IN VITRO STUDIES OF THE HAEMOLYTIC EFFECT OF SUYA CONDIMENTS ON HUMAN RED BLOOD CELLS

BY

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DECLARATION

The work reported in this thesis has been carried out by the author under the supervision of Dr. Joseph A. Abalaka. It has not been accepted elsewhere for the award of higher degree or diploma. The works of other investigators are acknowledged and referred to therein accordingly.



..... Date 25-8-1988

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DEDICATION

This work is dedicated to the entire members of my family.

With a deep sense of gratitude, I thank all those that helped me in one way or the other towards the successfull completion of this work but who for lack of space, their names could not be mentioned.

Finally, I would like to express my deep sense of appreciation and indeptedness to all the members of my family, for their everpresent encouragement, support and patience throughout the entire period of this programme.

And the RBC turn-over rates due the mixture of all the Suya condiments obtained from Shagamu and Samaru village are $1.52 \times 10^{-4} min^{-1}$ and $1.25 \times 10^{-4} min^{-1}$ respectively.

The RBC from malaria patients were more susceptible to the haemolytic effect with a half-life ranging from 3652 mins (due to kuli-kuli from Shagamu) to 1203 mins (due to Jawa from Shagamu). The half-life for the RBC from malaria patients when treated with the Suya condiments from Samaru village ranges from 3698 mins (due to kuli-kuli) to 2605mins (due to Jawa).

The RBC from normal individuals were more resistant to the haemolytic effects.

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CHAPTER 1.

1. INTRODUCTION

"Suya" is a popular roadside barbecued beafsteak taken as snack by majority of the Nigerian populace, especially those in the Northern part of the country where it originated.

Two types of Suya exist, the red type and the brown type. The redtype is common in the Southern part of the Country, while the brown type predominates in the Northern part of the Country.

Recently, a peculair haemolytic anaemia in many Nigerians was reported by a group of Scientists (Akinyanju *et al*, 1983). They preceeded by the ingestion of Suya some two to four days earlier. They observed that only Suya covered with a layer of a reddish-orange powder, which was later shown to contain orange - RN, appeared to cause the syndrome. It also appeared that individuals whose red cells were deficient in glucose-6-phosphate dehydrogenase (G6PD) were especially susceptible to the haemolysis.

In Nigeria, the red powder was usually mixed with chillies before applying the mixture on the raw beef. The beef so treated often looked fairly reddish but quite attractive when raw but turned darkish brown on roasting. Nevertheless within a few hours after consumption of such products, the urine of all who consumed the Suya, no matter what their G6PD status, turned red (Akinyanju *et al*., 1983). Red Suya has existed for many years and the recent

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in many parts of the world. They are used as spices, carminative, stimulant aromatic and as drugs for the treatment of various ailments (Albert, 1952). A common denominator in all these condiments is that they are phenolic compounds. They also contain alkaloids.

Various experiments have been conducted aimed at finding out the mechanisms of actions of chemical agents on red blood cells. Thus the red cell membrane and intracellular contents of the red cells have been the target of study by various research workers. Various enzymes within the erthrocyte which participate in the metabolic processes of the erythrocytes have been discovered. These enzymes are maintained at a certain level and their deficiency could cause or lead to haemolysis (Carson *et al*., 1956)

The discovery of G6PD enzyme deficiency as a common feature in persons subject to haemolytic episodes as the result of the administration of certain drugs e.g. the anti-malarial primaquine, provided the first strong evidence that enzymatic deficiencies (which are specific and can be inherited) could be responsible for haemolytic reactions of drugs. From the results of intensive study, Carson *et al* (1956) stated categorically that G6PD deficiency is the usual cause of the haemolytic anaemias. This hypothesis has gained wide acceptance and it is now used to explain unusual reaction to drugs like hypersensitivity.

Other investigators have established a relationship between the erythrocyte reduced glutathione (GSH) and the haemolytic processes caused by oxidant drugs. The thiol groups are involved in the

maintenance of the integrity of the red blood cell (Grimes, 1980) and they are easily oxidized under physiological conditions.

Recent research has shown that selenium combined with an amino acid is an essential component of the prosthetic groups of several enzymes particularly glutathione peroxidase, which functions together with glutathione to protect cells against the destructive effects of hydrogen peroxide (Hoekstra, 1974).

In red blood cell, the haemoglobin iron normally exists in the ferrous (Fe II) form but it is readily oxidized to the ferric (Fe III) state by hydrogen peroxide to yield methaemoglobin, which is inactive in carrying oxygen. Thus glutathione peroxidase protects the erythrocytes against the formation of methaemoglobin by consuming hydrogen peroxide in the reaction:

$$2GSH + H_2O_2 \longrightarrow GSSG + 2H_2O$$

Haemolysis can result from haemoglobin defects, intracellujlar enzyme abnormalities or membrane alteractions, all of which are inter-related (Weed and Reed, 1986). In terms of erythrocyte glycolysis, the pentose - phosphate pathway is essential for the maintenance of intracellular GSH and hence, of proteins containing thiol groups. GSH is also of importance in the maintenance of membrane thiol groups (Grimes, 1980). Defects in the pentose phosphate pathway (G6PD deficiency, GSH deficiency) may give rise to haemolytic states when the red blood cells are exposed to the appropriate oxidant stress (Grimes, 1980).

Recent studies revealed that there are specific sulfhydryl groups that are intimately related to membrane structure and are

relatively sensitive to *in vivo* and *in vitro* oxidation and that the maintenance of these groups in the reduced state is dependant upon the metabolic processes within the erythrocytes (Grimes, 1980).

The present investigation focussed on the various Suya condiments used in the northern part of the country. The previous reports of "red Suya" Syndrome had been confined to those condiments used predominantly in the Southern part of the country. No study has been extended to the northern part where the consumption of Suya is far more common than in the Southern part.

As an extention of this investigation the effect of the various Suya condiments on normal individuals and those afflicted by certain diseases (malaria, sickle cell anaemia and diseased liver) would be investigated with a view to establishing some safe ranges of the condiments for the various categories of human subjects.

make it capable of containing enzyme sites for specific functions and, especially for red cells, to undergo changes of shapes.

.Physically the membrane is a minor component of the cell. In a red cell it weighs only about 10 - 12g and so comprises a mere 2% of the cell weight (Grimes, 1980).

2.1.2 Membrane Composition And Structure.

An early contributor to the study of membrane composition was Overton (Grimes, 1980) who demonstrated that red cells could take up lipid - soluble material. This raised the idea that the membrane consisted of lipid, a view confirmed by other workers (Schanker et al., 1964) with the demonstration, for example, that the rate of entry of organic acids into red cells is approximately proportional to their partition coefficients in a lipid - water system. Many other homologous series followed this rule and it is now generally understood that the action of a number of drugs, particularly anaesthetics, depends upon their solubility in the lipid component of the membrane.

A major advanced concept came with the proposition that the lipid exists in the cell as a double layer. This idea, and the probable configuration of the lipid, arose from experiments using the Langmuir trough technique (Grimes, 1980). The tadpole - shaped lipid moleules arranged themselves with their polar heads in the aqueous phase and the nonpolar tails sticking vertically out of the aqueous layer. It appeared that the tail length made no difference to the limiting area occupied by the monomolecular layer. Thus the polar heads of fatty acids or phospholipid

molecules always dispose themselves in an aqueous or hydrophilic phase while the nonpolar, fat - soluble tails point away from the hydrophilic region into a nonionic or hydrophobic region if one iws available.

This finding has a key significance on all the models of membrane structure that were to be proposed. Evidence that the membrane contained lipids arranged in this manner, but as a double layer, was obtained by Gorter and Grendel (1925). They extracted the lipid component of red cells and determined the area occupied by the lipid when close - packed as a unimolecular layer in a Langmuir trough; the value observed was compared with the surface area of the red cell membrane from which the lipids were extracted. They calculated that the lipid must exist as a layer of two molecules thick, in a two - dimensional array or sheet around the membrane.

It has since been remarked (Bar, *et al*., 1966) that in these experiments no values were given for the recovery of lipid from extracted membranes but that acetone extraction would probably have removed about 70%. Also the value of 99μm^2 used by Gorter and Grendel (1925) for the surface area of the cell is too low, and more realistic value would be 145 + 8μm^2. It is likely, therefore, as suggested also by Hendler (1971) that these two errors cancelled out to yield a value that implied that the lipid must exist in the membrane as a bilayer.

It appears that a lipid bilayer in which the polar heads point outwards into the external polar environment with the tails is an inner hydrophobic region in a arrangement that is thermodynamically

favourable (Grimes, 1980). Grimes (1980) remarked that such a bilayer is possible because the constituent lipids are amphipathic, that is each lipid molecule has both hydrophilic and hydrophobic properties. He pointed out also that the protein component of the membrane behaves in an amphipathic manner although this has been more difficult to establish.

Membrane which consist of lipid alone woulde lack the mechanical strength necessary to withstand the trauma of existence, particularly if they are blood cells. On the other hand, if they contain protein alone the electrical conductivity would be higher than the known values. That they contain a mixture of both was suggested from early surface tension studies (Davson and Danielli, 1952) which showed low values that were incompatible with a purely lipoidal membrane but acceptable if protein is assumed to be a component also.

This important contribution to membrane structure arose from work on marine eggs (Cole, 1932) in which the surface tension values were too low (1 dyne/cm^2) to assume that lipid alone was present. A similar finding emerged from the work of Harvey and Shapiro (1934) on oil droplets suspended inside starfish eggs. Again, unexpectedly low values were found for the surface tension in oil extracted from mackerel eggs (Danielli and Harvey, 1935, Danielli and Davson, 1935) which suggested that contaminating protein was absorbed onto the polar heads at the lipid surface.

Further evidence that material in addition to lipid was present has come from studies of artificial membrane (Finkelstein, 1972).

Such membrane prepared from lipid alone had little mechanical strength.

However, proteins form stable monomolecular films at poil - water interfaces and globular protein normally uncoil and become less soluble (Danielli, 1938). It was envisaged that such protein could be absorbed as a sheet onto the lipid layer of a cell membrane with the nonpolar residues embedded in the lipid and the polar groups facing the aqueous exterior. Such a combination of protein with lipid could give the necessary mechanical strength to the membrane.

2.1.3. Protein - Lipid Association

The amount of protein present in a red cell membrane is about 10^{-12}g and the ratio of protein to lipid is about 1:1 (Grimes, 1980). Other cell or organelle membranes have characteristic ratios which are markedly different from the human red cell and which are a reflection of their function.

The protein and lipid in cell membrane are in intimate association (Schmitt *et al.*, 1938) but can be separated under the relatively mild treatment afforded by organic selvents or detergents.

Membrane proteins fit into two broad - based classes, usually based on the method used in extracting protein and consequently the location of the proteins (Singer and Nicholson, 1972). One of the classes are peripheral proteins and the other are integral proteins. The former class consists of proteins that are only loosely bound to the membrane matrix and can easily be removed by ionic manipulations (Varying pH conditions, chelating agents,

detergents). The latter (integral proteins) are those that interact with the lipid bilayer.

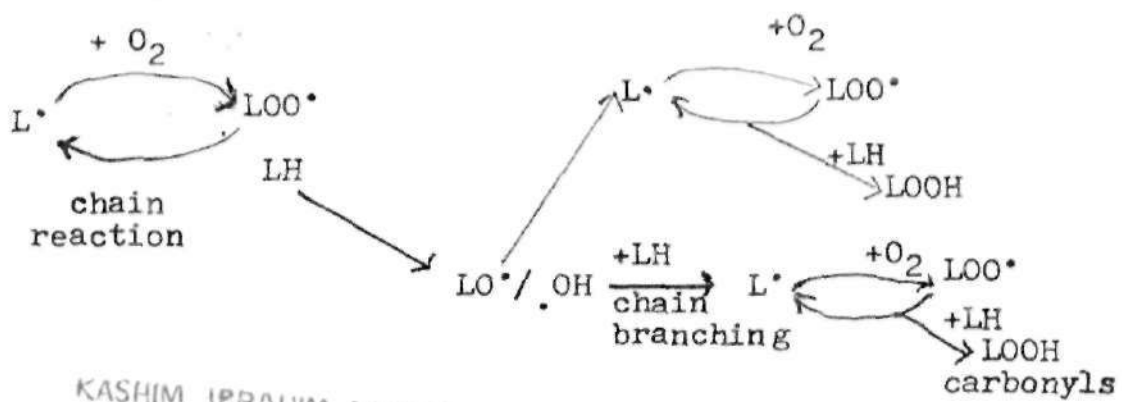
MEMBRANE COMPOSITION

Component	Intact Membrane (%) w/w
Protein	49.2
Lipid (total)	43.6
Phospholipid	32.6
Cholesterol	11.1
Carbohydrate (total)	7.2
Neutral Sugar	4.0
Hexosamines	2.0
Sialic acid	1.2

Adopted from Rosenberg and Gujidotti (1968).

2.2 Lipid Peroxication And Red Cell Integrity

Lipid peroxidation involves the production of semistable peroxides from free radical intermediates generated through direct reaction of oxygen with unsaturated lipids. The following are free radicals involved in the reaction:-



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Peroxy radicals - LOO
alkoxy radicals - LO
Hydroxy radicals - OH
} Free radicals

Adapted from Benjamin and Arstila, 1975.

Valuable background information for the application of autocatalysis to non biological studies has come from studies of peroxidation reactions in non biological olefinic compounds (Scott, 1965). The production of free radicals during lipid peroxidation leads to subsequent damaging reactions.

Free radicals have unpaired electrons which react very strongly and initiate relatively nonspecific hydrogen abstraction and chemical addition reactions. Free radicals produced during lipid peroxidation are chemically similar to the damaging species produced in radiation (Benjamin and Arstila, 1975).

Based on measurements of moles of enzyme inactivated per mole of free radical, lipid peroxidation is one-tenth as damaging as ionizing raciation (Chio and Tappel, 1969). Lipid peroxication is one of the most deteriorative reactions that results in damage to membranous cellular constituents.

Red cell membranes contain a high level of polyunsaturated fatty acids in their phospholipids, and they are exposed directly to molecular oxygen. For these reasons these membranes are quite susceptible to lipid peroxidation (Hochstein, 1966). During hyperoxia, the red blood cells of vitamin-E deficinent mice will undergo lipid peroxidation *in vivo* leading to haemolysis of the

cells (Kahn and Mengel, 1966). Combs *et al.*(1976) have projected that the mechanisms of haemolysis involve both direct damage to the lipid structure of the membrane and to the inhibition by lipid peroxides of the erythrocyte acetylcholinesterase activity.

Among the deteriorative reactions basic to the mechanism of membrane and cellular aging processes, *in vivo* lipid peroxidation (Barber and Bernhein, 1967) has been identified. Lipid peroxidation also has been implicated or indentified as the damaging reaction that occurs in air-pollution oxidative damage to cells (Goldstein and Balchun,1967).

2.2.1. Role of Molecular Oxygen In Lipid Peroxidation

The role of molecular oxygen as a precursor of lipid peroxidation lies not so much in its own reactivity, which is surprisingly low, but rather in the reactivities of the chemical species to which oxygen can give rise to. The reduction of oxygen leads to the production of free intermidiates, superoxide, hydrogen peroxide and hydroxyl radical (Quagliariello *et al.* 1974):

$$O_2 + e' \rightleftharpoons O_2^{\cdot-}$$

$$O_2^{\cdot-} + e' + 2H^+ \rightleftharpoons H_2O_2 \text{ (hydrogen peroxide)}$$

$$H_2O_2 + e' + H^+ \rightleftharpoons H_3O_2 \rightleftharpoons H_2O + {}^{\cdot}OH \text{ (hydroxy radical)}$$

$${}^{\cdot}OH + e' + H^+ \rightleftharpoons H_2O$$

Superoxide and hydroxyl moieties are free radicals and as such are intrinsically very reactive. One of these radicals, OH, is the most potent oxidant known in chemistry, and it will react with lots of organic substances at rates that are close to the theoritical limit set by diffusion (Quagliariello *et al.* 1974). Hydrogen peroxide is

a powerful oxidant whose behaviour is incompatible with the delicate molecular fabric of living cells.

Hydrogen peroxide will oxidize thiol (-SH) groups and methionyl residues in proteins and can cause the peroxidation of polyunsaturated fatty acids, but these reactions proceed at reasonable rates only when the concentration of hydrogen peroxide (H_2O_2) is much higher than that achieved by living systems (Quagliariello *et al.* 1974). Yet aerobic cells have gone to great lengths to prevent the accumulation of H_2O_2. Catalases and peroxidases which are common in such cells catalyze H_2O_2 breakdown to water.

One of the greatest dangers of H_2O_2 lies in its ability to generate the terribly reactive hydroxyl radical by interaction with O_2.

2.2.2 Involvement of Vitamin E And Selenium in Lipid Peroxidation

The interaction between selenium, vitamin E, unsaturRWS LIPIDS, ONE sulfur-containing amino acids posed problems in postulating the metabolic functions of vitamin E and selenium. Two main ideas attracted the most attention.

One idea as proposed by Schwarz (1957) was that both nutrients functioned separetely in a co-factor type, probably enzymatic role, but at sites of metabolism which were closely related. The other idea as proposed by Tappel (1962), was that both nutrients were lipid antioxidants or were precursors of anti-oxidants (e.g Selenite is itself not an anti-oxidant, but the Se-containing

proteins derived from it were envisaged to carry out such an antioxidant function).

Fairly lately (Glavind, 1973), in a review of the antioxidant theory, it was stated: "The whole theory was founded upon assumptions and indirect evidence, and complications emerging from the observation that selenium protects against vitamin E deficiency symptoms were reconciled with the doctrine by assigning powerful antioxidant properties to Selenium" and "it seems awkward to interpret the protective properties of trace amounts of selenium against certain manifestations of vitamin E deficiency by attributing exceptional antioxidant properties of Selenium compounds".

A satisfying aspect of recent discovery of selenium as an essential component of glutathione peroxidase is that it reconciles this dilemma and encompasses both a "co-factor type" role for selenium as well as an "antioxidant-like" role of the entire selenium- dependant enzyme system (Hoekstra, 1974).

2.2.3 (see the following page for details)

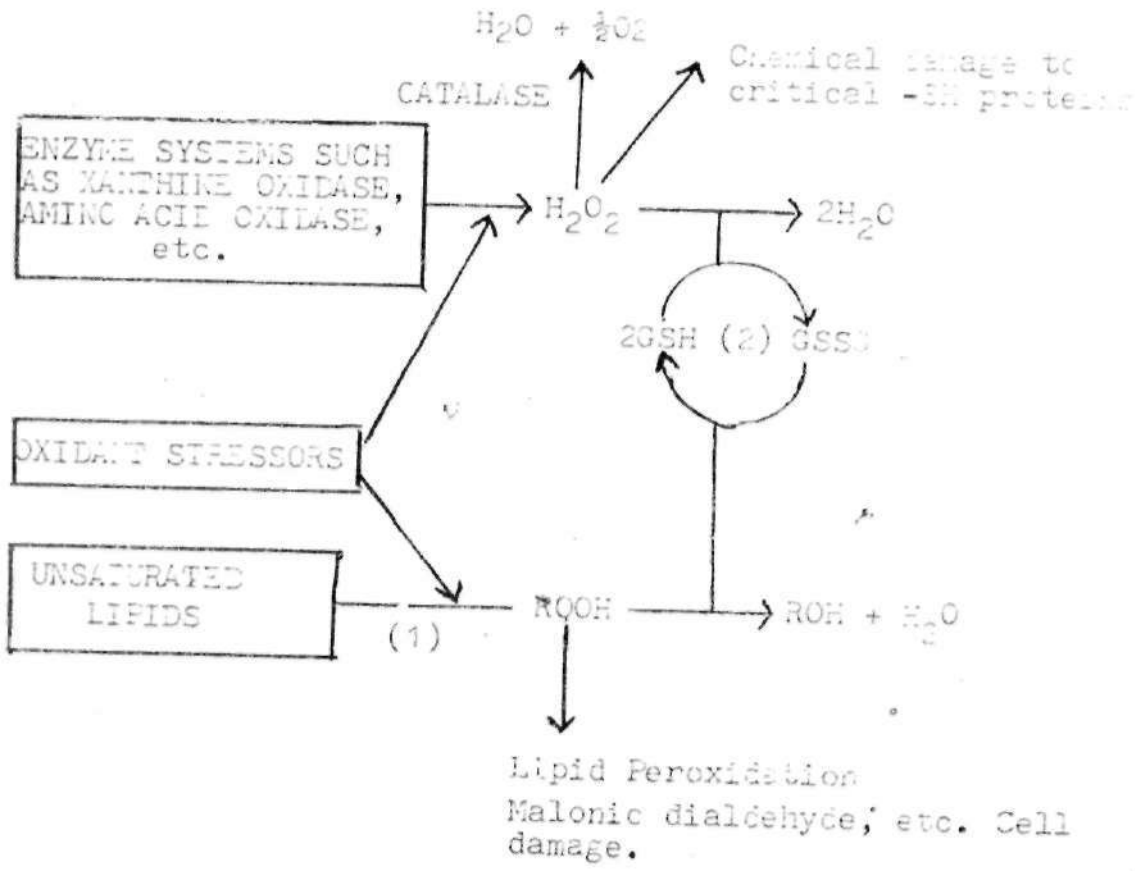
Vitamin E "blocks" reaction (1)

Selenium, as component of GSH-peroxidase, catalyses reaction (2)

N.B. Scheme showing the postulated mechanisms of interaction between Se and Vit-E in preventing oxidant damage.

The vitamin-E-Selenium interaction is viewed as that in which the vitamin prevents lipid hydroperoxide (ROOH) formation and the consequent autocatalytic lipid peroxidation and cell damage. The Selenium is seen, as a component of glutathine peroxidase, that

2.2.3. Functional Interrelationships Involving Selenium, Vitamin E, Unsaturated Lipids, Sulfur - Containing Amino Acids And Oxidant Stressors



Adapted from Hoekstra, 1974.

converts such hydroperoxides, to the less damaging alcohols, whose ultimate fate is not yet understood (Chow *et al* 1975).

2.2.4 Protection of Erythrocyte Plasma Membranes by Selenium Annd Vitamin E

Erythrocyte plasma membranes are susceptible to lipid peroxidation due to their high polyunsaturated fatty acid content and to their direct exposure to molecular oxygen (Hochstein, 1966).

Peroxidation leads to cell haemolysis. Horn *et al*., (1974) have shown that the rabbit erythrocyte, normally resistant to peroxidative haemolysis, can be rendered sensitive by increasing both the unsaturated fatty acid content of the membrane and also renders it susceptible to peroxidative haemolysis.

Clinical anaemias have been reported in chicks, in monkeys, in fish, in humans with protein energy malnutrition (PEM) and in premature human infants (Chow *et al*., 1973, all of which are associated with increased erythrocyte fragility. These anomalies are responsive to vitamin E treatment. Dietary Selenium has been shown to prevent peroxidative haemolysis in the vitamin E deficient rat (Combs *et al*., 1975). Combs *et al*., (1975) demonstrated a selenium dependent factor in vitamin E deficient rat erythrocytes that protected those cells from haemolysis in the presence of glucose. This finding led the investigators to the important discovery that selenium is an essential constituent of the enzyme glutathione peroxidase, an enzyme that utilizes reducing equivalents from glutathione to destroy hydrogen peroxide and fatty acid hydroperoxides that could otherwise initiate oxidative

degradation of cellular components including erthrocyte membrane lipids (Combs *et al*., 1975) Thus, peroxidative damage to the erythrocyte plasma membrane is prevented by glutathione peroxidase and this activity is dependent on the level of selenium in the diet (Combs *et al*., 1975).

Protection of the erythrocyte from peroxidative haemolysis by vitamin E has been well established (Combs *et al*., 1975). This function of vitamin E differs from that of selenium: Whereas the selenium containing enzyme glutathione peroxidase is associated primarily with the aqueous phase of the cytosol and pllasma (Nouguchi *et al*., 1973), vitamin E is present within the membrane itself (Combs *et al*., 1975).

Thus, dietary selenium protects against both erythrocyte haemolysis and haemoglobin oxidation under conditions of oxidative stress, while vitamin E protects only the erythrocyte plasma membrane under these conditions (Combs et al., 1975). The action of vitamin E in the erythrocyte plasma membrane appears to be that of lipid-soluble antioxidant with biological specificity conferred on it by stereochemical properties that enable it readily to become part of biological membrane structure (Combs et al., 1975).

2.3. Red Cell Enzyme Deficiency In Haematological Diseases Associated with Haemolytic Anaemia

2.3.1. Glucose-6-Phosphate Dehydrogenase (G-6-PD) Deficiency

Glucose-6-Phosphate dehydrogenase (G-6-PD) is the enzyme that catalyses the first oxidative reaction in the pentose phosphate shunt (Eugene, 1970). In the first 12 years of the discovery that a

deficiency in this enzyme predisposed individuals to haemolysis on ingestion of primaquine (Carson *et al*., 1956) several hundred papers and several excellent reviews have appeared (Beuler, 1966).

Glucose-6-PD deficiency is due to an abnormality of a gene associated with the x-chromosome. It is estimated to affect more than 100 million people of all races throughout the world (Eugene, 1970). In the United States of America alone, nearly 3 million people carry the gene for this disorder (Eugene, 1970). G-6-PD deficiency is associated with about 30% of cases of chronic nonspherocytic haemolytic anaemia (Eugene, 1979). About 2% of subjects with G-6-PD deficiency have a chronic haemolytic anaemia (Eugene, 1970).

Interest in the red-cell Enzyme arises as the result of its importance in drug-induced haemolytic anaemias and in congenital non-spherocytic anaemias (Carlson *et al*., 1956) and with the occurrence of electrophoretic variants (Beutler, 1966).

Next to ABO RH blood-group incompatibility this metabolic error is the commonest predisposing factor for haemolytic disease in man (Eugene, 1970). The susceptibility of cells to haemolysis with G-6-PD deficiency is believed to be due to a decrease in their capacity to generate sufficient amount of NADPH to protect the cell against oxidative agents (Eugene, 1970).

2.3.2 Glutathione Reductase (GSSG-R) Deficiency

Glutathione reductase GSSG-R) reduces oxidized glutathione to reduced glutathione. It requires NADPH and generates NADP.

G-S-S-G + NADPH H +....2GSH + $NADP^+$ (Grimes, 1980).

Reduced glutathione plays an important role in protecting the cell against oxidative agents. Deficiency of this erythrocyte enzyme has been reported in patients with hereditary chronic nonspherocytic haemolytic anaemia, sometimes associated with thrombocytopemia and neurological complication (Waller, 1968). Just like in patients with G-6-PD deficiency to certain drugs may precipitate a haemolytic anaemia. The inheritance appears to be autosomal, but genetic studies are limited (Waller, 1968).

2.3.3. Glutathione Peroxidase (GSH-PX) Deficiency

Glutathione peroxidase catalyses the degradation of hydrogen peroxide. It provides protection in the cell against peroxidation (Eugene, 1970). This reaction is linked to the pentose phosphate pathway through its role in glutathione oxidation. A partial deficiency in GSH-PX has been associated with transient mild haemolysis in the neonatal period (Nechelles et al., 1967). Nechelles and his associates (Nechelles et al., 1967) reported a Peurto Rican male presumed homozygous for GSH-PX deficiency (erythrocyte activity of about 25% of normal), both of whose parents and one sibling apparently were heterozygous for the deficiency. The patient had an acute haemolytic reaction during a transfusion and following recovery he had what appeared to be compensated haemolytic anemia.

2.3.4. Glutathione (GSH) Deficiency

A series of studies in a Dutch Kindred had demonstrated decreased to absent red cell glutathione levels associated with a compensated

chronic congenital nonspherocytic haemolytic anaemia (Prins *et al.*, 1968). The transmission was considered to be autosomal. The red cells of these subjects are susceptible to primaquine-induced haemolysis. These patients are believed to have a defect in GSH synthesis (Glutathione synthetase) (Prins et al., 1968).

2.4 Red Cell Destruction In Malaria

In malaria, as in certain drug-induced diseases, anaemia is the result of enhanced erythrocyte destruction, which is in turn a prime contributor to the pathogenesis of the disease (Julius, 1980).

2.4.1 . Mechanism of Erythrocyte Destruction

A common denominator in all haemolytic anaemias is the shortened life span of circulating erythrocytes. This can be attributed to (a) Intrinsic cellular defects (b) extrinsic defects in the cell environment (Prankerd, 1961) (c) a combination of the two; that is intrinsically defective cells circulating within a defective environment. Malaria as a haemolytic disease falls into the latter category (Julius, 1980).

Numerous investigators have found that the life span of circulating radioactively labelled erythrocytes is shortened during malarial infection (Stohlman et al., 1963; Rosenberg et al., 1973). To a great extent this is the result of intrinsic cellular defects which enhance sequestration of intact erythrocytes, promotes cell fragmentation, and causes haemolysis (Julius, 1980). A variety of indirect experimental studies done by various investigators (Seed, 1972). Schnitzer *et al.* (1973) indicated that erythrocytes containing

large parasites are more likely to be sequestered than those containing small parasites.

Erythrocyte lesions attributed directly to the parasites can be divided into those associated with (a) Merozoite penetration of the host cell and (b) the presence of mature Schizonts (Ladda *et al.*, 1969). The physical processes involved in red cell penetration by merozoites are well described (Aikawa *et al.*, 1978) and include specific events which occur in an ordered sequence, (a) Merozoite recognition and attachment to susceptible host cells (Miller *et al.*, 1979), (b) Junction formation. (c) progressive invagination of the red cell membrane by the invading merozoite, and (b) complete sealing of the formed vacuole over the penetrating parasite.

The extent of damage caused to the penetrated red cells by merozoites during infection is not known. Kreier *et al.* (1972) speculated that the excess red cell destruction seen during the course of these infections might be attributable at least in part to red cell damage due to ineffective merozoite penetration. Haemolytic agents have been isolated from intraerythrocytic parasites and are thought to be derived from the specialized apical organelles of merozoites (Kilejian, 1974). These haemolytic agents are believed to function in host cell penetration (Kilejian and Jeensen, 1977). The action of these agents could damage the erythrocyte membrane sufficiently to cause lysis under some circumstances.

From several studies (Seed *et al.*, 1972 and Aikawa, 1977) a general conclusion was drawn that the gross abnormalities in

erythrocyte shape occurred only when the parasite developed beyond the trophozoite stage.

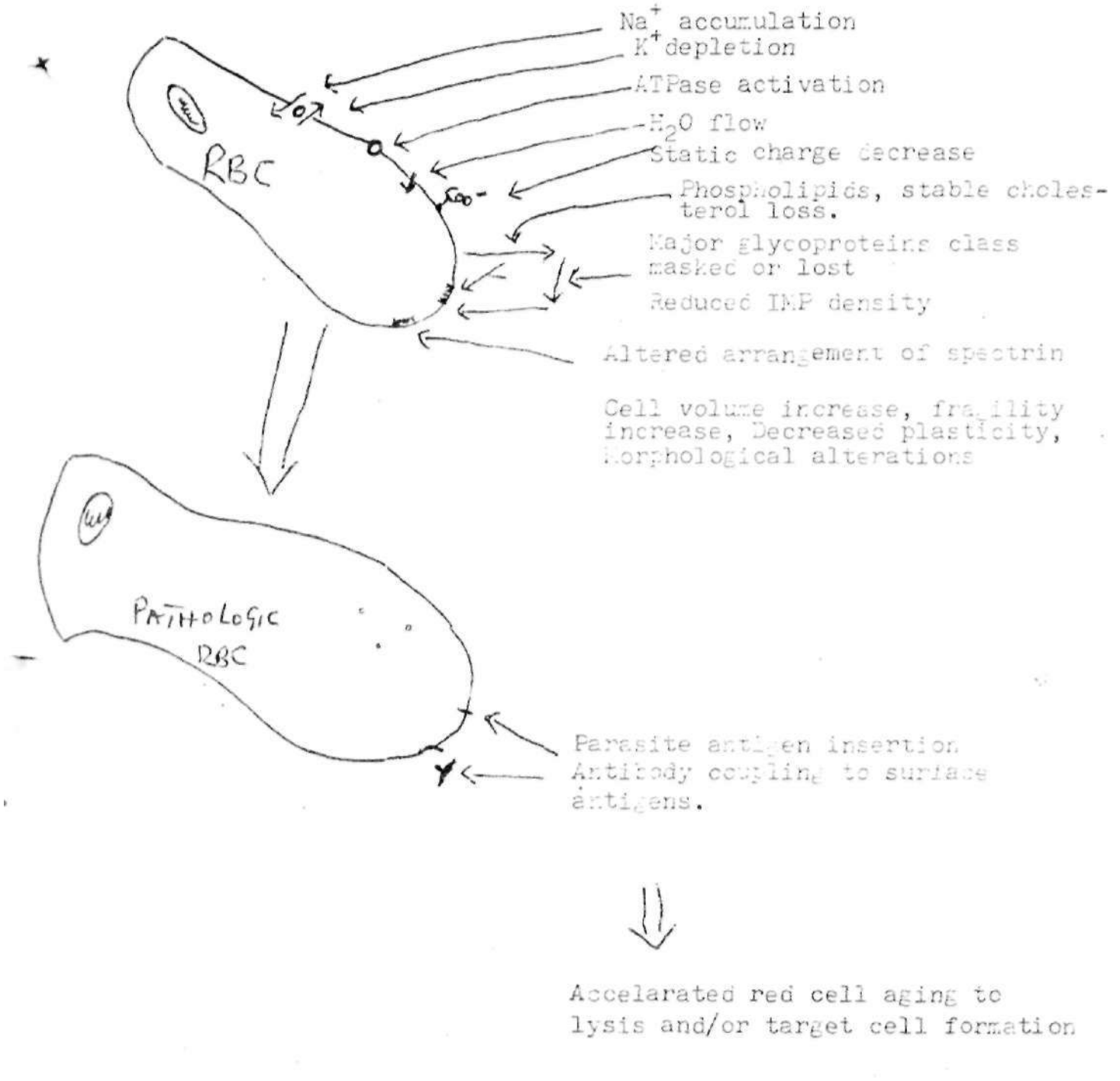
Various types of erythrocyte dysfunction have been incriminated in red cell destruction (Julius, 1980). These include (a) abnormalities in erythrocyte size, shape and elasticity, (b) metabolic abnormalities, and (c) membrane abnormalities (Julius, 1980). The changes in red cell shape as a consequence of parasitization are undoubtedly not the result of a single cellular defect but of a cascade of molecular lesions which are responsible for physiological dysfunctions (Julius, 1980). A list of these disorders and a hypothesis of how these processes interact is given on page 24.

2.5 Sickle Cell Disease And Red Cell Membrane

Sickle cell disease afflicts individuals who are homozygous (Hbss) for sickle cell haemoglobin, Hbs. In Hbs the glutamate residues at the sixth position of the normal haemoglobin B-chain is substituted by valine. At the low oxygen tension (PO_2) that occur in tissues, this replacement of a negatively charged group by a large apolar one leads to formation of large, parallel, rod-like, hydrophobically stabilized intermolecular aggregates, which cause erythrocyte deformation or sickling.

As summarized in an earlier commentary on sickle cell disease as an example of membrane pathobiology (Wallach, 1973), a variety of early studies indicated that the erythrocyte membrane may be deficient in sickle cell disease.

Homozygous sickle cells differ from normal in their high intracellular calcium (Palek, 1977). Several abnormal



A partial listing of red cell alterations which occur as a consequence of malarial parasitization: Cumulatively these modifications are responsible for premature aging and sequestration.

(Adapted from Julius, 1980).

Consequences of high intracellular calcium have been discussed (Weidekamm *et al.*, 1977). They include: Cross-linking of spectrin components, aggregation of spectrin with or without other proteins in association with the membrane, segregation of spectrin, activation of phosphatidic acid synthesis, red cell protease activity and transglutaminase activity (Wallach, 1979). An important consequence of all of these processes appears to be a disturbance of the red cell cytoskeleton and an increase in erythrocyte fragility (Wallach, 1979).

2.6. Chronic Liver Disease And Red Cell Membrane

The liver holds a unique position in the body because of its anatomical connections and the multiple and varied functions it performs. Alterations in structure may result in portal hypertension, ascites, Jaundice, increased bleeding tendency and complex metabolic changes affecting most body constituents.

Strictly speaking, chronic liver disease is not directly associated with erythrocyte membrane defects (Akinyanju, personal communication).

2.7 Plant Materials Used In The Preparation Of Suya Condiments

2.7.1 Ginger

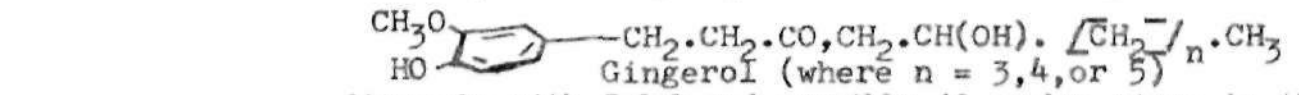
Ginger (Zingiber; *Radix Zingiberis*) is the rhizome of *Zingiber officinale* (Fam. *Zangiberaceae*), which has been scrapped and dried in the sun. *Zingibar officinale*, a reed-like plant, is grown in many parts of the world, including Jamaica, India and Africa (Trease and Evans, 1978).

Ginger is the most important of the species obtained from roots. It has had a long and interesting history. A native of south Eastern Asia, it was early used in China and India, and was brought by Caravans to Asia Minor before the time of Rome (Albert, 1952). It was among the first of the oriental spices to be known in Europe, where it was prominent early in the Middle Ages (Albert, 1952). For many years it was an important drug.

2.7.1.a. Constituents

Ginger contains about 1-2% of volatile oil and between 5 and 8% of resinous matter, starch and mucilage (Trease and Evans, 1978). Oil of ginger, to which the drug mainly owes it aroma, contains terpenes [(+)- Camphene and B- phellandrene)], sesquiterpenses (Zingiberene), bisabolene, cineole, citral and borneol.

The pungency of ginger is due to gingerol, an oily liquid consisting of homologous phenols of the following formula:-



Gingerol (where n = 3,4,or 5)

Gingerols with 7,8,9 and possibly 16 carbon atoms in the side chain have been reported by Japanese Scientists (Trease and Evans, 1978) new minor constituents of an extract found, included gingediols, Methylgingediol, gingediacetates and methylgingediacetates (Trease and Evans, 1978).

2.7.1.b. Uses

Ginger is used more as a condiment than as a spice (Trease and Evans, 1978). It dilates the blood vessels in the skin, causing a feeling of warmth, and increases perspiration, with an accompanying

drop in temperature (Albert, 1952). For this reason it is much used in warm countries.

In medicine, ginger is used as a carminative, and a digestive stimulant. It is extensively used in culinary preparations, such as soups, puddings, pickles, ginger bread and cookies, and is an ingredient of all curries, except those used with fish (Albert, 1952).

Ginger is exceedingly popular for flavouring beverages such as ginger ale. Its oleoresin is extracted and used in medicine and flavouring. The essential oil is also extracted. As this lacks pungency, cayenne pepper is usually added when it is used for flavouring purposes.

2.7.1.c. Other Uses

While it was being screened as anthelmintic agents ginger was discovered to have biological effects which prevent the hatching of schistosome eggs (Kucera *et al.*, 1975). The pungent phenolic principles (consisting of phenylpropane derivatives) of ginger have been shown to be responsible for this biological property and, in particular, the ketonic fraction (probaly Zingerone, Methyl Zingerone, etc) of ginger extract has been implicated (Kucera *et al.*, 1975).

Tablets of ginger root powder and of ginger aqueous extract, as well as capsules of the Ketonic fraction, have been given to volunteer school children suffering from Schistosomiasis (Kucera et al., 1975). The pupils' bloody urine was stopped by this treatment; the schistosome egg count in their urine also dropped, indicating

some *in vivo* efficacy of ginger in schistosomiasis. This requires more detailed study.

2.7.2. Cloves

Cloves (caryophyllum) are the dried flower buds of *Syzygium aromaticum* (*Eugenia caryophyllus*), (Fam. *Myrtaceae*), a tree of 10-20m high which is indigenous to the Molucca or Clove Islands (Trease and Evans, 1978).

Cloves are one of the most important and useful spices. They were in use as early as the third century B.C. in China, were well known to the Romans and they reached Europe during the Middle Ages (Albert, 1952). Their source and place of origin were unknown until the Portugues discovered the Molucca Islands in the sixteenth century. For a time cloves were a Portuguese and later a Dutch monopoly. Today they are grown in many tropical countries in both the old and new worlds.

2.7.2.a. Constituents

Cloves contain about 14-21% of volatile oil, 10-13% of tannin, various triterpene acids and esters, and the glucosides of sitosterol, stigmasterol, and campestrol (Trease and Evans, 1978).

2.7.2.b. Uses

Cloves are very aromatic and fine flavoured and impart warming qualities. They have almost endless uses, both whole and in the ground state as a culinary spice, for the flavour blends well with both sweet and savoury dishes. They are used for flavouring pickles, curries, ketch-up, and sauces; in medicine; and for perfuming the breath and the air in rooms (Albert, 1952). Cloves

have stimulating properties and are one of the ingredients of betel-nut chew. Cloves cigarettes are smoked in Java (Albert, 1952).

The essential oil, which is obtained by distilling cloves with water or steam, has even more uses. It is used in medicine as an aid to digestion and for its antiseptic and antispasmodic action (Albert,1952) It is often used as a local antiseptic in toothache. Externally it has a counterirritant action, It is an ingredient of many toothpastes and mouthwashes.

The oil has many industrial applications and is extensively employed in perfumes, in scenting soap, and as a clearing agent in histological work (Albert, 1952). The chief constituent of the oil, Eugenol, is extracted and used as an imitation carnation in perfumes and for the formation of artificial Vanilla.

2.7.3 Black Pepper

Black pepper (*Piper nigrum*) consists of the dried, unripe fruits for Piper nigrum (Fam. *Piperaceae*), a perennial climbing plant cultivated in the Malay Archipelago, Southern India, South America and the West Indies (Trease and Evans, 1978). Large quantities are obtained from Indonesia, Sarawak and Brazil. Other species include *P. officinarum*, *P. longum*, *P. cubeba* and *P. guindense* (Trease and Evans, 1978).

2.7.3.a. Constituents

Pepper contains from 1-25% of volatile oil, from 5-9% of the crystaline alkaloids piperine and piperetiene and a resin (Trease and Evans, 1978). The aroma of the spice is due to the volatile

oil, which consists largely of terpenes (α-and β-penene, phellandrene, dipentene and sequiterpenes), while the pungency is ascribed to piperine and the resin (Trease and Evans 1978). Piperine ($C_{17}H_{19}O_3N$) was isolated by Oersted in 1819. It has since been found in long pepper (1-2% and in Ashanti pepper, the fruits of *Piper guineense*.

2.7.3.b. Uses

The West African black pepper (P. guineense) is a medicinal plant of varied use in West Africa. Recent phytochemical studies have shown it to contain several amide alkaloids as well as sterols and lignans. Some of these constituents have been shown to have antimicrobial, anticonvulsant, antihypertensive, sedative, tranquilizing and insecticidal properties (Adae-Mensah et al;. 1977).

Pepper is now little used in medicine but large quantities are used as a condiment (Trease and Evans, 1978).

2.7.4 Capsicum

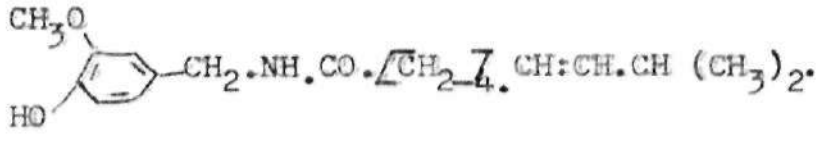
Fruits of capsicum (Chillies; Red peppers); *C. frutescens* (Fam. *Solanaceae*) and the Japanese variety are sold in England as chillies, while the larger but less pungent Bombay and Natal fruits are known on the London market as capsicums (Trease and Evans, 1978).

Very large capsicum fruits resembling tomatoes in texture and practically non-pungent, are widely grown in South Europe as vegetables. The degree of relationship between the plants yielding the above fruits is still somewhat doubtful; some botanists

separate as distinct species what others regard as cultural varieties (Trease and Evans, 1978).

2.7.4.a. Constituents

A pungent phenolic compound, capsaicin from capsicum fruits was extracted by Thresh in 1876 (Trease and Evans, 1978.)



Capsaicin (Vanillyl) amide of isodecenoic acid)

The pungent phenolic fraction of capsicum also contains a proportion of 6,7-dihydrocapsaicin. The capsaicin content of fruits varies appreciably and this is much influenced by environmental conditions, and age of the fruit. It occurs principally in the dissepiments of the fruits, for example, entire fruit 0.49;pericarp 0.01; dissepiment 1.79; seed 0.07 (Trease and Evans 1978).

The pungency of capsicum is not destroyed by treatment with alkalis (distinction from gingerol, which also contains the vanillyl group) but is destroyed by oxidation with potassium dichromate or permanganate. Chillies also contain ascorbic acid (0.1-0.5%), thiamine, red carotenoids such as capsanthin and capsorubin and fixed oil (about 4-16%) (Trease and Evans, 1978).

They yield about 20-25% of alcoholic extract ("capsicin") and about 5% of ash (Trease and Evans, 1978).

2.7.4.b. Uses

Capsicums are used as a condiment under the name of cayenne pepper. The drug is given internally in atomic dyspepsia and

flatulence (Trease and Evans, 1978). It is used externally as a counter-irritant, in the form of ointment, plaster, medicated wool, etc. for the relief of rheumatism, lumbago, etc. (Trease and Evans, 1978).

2.7.5 Groundnuts

Groundnuts (*Arachis hypogaea*; also called peanuts, mokey-nuts, etc) are grown for food in practically all tropical and subtropical regions. The genus *Arachis* comprises twelve species of which only a single one, *A. hypogaea*, the groundnut, is of economic importance (Arnon, 1972). They belong to the leguminosae and constitute one of the leading agricultural crops of the world.

It is generally agree that the cultivated forms of groundnuts originated in the Granchaco area of tropical South America, and from there spead throughout the tropical and subtropical regions of both South and North America (Higgins, 1951). Groundnuts were probably brought to Africa by the slave traders (Arnon, 1972).

2.7.5.a. Utilization

Groundnuts are rich in oil and protein and form one of the principal oil crops in the world. The shell constitutes 40-45% of the weight of the whole fruit. The kernels consist of approximately equal parts of fatty and non-fatty constituents (Woodroof, 1966).

Shelled nuts contain about 26% protein and 45-50% oil (Woodroof, 1966). The kernels are also rich in phosphorous and are an important source of vitamins (in particular, thiamine, riboflavin, and niacin) (Arnon, 1972).

2.7.5.b. Groundnut For Direct Consumption

Groundnuts have a very high caloric value and are easily digestible. The protein is of very high quality, and its biological value is among the highest of the vegetable proteins. Groundnuts for human food are used in a number of ways:

(a) Roasted in the shell for out-of-the hand eating;

(b) Roased and salted, after mechanical shelling, or used by chocolate manufacturers;

c) As peanut butter, which is the major use in the United States of America (Wren shall,1940). The "butter" is produced by grinding roasted peanuts and mixing them with added ingredients (Wrenshall, 1949).

For human consumption, big kernels with rose-coloured or pale-brown testas are preferred. For non-shelled groundnuts, the appearance of the shell is also extremely important - a light, creamy colour of the shell being widely favoured (Kay,1965).

2.7.5.c Oil Production

About two-thirds of the world's groundnut crop is used for oil production (Woodroof, 1966). The oil extracted from groundnuts is used in the preparation of hydrogenated cooking-fats and frying oils; a fraction that settles out during the manufacturing process is used for soap making (Arnon, 1972).

2.7.5.d. Livestock Feed

Oil cake the residual cake left after removal of the oil contains over 50% protein, and is widely used in concentrate rations of livestock (Arnon, 1972).

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2.7.6 Jawa

Jawa is a red dye that enjoys a wide range of usability in Hausa society. It is used as a dyestuff for dying locally wooven threads and other textile materials. It is used as a colouring agent in certain mineral drinks that are prepared locally for children.

It is sold in the local markets by a group of trademen called "Yan Koli (Hausa). But our source is not able to define the dye by its origin. Hence our inability to describe the dye in terms of its chemical constituents and other relevant parameters.

CHAPTER 3

3. MATERIAL AND METHODS

3.1 Subjects

3.1.1. Normal Subjects

Blood samples from ten (10) medical students of Ahmadu Bello University, Zaria and ten (10) Students of University of Sokoto, Sokoto, were collected. Their age ranges from 20-27 years and five (5) out of them were females.

This group represents individuals who do not show any apparent signs of diseased conditions and their red blood cells show normal activity of glucose-6-phosphate dehydrogenase (G6PD).

3.1.2 Sickle Cell Disease Subjects

Blood samples from twenty (20) sickle cell patients that attended the weekly clinic at Ahmadu Bello University Teaching Hospital (ABUTH), Zaria, were obtained. All of the patients had the genotype SS and Seven (7) out of them came to the hospital during crisis state.

Their age ranges from 11-30 years and six (6) out of the patients were female.

3.1.3 Malaria Subjects

Blood samples from fifteen (15) out-patients with malaria fever were collected at ABUTH, Zaria. Five (5) more blood samples from

out-patients with the same disease were collected at Sokoto University Teaching Hospital (SUTH), Sokoto.

Their age ranges from 10-35 years and eight (8) out of the total number of the patients considered were female.

3.1.4 Diseased Liver Subjects

Blood samples from twenty (20) out-patients with chronic liver diseases were collected at ABUTH, Zaria. Their age ranges from 20-40 years and five (5) out of them were females.

3.2 Suya Condiments

3.2.1 Kuli-Kuli

Kuli-Kili is the local name (Hausa) of the powder made out of the groundnut oil cake to which cayenne pepper and salt have been added.

The condiment was obtained from Samaru village in Kaduna State and Shagamu in Lagos State.

3.2.2. Yaji

Yaji is the Hausa name of the powder prepared from the mixture of the fruits of ginger, capsicum, black pepper and cloves. The ratio of these spices that make up yaji is not standardized and is left to the discretion of the suya maker. This accounts for the qualitative differences that exist among suya from different suya makers.

The condiment was obtained also from the same sources as above.

3.2.3 Jawa

Jawa is the hausa name of a red dye that is used for various purposes in hausa society. Its nature is ill-defined. This dye was also obtained from the same sources as above.

3.2.4. "Jawa Mix"

This phrase was coined by the author of this work to refer to the mixture of jawa and powdered chillies (ratio 3:7). "Jawa mix" is mainly used by housewives with the sole aim of enhancing the organoleptic qualities of their soups. Some suya makers also make use of this mixture.

This condiment was also obtained from Samaru village main market and at Shagamu.

3.2.5. Mixture of Kuli-Kuli, Yaji and "Jawa Mix"

In Suya making, it is the mixture of all the above mentioned condiments (Jawa excluded) that is used in coating the meat. Therefore, Suya makers were contacted to obtain this mixture.

The ratio of the condiments used in this mixture varies from one Suya maker to another. Thus, besides the Yaji factor, this also is another point of divergence in terms of organoleptic properties of Suya from different sources.

The mixture was obtained also from the same sources as above.

3.2.6. Condiments From Roasted Meat

Suya was bought and the condiments scrapped from coats of the roasted Suya and stored for use in the investigation.

This was obtained from Samaru village.

3.3 Chemical Reagents

Sodium chloride (ANALAR), EDTA (calcium salt), and Triton-X 100 were used during the ivestigation. These chemicals were made by Hopkins and Williams, Chadwell: Heath, Essex England.

Disodium hydrogen orthophosphate, and dihydrogen sodium orthophosphate both from BDH were also used.

All other chemicals used in the course of the investigation were either obtained from Sigma chemical co., St, Louis, Mo USA or May and Baker Dagenham, England.

3.4. Spectrophotometric Method of Monitoring Red Blood Cell Lysis: *In Vitro* Experiments

Human blood was collected in reagent bottles containing EDTA as anticoagulant, at both Ahmadu Bello University Teaching Hospital, Zaria and Sokoto University Teaching Hospital, Sokoto.

Five millilitres of the Blood was centrifuged at 6,000 r.p.m. for 15 minutes. The plasma was discarded, and the region of white buffy coat of the white blood cells was removed by aspiration using a micropipette.

The erythrocyte were then washed in 40ml of 0.9% Nacl/ml of whole blood.

The washed cells (0.10ml) were suspended in 0.5ml of 0.01M KPO_4 buffer, PH 7.4, and 4.5ml of isotonic saline, To the mixture, 1g/l of Triton-x 100 solution was added and incubated for 30 minutes at 37°C and the absorbance at 540nm of the supernatant after *contrifuging was noted and recorded.*

This same procedure was followed using verying concentrations of the detergent (Triton-x 100), i.e. 2g/l, 3g/l, 4g/l, up to 10g/l and the various absorbance values were noted. The absorbance values increased with increasing concentration of the detergent up to a certain concentration at which point an increase in the detergent concentration caused no increase in the absorbance. This corresponds to 100% lysis due to the detergent.

The solution of the condiments were then incubated separetely with same amount of the preparations, i.e.0.1ml of RBC, 0.5ml of 0.01M Kp04 buffer of PH 7.4, and 4.5ml of isotonic saline. The amount of condiments incubated with RBC was determined and noted. These various concentrations of the condiments (5%, 10%, 15% and 20% w/v) were then incubated with the RBC differently over a time period of 2 hours.

At 30 minutes intervals, the tubes containing the RBC and the condiments were removed, centrifuged at 6,000 r.m.p. for 15 minutes and the absorbance at 540nm noted and recorded. This was repeated over an incubation period of 2 hours. In all eighty (80) different RBCs samples were determined and in each cases the percentage haemolysis was recorded.

This procedure was then followed using the different blood samples (normal and subjects with various diseases) and the solutions of the condiments and the absorbance values noted. Percentage haemolysis was then calculated in each case.

A control was also set up in each case whereby a mixture of the RBCs contained in the buffer and normal saline but no condiments were incubated under the same conditions and length of time. The percentage haemolysis was then computed and recorded in each case.

3.5 Calculation of Percentage Haemolysis

Samples calculation for % haemolysis:-

a) Absorbance corresponding to 100% lysis due to triton x 100=x.

b) Absorbance due to (say 20%) solution of Kuli-Kuli as introduced to the RBC solution from normal blood sample = Q.

c) Therefore, % haemolysis $= \frac{Q}{x} \times 100 = Z\%$. This procedure was then adopted for all the absorbance values noted and recorded.

CHAPTER 4

4. RESULTS AND DISCUSSIONS

The results of the studies indicate that all the Suya condiments considered during the course of the investigation were capable of inducing certain degree of haemolysis, over and above the haemolysis endogenous to the particular type of Red blood cells considered (figure 2 through figure 5 where it is shown that percentage haemolysis at Zero concentration of condiments for normal red blood cell ranges from 16-17%, diseased liver 15-17%, sickle cell disease 41-43%, and malaria 35-42%).

Amongst the Suya condiments obtained from Shagamu, Jawa proved to be the most haemolytic irrespective of blood type (Table 3), with a red blood cell (RBC) turn-over rate of between 3.50 x $10^{-4}min^{-1}$ and $5.76x10^{-4}min^{-1}$. The turn-over rate is the first order rate constant for the haemolytic process. It serves as a measure of the intrinsic tendency of the RBC to haemolyse under a given condition. From the plot of log % unhaemolysed RBCs Vs incubation period (figures 6 and 7) a straight line was obtained, indicating that the RBC lysis conforms to a first order kinetic process. Hence the first order rate constant.

In the case of Suya condiments obtained from Samaru village, Jawa also proved to be the most haemolytic irrespective of blood type with a RBC turnover rate of between $2.0x10^{-4}min^{-1}$ to

$2.72x10^{-4}min^{-1}$ (Table 1). Kuli-Kuli appeared to exert the lowest haemolytic effect with RBC turn-over rate of between $1.48x10^{-4}min^{-1}$ to $1.90x10^{-4}min^{-1}$ from Shagamu (Table 3). This also was the case with Suya condiments obtained from Samaru (Table 1) with a RBC turn-over rate due to Kuli-Kuli of $1.29x10^{-4}min^{-1}$ to $1.87x10^{-4}min^{-1}$.

On individual condiments basis, all the condiments obtained from Shagamu appeared to be more haemolytic than the condiments obtained from Samaru village. This is evident from the results of the turn-over rates due to each of the condiments (Table 1 and 3). The RBC turn-over rate due to Kuli-Kuli from Shagamu is $1.48-10^{-4}min^{-1}$ as against that of Kuli-Kuli from Samaru village of $1.29x10^{-4}min^{-1}$. The turn-over rate due to Yaji from Shagamu is $3.0x10^{-4}min^{-1}$ as against that of Yaji from Samaru of $1.91x10^{-4}min^{-1}$. The RBC turn-over rate due to Jawa from Shagamu is $3.5x10^{-4}min.^{-1}$ as against that of Jawa from Samaru village of $2.00x10^{-4}min^{-1}$. In the case of "Jawa mix", the RBC turn-over rates due to that obtained from Shagamu and Samaru village is $3.28x10^{-4}min^{-1}$ and $1.93x10^{-4}min^{-1}$ respectively. And finally, the RBC turn-over rates due to the mixture of all the Suya condiments obtained from Shagamu and that of Samaru village is $1.52x10^{-4}min^{-1}$ and $1.25x10^{-4}min^{-1}$ respectively.

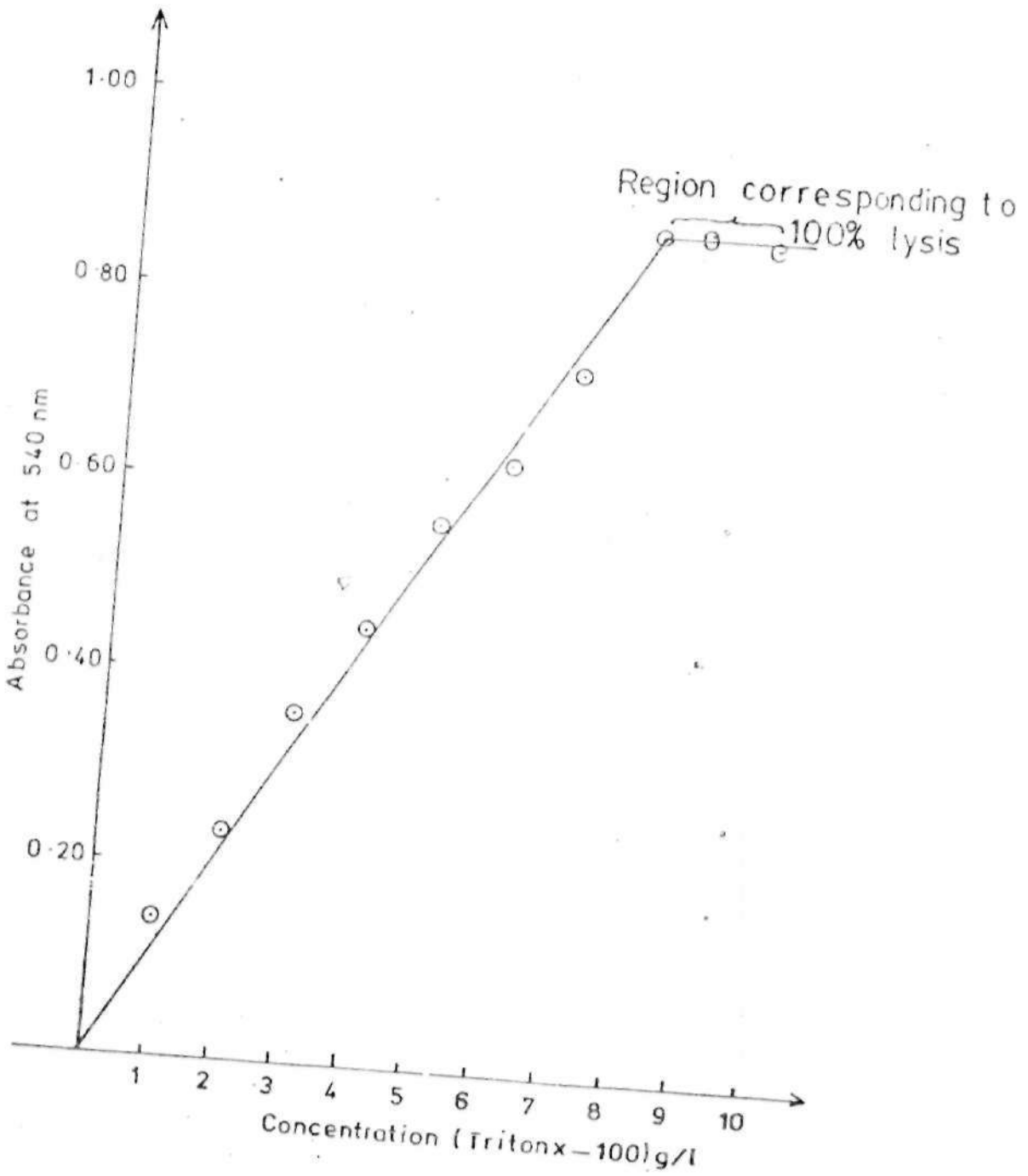


FIG.1. STANDARD CURVE FOR ABSORBANCE OF RED BLOOD CELLS VERSUS TRITONX–100 CONCENTRATION

S C D = Sickle cell disease

DL = Diseased liver

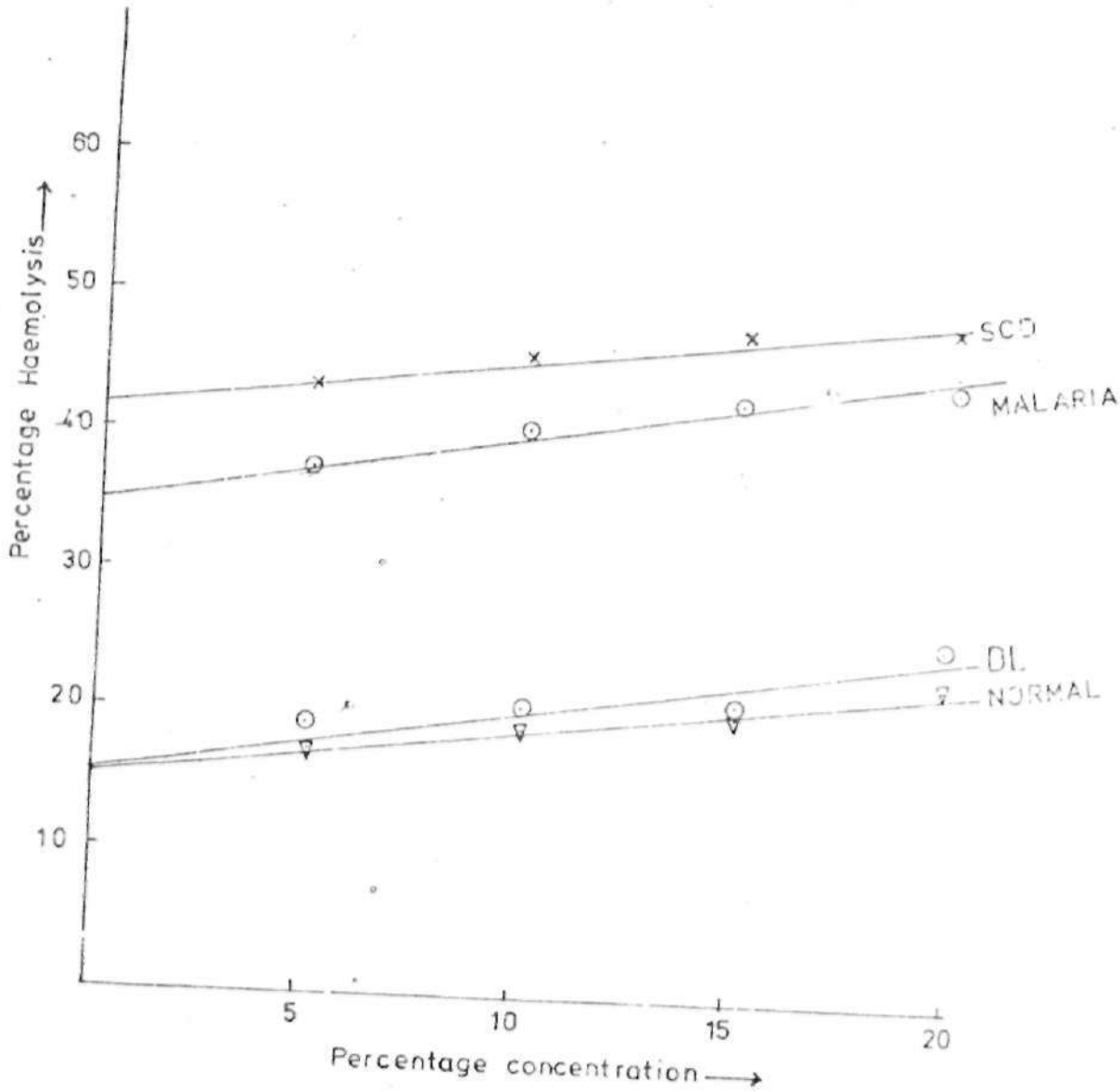


FIG. 2 VARIATION OF HAEMOLYSIS WITH VARYING CONCENTRATIONS OF SAMARU KULIKULI (INCUBATION PERIOD OF 60Mins.)

SCD = Sickle cell disease

DL = Diseased Liver

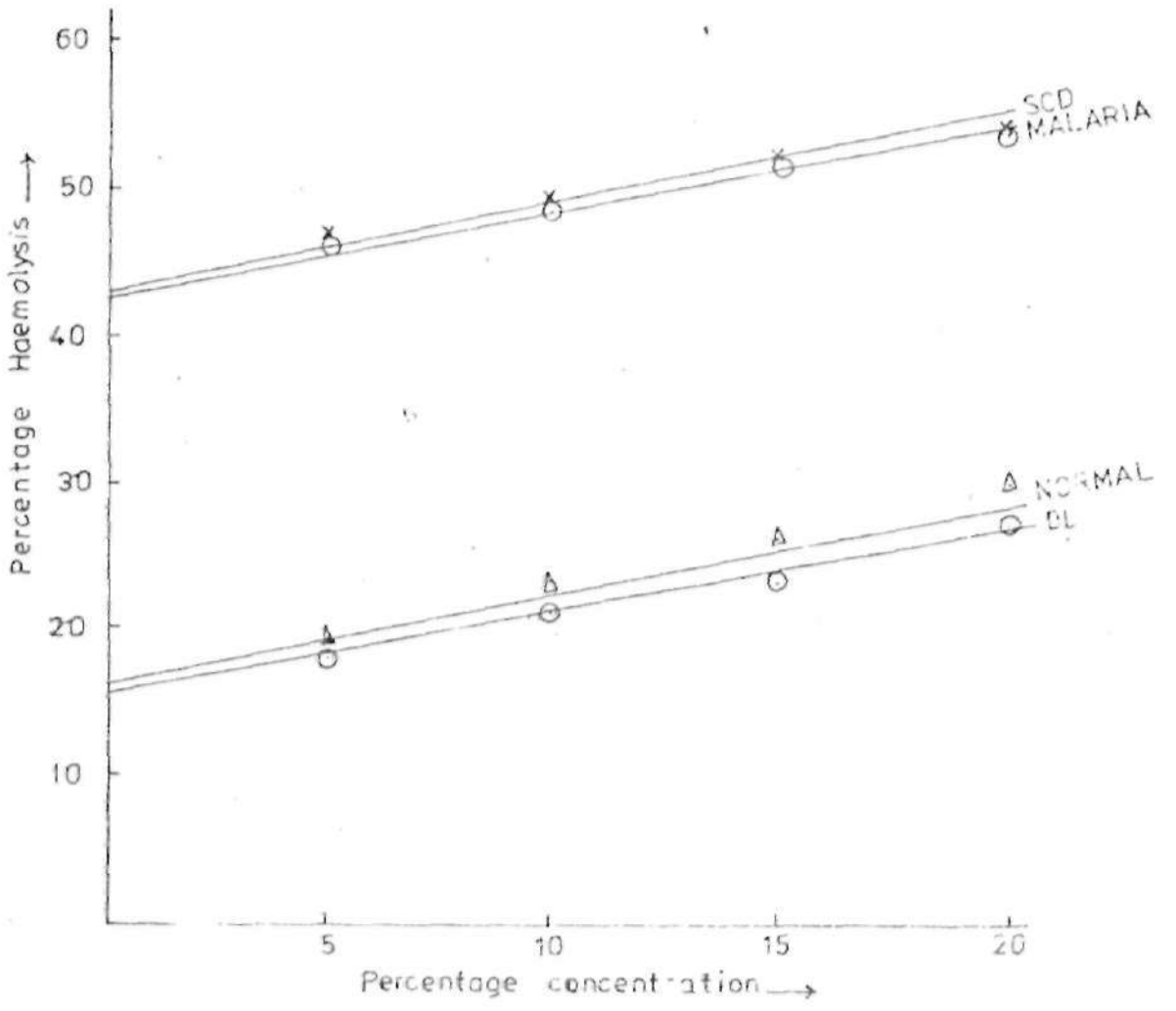


FIG. 3. VARIATION OF HAEMOLYSIS WITH VARYING CONCENTRATIONS OF SHAGAMU KULIKULI (INCUBATION PERIOD OF 60Mins)

SCD = Sickle cell disease

DL = Diseased Liver

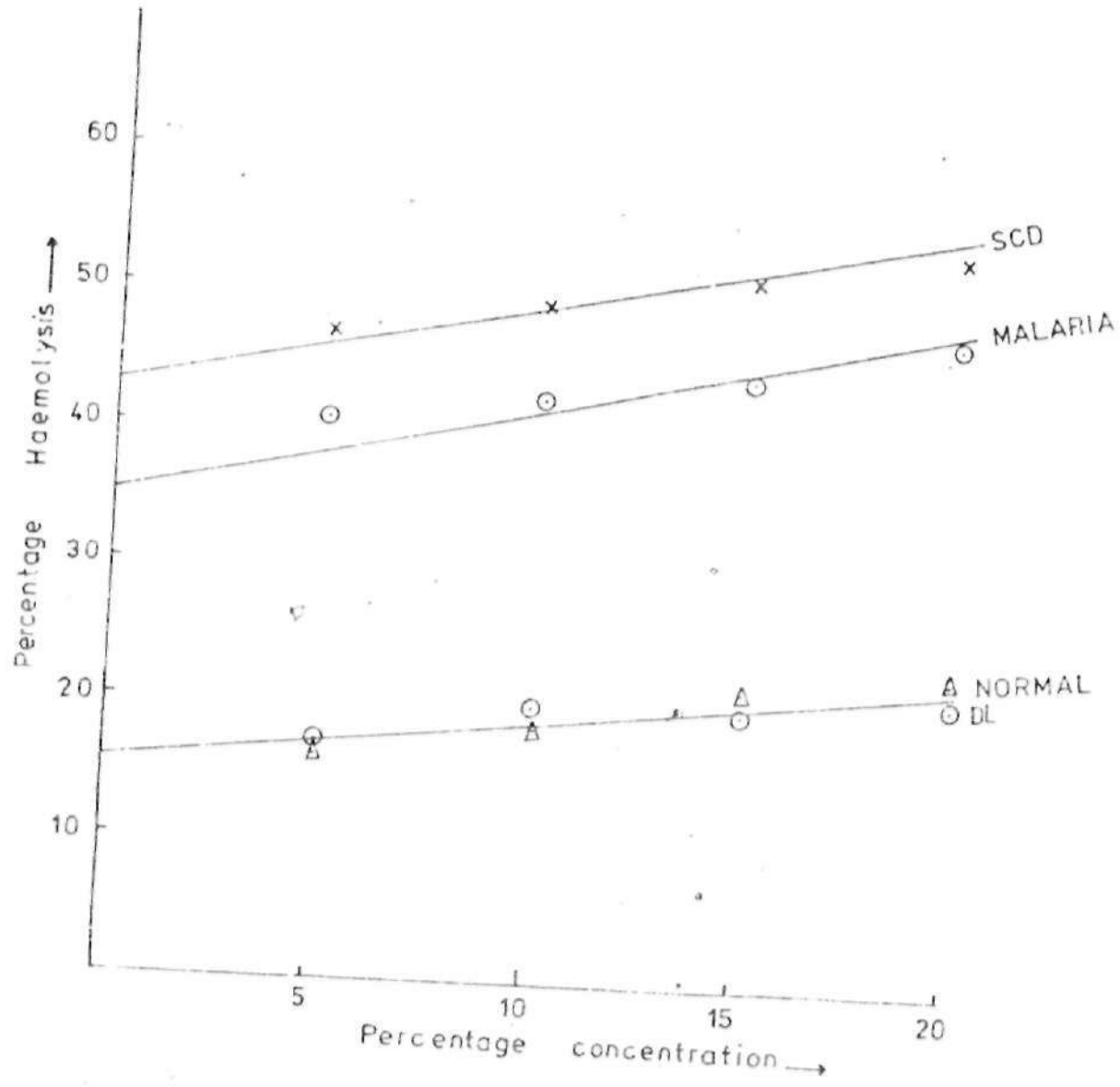


FIG. 4. VARIATION OF HAEMOLYSIS WITH VARYING CONCENTS. OF SAMARU JAWA (INCUBATION PERIOD OF 60 MINUTES)

S C D = Sickle cell disease

D L = Diseased Liver

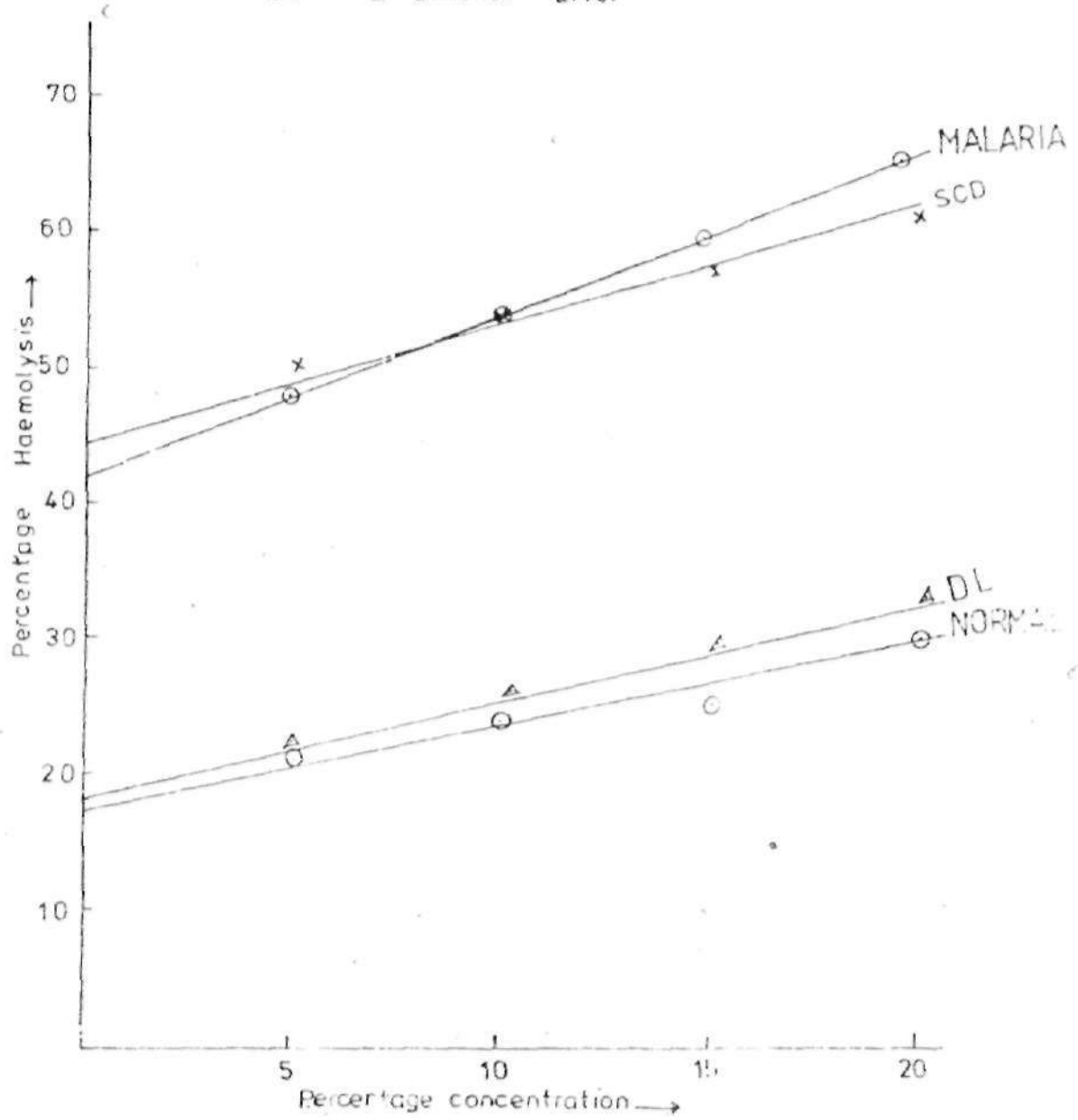


FIG. 5. VARIATION OF HAEMOLYSIS WITH VARYING CONCENTS OF SHAGAMU JAWA (INCUBATION PERIOD OF 60Mins)

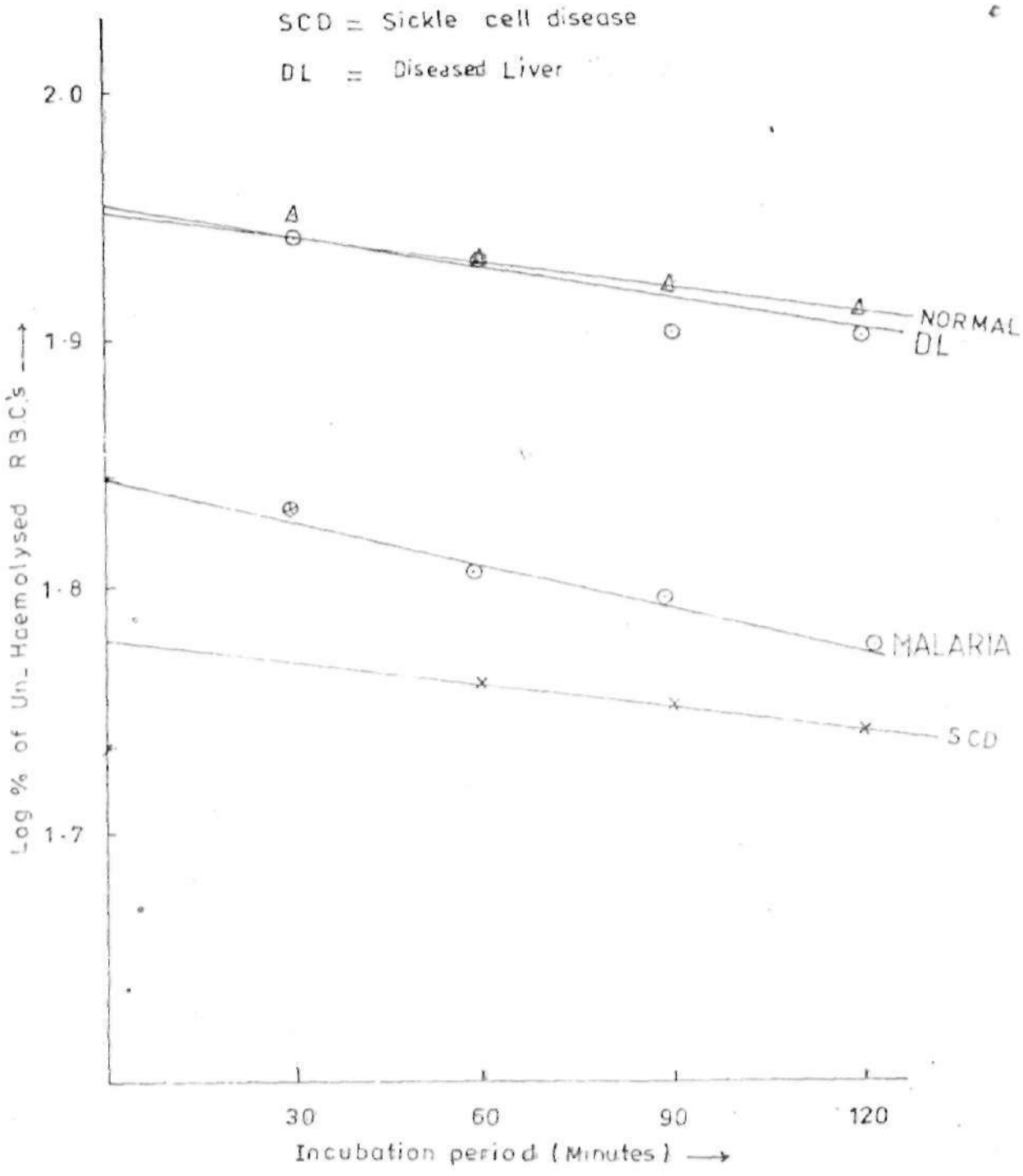


FIG.6. LOG. % OF UN_HAEMOLYSED R.B.C.'s VERSUS. INCUEATION PERIOD IN MINUTES (CONTROL FOR SAMARU CONDIMENTS).

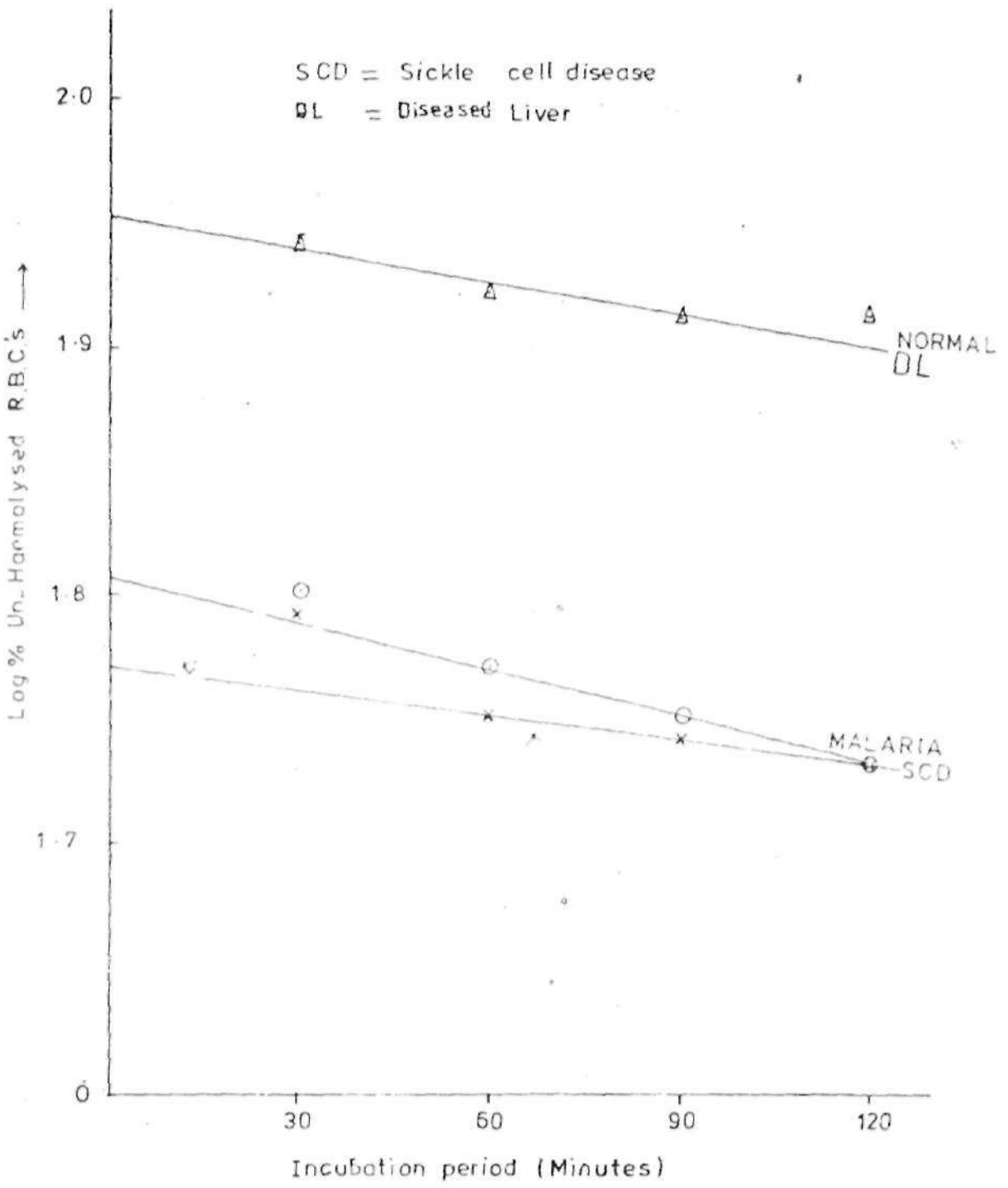


FIG.7. LOG.% OF UN-HAEMOLYSED R.B.C's VERSUS INCUBATION PERIOD IN MINUTES (CONTROL FOR SHAGAMU CONDIMENTS).

Table 1: TURN-OVER NUMBER OF RBC's INCUBATED WITH RED SUYA CONDIMENTS DERIVED FROM SAMARU VILLAGE.

CONDIEMENTS	TURN-OVER NUMBER k x 10-4(MIN-1)			
	Normal	Diseased Liver	Sickle-Cell Disease	Malaria
KULI	1.29	1.30	1.52	1.87
YAJI	1.91	2.00	2.48	2.53
JAWA	2.00	2.02	2,72	2.66
JAWA MIX	1.93	2.00	2.58	2.61
MIXTURE	1.25	1.33	1.58	1.88
ROASTED	1.65	1.74	2.02	2.12
CONTROL	1.23	1.25	1.40	1.72

TABLE 2: HALF-LIFE FOR RBC's INCUBATED WITH RED SUYA CONDIMENTS DERIVED FROM SAMARU VILLAGE

CONDIMENTS	HALF-LIFE (MINUTES)			
	Normal	Diseased Liver	Sickle-Cell Disease	Malaria
KULI	5408	5335	4563	3698
YAJI	3622	3533	2793	2742
JAWA	3504	3436	2552	2605
JAWA MIX	3598	3479	2685	2655
MIXTURE	5530	5220	4395	3682
ROASTED	4203	3987	3425	3272
CONTROL	5632	5547	4958	4036

TABLE 3: TURN-OVER NUMBER OF RBC's INCUBATED WITH RED SUYA CONDIMENTS DERIVED FROM SHAGAMU

CONDIMENTS	TURN-OVER NUMBER, k x 10-4(min-1)			
	Normal	Diseased Liver	Sickle-Cell Disease	Malaria
KULI	1.48	1.54	1.86	1.90
YAJI	3.00	3.02	3.48	4.13
JAWA	3.50	3.57	3.94	5.80
JAWA MIX	3.28	3.29	4.02	5.21
MIXTURE	1.52	1.55	1.88	2.09
ROASTED	1.95	2.00	2.68	3.05
CONTROL	1.32	1.33	1.47	1.64

TABLE 4: HALF-LIFE FOR RBC's INCUBATED WITH RED SUYA CONDIMENTS DERIVED FROM SHAGAMU

CONDIMENTS	HALF-LIFE (MIINUTES)			
	Normal	Diseased Liver	Sickle-Cell Disease	Malaria
KULI	4640	4503	3730	3652
YAJI	2330	2295	1990	1679
JAWA	1985	1940	1760	1203
JAWA MIX	2115	2104	1722	1330
MIXTURE	4560	4479	3690	3320
ROASTED	3559	3498	2590	2270
CONTROL	5250	5203	4730	4223

There seems to be revealed also a varying synergistic pattern amongst the different combinations of the Suya condiments. In the case of "Jawa mix", which is a mixture of Jawa and Yaji in the ratio of about 3:7, the result of the RBC turn-over rate was $1.94x10^{-4}min^{-1}$, the same value as that obtained if the sum of the individual effects of the components of the "Jaw mix" were considered (Table 1). A positive synergistic pattern unfolds when "Jawa Mix" obtained from Shagamu was considered (Table 3), i.e. $3.28x10^{-4}min^{-1}$ is the RBC turn-over rate due to "Jawa mix" which is higher than the sum of the individual contributions due to the individual components of the "Jawa mix" of $3.15x10^{-4}min^{-1}$.

But a negative synergistic pattern was observed for the mixture of all the condiments (Tables 1 and 2). The mixture is made from the combination of Kuli-Kuli, Yaji and "Jawa mix"in the ratio of about 7:2:1 respectively. The RBC turn-over rate due to the mixture of the condiments obtained from Samaru village is $1.25x10^{-4}min^{-1}$. This value is lower than the turn-over rate obtained due to the sum of the contributions of the individual components of the mixture of $1.48x10^{-4}min^{-1}$. In the case of the mixture of the condiments from Shagamu, the RBC turn-over rate is $1.52x10^{-4}min^{-1}$ as against the value of $1.96x10^{-4}min^{-1}$ obtained if the sum of the individual contributions of each condiments in the mixture is considered. This pattern holds true in all cases considered.

It appeared from the results of the investigation also that the ability of the Suya condiment mixture to cause haemolysis is enhanced when roasted (Tables 1 and 3). The roasting process is

known to deposit some constituents of the smoke of the fire on the meat and these are hazardous (Tilgner *et al*., 1963). Period of smoking determines the quantity of the constituent on or in the meat. Benzo- (a)- Pyrene and some other Polycyclic aromatic compounds have been reported (Tilgner *et al*., 1963) to be present in smoke. And the composition of smoke is affected by type of wood, type of generator, moisture content of wood, temperature of combustion and air supply (Draudt, 1963). But whether these or other constituents of the smoke are capable of causing haemolysis remains to be determined.

The results of the RBC lysis rate for the different category of subjects considered on incubation with the different Suya condiments revealed that blood from normal individuals lyse at a slower rate than the blood cells obtained from the patients considered (Tables 2 and 4). This could be attributable to the normal activity of the enzyme, glucose-6-phosphate dehydrogenase (G6PD) in the red blood cells of these subjects. And since normal activity of this enzyme is required for cellular integrity, especially under conditions of oxidant stress (Carson *et al*, 1956), one could venture to attribute the observable degree of haemolysis exerted by these condiments to be oxidative in nature.

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is one of the two most common enzyme deficiencies known to cause non-spherocytic haemolytic anaemia (Beutler, 1979). Erythrocytic G6PD activity occurs as a genetic polymorphism in the Nigerian population (Porter *et al*., 1964) and about 20% of the male population have G6PD deficiency (Luzzato *et al*., 1968).

In a recent, but similar studies, Akinyanju *et al*. (1984), reported an incedence of a Syndrome of acute intravascular haemolysis following the ingestion of red Suya. it seems that only individuals whose red blood cell is deficient in G6PD are prone to this haemolytic crisis (Akinyanju *et al*., 1984). None had any illness or were exposed to any oxidant agent before the onset of the haemolytic crisis or its prodromal symtoms (Akinyanju *et al*., 1984).

The RBC from patients suffering from malaria lyse at a faster rate (Tables 2 and 4) than those of the other cases considered. Motulsky (1965) reported a similarity in the geographical distribution of falciparum malaria and the gene frequency for G6PD deficiency and went further to suggest that this deficiency confers some protection against malaria. Though this present study does not confirm or otherwise a deficiency of G6PD in the malaria patients studied, a deficiency of the enzyme could be a likely reason for the enhanced susceptibility of these red blood cells to the haemolytic action of the condiments. This is similar to the haemolysis induced by a variety of drugs in the wake of G6PD deficiency (Dern *et al*., 1955).

The RBC of individuals with malaria are also known to contain parasites which elaborate some lytic compounds and thereby destroying the RBC's (Holz, 1977). Malaria is a recognised cause of haemolytic anaemia, due principally to alterations of the cell membrane of parasitized RBCs. Prankerd (1961) asserted that the circulating erythrocyte in malaria is intrinsically defective and the environment in which they are circulating is also defective.

Haemolytic octadecanoic fatty acids have been isolated from both normal and infected blood. Laser (1950) reported increases of 25-to-75 fold in plasma concentrations of octadecanoic acid as a consequence of active *Plasmodium knowlesi* infection. Holz (1977) implicated both oleic (18:1 (n-7)) and cis-vacenic (18:1 (n-7)) acids in the prehaemolytic and haemolytic episodes associated with *P. Lophurae* infections in ducks. The mode of action of these haemolytic fatty acids suggested by Holz (1977) appeared to be specific modification of plasma membrane fluidity by an alteration in the fatty acid composition of the membrane phospholipids and by the direct influence on adenyl cyclase activity. These changes in turn affect membrane protein conformation and ultimately membrane transport.

Sickle cell anaemia is also a haemolytic anaemia and as reflected in this present study, the RBC's obtained from sickle cell patients are next to RBCs obtained from malaria patients in terms of susceptibility to the haemolytic effects of the Suya condiments (Tables 2 and 4).

Sickle cell haemoglobin is found in all forms of sickle cell disease (Douglas, 1975). In sickle cell haemoglobin, valine replaces the normally occurring glutamic acid at residue B6 (A3); as a consequence of this substitution, the unliganded form of the haemoglobin tends to polymerize at high concentrations. The polymerization or aggregation of deoxyhaemoglobins leads to the formation of sickled cells, which aggregate in small vessels leading to tissue anoxia.

The nature of the polymers containing haemoglobins is a central question in the molecular pathology of sickle cell disease. It is clear that sickle cell haemoglobin <Hbs) polymerizes only in the unliganded form (Douglas, 1975); hence circusratances that favour the oxy-oonformation of haemoglobin will Impair sickling, whereas when the deoxy-conformatlon is favoured, sickling is favoured.

The interactions of sickle cell haemoglobin, or indeed of normal haemogldbin (HbA) with the red cell membrane, are not well defined. The red cell membrane is distorted by the sickling process, and there is evidence (Douglas, 1975) that it becomes rigid even before the sickle shape can be identified.

In the anaemia, red cells usually carry about 95% or more haemoglobin S and the rest is foetal haemoglobin (HbF) and the distribution of these two haemoglobin is not uniform in each cell (Lehmann *et al.,* 1974). Foetal haemoglobin is particularly useful as a protector against sickling for, it does not interact when sickle cell haemoglobin <Hbs) precipitates (:Lehmann et a_l, . 1974). The more Hbp there is present in the cell,, the less the cell is likely to sickle. Irreversibly sickled cells have an unusually low content of HbF and this explains the important protective effect of "Hereditary Fersistance of HbF" (Lehamnn et *al,,* 1974), where the HbF is shared equally between the red cells.

Patients with sickle cell disease may also be deficient in G6PD (Lehmann ej; al. , 1974). This could also be a very important factor as regards susceptibility of RBEs from sickled cell patients to the haemolytic effect of oxidant stressors. A variety

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of early studies indicated also that the erythrocyte membrane may be deficient in sickle cell disease. Homozygous sickle cells differ from normal in their high intracellular calcium content (Palek, 1977). Several abnormal consequences of high intracellular calcium have been reported (Weidekamm *et al.*, 1977) and they ultimately lead to a disturbance of the red blood cell cytoskeleton and an increase in erythrocyte fragility. However, uncomplicated chronic liver disease is not a usual cause of haemolytic anaemias.

Red Suya is made by coating raw meat, with a mixture of pepper and dried grounded vegetables. The coated meat is dabbed with vegetable oil before being barbecued round an open wood fire (Akinyanji *et al.*, 1984). The phenolic compounds present in the smoke are generally considered to be of great importance in the production of flavour and preservation action (Feron, 1972). The colouring effect is due to the reaction between the dicarbonyl compounds in wood smoke and the imino groups of amino acids of proteins (Feron,1972).

Recently, the quantity of pepper in the mixture has been reduced in order to lessen the hotness of the mixture, but the red attractive colour has been maintained by adding a red powder which was identified as an azo dye called orange-RN (Akinyanju *et al.*, 1984). Orange-RN is a known food colouring agent used in soft drinks, Sausages, etc (Akinyanju *et al.*, 1984). The dye had been reported to cause haemolytic anaemia in rats and Pigs (Hansen *et al.*, 1978) and a dose related, liver enlargement and fibrosis, and bile duck proliferation in pigs (Hasselager, 1978).

It was suggested that the dye has been smuggled into Nigeria from Eastern European countries (Akinyanju *et al*., 1984).

The ability of the RBC to haemolyse is enhanced when the RBC is exposed to certain oxidant stress. These could be in a form of oxidant drug or substance consumed (Motulsky, 1965). This is apart from variation in the susceptibility of the G6PD phenotypic variants (Beutler, 1969). In addition, the haemolytic effect of a drug may vary with its rate of metabolism in deficient individuals (Woolhouse, 1982), particularly if the oxidant is a metabolite rather than the parent compound (Brewer, 1980).

Haemolytic episodes as a result of oxidant stress is also precipitated in vitamin E deficiency. It is clearly established that in man and in animals with low serum levels of vitamin E there is a tendency for the RBCs to undergo lysis when exposed *in vitro* to H_2O_2. The site of action of vitamin E as an antioxidant is on the outer surface of the membrane were it serves to protect lipid against oxidation (Grimes, 1980).

A deficiency of vitamin E does not occur in man except in malnutrition; neverthless low levels in serum are encountered in the absence of clinical indications of malnutrition and, when they occur, they are frequently of haematological interest (Grimes, 1980). Of further interest was that GSH and intact -SH groups were also necessary in the RBCs to prevent lysis (Grimes, 1980).

CONCLUSIONS

It has been observed in this work that all the Suya condiments were capable of inducing haemolysis on human RBCs *in vitro*. However, haemolytic crisis is n ot attributable to a single factor. Its causes are numerous and overlapping. Certain inheritable enzyme deficiencies (e.g. G6PD deficiency), disease conditions and vitamin E deficiency in malnutrition are only a few of the culprits that could render individuals susceptible to haemolytic episodes when exposed to the appropriate oxidant stress.

It has also been shown that the Suya condiments proved to exert a higher degree of haemolytic effect on the RBCs derived from individuals with malaria and sickle cell diseases. And the haemolytic effect is more on RBC derived from malaria patients.

A general pattern in the haemolytic ability of the individuals Suya condiments also revealed that Jawa is the most haemolytic with Kuli-Kuli showing a milder tendency to cause haemolysis.

In conclusion, we should be able to appreciate the public health hazard associated with widespread sale of red Suya in a country with a reportedly large population of G6PD deficient individuals.

Suggestions for Further Research

Efforts should be geared towards defining the chemical nature of the red dye, Jawa. This would enable us to know whether or not it is composed of substances that are deleterious to our health.

A diet should also be designed that excludes any lytic substances. This diet would allow us carry out *in vivo* studies of the Suya condiments for a more practical and conclusive interpretation of our results.

The activity of the enzyme, G6PD, should also be determined in both sickle cell patients and malaria patients. This would enable us confirm whether or not the enhanced susceptibility of the RBCs of these patients to the haemolytic effect of the Suya condiments is due to a deficiency of the enzyme, or to other intrinsic defects of the patients RBCs.

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APPENDIX 1

CALCULATION OF TURN-OVER RATE, K (MIN-1)

The turn-over rate is the first order rate constant, K, for the haemolytic process. It serves as a measure of the intrinsic tendency of the red blood cells to haemolyse under a given condition.

From the irreversible first order rate equation:

$$\text{Log}\ \frac{(a-x)}{a} = -2.303kt$$

where a = no of initial RBCs population,

x = no of haemolysed RBCs at the moment t,

k = rate constant, and

t = time;

A plot of $\text{Log}\ \frac{(a-x)}{a}$ (Log Percent unhaemolysed RBCs) against time, t, will give a straight line whose slope is given by -2.303k, from which K can be evaluated.

APPENDIX 2

CALCULATION OF HALF-LIFE (MINUTES)

The half-life ($t_{½}$) of the RBCs is given by:

$$\frac{\ln 2}{k} = \frac{0.693}{k}$$

where k is the turn-over rate (min^{-1}).

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