10^{-3}M) labilized the red cell membrane, though the same report claims concentrations less than 10^{-4}M stabilized the membrane. Apparently, the possibility of chloroquine inducing a primaquin-like hemolysis cannot be ruled out. Lending weight to this argument is the recent report by Cotton and Sutorius (1971) that, on equimolar basis of 10^{-3}M, chloroquine inhibits G6PD of human red cells by 47.5% whilst primaquine causes inhibition of 28.1% - a difference of 19.4.

From the foregoing discussions, it can be seen that certain concentrations of chloroquine Phosphate induce hemolysis of red blood cells; a threat that warrants biochemical investigations.

In the present thesis, therefore, in vitro experiments have been conducted to study the effect of chloroquine phosphate on the rat red blood cells, under variable conditions, viz: in presence and absence of glucose and ATP respectively. The parameters studied are: The effect of chloroquine phosphate on:

a) the stability of the red cells to hemolysis.

b) enzyme systems in the red blood cells,

c) osmotic resistance of the red blood cells.

The aims of these experiments are to investigate a probable chloroquine-induced hemolysis, and a possible mechanism for such a hemolytic episode.

PART I

LITERATURE REVIEW OF THE RED BLOOD CELL

A. Formation and History of the Red Blood Cell: The formation and maturation of the red blood cell have been discussed by Bell et al (1966), and Harris et al, (1970).

The precursor of the red blood cell, the multipotential nucleated stem cell is formed in the marrow - skull, ribs and to a less extent in children, the ends of the long bones of the ribs, and passes through many stages before it is discharged into general circulation. The vast majority of erythrocytes develop by imperceptible gradations, through the orderly controlled sequence arbitrarily divided into pro-erythroblast, early erythroblast, late erythroblast, normoblast and reticulocyte to mature red cells with about three mitotic divisions within a period of 4 to 6 days (Erslev and Silver, 1967). In its early stages, the normoblast has a large nucleus with an open network of chromatin and basophilic cytoplasm. At the late or erythrochromatin normoblast stage, the cell becomes pyknotic, and is unable to carry out DNA synthesis and consequently incapable of further division (Cronkite et al. 1959). Hemoglobin then appears in the cytoplasm and the nucleus disappears from the cell by extrusion (Pease 1956, Simpson and King, 1967, Awai et al. 1968). The matured red cell therefore has neither a nucleus nor RNA and DNA.

B. Anatomy: The normal mature red blood cells are biconcave

discs having mean diameter of about 8μ and a thickness at the thickest point of approximately 2.4μ and in the centre of 1u or less. The average volume of the red cell is about 87 cubic microns. (Guyton, 1967). The red cell is composed of water, hemoglobin, lipids such as cholesterol, lecithin, etc. (Moskowits and Calvin, 1952; Perutz et al. 1960) and Carbohydrates, salts, enzymes, proteins, etc. (Ludwig, 1960).

The Colorless framework of red blood cells remaining after hemolysis has been referred to as ghosts, stroma, stromata, membrane or post-hemolytic residues (Van Deenen and De Gier, 1964). Shape controlling factors have been placed both within the interior of the cell (Shrivester and Burton, 1969) and in the membrane, but the membrane is generally considered to be responsible for the biconcave shape (Weed et al. 1963). The lipid components interact with the proteins in the structural organization of the membrane (Hanahan, 1969). The stroma itself has been shown to be birefringent, or doubly refractile, indicating a patterned molecular structure (Moskowitz and Calvin, 1952). Electron microscopy shows the surface of the human red cell to be covered by a layer of circular plaque-like structures approximately 100-500A° in diameter and 30A° thick (Hillier and Hoffman, 1953). The interstices of the plaques are thought to represent channels, or pores that allow for the egress and ingress of water, and electrolytes, etc. The composition of these plaques is not certain, but at least one model proposes

these plaques to be composed of lipid carbohydrate-protein complexes including a fraction termed clinin (Hillier and Hoffman; 1953; Whittam, 1964). Optical studies have demonstrated that (a) the lipoprotein complex in the red cell membrane has an intrinsic radial birefringence and (b) the proteins have a birefringence which together indicates a parallel arrangement of the proteins at right angles to the lipids (Moskowitz and Calvin, 1952).

C. Metabolism of the Red Cell:

(i) The developing nucleated red cell: With all the necessary subcellular components and enzyme systems for replication, maturation and differentiation at its disposal, the red cellat this stage is capable of synthesizing many different kinds of compounds and therefore adds to its structural material and enzyme components (Harris, 1970). The main effort of the cell at this stage appears to be the production of heme and globin. The cell has available to it, the TCA cycle, the Embden-Meyerhoff pathway and the Pentose Phosphate Pathway to meet its energy requirements (Bessis, 1956, 1961; Theorel 1968). The normoblast ceases to synthesize DNA and cannot reproduce by mitotic division any longer.

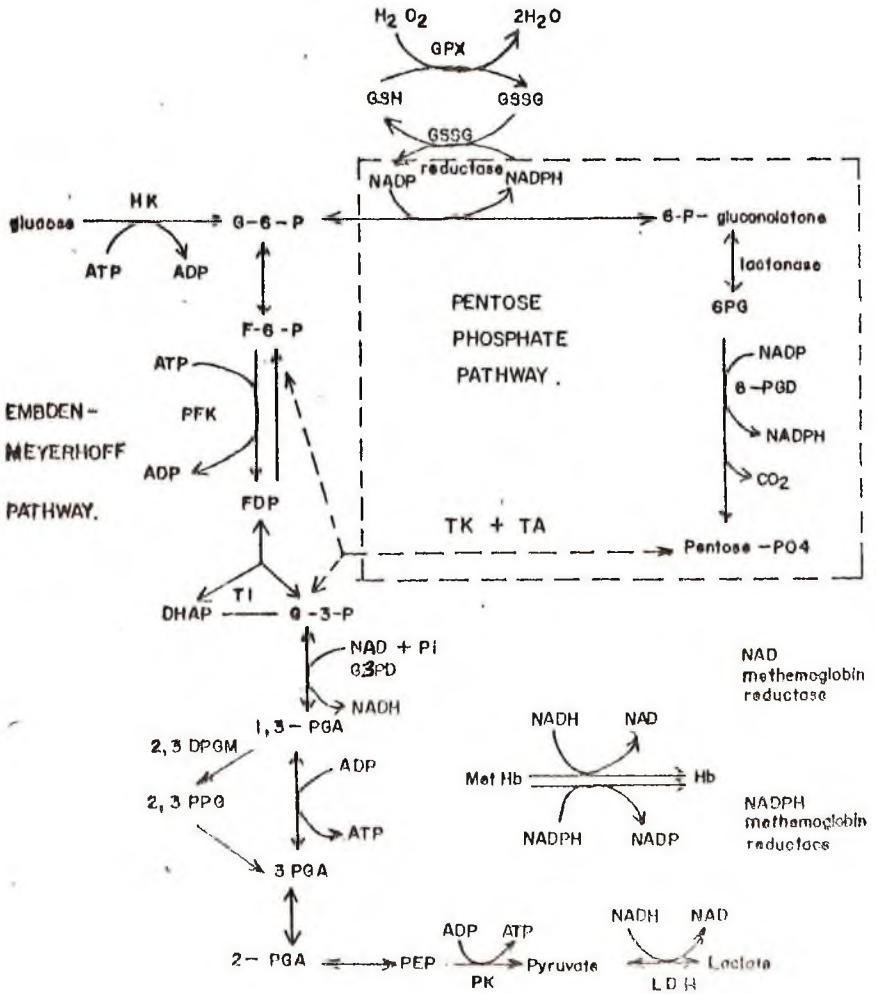
(ii) The reticulocyte retains the capacity to synthesize proteins (Lowesteine, 1959) in spite of the absence of nucleus and its inability to multiply and divide. Synthesis of heme and the incorporation of iron into hemoglobin also takes place at this stage. The reticulocyte contains the enzymes necessary for

the de novo production of compounds containing considerable potential energy for cell metabolism - the purine and Pyrimidine nucleotides. Both the Embden-Meyerhoff pathway and the pentose phosphate shunt are active at this stage of development. The TCA cycle and cytochrome system are intact and to some extent functional for electron transport (Enzymes, 1958). There is some evidence that lipids can be synthesized by the reticulocytes (Marks et al. 1960) and that the cell and plasma are in a dynamic state of exchange with respect to lipids and phospholipids (James et al, 1959; Reed 1959; Lovelock et al. 1960 ; Mendelsohn, 1961a, b).

(iii) The circulating mature red blood cell: The red cell at this stage of development has no RNA and is therefore incapable of forming heme, or synthesizing proteins, lipids and phospholipids (Buchanan, 1960; Darling and Roughton, 1942; Van Deenen and de Gier, 1964; Reed, 1968b). There is however a rapid exchange of phospholipids that requires no energy (Reed, 1968b). The TCA cycle no longer functions though a vestige of its enzymes exist (Dajani and Orton, 1958). In spite of its limited resources, the mature red blood cell does maintain itself by utilizing glucose through the Embden-Meyerhoff, Pentose phosphate, and glycogen synthesis pathways (Barlott, 1958; Murphy, 1960a b; [illegible] ; Mills, 1969; Yunis and Yasminsh, 1969).

Glucose metabolism in the red cell provides energy for two general activities essential to viability;

DIAGRAM I : A SCHEMATIC REPRESENTATION OF THE MAJOR KNOWN AND APPENDAGE REACTION OF ERYTHROCYTE CARBOHYDRATE METABOLISM.



Reactions in box of broken lines represent the Pentose Phosphate Pathway.

1) maintenance of oxidation - reduction processes so as to preserve hemoglobin and other proteins in their functional state and 2) maintenance of the physical attributes of the red cell including volume, shape, plasticity etc. (Jandl, 1966).

The Embden-Meyerhoff and the Pentose phosphate pathways differ with respect to the forms in which energy is made available for the maintenance of the red cell's integrity. Energy in the form of ATP is derived only from the reactions of the Embden-Meyerhoff pathways - specifically reactions catalysed by the enzymes phosphoglycerate kinase and pyruvate kinase, (Diagram I). Energy in the form of reducing power is generated by both pathways - the reactions of the Embden-Meyerhoff pathway generating uniquely NADH; and the oxidative reactions of the pentose phosphate pathway generating NADPH in the mature red blood cell. Subsequent reactions of the pentose phosphate shunt are non-oxidative and involve the formation of pentose phosphates. Isotopic studies indicate that the pentose phosphate pathway is a cycle in the intact red blood cells (Brin and Yanemoto, 1958; Szeinberg and Marks, 1961). The overall rate limiting step in the catabolism of glucose by the red cells appears to be hexokinase (HK) (Rapoport, 1966).

D. Enzymology of the red blood cell: In addition to enzymes with known functions (as those involved in glucose metabolism); many enzymes are present in the mature red bells whose functions are still obscure. Such enzymes include acetylcholineesterase,

which is suggestively associated with lipoprotein components on the outside surface of the stroma (Sabine, 1959). Human red blood cells also contain the necessary synthetic enzymes (GSH synthetase and 5-glutamyl cysteine synthetase) in adequate quantity to synthesize glutathione (Jackson, 1969). Among the enzyme systems of pathological significance in glucose metabolism are G6PD, 6PGD, Glutathione reductase and glutathione peroxidase.

G6PD: Red blood cell glucose-6-phosphate dehydrogenase (G6PD) has been a subject of considerable interest during the past two decades. This interest derives from the fact that a genetically determined deficiency of this enzyme is associated with an increased susceptibility of red cells to hemolysis; and also that the activity of this enzyme decreases as the cells age *in vivo* - both believed to be factors determining the survival of the red blood cells (Marks, 1967).

G6PD catalyses the first of the two oxidative reactions of the Pentose phosphate pathway (Diagram I) which are major sites of NADPH generation in the red blood cells. An evaluation of the importance of the G6PD reaction to the survival of the red blood cells can be made by considering the various metabolic functions of Pentose phosphates and the NADPH generated through the reactions of the Pentose Phosphate pathway. Ribose-5-phosphate, a constituent of various substances important in erythrocytes such as the Pyrimidine nucleotides, ADP and ATP (Bonsignore *et al.* 1964; Preiss

and Handler, 1957) is generated in the pentose phosphate shunt. The NADPH serves as an essential factor in reactions important in maintaining the integrity of the red cell, Mills (1959, 1960) suggested that this is important in maintaining the integrity of the red cell primarily because it is required to reduce oxidised glutathione.

Investigations of the enzyme in different human populations have shown the existence of many genetic variants (WHO, 1967). Some variants are associated with normal enzyme activity and are therefore not accompanied by clinical manifestations (e.g. A type in negroes). Another variant causes enzyme deficiency, but requires exogenous agents such as drugs, infections or fava beans for hemolysis to occur (i.e. A^- variant in negroes and G6PD deficiency in mediterranean populace). Other variants cause severe instability of the enzyme and are associated with chronic hemolytic disease even in the absence of exogenous agents (i.e. G6PD Chicago, Oklahama, etc.) (Yunis, 1969).

Motulsky et al, (1966) postulated that the A^- variant in negro owes its frequency to selective advantage, viz-a-viz Falciparum malaria.

The locus for G6PD is situated on the X-chromosomes (Beutler et al, 1968). Variants of G6PD presumably representing single amino acid substutions are caused by specific base pair mutations within the structural gene for G6PD. So far, the exact nature of the amino acid substitution has only been fully demonstrated for

the A mutations (asparagine to aspartic acid). This is not associated with enzyme deficiency and is commonly seen in persons of African descent (Yoshida 1967a, b).

6-Phosphogluconate dehydrogenase (6PGD) catalyses the second oxidative reaction in the pentose phosphate pathway, subsequent to the G6PD reaction (Diagram I). The importance of the 6PGD reaction lies in the inherent products of the pentose phosphate shunt - Pentose phosphates and NADPH.

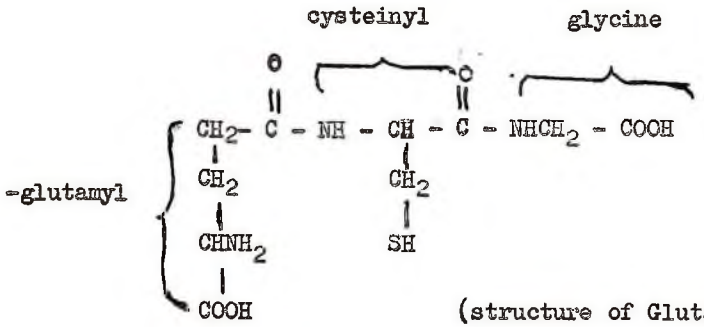
A deficiency of 6PGD has been reported to be present in the red cells of several individuals (Brewer and Dern 1964a, b; Parr and Fitch, 1964; Lauseckler, et al, 1965; Scialom, et al, 1966). This deficiency has several qualitatively and quantitatively (30-90% of normal) abnormal representations (Brewer, 1969). The individual initially reported with a deficiency of 6PGD did not have associated hemolytic process (Brewer, 1964b), however, red blood cells of this individual had a small decrease in survival time in vivo following the daily ingestion of 60 mg of primaquine. In only two cases of the abnormality was there evidence of accelerated hemolysis in absence of drug (Lausecker, et al, 1965; Scialom et al, 1966). In addition to the 50% decrease in activity, Lausecker et al, (1965) found a 50% decrease in NADH, NADPH, and ATP content of the erythrocytes. Isotopic studies indicate that the decrease in G6PD activity produces a minimal impairment of the functional activity of the Pentose phosphate pathway in the erythrocytes.

Glutathione-Reductase catalyses the transfer of electrons from NADPH to oxidised glutathione (GSSG) to form two molecules of GSH (Diagram I). Glutathione reductase in human red cells is apparently controlled by an autosomal gene and Brewer (1969) and Long (1967) have described two rare qualitative variants. Many authors have reported variants with increased activity (Long and Carson, 1961; Long 1962; Schrier et al. 1958) and other variants with low activity (Carson et al. 1963; Lohr and Waller, 1962; Waller, 1968). A deficiency has been reported in several individuals (Carson et al, 1961a, Lohr and Waller, 1962; Waller, 1968), and a few of such patients develop hemolytic anaemia in association with the ingestion of oxidant drugs (Carson et al. 1963, Carson and Frischer, 1962).

Glutathione reductase activity may be increased 20 to 30% in patients with G6PD deficiency (Schrier et al. 1958), gout (Long 1962), and diabetes mellitus (Long and Carson, 1961). Nitrofurantoin which causes hemolysis in G6PD deficient individuals, has been reported to cause a reduction of glutathione reductase activity in vitro, at concentrations that are likely to occur in vivo with therapeutic dosages of the drug (Buzard et al. 1960). In vitro, glutathione-reductase has been found to increase by the formation of methemoglobin (Michot and Marti, 1966) and by incubation of hemolysate with stroma (Carson et al, 1961b).

Individuals with a nutritional deficiency of riboflavin have a significant decrease in the activity of this enzyme. (Bamju, 1969).

Glutathione: Reduced glutathione (GSH) exists in human red Cells in high concentration compared with other sulfhydryl compounds. It is a compound composed of three amino acids with a free sulfhydryl group on cysteine, and it is presumed that this compound could be used to make proteins or act as a source of electrons to serve as a "buffer" against oxidising agents and free radicals which might otherwise damage vital parts of the red cell - particularly the membrane.



(structure of Glutathione (GSH)

It is firmly established that reduced glutathione is bound to the enzyme glyceraldehyde-3-phosphate dehydrogenase (G-3-PD) (Krimsky and Racker, 1952). However, it is of note that Sternschus et al. (1961) observed no decrease in G3PD activity in erythrocytes

whose GSH concentrations were decreased to undetectable levels by incubation with acetylphenylhydrazine.

Five members of a large Dutch family, resulting from consanguineous marriage, were found to have a marked deficiency of reduced glutathione (GSH), in their red blood cells (Prins et al, 1966, 1968). All five individuals had a compensated hemolytic process that was absent in other family members without the deficiency. The hemolytic process was accelerated by primaquine.

PART II

LITERATURE REVIEW OF ANTIMALARIALS

A. General Discussion: Remedies for treatment of malaria have been used since the dawn of civilized man's history. Of these herbal remedies only one, quinine, survives today. Others of much more recent vintage includes synthetic drugs like primaquine, chloroquine, quinacrine, etc.

B. Terminology of malaria and malaria Eradication: Two terms that are used to reflect the mode rather than the site of drug section are:

Plasmodicidal: Killing of malaria parasites.

Plasmodistatic: Arresting malaria parasite development.

Drugs used in the treatment, prevention and cure of malaria have been classified into the following categories:

1) a) Blood Schizontocide: A drug which acts on the asexual parasites in the blood.

b) Tissue Schizontocide: A drug which acts on the asexual parasites in tissues.

2. Gametocytocide: A drug which destroys the sexual forms of the malaria parasite.

3. Sporontocide: Drugs which when given to malaria-infected vertebrate host prevents or interrupts the development of the parasite in mosquitoes feeding on that host.

The following terms are also used in reference to the function of antimalarials as defined by Covell et al (1953) and later modified by a WHO Committee (WHO, 1963;

a) Treatment - Suppressive: Treatment aimed at preventing or eliminating clinical symptons and/or parasitaemia by early destruction of erythrocytic parasites. It does not necessarily prevent or eliminate the infection, and overt malaria may develop after drug withdrawal.

b) Treatment - radical: Treatment adequate to achieve radical cure. In vivax, malariae and ovale infections, this implies the use of drugs which destroy the secondary tissue stages of the parasite.

c) Prophylaxis - Casual: Complete prevention of erythrocytic infection by administration of drugs that destroy either the sporozoites or the Primary tissue forms of the malaria parasite.

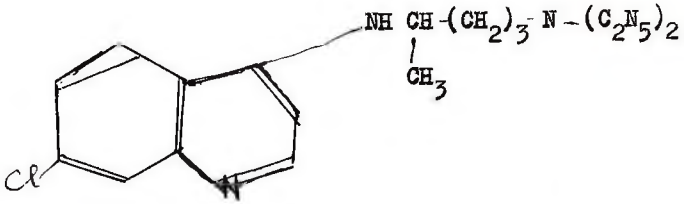
C. Chemistry and Pharmacology of Chloroquine: The pharmacology of chloroquine has been reviewed by several authors (Rollo, 1966, Williams R.T., 1959, M. Sams, 1967 and Olatunde, 1971).

Chloroquine: (Aralen, Avoclor, Nivaquine, Resochin, Malarex etc). Chloroquine is a potent Schizontocidal antimalarial agent - a drug of first choice in the treatment of an overt attack of falciparum as well as other species of malaria in man (Olatunde, 1971).

The benefits derived from this remarkable drug are not limited to malaria alone. It is frequently employed for the treatment of collagen diseases, especially rheumatoid arthritis and discoid lupus erythematosis (Goldman and Preston, 1957) and in addition it enjoys a good record in the treatment of extraintestinal amoebiasis (Conan, 1948; Lane 1951), Clonorchis infection and several other parasitic diseases. Its use in the treatment of broncial asthma (Juul Moller, 1961, Knoryavstaer, 1966) and in the control of epilepsy (Burns, 1966) has also been reported.

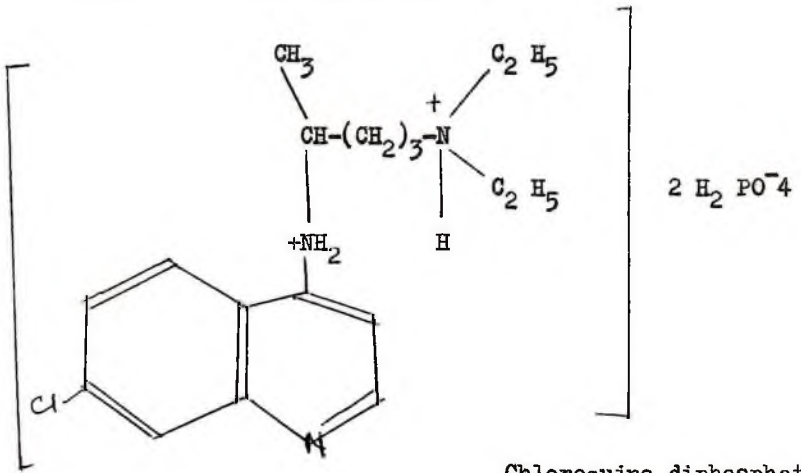
For suppression of malaria, an oral dose of 0.5 g is administered on the same day of each week. For treatment of an acute attack of P. vivax or P. falciparum, and initial dose of 1.0 g is administered followed by 0.5 g after 6-8 hours on each of two consecutive days to make a total of 3 g in three days (British Pharmaceutical Codex, 1968). As an antimalarial prophylactic, about 5mg of base per kg body weight is taken once weekly for many years. In the treatment of collagen diseases which usually extends over many months, the daily dosages are 3.5-14mg/kg body weight (Chinyanga et al. 1971).

II. Chemistry: Chloroquine is one of the derivatives of the 4-aminoquinolines, and has the chemical formula ($C_{18}H_{20}ClN_3$).



7-Chloro-4(4-diethylamino-1-methyl-butylamino)quinoline.

Chloroquine may exist in the sulfate or phosphate forms. Chloroquine in either form is a salt and in the case of the phosphate has the structural formula:



Chloroquine diphosphate.

The phosphates are available both as white tablets and as injections. The tablets are odourless, have a bitter taste and slowly decolourize on exposure to light. Chloroquine is freely soluble in water and aqueous solutions have pH of about 4.7, however it is insoluble in alcohol (Am. Prof. Pharma, 1946).

Chloroquine phosphate may be prepared by condensing 4, 7-dichloroquinoline with 1-diethylamino-4-amino pentane. The resulting free base is treated with $H_3 PO_4$ in methanol (Surrey and Hammer, 1946). The colourless crystals that separate have m.p. of 193-218°C.

Acid-Base Properties: At physiological pH, chloroquine accepts two protons to form doubly charged species which may be sympolised as $H\overset{+}{B} - \overset{+}{B'}H$ where B and B' represent the amino nitrogen and the diethylamino nitrogen respectively.

The ionization constants of chloroquine have been identified by Irvin and Irvin (1947):

i) $H\overset{+}{B} - \overset{+}{B'}H$ $\qquad B \longrightarrow \overset{+}{B'}H + H + pKa = 8.1$

ii) $B - B'H$ $\qquad B' \longrightarrow B = H + pKa = 10.2$

III. Absorption: Chloroquine is rapidly and almost completely absorbed from the gut when taken by mouth (Berliner et al, 1948; Loeb 1946), less than 10% is excreted. The rate of absorption suggests that absorption takes place mostly in the stomach and upper small intestine. Olatunde (1971a) suggests that the efficiency of absorption from the alimentary tract does not call for parenteral therapy except in cases where oral adminstration is marked by conditions like vomitting, unconsciousness etc.

Intramuscular injection has been recommended in highly imperative conditions (Jelliffe 1966; Adams and Maegrath 1960; Gilles, 1966). Adequate levels are reached within 15 mins of intramuscular injection and one to two hours following oral adminstration.

IV. Distribution: Several workers have studied the distribution of chloroquine in the tissues. The concentration in red blood cells is about twice that in plasma (Berliner et al, 1948), but much higher levels have been reported for leucocytes (Wiselogle 1946). This latter observation demonstrates chloroquine's higher affinity for white blood cells.

Berliner et al. (1948) reported plasma levels to be not greater than 10^{-6}M. Red cells containing chloroquine-sensitive malaria parasites have been shown to concentrate the drug to higher levels. The concentration relative to that of non-infected cells have been estimated variously as 100:1 (Macomber et al. 1966): 300-500:1 (Polet and Barr, 1968) and 40:1 (Fitch 1969). Erythrocytes containing Chloroquine-resistant parasites have been reported to accumulate considerably less of the drug than sensitive ones (Macomber et al, 1966). Chloroquine and mepacrine are stored in liver parenchyma cells where high concentrations may build up, thus providing storage facilities to prolong drug action. In the liver, concentrations build up to 400 times, while in the lungs and kidneys the tissue levels may exceed 600 times those in the plasma (Berliner et al, 1948).

Rubin et al (1963, 1965) found very high levels of the drug in ocular tissues in pigmented rats. It has been shown that chloroquine and related compounds have a strong affinity for melanin containing cells and tissues (Zvaifer et al, 1963; Sams and Epstein, 1965). Berstein et al,(1963), for example, showed that in rats chronically treated with chloroquine, the liver contained 240 µg/g wet weight while the iris contained nearly 21.00µg/g! The choroid, retina, brain and hair had levels of 7,820, 312, 15 and 780µg/g respectively.

In all these cases, most of the drug was reportedly stored unchanged and only a small proportion as the metabolites.

Shaffer et al (1958) had shown that chloroquine is concentrated about 5 to 15 times more in the epidermis than in the corium. However Tuffaneli et al (1963) found no difference between the concentrations of chloroquine in the skin of negro and white patients. Olatunde (1971) found higher concentrations of chloroquine and lower levels of metabolites in skin from patients prone to chloroquine-induced pruritus than in skin from other patients. He therefore suggested that increased liability to chloroquine-induced pruritus may be associated with a lower rate of chloroquine metabolism.

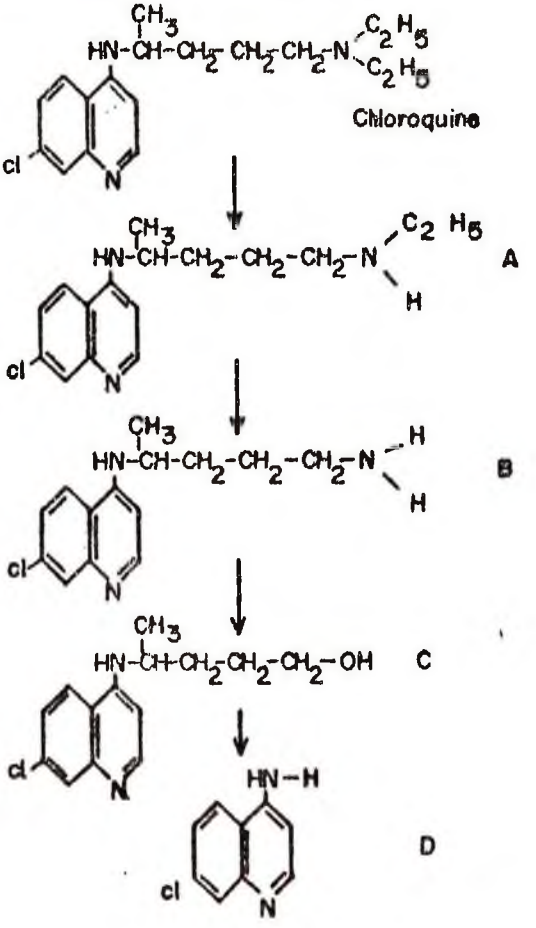
Protein binding in the plasma occurs to the extent of 55% (Berliner et al. 1948). Parker and Irvin (1952) have also shown that chloroquine has some affinity for the highly polymerized deoxypentose nucleate and nucleoprotein of beef spleen. It has actually been confirmed that chloroquine combines with DNA (Kurnick, 1956, Hahn et al 1966), and this has led to the idea that such binding determines the intracellular distribution of the drug in vivo. particularly the suggestion that such interaction may be important in the distribution of the drug among various tissues such as the spleen, white blood cells; and parasitized red blood cells. Varga (1968) could however not confirm this suggestion since he found no relationship between chloroquine and nucleic acid content of various cell particles. His argument was in accord with the histological findings of Baumer and Coworkers (1968) that in the

white blood cell of chloroquine-treated rabbits, the majority of the drug can be found in the cytoplasm whereas the nucleus contained only insignificant amounts.

V. Metabolism: Titus et al (1948) investigated the metabolism of chloroquine and several of its analogues in man, partly in an attempt to identify metabolites with higher levels of antimalarial activity. They succeeded in identifying two processes of detoxification, the first involving conjugation to an uronic derivative and the second the successive removal of side chain groupings down to the quinoline nucleus which remained intact. Chloroquine is slowly degraded in man, and plasma levels up to 52% of that measured at 3 hrs after a dose were found 5 days after the same dose (Alving et al, 1948; Berliner et al, 1948). Prouty and Kuroda (1958) utilized a spectrophotometric method to determine the chloroquine levels in various tissues obtained at post-mortems from men who had died in Korea. Kuroda (1962) later investigated the same specimen further by means of paper Chromatography and U.V. spectroscopy in order to determine the presence and nature of chloroquine metabolites. The four metabolites he detected confirmed the general conclusions of Titus et al (1948). The deethylated derivative (metabolite A in diagram II) has been shown to be the major metabolite)McChesney et al, 1954).

Muting (1962) compared the metabolism of the sulfate and the diphosphate of hydroxy chloroquine in patients with chronic poly-

DIAGRAM II. METABOLISM OF CHLOROQUINE (Kuroda, 1962).



arthritis, and found that serum concentrations of the sulfate various times up to 48 hours of adminstering a single dose of 400 mg were about 3 times as high as those following the same dose of the diphosphate. In each case peak levels were reached after 2 hours but the parent compound showed levels of only 2-3 mg %, whereas metabolites accounted for some 10-20 mg%. These findings differ from those of Kuroda (1962) whose analyses revealed that, at least in solid tissues, the main compound present was the parent substance - chloroquine. William (1959) pointed out that not only chloroquine but also some of its metabolites are potent antimalarials and probably the latter contribute to its total schizontocidal activity. McChesney (1954) followed the trials further and reported that the final degradation product 4-amino-7-chloroquineline(D) is a relatively minor metabolite in man.

VI. Excretion: The urinary excretion of chloroquine is slow and discontinuous (Zanca and Benatti 1959). It was estimated that plasma levels fall by an average of about 50% per week after stopping drug administration (Zvaifler, et al 1963). Three to five years after the last known injection of chloroquine patients were found excreting the drug in urine and to have measurable concentrations in the blood (Rubin et al, 1963). The proportions of drugs and metabolites excreted in the urine following daily administration to man of chloroquine or hydroxy-chloroquine have been estimated as parent compound 60%; secondary amine(s) (A) 37%, and primary

amine 3% (McChesney et al 1966). In a later report, McChesney, Fasco and Banks (1967) showed that plateau plasma level of 125 ug/litre of chloroquine was reached after daily oral administration of 310 mg chloroquine base for 14 days. Some 10% of the dose was excreted in the faeces and 56% in the urine, but 34% was not recovered and its fate in the body remained obscure. Of the material excreted in the urine; 70% was parent compound and 23% deethylchloroquine. Commenting on the kinetics of chloroquine metabolism, the authors pointed out that, although no further deposition of the drug in the tissues would logically be anticipated once a steady balance had been achieved between plasma levels and urinary output, their experimental data did not support this concept.

The administration of intramuscular dimercaprol (BAL) was found to increase the renal output of chloroquine (Rubin et al. 1963).

VII. Toxicity and side Effects: Chloroquine may occasionally give rise to headache, gastrointestinal disturbances and blurring of vision (Alving et al. 1948; McChesney and Coulson, 1963). In children aged 14 months to 3 years ingesting large doses (0.75 - 2 gm chloroquine phosphate tablets), signs and symptoms of toxicity appeared within 45 minutes and death occurred under 2½hrs (Cann and Verhulst, 1961). Burns (1966) points out that permanent loss of vision from retinopathy may be a most serious sequel of prolonged chloroquine therapy, and advocates sporadic check up by opthalmologists on patients requiring chloroquine.

Chloroquine-induced retinopathy has been attributed to accumulated levels in occular tissues, and, Read, (1968b), reviewing his own and others' data has questioned the wisdom of using anti-malarials at all in collagen diseases, the therapeutic value of which is in any case open to dispute.

Cases of severe muscle weakness ("neuromyopathy") have been reported (Du Bois 1956; Loftus 1963; Whisnaut, 1963; Begg, 1964; Merwin, 1965, and Eadie, 1966). This chloroquine-induced neuromyopathy was observed to be clinically completely reversible within a period of six months after withdrawing the drug (Blom, 1965). Chloroquine has been reported to have a direct relaxant action on isolated guinea pig ileum, inhibitory to the spontaneous pendular movements of the isolated rabbit duodenum (Olatunde 1970b), and antagonize histamineacetylcholine and serotonin induced constractions of the isolated guinea pig ileum (Olantunde 1970b).

Eadie and Ferrier (1966) who observed glycogen accumulation in miscles of a patient receiving continuous chloroquine therapy for 6 months had suggested that chloroquine injures muscles by inhibiting enzymes involved in glycogen metabolism. Chloroquine also causes beaching of the hair.

One of the enzyme systems concerned with melanin production, the tryrosine oxidase system, is inhibited by chloroquine in tadpoles and an abnormal brown pigment is produced in treated animals.

Hopkinson and Jackson (1964) have suggested that this property might be related in some way to the abnormal pigmentation that is seen in some patients on chloroquine therapy for collagen diseases.

In view of the inherent abnormal tyrosine metabolism in rheumatoid arthritis and other collagen diseases, these workers have suggested that chloroquine might be producing some beneficial effects in such conditions by its effect on tyrosine metabolism or other amino acids. Hopkinson & Jacobson also showed that chloroquine inhibits tyrosine uptake by certain mammalian tissues and hence the incorporation of this amino acid into proteins. This they believed is a possible mechanism by which chloroquine inhibited the growth and metamorphosis of tadpoles in their experiments. Intravenous injection of chloroquine may cause a fall in blood pressure (Scott, 1950; Arora et al, 1955). A boy aged 1½ yrs weighing 10 kg and in good condition died about 10 mins after the intramuscular injection of about 300 mg base. Death was believed to be due to sudden pronounced hypotension caused by an overdose of the drug (Harris, 1955).

In humans, a single large dose of chloroquine causes depression in the respiratory rate, difficulty in breathing and swallowing, analgesia and paresthesia of the face and neck (Kjaor, 1955).

Nelson and Fitzhugh (1948) described microscopic pathological changes in albino rats fed with free chloroquine base at various levels for 2 years. The lesions observed grossly were humping of the

back, slight roughness of the liver surface, cardiac atrial thrombosis, hydrothorax, testicular atrophy, pitting of kidney surface and brown uteri.

Of the microscopic changes caused by chloroquine, the most damaging was a relatively slow necrosis on voluntary muscle, with destroyed muscle being replaced by fibrous tissue. The liver was moderately damaged. Slow necrosis of the hepatic cells led to centrilobular necrosis and distortion of structure. It also caused a slight to moderate increase of fat in the liver, kidney, spleen, uterus and the pancreas. Mendel (1960), noted that chloroquine possesses local anaesthetic and in high concentrations anti-coagulant properties; Madow (1960) reports that chloroquine has a desludging effect on blood. Chloroquine is particularly useful in the treatment of photoallergic reactions (Rollo, 1965), and Knox and Freeman (1963) showed that chloroquine decreases the incidence in humans of cutaneous carcinogenesis caused by sunlight. The drug has been found to inhibit the enzyme succinate dehydrogenase, possibly decreasing the energy available for the conduction of cardiac impulses (Bellet S., 1961).

It has been suggested that chloroquine is beneficial in rheumatoid arthritis andsystematic lupus erythematosus because it interferes with the ability of host to form antibodies. Thompson and his co-workers (1962) supported this hypothesis by demonstrating a slight decrease in the serum-globulin levels of patients

with rheumatoid arthritis on hydroxychloroquine therapy. In a later study, Thompson and Bartholomew (1964) found that chloroquine had absolutely no effect on rabbits' ability to produce circulating antibody to typhoid C and H antigens and to bovine serum albumin. Further work by Kalmanson and Guze (1965) suggests that chloroquine may not suppress immune mechanisms to any measurable degree. Stephen et al (1965a) found no significant differences in steroid levels on a single Resochin infusion. However, they reported slightly increased levels after adrenocorticotrophic hormone (ACTH) infusion. Long term administration apparently regulated sodium and potassium metabolism in patients with articular diseases. They proposed from their experiments that chloroquine probably interferes with the regulatory mechanisms of adrenal cortical hormones.

Whitehouse and Conway (1966) demonstrated that chloroquine inhibits the protease induced release of peptides from incubated cartilage slices. The possibility that this effect could be due to lysosomal stabilization was eliminated when the authors demonstrated that lysosomal labilizers like Triton X-100 or repeated freezing and thawing had no effect on the chloroquine-sensitivity of the protease. Chloroquine has also been found to significantly retard the release of enzymes from rabbit liver lysosomes in vitro irrespective of whether the condition is effected by toxins, steroids or vitamin A (Weissman 1964). The drug has also been shown to protect lysosomes and leucocytes from disruption by streptolysin S

or lysolecithin. Allison and Young (1964) using fluorescence microscopy have shown the localization of chloroquine to cytoplasmic organelles of macrophages, organelles which they assumed to be lysosomes. Of more interest to the dermatologist however is that chloroquine greatly retards the U.V.-induced release of enzymes from lysosomes (Weisman 1964).

These correlations are so challenging that the possibility is suggested that the action of chloroquine on lysosomes is at least in part responsible for their biological activity.

D. Mechanism and mode of antimalarial action: Partition profile studies have shown that the accumulation of basic antimalarials within the red cell is dependent upon the difference between pH of the plasma and that within the red cell (Rollo 1968, 1969). It has therefore been suggested that lactate production by the actively metabolising parasites decreases the pH of the red cell, thus increasing the pH gradient and facilitating the entry of more drug (Peters 1970).

It thus follows logically that at physiological pH when 18% of chloroquine exists in the monoprotonated form (Allison and Young, 1964), which is lipid soluble, the drug would pass from membrane plasma into the more acid cytoplasm with the production of the doubly protonated form which is incapable of passing back.

An unusually large number of food vacuoles and mitochondrion-like whorled membranes found in chloroquine resistant P. berghei

(Peters et al. 1965) suggest a relatively higher metabolic and respiratory activity in resistant parasites than in normal ones (Howells et al. 1968). This in turn implies a greater, or at least, more effective diffusion of solutes across the parasite membrane, so that if chloroquine enters the sensitive parasite by a non-selective process as proposed above, one would anticipate that more, rather than less drug enters the resistant parasite. However Macomber et al. (1966) have shown that this is not the case; and the corollary therefore could be that chloroquine enters the parasites by some selective process.

Chloroquine itself, as already discussed, has the ability to stabilize certain types of membranes including that of the red cell envelope and of lysosomes. Allison and Malluci (1964) showed that the stabilization of lysosomes by chloroquine in dividing lymphocytes and liver parenchymal cells in tissue culture inhibited the normal process of cytoplasmic division which, they suggested, was normally proceeded by dissolution of lysosomal membranes and release of lysosomal isoenzymes. Following Allison and Young's (1964) demonstration that other antimalarial blood schizontocides including mepacrine and quinine were concentrated in mammalian lysosomes, Warhurst (1967) and his co-workers directed their efforts towards demonstrating the existence of lysosomes in malarial parasites. Warhurst and Hockley (1967) in England and Macomber et al.(1967) in Washington simultaneously published Ultrastructural data on the in vivo action of chloroquine.

Both groups of workers showed that the first action of chloroquine (as known from light microscopic observations) was to bring a conglomeration of haemozoin granules in what were clearly a type of secondary lysosomes (cytolysosomes and phagosomes). Warhurst and Hockley (1967) confirmed that pigment conglomeration in cytolysosomes of P. berghei and P. cynomolgi took place in developing trophozoites and not in mature merozoites. Upon contemporary reports that chloroquine could form a complex with certain porphyrin derivatives of hemoglobin, Macomber et al (1967) suggested that it was actually the concentration of chloroquine by such a pigment in the parasite that formed the basis of action of the drug. They therefore suggested that chloroquine resistance in P. berghei was a result of reduced haemozoin formation that occurs in resistant trophozoites. Although they agreed with the general principle that chloroquine was associated with sites of pigment formation. Warhurst and Hockley (1967) suggested that, in order to penetrate the remainder of the parasite cytoplasm where its toxicity could be exerted, it would be necessary for the drug to be released at some stage from the lysosomes (a similar conclusion was reached by Aikawa and Beaudoin 1969 on the basis of their studies with P. gallinaceum). This, they proposed, could occur if the action of chloroquine on lysosomal membranes could be shown to be biphasic, i.e. that low concentrations

labilize the membranes. Inglot and Wolna (1968) have already published evidence in support of this contention.

Polet and Barr (1968) have suggested that initial uptake of the drug is energy-dependent and therefore due to active transport though this could be followed by a slower energy independent phase. Fitch (1969, 1970) however proposes binding sites in the malarial cell as responsible for the accumulation of the drug. Homewood et al (1972) demonstrated that simply increasing pH of the medium in which parasitized erythrocytes were suspended caused the same morphological effects as exposure to chloroquine. Relating their findings and Rollo's theory to the lysosomal organelles of a cell which are considered to be at more acid pH than the general cytoplasm, the authors postulated the following mechanism of action for chloroquine.

Chloroquine acts by initially raising the pH of the malarial food vacuoles thereby reducing the digestion of hemoglobin by the parasites and preventing its growth. In this connection, they explain that the impotence of chloroquine on sporogonic and exoerythrocytic stages of the parasites is due to absence of digestive vacuoles. The decreased concentration of chloroquine in erythrocytes containing resistant parasites has on this basis been attributed to reduction of acidity of food vacuoles of resistant parasites - thus reducing uptake of chloroquine, and consequently, preventing an effective hemoglobin digestion by enzymes requiring an acid pH

for maximum activity.

Parker and Irvin (1949, 1952) first reported the interaction of chloroquine with yeast RNA; and later demonstrated the reversibility of binding between chloroquine and nucleic acids, particularly the highly polymerized deoxypentose nucleate (Parker and Irvin, 1952). They reasoned that this could explain the accumulation of chloroquine in cells such as those of the liver, heart, leucocytes and the red blood cells. The implication of this phenomenon received further clinical attention when Levin and Pinkus (1961) demonstrated the effectiveness of chloroquine therapy in a patient with autosensitivity to DNA. Cohen and Yielding (1963) observed that chloroquine protects the double-stranded DNA helix from heat denaturation. In addition, other biological properties of DNA are reported to be altered by its interaction with chloroquine. Such binding can:

1) retard or inhibit enzymic depolymerization of DNA(Kurnick and Radcliffe, 1962),
2) interfere with its function as a primer for the DNA and RNA polymerase reaction (Cohen and Yielding, 1964),
3) reduce the bacterial transforming activity of DNA (Stollar and Levin, 1963).

Using nucleic acid polymers, as well as monomers of certain Purines and pyrimidines, Cohen and Yielding (1965) studied the molecular nature of the chloroquine-DNA complex. Briefly, the binding results

from electrostatic forces and involves two separate portions of both molecules.

There appear to be few reports of chemical studies aimed at the direct evaluation of chloroquine activity on nucleic acid metabolism in malarial parasites. One morsel of direct evidence of chloroquine action on nucleic acid is the demonstration of Warhurst and Williamson (1968) and Warhurst (1969) that there is a change of parasite ribosomal RNA following exposure of P. Knowlesi erythrocytic parasites to chloroquine. Light ribosomal RNA (17-18S) decreased in relative proportions to yield in its place particles of 15S size. These, they assumed, represented degradation products formed by the action of parasite ribounclease in the cytolysosomes, that Warhurst and Hockley (1967) showed are produced by the action of chloroquine.

Following the uptake of ^{3}H-orotic acid in the presence of chloroquine in vitro, Warhurst (1969) demonstrated that drug concentration of approximately 4 x 10^{-4}M caused a 50% inhibition of DNA synthesis in P. Knowlesi; but that 2 x 10^{-3}M was needed to produce this degree of inhibition of RNA under similar conditions. DNA synthesis was inhibited by 13% at as low a concentration as 10^{-6}M which did not affect RNA. Examining the effect of chloroquine and dihydroxychloroquine upon P. Knowlesi in vitro, Polet and Barr (1969) concluded that the primary mechanism of action of both drugs was inhibition of DNA replication, which results in inhibition of

not only DNA but RNA and subsequently protein synthesis. This supports to some extent Warhurst's (1969) observations.

Ladda and Pious (1968) approaching the problem morphologically demonstrated in culture of human fibroblasts that chloroquine produces a disorganisation of the endoplasmic reticulum and separation of the ribonucleo protein (RNP) particles from it.

Lysosome-derived structures filled the cells and mitochondrial and nucleolar changes appeared. They suggested that the main site of action of chloroquine involves RNP and the endoplasmic reticulum.

PART III

PRIMAQUINE AND DRUG-INDUCED HEMOLYSIS IN THE RED BLOOD CELLS

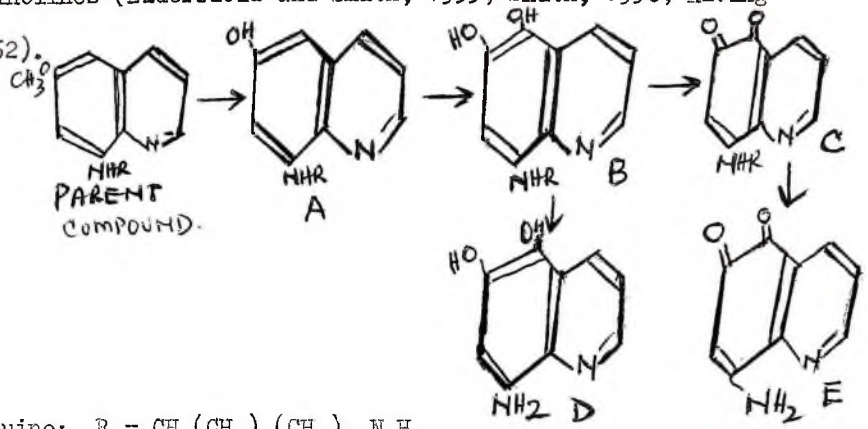
A. Primaquine: An 8-aminoquinoline derivative, primaquine is one of the most effective drugs for use against tissue stages of the malaria parasites. Though a poor blood schizontocide, primaquine is highly active against gametocytes of all species, and is used in curtailing transmission. It is not used alone for prevention of malaria.

Weekly adminstration of 45 mg primaquine together with 300 mg chloroquine base have been recommended for prophylaxis; in which case the primaquine suppsedly exert an effect upon persisting tissue forms (WHO, 1967).

In vitro, primaquine and its cogeners are inactive, but are markedly antiplasmodial in vivo. It has been suggested that they are metabolised in man to compounds of high oxidant properties which though active antimalarials, are very toxic, and induce hemolysis in individuals lacking G6PD.

Diagram III

Proposed pathway for metabolism of primaquine and related 8-aminoquinolines (Elderfield and Smith, 1953; Smith, 1956; Alving et al, 1962).



For Primaquine: R = CH (CH_3) $(CH_2)_3$ N H_2

The 5,6-quinoline quinone (structure C in diagram III) is presumed to be the major metabolite which exerts the antimalarial and toxic effects of the 8-aminoquinlines. A likely site of hydroxylation of aromatic substances in vivo is in the liver where microsomal hydroxylating system is present (Mitoma et al, (1956).

B. Hemolysis and Primaquine-induced Hemolysis: De Duve aptly remarked that life is characterised as much by death and degradation as by birth and proliferation, the former characteristic being every bit as essential to living matter as the latter, by allowing for replacement and regulation. With the red cell, loss of regulation as encountered in the anaemias is attributable to either disorders of birth (erythropoiesis) or of death (Hemolysis). Disorders of erythropoiosis has been generally classified into three pathogenetic kinds namely:

1) Aplastic anaemia: involving simple suppression of prolifera-

tion at an early level.

2) Megaloblastic anaemia: arising from impaired DNA synthesis, and 3) Hypochromic anaemia: the sequel of impaired hemoglobin synthesis.

In a sense, these disorders represent a failure of erythropioesis in which inadequate numbers of mature red cells enter circulation.

Hemolysis in vivo is generally defined as the shortening of the life span of the red blood cells. Hockwald (1952) first reported primaquine-induced hemolysis in American negroes. His findings initiated extensive routine hematological studies including morphological observations. The only abnormality noted was the presence of darkly stained $\frac{1}{2}$ to 1μ coccoid bodies called Heinz bodies; later identified as nothing but denatured hemoglobin. Beutler (1955) reported that the glutathione (GSH) content of primaquine sensitive red blood cells was lower than normal and that it fell further during the course of primaquine-induced hemolysis. Later, Carson (1956) demonstrated that G6PD deficiency is the primary defect of primaquine sensitive red blood cells. In another search for further biochemical defects, Tarlov (1961) reported a remarkably low catalase activity in most primaquine-sensitive individuals. Catalase catalyses the decomposition of H_2O_2 in the reaction; $2H2O_2 \longrightarrow 2H_2O + O_2$

Other metabolic defects of erythrocyte primaquine-sensitive negroes include the following:

1) Decreased oxygen consumption.

2) Diminished Pentose formation

3) Fall in rate of glucose utilization.

4) Diminished rate of methemoglobin reduction.

5) Vulnerability of glutathione (GSH) to oxidation.

6) Fall in ATP content *in vitro* with acetylphenylhydrazine.

C. Mechanism of Primaquine-induced Hemolysis: No definite mechanism of hemolysis of G6PD deficient erythrocytes has yet been formulated. It is however apparent that G6PD deficiency is not only the most striking biochemical defect in susceptible cells, but presumbaly, it is the primary biochemical lesion.

It has been proposed that primaquine and similar drugs produce an oxidant effect that is deleterous to the cell (Cohen *et al.* 1964; Jandl *et al.* 1960). It is also proposed that the metabolites of primaquine are redox agents that can link the oxidative potential of oxygen (oxyhemoglobin) to vital cellular components, perhaps by reacting with oxyhemoglobin to form the oxidant H_2O_2 (Fraser and Vassell, 1968).

Jandl *et al* (1960) demonstrated *in vitro* that oxidant drugs capable of acting as redox intemediates do oxidize hemoglobin, first reversibly to methemoglobin and eventually irreversibly to oxidation products resembling Heinz bodies. They further demonstrated that the oxidation of sulfhydryl groups in the red cell membrane resulted in increased osmotic fragility and the rapid

removal of these red cells from circulation, when the drug is injected *in vivo*. Hochstein and Cohen (1961) were able to demonstrate that many of the drugs which induce hemolysis in primaquine-sensitive individuals will result in the formation of H_2O_2 when incubated *in vitro* with human red cells containing oxyhemoglobin. It has therefore been presumed that these agents serve as reducing agents in the formation of H_2O_2. Furthermore, they demonstrated that the H_2O_2 produced in the erythrocytes was neutralized by GSH in a reaction catalysed by glutathione peroxidase:

$$2H_2O_2 + GSH \xrightarrow[\text{peroxidase}]{\text{glutathione}} 2H_2O + GSSG.$$

Following this finding, Mengel (1968) suggested that H_2O_2 will undergo hemolytic cleavage to form a free radical HO· which could capture a hydrogen ion from unsaturated fatty acids and lead to the formation of lipid peroxides. Little and O'Brien (1968) advanced the argument further by assuming the lipid peroxides so produced could oxidize other components of the cell including protein and non-protein thiols, or could result in the cleavage of the fatty acids, perhaps producing a defect in the cell membrane (Recknagel and Ghoshal 1966). If indeed, H_2O_2 does play a role, a specific site of damage within the membrane, or an enzyme system remains to be identified.

The possible neutralisation of H_2O_2 by catalase of the red cell is ruled out by Hochstein and Cohen's suggestion that the H_2O_2

produced is too low to satisfy the low affinity of catalase for it. This is further supported by lack of drug-induced hemolysis in patients lacking catalase in their erythrocytes; and the occurrence of hemolytic process associated with the congenital lack of glutathione peroxidase(Necheles et al. 1968; 1969).

Thus in a situation where the human red cell would be challenged by drugs capable of incuding hemolysis, H_2O_2 would be formed which would be neutralised by protons and electrons supplied by GSH. If the red cell is incapable of quickly reducing the oxidized glutathione which is formed in the neutralisation of the H_2O_2, as would be the case in G6PD deficient subjects, the H_2O_2 would accumulate and direct its oxidative potential against the cell membrane or intracellular proteins including hemoglobin and enzymes. It has been shown that in vitro incubation of primaquine-sensitive erythrocytes and hemolytic drugs results in the impairment of ATP production (Faulkner et al. 1968). One might assume that the impaired ATP production is a result of oxidative potential of H_2O_2 formed by the interaction of oxyhemoglobin and the drug directed against enzymes in the Embden-Meyerhoff pathway.

D. Clinical significance of Primaquine-sensitive hemolysis:

Many more drugs such as Sulphanilamide, aspirin and certain vegetable foods, notably fava beans have been found to induce hemolysis in primaquine sensitive individuals and herein lies the

significance of this inborn error of metabolism. Though hemolysis induced in healthy primaquine-sensitive individuals by therapautic dosages of most drugs is reportedly mild, several factors have been identified which influence the degree of hemolysis.

1) The blood level and the chemical character of the hemolytic drug have an influence. The blood level in turn depends upon the dosage, catabolism and excretion of the agent. For example patients with renal diseases and G6PD deficiency may experience more marked hemolysis due to a decreased excretion and a consequent high blood level of drug. It thus follows that hemolysis will occur in a normal individual if dosage of drug is sufficiently high as would pertain in places like Ghana where inordinate use of drugs is not checked.

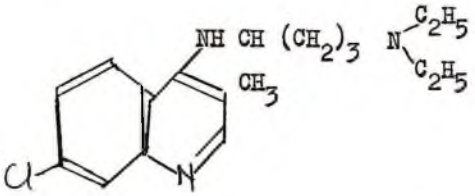
2) Certain disease states including viral and bacteriological infections, and hypoglycemia, have been observed to either induce or enhance the hemolytic effect in these patients.

3) The severity of the hemolysis has also been found to vary directly with the level of G6PD activity in the red blood cells and the biochemical characteristic of the enzyme - these characteristics are primarily under genetic control. For example heterozygotes are less susceptible to hemolysis than homozygotes or individuals with full expression of the phenotype.

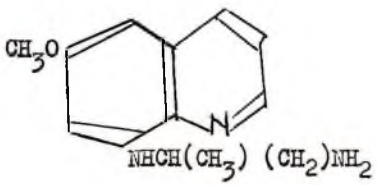
A question worth asking in blood-banking when choosing blood to give to a patient undergoing an acute fulminating hemolysis

secondary to G6PD deficiency, is whether it is necessary to screen donors for G6PD deficiency. Even though McCurdy (1969) claims experience has not yet precipitated the necessity to screen routine blood bank donors it could be an exciting and useful venture to undertake.

E. Primaquine and Chloroquine: Primaquine bears some structural resemblance to chloroquine in that both drugs have the quinoline nucleus; but for the position of the akyl side chain. (4-position for chloroquine; whilst with primaquine it is position 8) and the fact that chloroquine has a chlorine atom instead of a methoxy nuclear substituent. The metabolite of



I. Chloroquine.



II. Primaquine.

primaquine which is presumed to exert its toxic and antimalarial action is a quinone (structure C in diagram III) and could be assumed to have higher oxidant properties than the active form of chloroquine - the parent compound (I).

INDEX OF MATERIALS AND METHODS

GENERAL METHODS

Materials and Reagents: Reagents used were of analytical grade where possible and purchased from B.D.H. Chemicals Ltd., England. Drabkins reagent was purchased in capsules from the Diagnostic Reagents Ltd., Thames Oxon. England. Vials of aqueous chloroquine phosphate solution obtained from the University Hospital, Legon, were purchased from Dumex Ltd., Copenhagen, Denmark.

Animals: The experiments were performed with blood from adult male albino rats weighing 200 - 400g. The rats were obtained from the animal breeding sections of the Korle Bu Teaching Hospital and the Biochemistry Department of the University of Science and Technology, Kumasi.

Methods I (MI)

Preparation of Heparinised Bottles

Two millitre aliquots of a 20mg/100ml heparin solution were pipetted into blood collecting bottles (25ml capacity). The bottles were swirled to wet the inside of the bottles; and dried in an oven at 80°C overnight, cooled and used later.

Method II (MII)

Collection of Blood Samples

Buffer solutions: Stock solutions were prepared as follows:

(a) 0.155M or 310 milliosmolar NaH_2PO_4: 29.185g $NaH_2PO_4.2H_2O$ were dissolved in 400ml distilled water in a 500ml volumetric flask and made up to volume.

(b) 0.103M or 310 imOsm Na_2HPO_4; 36.901g $Na_2HPO_4.12H_2O$ per litre of distilled water.

Isotonic phosphate buffer, pH 7.4, was prepared by mixing 857ml stock solution of 0.103M Na_2HPO_4 and 143ml stock solution of 0.155M NaH_2PO_4 according to the Henderson - Hasselbalch equation. Isotonic phosphate buffer (pH 7.4) prepared this way has been used by Dodge et al (1963) to prepare and characterise Hemoglobin - free Ghosts of human red blood cells.

Experimental:

Under superficial ether anaesthesia, the animals were bled from the carotid arteries and the jungular veins. Blood (7-10mls) was collected into heparinised bottles with gentle swirling to mix contents. The blood was centrifuged for 10 minutes at 3,000g at 25°C, and plasma and buffy coat were removed by aspiration. The red blood cells were washed twice by suspension in cold isotonic phosphate buffer, (pH 7.4), centrifugation for 10 minutes at 3,000g and removal of the supernatant by aspiration. The red blood cells were finally suspended in an equal volume of isotonic phosphate buffer, pH 7.4.

Method III (MIII)

Determination of Hematocrit

The hematocrit or packed cell volume (PCV) was determined by Wintrobe's method (Wintrobe, 1956). Wintrobe tubes (100mm in length)

were filled with the blood suspension prepared as described above and sealed at one end. The tubes were centrifuged for 30 minutes at 3,000g at 25°C. The height of the sedimented red blood cells in the Wintrobe tube was meausred and the PCV expressed as a percentage of the total volume of blood, including plasma.

METHOD IV (.IV)

Estimation of Percentage Homolysis

Percentage hemolysis of various experimental samples was determined by measuring the amount of free hemoglobin present in the supernatant of the centrifuged red cell suspension after incubation, and dividing this by the amount of hemoglobin released after complete lysis. Hemoglobin was measured by conversion to the stable cyanide derivative cyanohemoglobin using Drabkin's reagent.

0.1ml of blood sample was added to 3.0ml Drabkin's solution. The solution was mixed by gentle shaking , and left to stand for five minutes. The extinction of the solution was measured at 540mμ against Drabkin - distilled water blank.

Glutathione (GSH) Estimation

Estimation of reduced glutathione (GSH) was made using a method based upon the development of a relatively stable yellow color when 5,5' - dithiobis - (2 nitrobenzoic acid) is added to sulfhydryl compounds (Beutler et al, 1963). The extinction

was measured at 412mμ. This method was preferred to a more widely used one of Grunert and Phillips (1951), which makes use of the formation of colored complexes when a solution of nitroprusside is added to glutathione and other sulfhydryl compounds, as the color was found to be unstable. However, it is interesting to note that 5,5' dithiobis - (2 nitrobenzoic acid) gives essentially the same extinction coefficient with cysteine as with GSH.

Reagents

1. Protein Precipitating Solution:

   (a) 10g of metaphosphoric acid (sticks) was dissolved in water and made up to 500ml

   (b) 150g sodium chloride was dissolved in 450ml of boiling distilled water and then, 1.0g of sodium EDTA was added. On cooling, 41.7ml of solution (a) were added. The solution was stable up to 4 months.

2. Phosphate Buffer:

   0.3M disodium hydrogen phosphate was prepared by dissolving 54.4g $Na_2HPO_4.2H_2O$ per litre of distilled water.

3. Disodium EDTA 1.0g/litre

4. Trisodium Citrate 10g/litre

5. Color Reagent - DTNB (5,5' - dithiobis -(2 benzoic acid).

   40mg DTNB were dissolved in 100ml trisodium citrate solution (4)

Working Solution for Calibration Curve

50mg GSH were dissolved in 100ml of a mixture of 40ml water and 60ml protein precipitating solution (solution b). 20ml of this stock solution was made up to 100ml with distilled water giving a solution of concentration 10mg/100ml. Dilutions of this solution was prepared as follows:

Table I. Working Standard Solutions

Tube No.	1	2	3	4	5	6
Glutathione Solution 10mg/100ml (ml)	0.5	1.0	2.0	3.0	4.0	5.0
Diluting Solution - di Na EDTA metaphosphoric acid-water ml	9.5	9.0	8.0	7.0	6.0	5.0
Glutathione ug/ml	5.0	10.0	20.0	30.0	40.0	50.0

Procedure: (a) A calibration curve (10-50ug) glutathione was prepared by adding 4.0ml phosphate buffer and 1.0ml DTNB reagent to 2.0ml portions of the five solutions obtained using the dilutions given under working standard (Table I).

(b) Estimation of GSH of incubated blood

(i) To 3.0ml of distilled water was added 1.0ml red blood cell suspension. The solution was left to stand for 5 minutes to hemolyse.

(ii) 6.0ml of the protein precipitating reagent were added.

The solution was mixed and allowed to stand for 10 minutes after which it was centrifuged for 20 minutes at 3,000g at 25°C, and filtered through Whatman No.4 filter paper.

(iii) To 2.0ml of supernatant fluid from (ii) were added 4.0ml phosphate buffer and 2.0ml DTNB reagent. The extinction was measured at 412mμ using 1 x 2cm silica cuvettes.

(iv) A reagent blank was prepared by adding 4.0ml phosphate buffer and 1.0ml DTNB reagent to a mixture of 1.2ml proteins precipitant (1) and 0.8ml EDTA solution (3) (under reagents, pages 3)

Calculation:

Concentration of reduced glutathione (μg/100ml red blood cells)

$$= 100 \times \frac{10}{2} \times \frac{100}{PCV} \times y$$

$$= \frac{50 \times 10^3}{PCV} \times y$$

where y = μg glutathione (obtained from calibration curve, Fig I);

= PCV = packed cell volume obtained previously.

Method VI (M VI)

Assay methods for the enzymes G6PD and 6PGD in the red blood cells

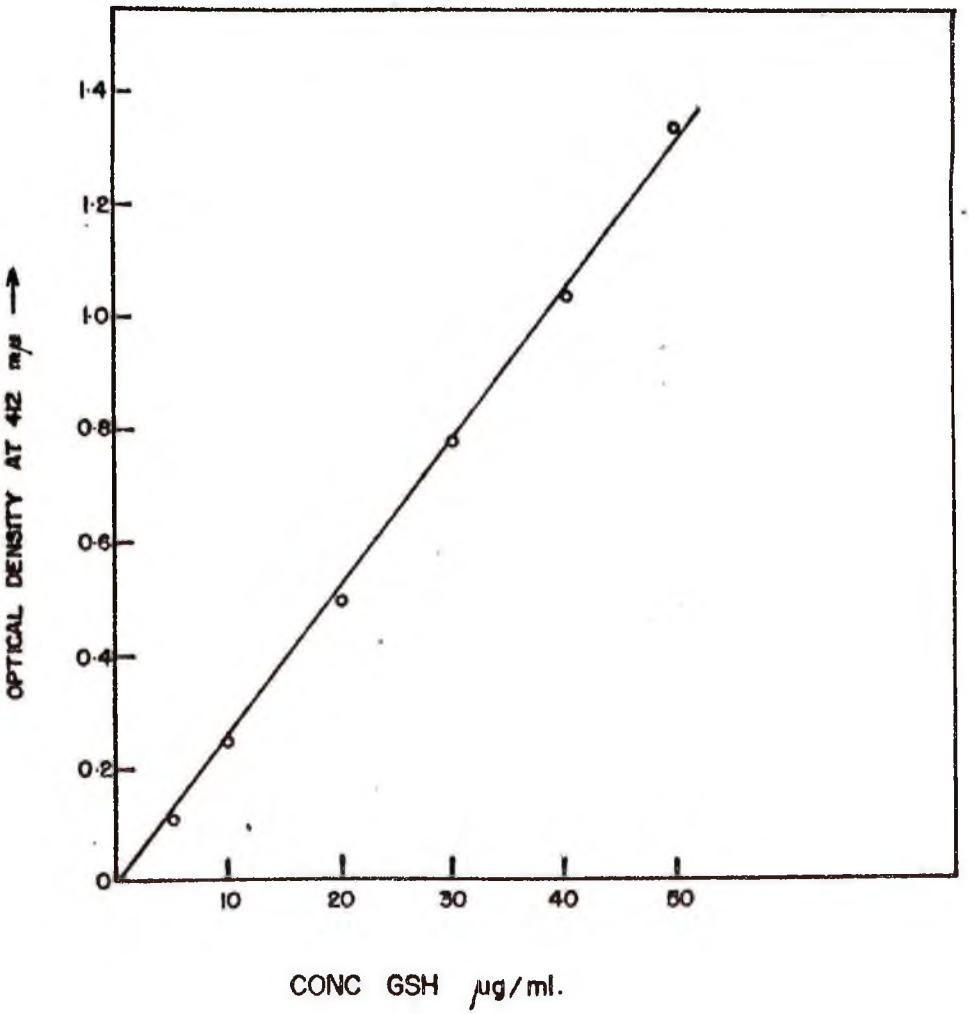
Both G6PD and 6PGD catalyse the oxidation reactions of the pentose Phosphate shunt involved in production of NADPH (Diagram I). The rate of formation of NADPH was therefore measured by following the

FIGURE I

STANDARD CURVE OF GSH

Optical Density at 412mμ was plotted against concentration of glutathione.

FIG. I CALIBRATION CURVE FOR GSH.



increase in absorption at 340mu, which is characteristic of NADPH. Since the G6PD reaction yields 6-phosphogluconate, which is the substrate for 6PGD, also present in the hemolysate (Glock and McClean, 1953); a three cuvette system was set up. Cell 1 measured 6PGD activity alone whilst cell 2 measured the combined G6PD + 6PGD activity. Cell 3 was the blank. True G6PD activity was obtained as the difference in enzymic activity between Cells 1 and 2.

Experimental

Reagents:

1. 0.2M Tris buffer. pH 7.4 was prepared by dissolving 3.63g of tris - (hydroxymethyl) amino methane, in 80ml of water. The pH of this solution was adjusted to 7.4 by adding 2N HCl. The volume was then made up to 100ml.
2. 0.1M $MgCl_2$: 2.03g of $MgCl_2.6H_2O$ were dissolved in 100ml distilled water.
3. 18mM 6PG was prepared by dissolving 12.6mg 6PG (disodium salt) in 2ml of distilled water.
4. 18mM G6P 12.6mg disodium G6P in 2ml distilled water.
5. 2mM NADP 6.0mg NADP (sodium salt) were dissolved in 4ml tris buffer solution pH 7.4.

Procedure (a): A 1:50 hemolysate of red blood cells was prepared by pipetting 0.1ml of the red blood cell suspension into 4.9ml distilled water. The mixture was left to stand for 10 minutes, and then centrifuged for another 10 minutes at 5,000g at 25°C. The clear supernatant was decanted and used as hemolysate for the assay. The reaction mixtures used for the assay of the enzymes G6PD and 6PGD are given in Table 2.

Table 2:

Reaction mixtures for testing the activities of G6PD and 6PGD in red blood cells.

Cell No.	1	2	3
0.3M tris buffer, pH 7.4	1.0ml	1.0ml	1.0ml
0.1M $MgCl_2$	0.3ml	0.3ml	0.3ml
18mM Sodium 6PG	0.1ml	0.1ml	-
18mM Sodium G6P	-	0.1ml	-
Water	1.0ml	0.9ml	1.2ml
1:50 Hemolysate	0.5ml	0.5ml	0.5ml

The hemolysate was added last. The reaction was started by the addition of 0.1ml 2mM NADP to cells 1 and 2 and the contents were mixed. After about two minutes when the reaction rate was constant, readings of the extinction at 340mu were made on an automatic

recording chart. For measurement of the hemoglobin concentrations, the extinction of test cells 1 and 2 was measured at 540mu against distilled water blank.

Procedure (b):

Studies on the activities of G6PD and 6PGD in rat red blood cell lysates exposed to Chloroquine Phosphate

A 1:50 hemolysate of rat red blood cells was prepared as in procedure (a). Reaction mixtures (Table 3 a & b) were prepared to study the effect of Chloroquine phosphate in the red blood cell lysate.

Table 3: Reaction mixtures for testing the effect of Chloroquine phosphate on G6PD and 6PGD activities in red cell lysates

(a) G6PD + 6PGD activity

Cell No.	1	2	3
0.3M Tris buffer (pH 7.4)	1.0ml	1.0ml	1.0ml
0.1M $MgCl_2$	0.3ml	0.3ml	0.3ml
18mM 6PG	0.1ml	0.1ml	-
18mM G6P	0.1ml	0.1ml	-
H_2O	0.9ml	0.8ml	1.0ml
1:50 Hemolysate	0.5ml	0.5ml	0.5ml
1mM Chloroquine	-	0.1ml	0.1ml

(b) 6-PGD activity

Cell No.	1	2	3
0.3M Tris buffer (pH 7.4)	1.0ml	1.0ml	1.0ml
0.1M $MgCl_2$	0.3ml	0.3ml	0.3ml
18mM sodium 6PG	0.1ml	0.1ml	-
18mM sodium G6P	-	-	-
H_2O	1.0ml	0.9ml	1.0ml
1:50 Hemolysate	0.5ml	0.5ml	0.5ml
1mM Chloroquine phosphate	-	0.1ml	0.1ml

Reaction mixtures were pre-incubated for 30 minutes at 37°C, after which the reaction was started by the addition of 0.1ml 2mM NADP to each of cells 1 and 2. Readings of the extinction at 340mμ was measured as in (a).

Calculation: Based on a millimolar extinction coefficient for NADP at 340mμ of 6.22 (Horecker and Kornberg, 1948), and an extinction of 8.58 at 540mμ for a one per cent hemoglobin (Donaldson et al. 1951), then the enzyme activity in international Units (i.u.) is calculated as follows:-

Let x = rate of increase in extinction at 340mu (graphically determined)

and y = extinction observed for hemoglobin at 540mu

Then Enzyme activity (i.u.)

$$= \frac{x}{y} \times \frac{8.58}{6.22} \times 100$$

$$= \frac{x}{y} \times 138 \text{ Units/g hemoglobin (Hb)}$$

One i.u.= 1mM NADPH produced/gHb.

Method VII (MVII)

Measurement of Osmotic Fragility of red blood Cell suspensions

The "Osmotic Fragility" test is a method usually employed to study the integrity of the red blood cell membrane. The method has been described by Dacie (1957). Hypotonic saline buffered to pH 7.4 was used, and the blood added to a range of hypotonic solutions in the proportion of 1nto 50 (with blood of PCV less than 20%). The test was carried out at room temperature (25°C) and hemolysis read spectrophotometrically.

Reagents: Stock solutions of buffered sodium chloride (equivalent to 10% NaCl in tonicity) were prepared by dissolving 90g NaCl, 17.12g $Na_2HPO_4.2H_2O$ and 2.43g $NaH_2PO_4.2H_{22}O$ in distilled water and adjusting the final volume to litre.

A 1% solution was made from the 10% stock solution, by dilution

with water. Dilutions equivalent to 0.90, 0.75, 0.65, 0.55, 0.525, 0.50, 0.475, 0.45, 0.40, 0.30, 0.20, and 0.10% NaCl were prepared.

Procedure: 0.1ml incubated blood mixture was added to 5ml volumes of each of the hypotonic solutions and immediately mixed. Tubes were allowed to stand for 30 minutes at room temperature (25°C), remixed and centrifuged for 10 minutes at 3,000g. The amount of hemoglobin in each tune was then compared with that in the 100% lysis tube (0.1% NaCl) by reading the absorption of the solution at 540mu. The supernatant from the 0.9% NaCl (with zero % hemolysis) tube was used as the blank.

Method VIII (VIII)

Spectrophotometric measurements

The extinction of solutions were read using a Unicam sp500 Spectrophotometer Model II. 1 cm cuvette was used unless otherwise stated.

PART IV

RESULTS

SECTION I:

EFFECT OF CHLOROQUINE PHOSPHATE ON THE STABILITY OF THE RAT RED BLOOD CELLS

The effect of chloroquine phosphate on the stability of the rat red blood cells was studied by measuring the percentage hemolysis and the packed cell volume of the red blood cells incubated with different concentrations of chloroquine at 37°C. Table 4 shows that 2.5 x 10^{-2}M chloroquine phosphate gave zero PCV while % hemolysis ranged from 44 - 69%. Higher chloroquine concentrations continued to give Zero PCV and increasing % hemolysis of the RBC. Hundred percent hemolysis was however obtained with 4.2 x 10^{-2}M chloroquine phosphate.

Table 4: The effect of Chloroquine Phosphate on the Rat red blood cells

Conc of Chloroquine	Zero time		After 4 hrs. incubation	
	PCV	% Hemolysis	PCV	% Hemolysis (Range)
OM	16	Zero	16	Zero
6.3 x 10^{-4}M	16	"	16	3 - 4
1.3 x 10^{-3}M	16	"	16	3 - 5
2.5 x 10^{-3}M	16	"	16	3 - 7
5.0 x 10^{-3}M	16	"	16	5 - 8
1.3 x 10^{-2}M	16	"	16	8 - 10
2.5 x 10^{-2}M	16	"	Zero	64 - 69
3.0 x 10-2_{M}	16	"	Zero	80 - 92
4.2 x 10^{-2}M	16	"	Zero	96 - 100

Range for 20 determinations in each case.

On the other hand chloroquine phosphate concentrations ranging from 6.3×10^{-4}M to 5.0×10^{-3}M caused only 3 - 10% hemolysis. No detectable change in the PCV was observed in this range of concentrations. It was considered from these results that PCV was not a sensitive method of estimating degree of hemolysis in the red blood cells under these experimental conditions used.

Fig2 curve(b) shows the time course of hemolysis induced by 2.5×10^{-2}M chloroquine phosphate. Hemolysis increased slowly during a 1-hour incubation period to 3% and then rapidly to 64% after 4 hours incubation.

The effect of various concentrations of glucose on such Chloroquine induced hemolysis after four hours incubation is shown in Table 5.

Increasing concentration of glucose starting from $1{:}89 \times 10^{-2}$M gradually reduced the % hemolysis due to 2.5×10^{-2}M chloroquine phosphate. Glucose concentration of 3.78×10^{-1}M reversed this chloroquine-induced hemolysis down to 3 - 7%; and this glucose concentration was used in subsequent experiments.

Table 5:

The stability of red blood cells incubated in presence of glucose and chloroquine

Concentration of chloroquine in the incubated system = 2.5×10^{-12}M. Red cell suspension was incubated in a 5ml system (pH 7.4 at 37°C.) Control contained no glucose, no chloroquine.

Conc of Glucose(M)	Zero time		After 4 hrs. incubation	
	PCV	% Hemolysis	PCV	% Hemolysis(Range)
0	18	Zero	Zero	66 - 70
1.89×10^{-2}	18	"	"	63 - 69
3.78×10^{-2}	18	"	"	56 - 64
7.56×10^{-2}	15	"	"	50 - 54
1.89×10^{-1}	14	"	5	26 - 32
3.78×10^{-1}	12	"	9	3 - 7
Control	18	"	18	Zero

Table shows range of values for 20 determinations for each concentration.

FIGURE 2

TIME COURSE PLOT FOR CHLOROQUINE-INDUCED HEMOLYSIS OF RED BLOOD CELLS AND THE EFFECT OF GLUCOSE ON SUCH HEMOLYSIS

A suspension of red blood cells in buffered saline (pH 7.4) was incubated with Chloroquine Phosphate, glucose or both at 37°C. Samples were taken at intervals for estimation of % Hemolysis.

Glucose = 3.78×10^{-1}M

Chloroquine Phosphate = 2.5×10^{-2}M

x - - - - - x Control. (a)

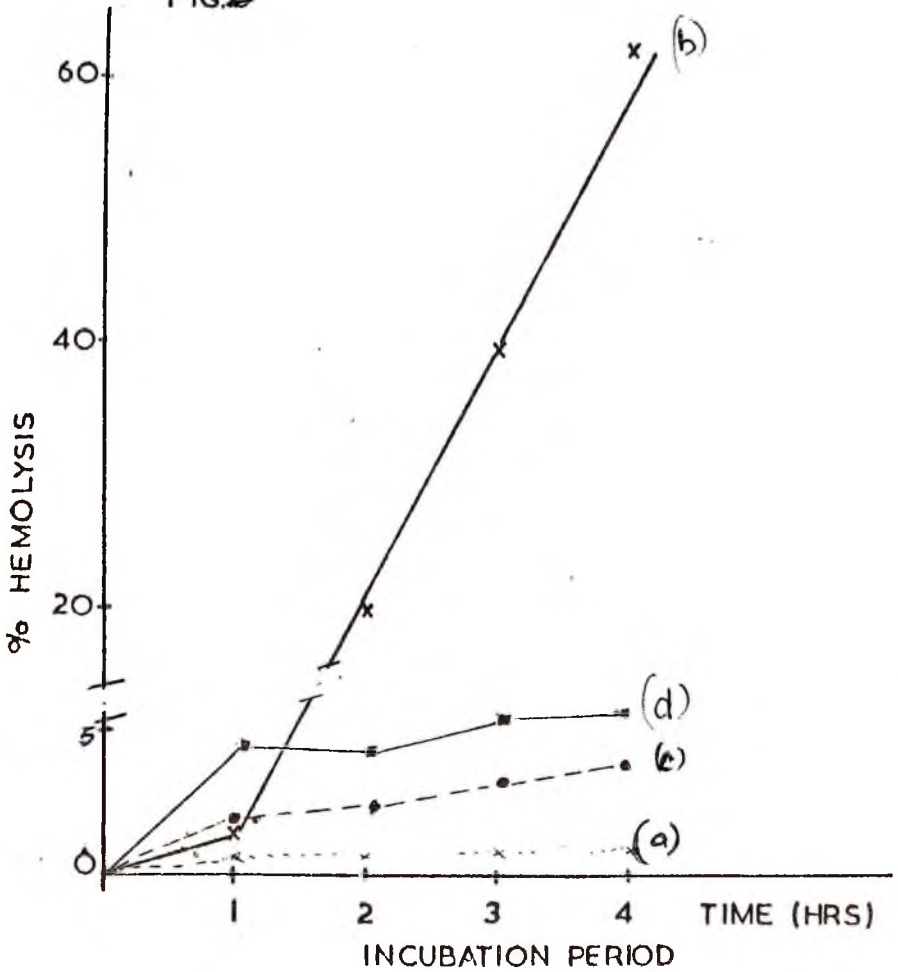
x——————x Chloroquine phosphate (b)

● - - - - ● Glucose (c)

■——————■ Glucose + Chloroquine Phosphate (d)

Control contained neither Chloroquine nor glucose.

FIG. 2



The time course for the red cell hemolysis in presence of glucose alone and glucose plus chloroquine phosphate is also shown in Fig.2, curve (c) and (d). It is found that hemolysis induced by 2.5×10^{-2}M chloroquine phosphate in presence of glucose increased to a relatively steady level of 4% after four hours. The same figure also shows that the presence of glucose alone caused 2% hemolysis in the red blood cells after four hours incubation at 37°C.

Table 5 shows that glucose at a concentration of 3.78×10^{-1}M reduced hemolysis from 66 - 70% to 3 - 7%; The PCV was however only 50% reversed.

Table 6: Effect of ATP on red cell suspensions incubated with chloroquine phosphate in presence or absence of glucose

(Chloroquine phosphate) = 2.5×10^{-2}M; (Glucose = 0.378M (ATP) = 0.02M. Cells were incubated in buffered medium pH 7.4, at 37°C. The control contained red blood cell suspension in isotonic buffer.

System	Composition	% Hemolysis after 4 hours incubation
1	Control	Zero
2	Chloroquine	66 - 74%
3	ATP	0 - 2%
4	Glucose + Chloroquine	3 - 7%
5	Chloroquine + ATP	7 - 12%
6	ATP + glucose + Chloroquine	Zero

Table 7 (Also in Fig 3)

Effect of Chloroquine Phosphate on the GSH Levels of the Rat Red Blood Cells in Presence or absence of Glucose

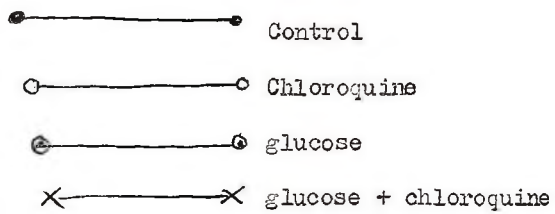
Treatment of RBC	Average (GSH) for 15 determinations($\mu g \times 10^{-4}$/ml RBC)		
	Zero time Incubation	After 2hrs. Incubation	After 4hrs Incubation
Control	3.26±0.05	3.22±0.10	3.20±0.11
Glucose	3.20±0.12	4.16±0.09	4.16±0.12
Chloroquine	3.19±0.11	2.74±0.06	2.06±0.10
Glucose + Chloroquine	3.21±0.06	4.51±0.06	5.7±0.16

The effect of chloroquine phosphate on the glutathione(GSH) levels in the red blood cells was studied in presence or absence of glucose. The aim here was to find a possible basis for the results observed in previous experiments. Fig 2 represents the standard calibration curve for estimation of reduced glutathione (GSH). Results of the experiments are recorded in Table 7 (also in Fig.3). Chloroquine phosphate concentration of 2.5 x 10^{-2}M caused a fall in GSH level from 3.19 to 2.06 x 10^{-4}µg/ml. RBC after 4 hours incubation. The same table also shows that in presence of both 3.78 x 10^{-1}M glucose and 2.5 x 10^{-2}M chloroquine phosphate, the GSH levels increased from 3.21 x 10^{-4}µg/ml RBC at zero time to a constant level of 4.16 x 10^{-4}µg/ml RBC after two hours incubation. The GSH levels in the control which contained neither glucose nor chloroquine phosphate remained unchanged at 3.20 x 10^{-4}µg/ml RBC after 4 hours.

Polet and Barr (1969) have suggested that uptake of chloroquine by the red blood cells could be partially energy-dependent; therefore it was considered that ATP levels might be important, and thus the effect of ATP on chloroquine-induced hemolysis was studied in presence or absence of glucose. Results of this experiment are shown in table 6. 0.02M ATP is shown in this table to reduced hemolysis caused by 2.5x 10^{-2}M chloroquine phosphate from 66 - 74% to 7 - 12%. The presence of both ATP and glucose reduced hemolysis induced by 2.5 x 10^{-2}M chloroquine phosphate to zero. ATP alone caused only 0 - 2% hemolysis in the rat red blood cells.

FIGURE 3 (TABLE 7)

EFFECT OF CHLOROQUINE PHOSPHATE ON THE GSH LEVELS OF RAT RED BLOOD CELLS IN PRESENCE OR ABSENCE OF GLUCOSE



where glucose is present, concentration = 0.378M

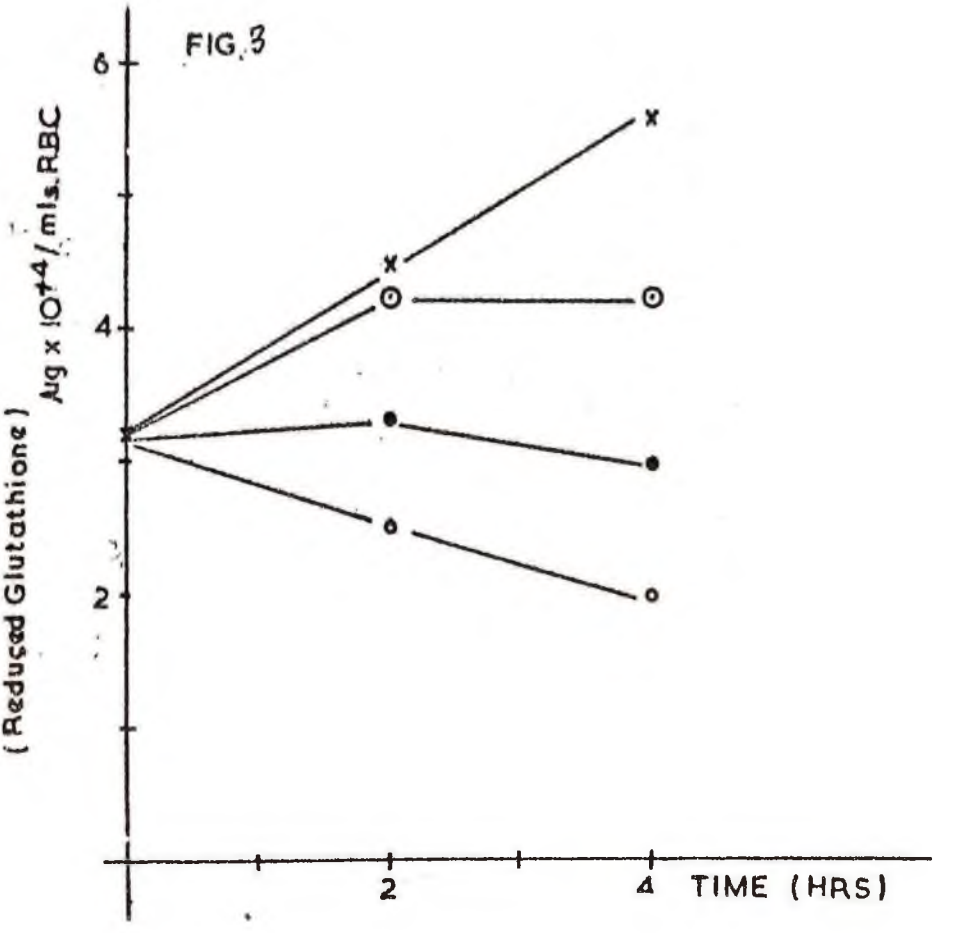
Chloroquine Phosphate = 2.5×10^{-2}M

Incubation temperature = 37°C.

Control contains neither glucose nor chloroquine

The average of 15 different experiments in each case.

FIG. 3



SECTION II

EFFECT OF CHLOROQUINE PHOSPHATE ON G6PD AND 6PGD LEVELS IN THE RAT RED BLOOD CELLS

Results in section I showed that 2.5×10^{-2}M chloroquine phosphate caused a fall in GSH levels in the rat red blood cells. In Vitro experiments were therefore performed to study the effect of chloroquine phosphate on the activities of the enzymes G6PD and 6PGD, which are involved in the maintenance of GSH levels in red blood cells (diagram I). The studies were made on both intact red blood cells and cell lysates.

For investigation of the activities of these enzymes in the intact red blood cells, a suspension of red blood cells at a final hematocrit of 16% was incubated with 2.5×10^{-2}M chloroquine phosphate in a 5ml buffered system, pH 7.4, at 37°C. After incubation for a 30 and 90 minutes respectively, the cells were washed twice in isotonic phosphate buffer pH 7.4 and finally suspended in an equal volume of the buffer. A 1:50 hemolysate was prepared as in procedure (a) in method VI and reaction mixtures in table 2 used for the assay. Procedure (b) for method VI and reaction mixtures in tables 3 (a) and (b) were used for studies of the activities of the enzymes in red blood cell lysates exposed to chloroquine phosphate.

Results: Figs 4(a) and (b) represent the activity curves for G6PD and 6PGD combined and that of 6PGD alone, respectively.

FIGURE 4

(a) JOINT ACTIVITY CURVE FOR THE ENZYMES GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND 6-PHOSPHOGLUCONATE (6-PGD) IN THE RAT RED BLOOD CELLS.

(b) ACTIVITY CURVE FOR THE ENZYMES 6PGD IN THE RAT RED BLOOD CELL HEMOLYSATE

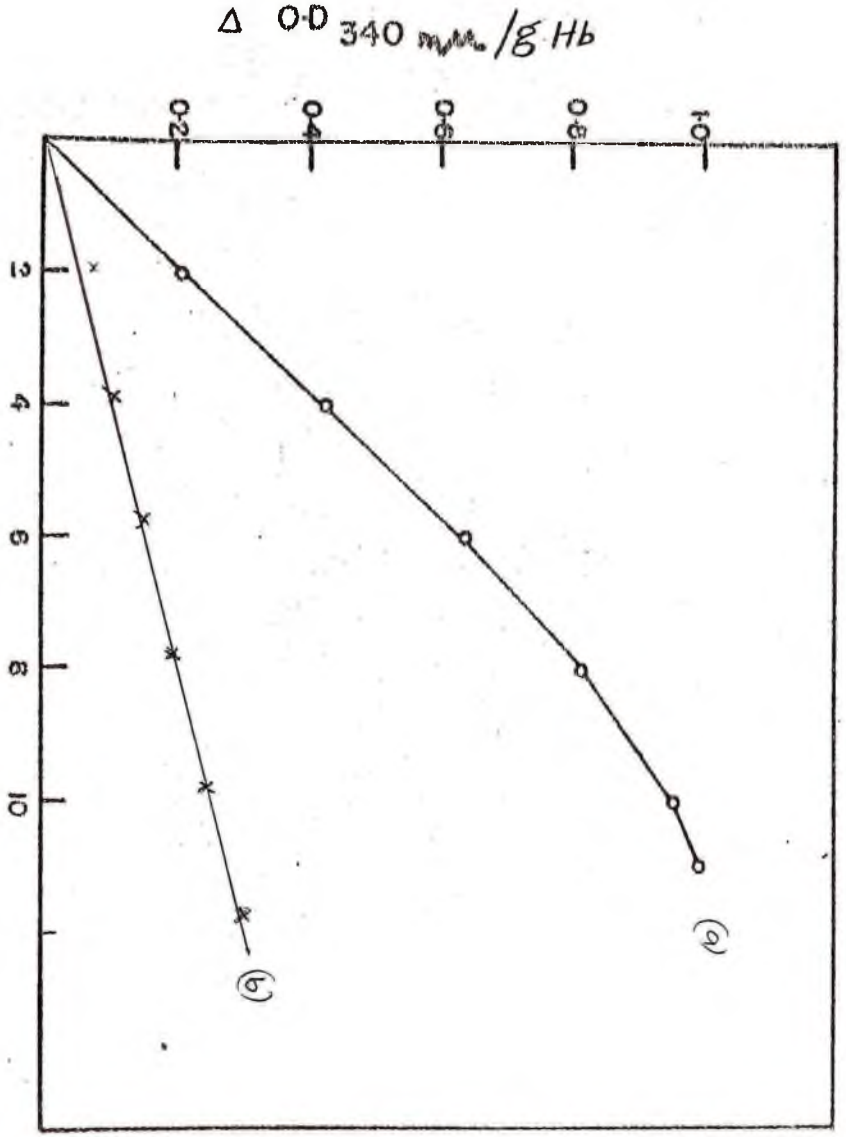


FIG. 4

Table 8 (a) shows the activities of the enzymes G6PD and 6PGD in the intact red blood cells incubated with 2.5×10^{-2}M chloroquine phosphate at 37°C. The activities of the enzymes 6PGD and G6PD are recorded both for the red blood cells exposed to chloroquine (the experimental system, E), and for cells incubated in absence of chloroquine (Control, C). The activity of G6PD in red blood cells exposed to chloroquine phosphate for 40 minutes was higher than that in cells incubated without chloroquine (control). There was however no significant change in the activities of G6PD and 6PGD of the intact red blood cells after 90 minutes incubation with 2.5×10^{-2}M chloroquine phosphate. Table 8 (b) shows the activities of G6PD and 6PGD of red blood cell lysates exposed to chloroquine phosphate at 37°C for 30 minutes, 2.5×10^{-2}M chloroquine phosphate had no significant effect on the activities of G6PD and 6PGD.

The activities of the enzymes have been expressed in millimoles of NADPH produced per minute per gram hemoglobin (i.e. in international Units).

Table 8(a) Mean activities of G6PDand 6PGD of the intact rat red blood cells exposed to Chloroquine Phosphate

No. of Expt.		Mean G6PD Activity µM NADPH/h Hb		Mean 6-PGD Activity µM NADPH/g Hb	
		After 40 mins incubation	After 90 mins incubation	After 40 mins incubation	After 90 mins incubation
1	E	8.3 ± 0.7	8.8 ± 1.3	3.8 ± 0.2	3.6 ± 0.5
	C	6.5 ± 0.6	8.0 ± 0.2	3.6 ± 0.1	3.7 ± 0.2
2	E	10.6 ± 0.3	10.0 ± 0.2	4.2 ± 0.2	4.0 ± 0.2
	C	9.3 ± 0.3	9.2 ± 0.4	4.0 ± 0.4	4.3 ± 0.3

Table 8(b) Activities of G6PD and 6PGD of rat red blood cell lysates exposed to Chloroquine Phosphate for 30 minutes at 37°C

No. of Expt.		G6PD Activity uM NADPH/g Hb	Mean 6PGD Activity uM NADPH/g Hb
1	E	10.8 ± 0.4	4.0 ± 0.2
	C	10.7 ± 0.5	4.2 ± 0.3
2	E	9.0 ± 0.5	3.9 ± 0.3
	C	9.2 ± 0.3	4.2 ± 0.3
3	E	10.5 ± 0.3	4.5 ± 0.5
	C	10.2 ± 0.2	5.1 ± 0.7

SECTION 3

STUDIES ON THE EFFECT OF CHLOROQUINE ON THE OSMOTIC FRAGILITY OF RAT RED CELLS

The effect of chloroquine phosphate on the osmotic fragility of red cells in presence or absence of Glucose or ATP or both was studied using Dacie's method (1957). Red cell suspensions were incubated at different times under specified conditions. After incubation, aliquots were taken for determination of the osmotic fragility.

Fig. 5 shows the osmotic fragility of red cells exposed to different concentrations of chloroquine. The osmotic fragility of red cells over a period of time of incubation with chloroquine phosphate was studied and results shown in Fig 6. A corresponding study in presence of glucose is shown in Fig. 8, whilst results on the effect of chloroquine phosphate on the osmotic fragility of red cells in presence of glucose or ATP is shown in Figs. 7 and 11 respectively. Fig. 9 contains results on the effect of different concentrations of chloroquine phosphate on the osmotic fragility of red cells in presence of glucose.

In Fig 7, glucose is shown to increase the osmotic fragility of the red cells though not to the same extent as chloroquine. In Fig. 9, the effect of glucose in increasing the osmotic fragility of the red cells is apparently checked by the presence of low levels of chloroquine phosphate (5×10^{-3}M). This is clearly shown in Fig. 10, in which red cells incubated with glucose alone are more

fragile than those incubated with both glucose and 5×10^{-3}M chloroquine phosphate. It is also interesting to note that the increase in osmotic fragility of red cells due to high levels of chloroquine phosphate (2×10^{-2}M) is reduced by the presence of glucose. (Fig. 9).

Fig 11 shows that ATP slightly increases the osmotic fragility of red cells. However, unlike the case of glucose, this effect of ATP is not affected by low levels of chloroquine phosphate. A summary of results is shown in Figs. 5 to 11 and tables 9(a) and (b). Table 9(a) shows a summary of the results on the effect of low and high levels of chloroquine phosphate on the osmotic fragility of red cells in presence of glucose. Table 9(b) shows a corresponding result for studies in presence of ATP. In column 3, in each table are recorded concentrations of saline causing 50% lysis or the median corpuscular fragility (M.C.F.). Column 4 contains the highest concentration of saline at which hemolysis is just detectable or the "minimum resistance"; and column 5, contains concentrations of saline in which hemolysis appeared to be complete or the maximum resistance.

FIGURE 5

OSMOTIC FRAGILITY OF RED CELLS EXPOSED TO CHLOROQUINE

Suspensions of rat red blood cells at a final hematocrit (PCV) of 16% were exposed each to different concentrations of chloroquine phosphate for 5 minutes. The Osmotic fragility of the cells in NaCl solutions was determined (M.VII). In the control, the red blood cells were incubated without chloroquine.

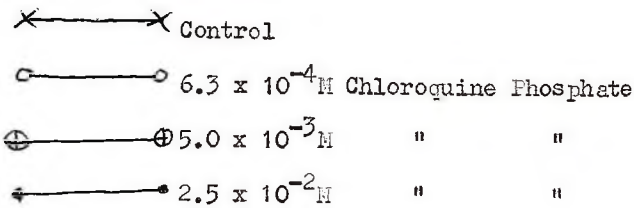
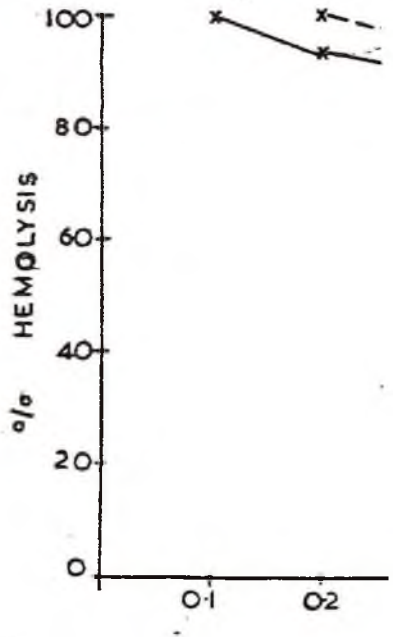


FIG. 6



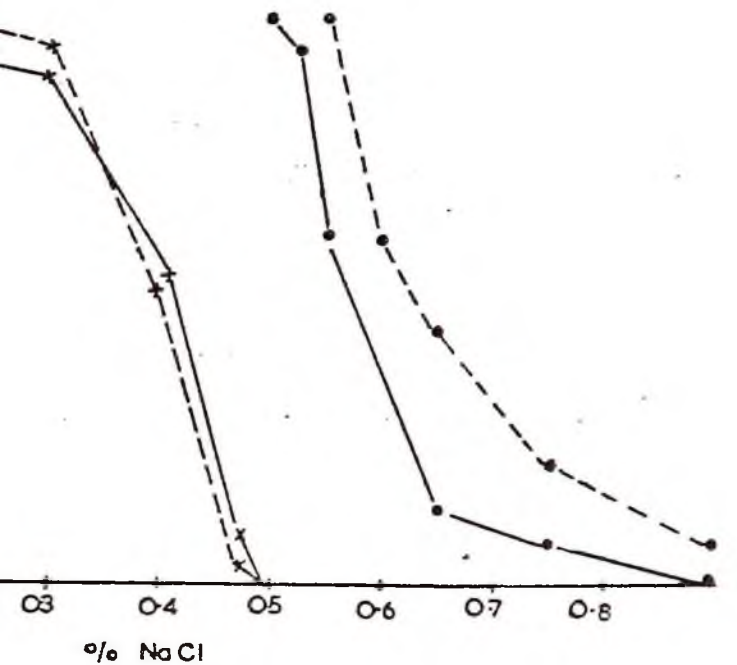
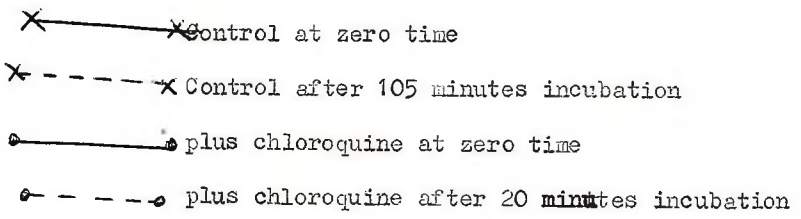


FIGURE 6

THE OSMOTIC FRAGILITY OF RAD RED CELLS OVER A PERIOD OF TIME OF INCUBATION WITH CHLOROQUINE PHOSPHATE

Suspensions of rat red blood cells at a final hematocrit of 16% were incubated with 2.5×10^{-2} M chloroquine phosphate. The Osmotic fragility of the cells containing chloroquine phosphate was determined at 20 minutes interval. Incubation temperature was 37°C. The Control contained red blood cells incubated without chloroquine phosphate.



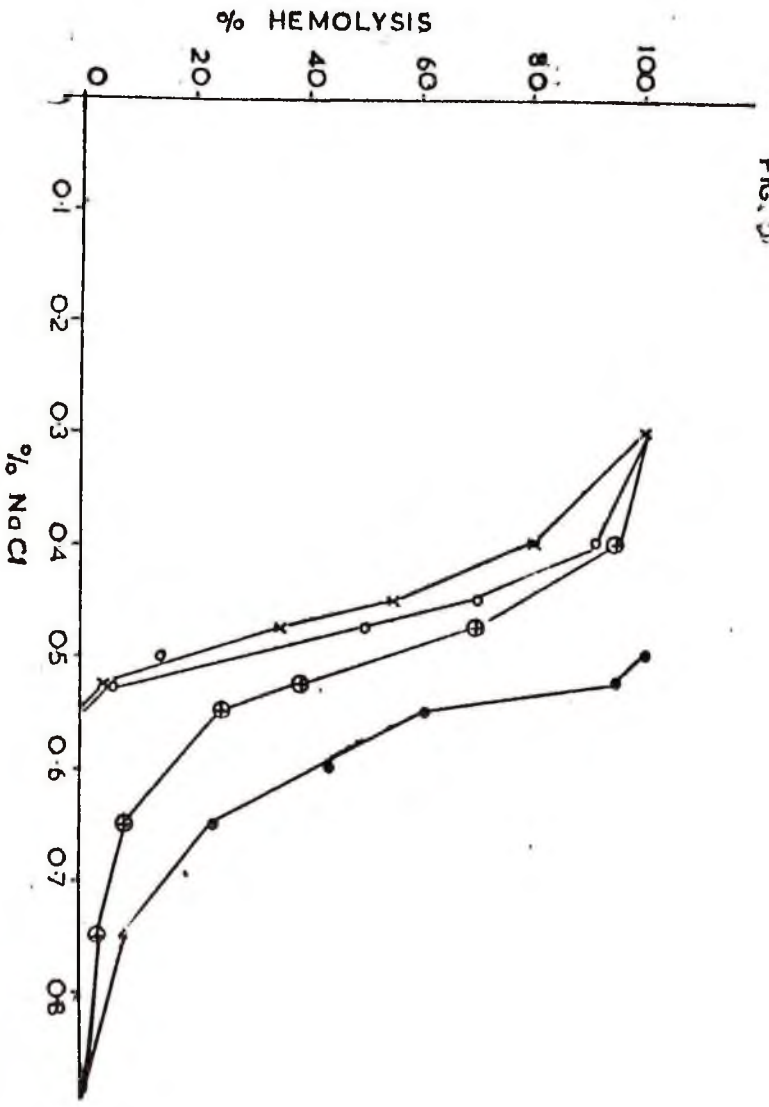
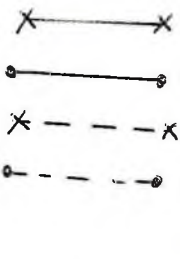


FIG. 5.

FIGURE 7

OSMOTIC FRAGILITY OF RED BLOOD CELLS INCUBATED WITH CHLOROQUINE PHOSPHATE IN PRESENCE OF GLUCOSE

Suspensions of rat red blood cells at a final hematocrit of 16% were incubated with 2.5 x 10^{-2}M chloroquine Phosphate and 3.78 x 10^{-1}M glucose at 37°C. Control in this experiment was red blood cell suspension containing only glucose (3.78 x 10^{-1}M). The Osmotic fragility was determined.



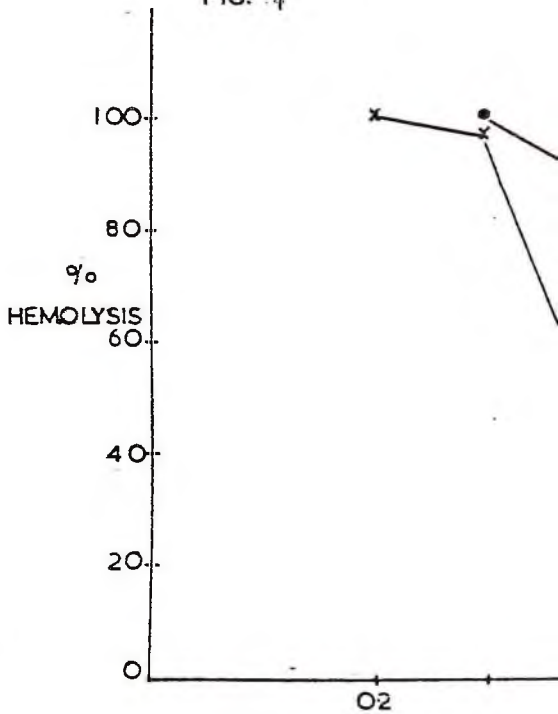
Plus glucose zero time

Glucose + Chloroquine phosphate at zero time

Plus glucose after 2 hrs. incubation

Glucose + Chloroquine phosphate after 2 hrs. incubation.

FIG. 7



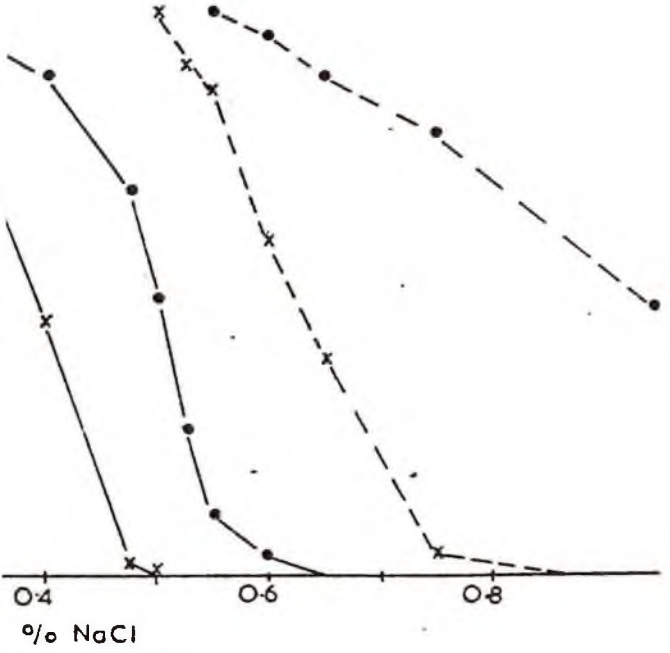
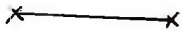


FIGURE 8

OSMOTIC FRAGILITY OF RED CELLS OVER THE PERIOD OF TIME OF INCUBATION WITH CHLOROQUINE PHOSPHATE AND GLUCOSE

Red cell suspension (pH 7.4) containing 2.5×10^{-2}M Chloroquine Phosphate and 3.78×10^{-1}M glucose was incubated at 37°C for 1½ hours.

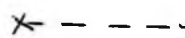
Aliquots of this suspension was taken at ½ hour interval and Osmotic fragility in NaCl solution was determined.



Osmotic fragility at zero time

" " after ½ hr.

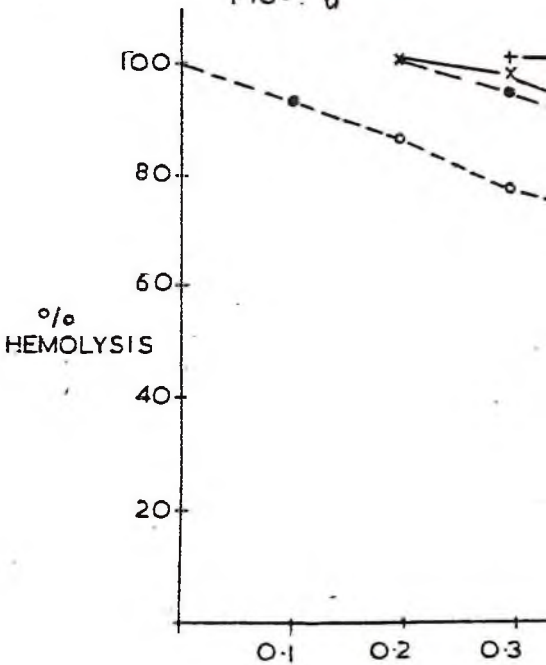
Incubation



Osmotic fragility after 1hr. incubation

Osmotic fragility after 1½hrs incubation.

FIG. 8



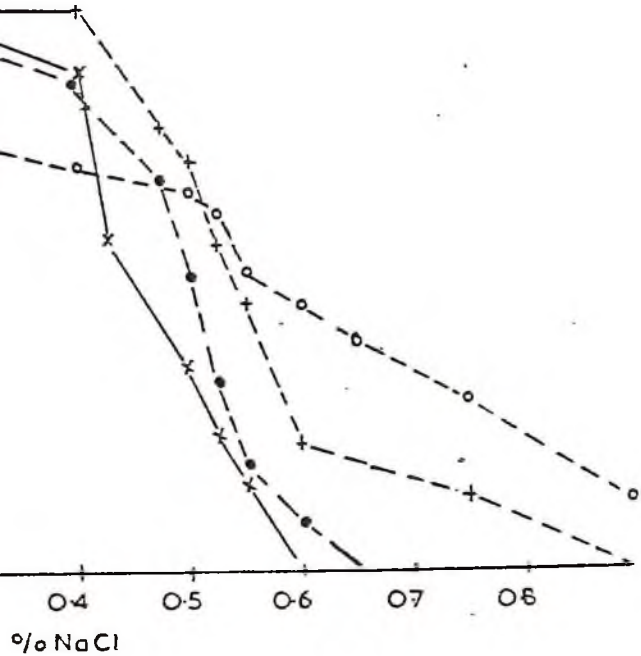


FIGURE 1.9

OSMOTIC FRAGILITY OF RED BLOOD CELLS EXPOSED TO VARIABLE CONCENTRATIONS OF CHLOROQUINE PHOSPHATE IN THEPRESENCE OF GLUCOSE

Suspensions of rat red blood cells at final hematocrit of 14% were incubated with different concentrations of chloroquine phosphate in the presence of glucose. Osmotic fragility was measured after one hour incubation period. Temperature 37°C. The control contained cells incubated in absence of chloroquine and glucose.

Glucose, = 3.78×10^{-1}M.

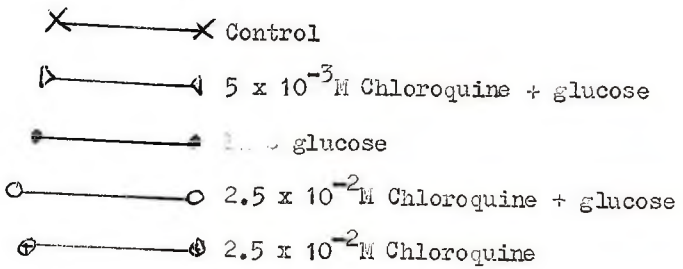


FIG. 9

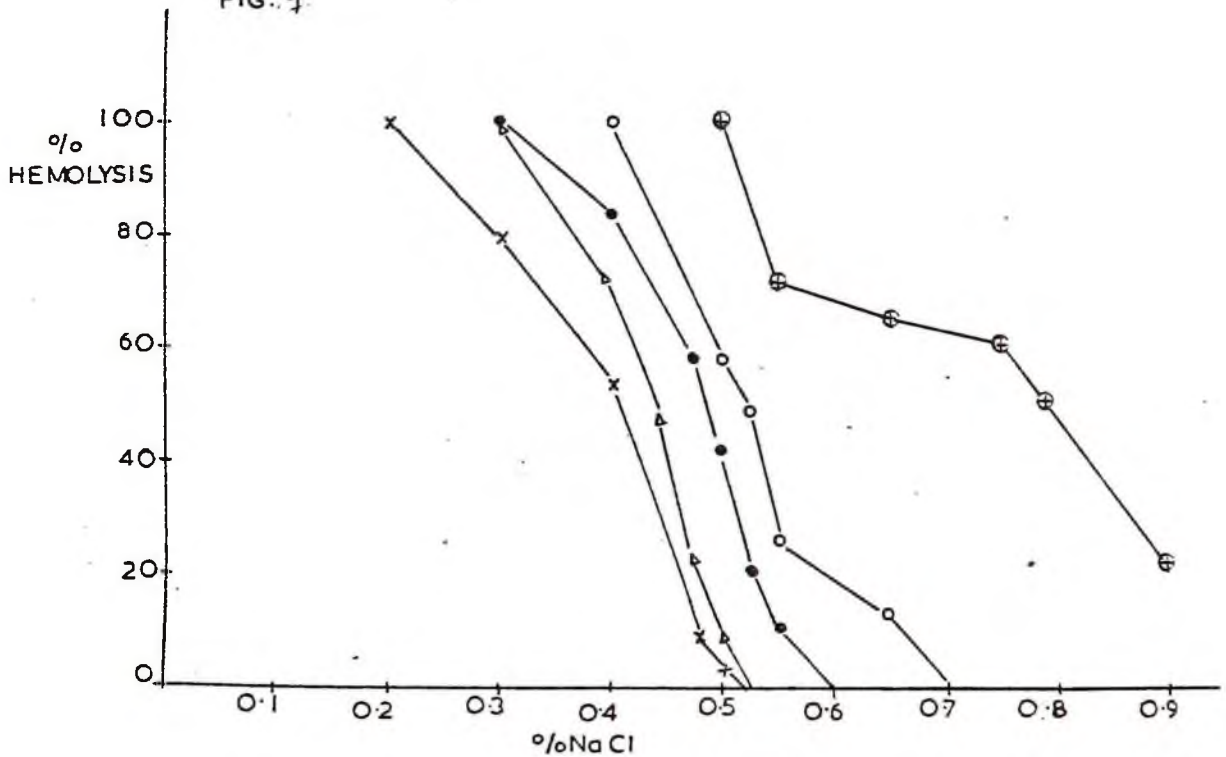


FIGURE 10

OSMOTIC FRAGILITY OF RED CELLS EXPOSED TO LOW LEVELS OF CHLOROQUINE PHOSPHATE IN PRESENCE OF GLUCOSE

Red cell suspensions at final hematocrit of 14% were incubated with (or without) 5×10^{-3}M Chloroquine phosphate in presence of glucose (3.78×10^{-1}M) for one hour at 37°C. The Control contains cells incubated without Chloroquine and glucose.

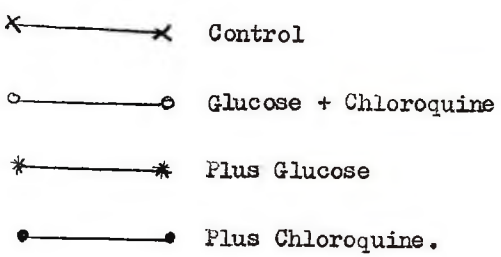


FIG. 10

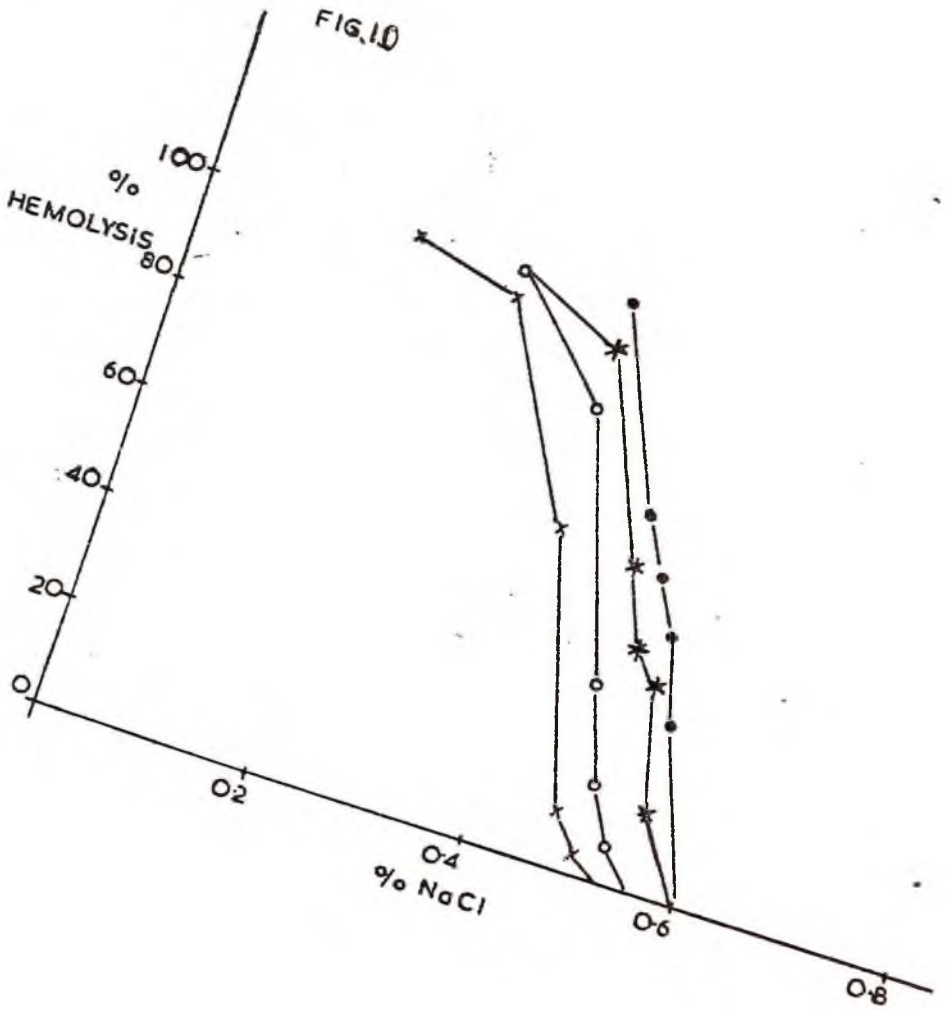
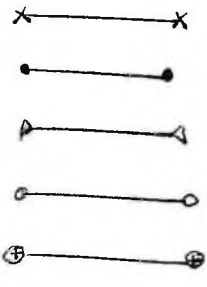


FIGURE 14

OSMOTIC FRAGILITY OF RED CELLS INCUBATED WITH CHLOROQUINE PHOSPHATE IN PRESENCE OF ATP

Suspensions of red cells at final hematocrit of 16% were incubated with 2.5 x 10^{-2}M or 5 x 10^{-3}M Chloroquine phosphate and 0.02M ATP at 37°C for one hour. The Osmotic fragility of each incubating system was determined at the end of incubation. Figure shows osmotic fragility curves after incubation.



Control (No ATP, No Chloroquine)

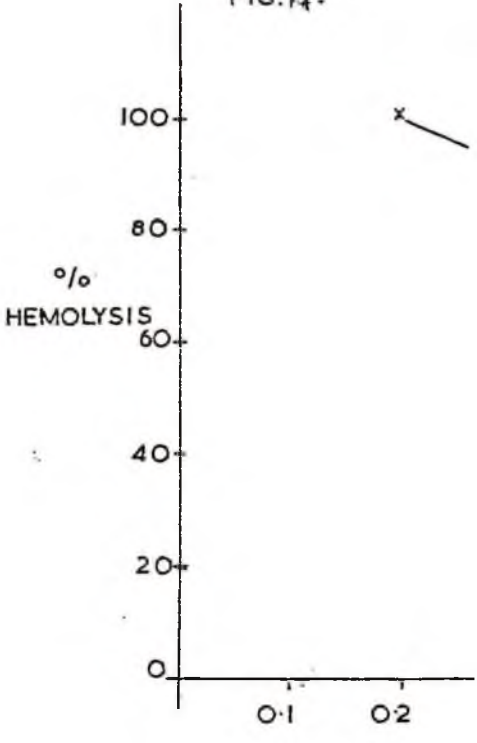
ATP present

ATP + 5 x 10^{-3}M Chloroquine

ATP + 2 x 10^{-2}M Chloroquine

2 x 10^{-2}M Chloroquine

FIG. 14.



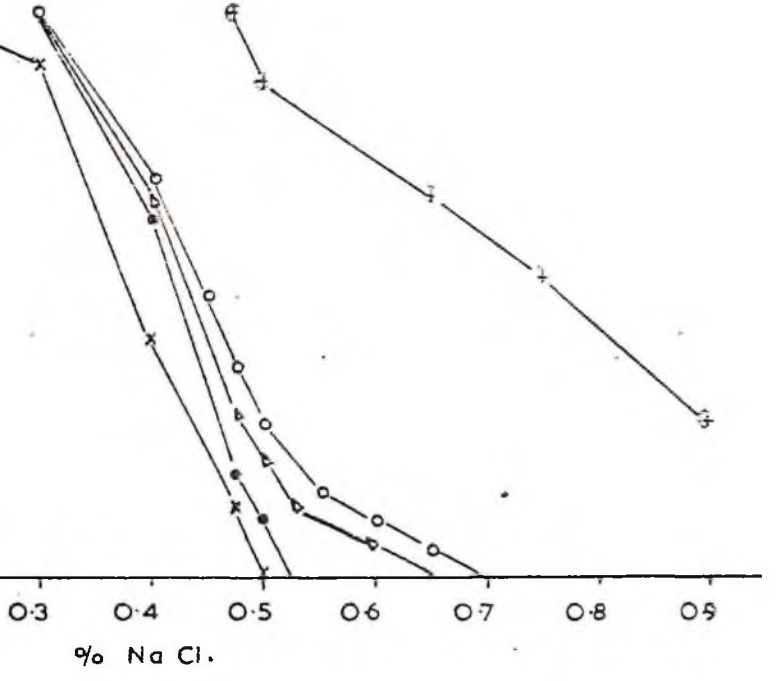


Table 9(a) Summary of the effect of chloroquine phosphate on the osmotic fragility of red cells in the presence of Glucose

Glucose = 3.78×10^{-1}M

Control contained cells incubated without chloroquine or glucose

System	Content	M.C.F. % NaCl	Minimum Resistance (% NaCl)	Maximum Resistance (% NaCl)
1	Control	0.42 ± 0.00	0.49 ± 0.02	0.20 ± 0.00
2	Glucose	0.49 ± 0.00	0.63 ± 0.02	0.30 ± 0.05
3	Glucose + 5×10^{-3}M Chloroquine	0.44 ± 0.00	0.50 ± 0.02	0.30 ± 0.00
4	5×10^{-3}M Chloroquine	0.48 ± 0.00	0.58 ± 0.02	0.30 ± 0.00
5	Glucose + 2.5×10^{-2}M Chloroquine	0.52 ± 0.00	0.69 ± 0.02	0.40 ± 0.00
6	2.5×10^{-2}M Chloroquine	0.79 ± 0.00	0.94 ± 0.00	0.59 ± 0.00

Table 9(b) Summary of results of the effect of Chloroquine Phosphate on the osmotic fragility of rat red cells incubated in presence of ATP

Control contained cells incubated without chloroquine or glucose

Glucose = 3.78×10^{-1}M (ATP) = 2.0×10^{-2}M

System I	Content	M.C.F.	Minimum Resistance	Maximum Resistance
1	Control	0.40 ± 0.02	0.50 ± 0.00	0.20 ± 0.00
2	ATP	0.43 ± 0.02	0.53 ± 0.02	0.30 ± 0.00
3	ATP + Glucose + 2.5×10^{-2}M Chloroquine	0.76 ± 0.02	0.68 ± 0.02	0.48 ± 0.04
4	ATP + 5×10^{-3}M Chloroquine	0.45 ± 0.02	0.65 ± 0.00	0.30 ± 0.00
5	ATP + 2.5×10^{-2}M Chloroquine	0.50 ± 0.02	0.68 ± 0.00	0.30 ± 0.00

Average for 15 determinations.

DISCUSSION

A remarkable feature in the results is that 2.5×10^{-2M} Chloroquine phosphate causes 64-69% homolysis in red blood cells incubated for 4 hours at $37^{0}C$ (table 4). Though Berliner et al (1948) have reported plasma levels of Chloroquine to be 10^{-6M}, the results in table 4 is still significant in view of the fact that parasitized red blood cells have been reported to accumulate higher levels of chloroquine. (Polet and Barr, 1968; Warhurst.1972). Even red blood cells infected with sensitive strains of plasmodia have been reported to accumulate chloroquine to levels higher than 10^{-4M} (Macomber et al,1966). It is therefore likely that parasitized red blood cells may accumulate chloroquine to higher levels enough, to cause hemolysis in patients on chloroquine therapy during parasitaemia. It would therefore be of interest to study chloroquine levels in parasitized red blood cells and find out whether chloroquine - induced hemolysis does occur in malaria patients on chloroquine.

In table 5, 3.78×10^{1M} glucose is shown to cause a 63% reversal of hemolyis due to chloroquine phosphate. This is also confirmed in figure 2, where glucose is shown to suppress chloroquine-induced hemolysis. Another salient observation is in table 6, in which 2.0×10^{-2M} ATP is reported to cause a 59-63% reversal of hemolysis induced by chloroquine phosphate.

The protective effect of both glucose and ATP on chloroquine-induced hemolysis in the red cells could be explained on the assumption that the mechanism of chloroquine-induced hemolysis is similar to that induced by primaquine. Upon such hypothesis, the site of hemolytic action of chloroquine phosphate and the protective effect of glucose and ATP could be located in systems involved in GSH production - presumably the G6PD/glutathione reductase system (Diagram I). For example, chloroquine phosphate could cause a fall in GSH levels by generating oxidants, or by acting as an oxidant itself could convert intracellular GSH to GSSG; or cause the formation of mixed disulfide linkages between intracellular GSH and membrane sulfhydryl groups (Jacob et al, 1967). If this happened the red cell membrane would be critically altered (Hrkal et al, 1967) with probable result of osmotic damage to the cells and eventual lysis.

The protective effect of glucose could be explained in the sense that exogenous supply of glucose provides the precursor of G6P, the substrate for the G6PD reaction. Glucose could be phosphorylated by intracellular ATP to G6P. With the formation of substrate G6P, and upon supposition that the rest of the Pentose Phosphate Shunt is not impaired, one would expect an increase in rate of Phosphate shunt

With a consequent production of enough GSH to quench the deleterious effect of chloroquine. This contention is supported by results in figure 3, in which glucose alone is shown to cause an increase of 33% in GSH levels after 2 hours incubation.

By similar argument, ATP might have prevented hemolysis by providing extra energy to phosphorylate intracellular glucose to the substrate G6P, for the smooth running of the pentose phosphate shunt with a concomittant production of GSH to neutralise the oxidative effect of chloroquine. If the above argument should have any backing, then one would expect an exogenous supply of both glucose and ATP to cause a much greater inhibition of chloroquine-induced hemolysis by producing more substrate G6P for the efficient running of pentose shunt and a substantial production of GSH. It has in fact been shown in these experiments that glucose and ATP caused a complete inhibition of hemolysis due to chloroquine phosphate (table 6). It has also been demonstrated that 2.5×10^{-2}M chloroquine phosphate even increases the activity of G6PD in the intact red blood cells after 40 minutes at 37°C, (table 8a columns 1,2). Thus with the efficient supply of substrate, an unimpaired pentose phosphate pathway and an increased G6PD activity, more GSH would be produced to offset the oxidative effect of chloroquine phosphate. One could also speculate the possibility that, the presence of the substrate G6P could inhibit chloroquine-induced hemolysis observed in these experiments. This was however not tested in these experiments owing to lack of the G6P.

The idea of using ATP and glucose in auto-hemolysis experiments is not new. Selwin and Dacie (1954) devised a method for screening and classifying congenital hemolytic anemia, the basis of which lies in the extent to which the addition of either glucose or ATP corrects autohemolysis of defibrinated blood incubated for 48 hours at 37°C. Normal hemolysis was induced in cases where hemolysis was mild and could be corrected by the addition of glucose, and pathological hemolysis in situations, where hemolysis was severe and was corrected by ATP alone or by ATP and glucose together.

Commenting on the role of glucose in such experiments, Yunis and Yasmineh (1969) suggested that glucose served as an extra supply of energy, while the ensuing formation of lactic acid lowered the pH with a consequent decrease in rate of glycolysis. Relating this argument to Rollo's (1968, 1969) theory that uptake of chloroquine by the red blood cell is enhanced at lower intracellular pH, one would have expected an increase in production of lactic acid, due to the addition of glucose to increase chloroquine-induced hemolysis in the red blood cells. The protective effect of glucose on hemolysis due to chloroquine phosphate found in these experiments, might be due to the fact that red blood cells in the experimental systems were well buffered to avoid pH changes due to lactic acid production.

Tanaka et al (1962) demonstrated that ATP corrects the autohemolysis of red blood cells deficient in pyruvate kinase, probably

by providing energy that could not be produced by the red blood cell.

Yunis and Yasmineh (1969) have reported that ATP being strongly ionic is not transported across the red blood cell. The same authors however believe that ATP is probably hydrolysed on the red blood cell membrane, ADP enters the red cell and is rephosphorylated to ATP. This assertion follows their demonstration that adenosine, AMP and ADP exert the same protective effect in autohemolysis experiments as ATP.

In the present thesis both ATP and glucose have been shown to inhibit chloroquine induced hemolysis; and if it should be proved that hemolysis occur in patients on chloroquine therapy during parasitaemia, it would appear advisable to consider giving glucose and ATP to malaria patients on chloroquine therapy.

Another interesting feature in the results is that 2.50×10^{-2M} chloroquine phosphate caused a 33% decrease in GSH levels in red cells incubated for 4 hours at $37^{\circ}C$ (Figure 3). This result can only be explained to agree with the early experimental results that G6PD is partly activated by chloroquine (table 8a), by demonstrating that chloroquine inhibits glutathione reductase. An attempt to study the effect of chloroquine phosphate on the activity of glutathione reductase using the method of Beutler et al (1957) was not successful. The measured activity for glutathione reductase was too low to allow

any detectable change in the activity of glutathione reductase due to chloroquine phosphate to be made. A demonstration that chloroquine phosphate inhibits glutathione reductase activity is therefore not only essential to explain the experimental results. It will also lend support to the possibility that chloroquine-induced hemolysis is mediated through a similar mechanism as primaquine. It is therefore imperative that further studies are performed to investigate the effect of chloroquine phosphate on glutathione reductase.

Furthermore, the report that chloroquine phosphate causes 47.5% inhibition in the activity of purified G6PD of human red blood cells (Cotton and Suttorius, 1971) is contrary to the apparent activation, observed in these studies (table 8a). The difference between their results and that reported in this thesis may be one of such cases in which findings in isolated systems differ markedly from that in the intact red blood cell. For example, acetylphenylhydrazine, which inhibits G6PD in an isolated system (Desforges et al, 1960) was shown to stimulate the pentose shunt by acting as an electron acceptor in the red blood cells (Szeinberg et al, 1961).

A third possible site of action of chloroquine phosphate could be on the red cell membrane. A method usually used to study the integrity of the red blood cell membrane is the "Osmotic Fragility"

Test. Dacie (1957) points out that one major factor that controls the cell's osmotic fragility is its shape, which in turn depends on its volume, surface area and the functional state of its surface membrane. This method has therefore been used to screen and identify patients suspected of suffering from hemolytic anemia. When a range of hypotonic solutions has been used as in these experiments, a "fragility curve" is drawn by plotting on graph paper the percentage of hemolysis in each tube against the corresponding concentration of NaCl solution. When the cells are normal, a normal sigmoid type of curve are found. In diseases, however, deviations from the normal type of curve are found, e.g. curves with long 'tails' due to some proportion of very fragile cells. An increase in osmotic fragility implies that the cells involved are very succeptible to lysis.

The capacity of primaquine and pentaquine to increase the osmotic fragility of the red blood cells has been well documented (Weed et al. 1961; George et al, 1966). Experiments were therefore performed to study the effect of chloroquine phosphate on the rat red blood cells and the results expressed in Figs. 5 - 11. In Fig. 5, the osmotic fragility of the red cells was shown to increase with increasing concentration of chloroquine phosphate. Figure 6 shows that the osmotic fragility of the red cells in the control containing cells without chloroquine remained virtually unaltered after 105 minutes incubation, whilst with chloroquine phosphate, a significant

increase in fragility was observed within the first 20 minutes of incubation. These results suggest that chloroquine phosphate increases the osmotic fragility of red cells during hemolysis.

The effect of ATP and glucose on the osmotic fragility of the red blood cells was also studied. Glucose alone increased the osmotic fragility of the red cells, but the increase was much higher when chloroquine phosphate was present either alone (Fig. 6), or together with glucose (Fig. 7). When the red blood cells were incubated with glucose and chloroquine phosphate, the cells appeared to react differently as shown by the deviation of the corresponding fragility curve from the ideal sigmoid curve for normal cells (Fig. 7). It appears from this results that osmotic fragility is not a good measure for susceptibility of cells to hemolysis. This is because cells incubated with both chloroquine and glucose are more fragile than cells incubated with chloroquine alone, and yet, it is the latter which undergo hemolysis.

A similar puzzling observation is made in figure 3, in which glucose and chloroquine together, are shown to cause a 70% progressive increase in red blood cell GSH levels after 4 hours incubation at 37°C. These results suggest a strong possibility that chloroquine acts also directly on the membrane, possibly by affecting membrane sulfhydryl groups.

Results in Fig. 8 show that cells incubated with both glucose and chloroquine phosphate for 1½ hours have an increased osmotic fragility, with the fragility curve spread over a wide range of tonicity, from 0% NaCl to 0.9% NaCl. The implication here is that under these conditions, a large percentage of the red cells become fragile after 1½ hours incubation with both glucose and chloroquine. Thus one hour was chosen as the time interval for incubation in subsequent studies involving both glucose and chloroquine.

Fig. 9 shows the osmotic fragility curves of cells incubated with 5×10^{-3}M and 2.5×10^{-2}M chloroquine phosphate - all in presence and absence of 3.78×10^{-1}M glucose. After one hour incubation period, the red cells incubated with lower levels (5.0×10^{-3}M) of chloroquine phosphate had a lower osmotic fragility compared to cells incubated with either glucose, chloroquine or both. This is confirmed in Fig 10, which shows the osmotic fragility curves of red cells exposed to low levels of chloroquine in presence of glucose. The order of stability with respect to osmotic fragility as shown in Fig. 10 is; control > chloroquine + glucose > glucose > chloroquine phosphate. Glucose is here shown to cause less fragility in the red blood cells than chloroquine phosphate and this also agrees with the early results that glucose prevents chloroquine-induced hemolysis.

The osmotic fragility of the red blood cells incubated with both ATP and 5.0×10^{-3}M chloroquine phosphate is higher than that due to the presence of ATP alone but lower than that due to chloroquine phosphate (Fig. 14). This observation also supports the early contention that ATP prevents chloroquine-induced hemolysis by providing extra energy to stimulate the pentose shunt and produce more GSH to offset the oxidative effects of chloroquine.

The mechanism of action of chloroquine phosphate on red cells is not clearly understood. However, consideration of results in this thesis suggests a strong possibility that chloroquine acts on intracellular sulfhydryl groups either directly on the membrane or indirectly by inhibiting glutathione reductase.

A recently proposed mechanism for primaquine hemolysis invokes the participation of red blood cell membrane phospholipids (Wittel, 1970). Their observation indicated 1) that primaquine accelerates the conversion of lysolecithin to lecithin in the intact red cells 2) the reduction of red blood cell lysolecithin content is accompanied by a marked increase in red blood cell osmotic fragility in presence of primaquine and (3) that the osmotic stability of such labilized cells can be restored by repletion of lysolecithin, and it was postulated that primaquine-induced hemolysis is mediated by an effective reduction in the steady state concentration of membrane lysolecithin (Wittels, 1971).

According to results presented for concentrations of chloroquine which are slightly higher than physiological levels, chloroquine seems to affect normal rat red blood cells in a way similar to primaquine-induced hemolysis, namely:

1) A fall in GSH content of the rat red blood cells, and
2) An increase in the osmotic fragility of the red blood cells.

It might therefore be useful to assess the capacity of chloroquine phosphate to modify the lysolecithin metabolism of the red blood cell as compared with its effect on the osmotic stability and GSH content of the red blood cells.

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