VITAMIN E AND SELENIUM STATUS IN HEALTH AND DISEASE

A Thesis Submitted in Partial

Fulfillment of the Requirments

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DEDICATED

To my Brothers and Sisters.

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TABLE OF CONTENTS

Page

Page

LIST OF TABLES

List of tables contd.

LIST OF FIGURES

ABSTRACT

Vitamin E and selenium status of 45 healthy and 90 unhealthy human subjects were assessed. Vitamin E status was assessed as erythrocyte hemolysis (%) while selenium as erythrocyte glutathione peroxidase activity. The mean values for healthy control subjects were 5.4± 0.59 for erythrocyte hemolysis (%) and 3.8 x $10^{-\pm}$ 0.34 units for glutathione peroxidase activity. There was no significant difference ($P> 0.05$) between these values and those for the unhealthy ones studied except for subjects with heart muscle diseases. Erythrocyte hemolysis (%) and erythrocyte activity were significantly lower ($p<0.05$) in subjects with heart muscle diseases compared to the controls. Erythrocyte glutathione and plasma ascorbic acid were also assessed in the subjects. In the control glutathione level was 38.± 4.03 mg per 100 ml and plasma ascorbic acid was 0.70 ± 0.14 mg per 100 ml. The values were not affected significantly by vitamin E and selenium status of the subjects as also by diseased status studied. A vitamin E deficient male subject had a mean of 18.5 and 2.76 x 10^{-2} units as erythrocyte hemolysis (%) and glutathione peroxidase activity respectively over a period of 5 weeks. These values dropped significantly ($P< 0.05$)

when the subject was given oral 60 mg d x-tocopherol per day for 3 days. This indicated a metabolic interrelationship of vitamin E with selenoenzyme glutathione peroxidase. This observation needs further investigations. As reported in earlier works, glutathione peroxidase activity in the rats studied, was located more with the cytosal than the mitochon-dria. Complete starvation for 48 hours in the rats increased the activity of the selenoenzyme significantly by 62.5% in the cytosol without any effect on **it's** mitochondrial activity.

INTRODUCTION AND LITERATURE REVIEW

Alpha-tocopherol and Selenium are among the essential nutrients that are needed for normal functioning of animal cell. In-spite of the extensive literature on these nutrients over the past few decades, much still needed to be under-stood about their functions and involvement in cell reactions. The literature on the amount of these nutrients in human subjects in this part of the world is rare. The only nation wide nutritional survey of Nigeria carried out by ICNND, in collaboration with Government of Nigeria in 1965, did not include data on these essentials.

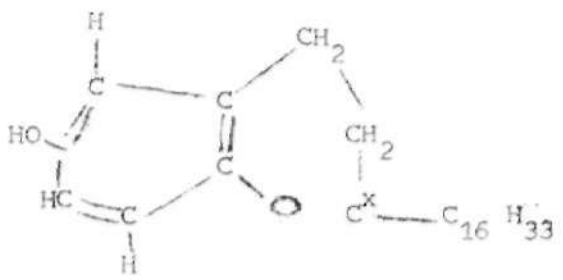
One of the important roles α-tocopherol and selenium together with glutathione play in the cell is the maintenance of the intergrity of the cell membrane, especially the erythrocyte membrane. They are involved in the protection of the membranes against oxidative damage by some physiological oxidants such as hydrogen peroxide, chemical toxicants such as ozone and oxidant stressors such as oxygen. Which are generated from enzyme systems and other reactions in the cell.

Alpha-tocopherol and selenium have also been implicated in a number of disorders involving the vascular, muscular and reproductive systems in the body of animals. Erythrocyle hemolysis

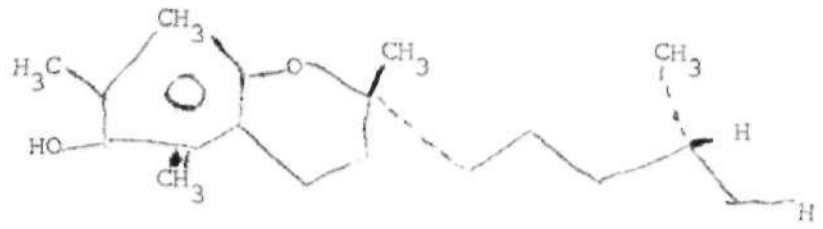
in man, muscular dystrophy and Testicular degeneration in rats and pigs, exudative diathesis in chickens, Hepatic necrosis in pigs and Embryogenesis defect in cow are some of the diseases that respond to treatment with α-tocopherol or selenium or both (42). The roles played by either of the nutrients in the pathogenesis of these diseases are discussed under separate section that follows.

VITAMIN E:

Vitamin E is a fat soluble vitamin which is required by animals for normal cell differentian and function. It belongs to the tocopherol group of compounds which are derivatives of G-hydroxychroman. Members of such groups have a 13-carbon side chain which confers on them lipid solubility. The chemical structure is given below:-



Tocopherol nucleus.



α-Tocopherol (Vitamin E)

Fig I.

Various forms of tocopherol occur depending on the degree of substitution of the methyl group in the tocopherol nucleus. Alpha, Beta, Gamma and Delta tocopherols and alpha and Gamma tocotrienols are all forms of tocopherols known. The d-isomer of α-tocopherol is the natural form and the most active physiologically, of all the tocopherols.

Blood Levels:- Normal plasma tocopherol level for human subjects varies between 0.36 and 1.80 mg per 100 ml of plasma (24). These values, however, represent total reducing materials including ubiquones, chromeols and antioxidants rather than tocopherol per se. Infact, only alpha form of tocopherol is found in reasonable quantity in the plasma. Other forms occur only in minute quantity and may not be present at all in the red blood cell. Chow (9) in his work on distribution of tocopherols in human plasma and red blood cell, observed that

alpha tocopherol accounts for 83 and 87 percent of all tocopherols in plasma and red blood cell respectively. Gamma form represent about 17 percent in each system. His work indicate that non-alpha tocopherols except gamma form seem to contribute little to the total tocopherols in human blood.

The tocopherols in plasma have been shown (6) to be associated with lipid proteins and distributed according to the fat content of each fraction of the plasma. In red blood cell the alpha tocopherol is localised in the membrane fraction (9). This suggests that alpha tocopherol may play a role in the membrane permeability. Plasma vitamin E level does not change significantly between 6 and 17 years of life but it is known that (63) there is a steady increase of amount of alpha tocopherol in plasma as the age increases. The plasma level of alpha tocopherol is not affected by sex difference or geographical location (63).

Dietary Sources and Recommended intake: The tocopherols occur in oils of vegetable seeds such as wheat, soya bean, cotton and corn. Animal fat also contain high amount of tocopherols.

Vegetable oils contain a high amount of the less active form of tocopherols. In animals fat, the more active alpha tocopherol predominates.

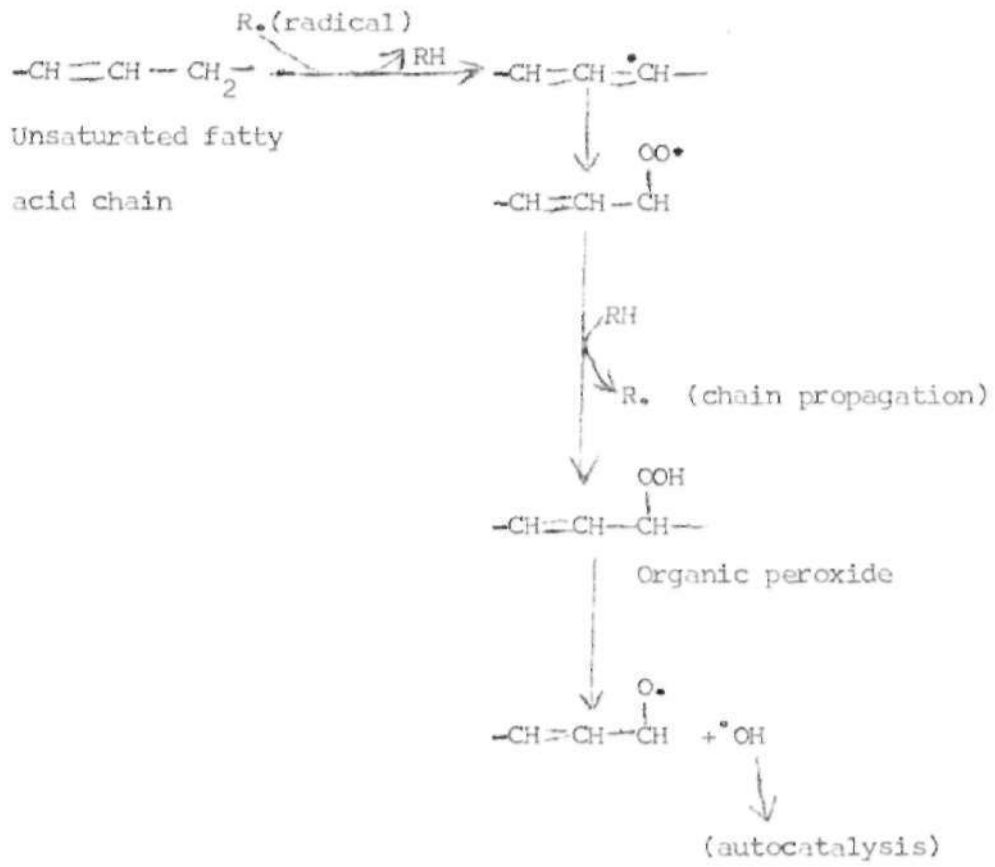
The requirement for alpha tocopherol by animals depends on a number of factors. Such factors include age and the amounts of polyunsaturated fatty acids taken in the diet (24). Adult require about 5 mg of alphatocopherol a day when polyunsaturated fatty acid intake is low (43). This value may increase to 14 mg a day when polyunsaturated fatty acid intake is high. An equivalent of about 0.6 mg of alphatocopherol is needed for each gram of polyunsaturated fatty acid taken in the diet by animals and Man (43). In rat the requirment for vitamin E increases with age (2) and at the rate of 7.6 percent a week after maturity.

Functions: Alpha tocopherol has been shown to be involved in a number of important reactions and processes in the body. Successful reproduction in female albino rats is known (2) to be enhanced by vitamin E supplementation and the vitamin E requirment for reproduction in these rats increases markedly with increase in age. It has also been observed (2) that abscence of vitamin E in the diet of rats during reproductive stage, leads to in-ability of the rats to carry their pregnancy to term. Instead abortion and foetal resorption occurs. In male rats deficient in vitamin E, degeneration of the testis occurs (42) indicating the involvement of vitamin E in the development of the reproductive system in rat.

Alpha tocopherol is also involved in the maintenance of the intergrity of the many system in the body. Rat and cow fed on vitamin E deficient diet for a long time develop a disease called defective embryogenesis (60). Chickens on such diet develop exudative diathesis which is a disease of the vascular system. Recent studies by Mustafa (36) indicate that vitamin E may even function by protecting the lung against damage by ozone. Mustafa observed that there was a greater response of lung mitochondria to exposure to ozone in rats on low vitamin E diet when compared to those on high vitamin E diet. The work suggests that the ability of animals to withstand oxidant stress can be increased by dietary supplementation of vitamin E. Ozone is the predominant oxidant of photochemical smog and exposure to it can cause injury to lung cell by initiating a number of reactions (36) in the body. Such reactions include oxidation of sulphydryl compounds and groups of enzymes and proteins and peroxidation of unsaturated lipids. Vitamin E might thus act as an important antioxidant against exposure to ozone and helps in the prevention of lung injury.

Alpha-tocopherol and cell Membranes: The maintenance of the intergrity of cell membranes (especially erythrocyte membrane) is the principal function of α-tocopherol in the cell. The erythrocyte membrane like other membranes has lipids as one of the Chief constituents. Phospholipids account for about 60 percent of the total lipid in the human erythrocyte (33). The phospholipid is made up of many fatty acids with unsaturated bounds which are open to attack from physiological and other chemical oxidants generated from enzyme systems in the cell.

Alpha tocopherol itself is a minor component of the membrane and it is now commonly accepted that tocopherol serves as an antioxidant for the unsaturated bonds of the phospholipid in the membrane (33) and protects them against attack by free radicals such as •OH (from hydrogen peroxide), generated by enzyme or non-enzymatic reaction catalysed by transition metals such as iron (Fe III) and Copper (Cu II). The radicals, if not trapped by alpha tocopherol, can initiate an auto-catalytic chain reaction of the type shown which follows.



The reactions regenerate radical R. and also produce an organic peroxide that can react further. This leads to chain cleavage reactions which may eventually lead to disruption of the lipid and finally membrane breakage. Even a minute quantity of alpha tocopherol stops the reaction by trapping the radicals to form more stable tocopherol radicals which may dimerise to terminate the chain.

Makenzie (32) has further shown that alpha tocopherol functions in the maintenance of the intergrity of erythrocyte membrane. Thus he observed that hemolytic effect of hydrogen peroxide (Membrane disruption) on red cells of full term infants can be completely inhibited, by the supplementation of alpha tocopherol or vitamin E in their diet. Furthermore red blood cells of premature infants, when given vitamin E supplement, showed a greater resistance to hemolysis (membrane breakage) by hydrogen peroxide. (21)

From the foregoing it appears vitamin E acts as a stabilizer of lipid structures (membranes) in animal tissues. It can also be considered as a physiological antioxidant. However, it has not so far been confirmed whether any enzyme reaction in the body has specific requirment for alpha tocopherol. Moreover many observed enhancements or inhibitions of enzyme reactions due to alpha tocopherol status of individuals have not been properly understood. It's action has further been complicated by it's partial or complete repleacibility by non-tocopherol molecules including selenium, sulphur amino acids and organic antioxidants. Diplock (12) has suggested that Vitamin E may even function by protecting microsomal, membrane bound, selenide containing non-heme proteins, against oxidation in vivo.

Deficiency of alpha-tocopherol: A variety of Vitamin E deficiency syndromes are observed in many animals. These syndromes vary from one specie of animal to another and also involve a variety of systems in their body.

Deficiency of vitamin E is not common in normal adult man because there is a considerable tissue storage of the vitamin in man(6) It is found in all tissues but in larger quantity in the muscle and adipose tissues which have high fat content (43). The stores of vitamin E in these tissues are known to take a long time to be depleted (6). In adult man deficiency could occur when there is a defect in ability to absorb dietary fat. This is because the vitamin being fat soluble is absorbed together with fat in the diet. In-adequacy of polyunsaturated fatty acid in diet may lead to deficiency of alpha tocopherols in individuals subsisting on such a diet for a long time. In such individuals there is an increase in susceptibility of their erythrocyte to hemolysis by physiological oxidants. Increase irritability and edema accompanied by anemia, are some of the clinical evidences that can be observed (5) in premature infants deficient in vitamin E.

Complete protection of erythrocyte against hemolysis is achieved in both adult and infants when serum level of alpha-

tocopherol is 0.5 mg per 100 ml or more (63). Extensive erythrocyte hemolysis in animals indicates a high probability of inadequacy of tissue stores of alpha tocopherol in such animals and man. Other symptons of vitamin E deficiency in animals include disorders of reproductive systems, muscle function, vascular system, bone marrow, brain and liver. For example muscle of rats on vitamin E deficient diet show abnormally high rate of oxygen uptake and also abnomalities on membrane of endoplasmic reticulum, when viewed with electron microscope (33). In rabbit, monkey, man and lamb (42), vitamin E deficiency causes muscular degeneration (nutritional muscular dyostrophy).

Vitamin E and Heart muscle disease: Despite the common occurence of skeletal muscle dystrophy in Vitamin E deficient animals, reports indicate that involvement of the heart is less common and less severe. However, in Lambs and Cows it has been observed (42) that cardiac disease can occur when diets deficient in Vitamin E are given. The cardiac disease usually show both functional and pathological expressions including electrocardiographic changes when the electorcardiograph is inspected. These changes may even lead to sudden death.

In rabbit the heart may also be involved leading to a disease called cardiomyopathy (33) and constriction bands may appear on the heart. The electro-cardiogram of such animals reveals a right axis deviation accompany by changes in T waves (42). Cardiac involvement has, however, not been observed in man suffering from vitamin E deficiency.

Assessment of Vitamin E Status: Evaluation of human vitamin E status is still inaccurate (5). The method of assaying vitamin E in the blood is rather difficult and very unreliable as an indicator of vitamin E status. This involves gas liquid chromatography (54). Serum level of alpha tocopherol does not reflect the true state of storage or distribution of vitamin E in the body (5). Also, only a portion of tocopherol estimated in the serum is biologically effective in maintaining the intergrity of red blood cells (32). Thus determination of serum, or plasma or erythrocyte alpha tocopherol does not give the true vitamin E status of subjects.

Since alpha tocopherol's main function in the cell, is to prevent lipid peroxidation damage to membrane by oxidants, a functional test; susceptibility of the red cell to hemolysis by H_2O_2 is now used as an indicator of vitamin E status of subjects. The test was first used by Gyorgy and Rose (22) in 1950.

The test is now known as erythrocyte hemolysis test and has been used by numerous workers (5, 6, 24, 54) for assessing vitamin E status of individuals. The degree of hemolysis of erythrocyte due to hydrogen peroxide is compared to the complete hemolysis due to water and the percentage of relative erythrocyte hemolysis is obtained. This test was used in this study to assess the vitamin E status of the human subjects.

Individuals with erythrocyte hemolysis (%) value between 0-10 are accepted to have adequate vitamin E in the body (6). Those with values between 10 and 20 were considered to have low or inadequate amount of vitamin E. Values greater than 20 indicate definite cases of vitamin E deficiency.

SELENIUM

Selenium is an essential trace element which is necessary for growth, fertility and prevention of certain disease conditions in animals and man.

Selenium in tissues and blood: In the cell and tissues of animals selenium is found in concentration that vary with the tissue and the chemical form of selenium in the diet of the animals. The liver and kidney usually carry the highest selenium concentration while much lower levels are found in the muscle, bone and the adipose tissues (60). The cardiac muscle has been reported to be richer in selenium than the skeletal muscles (60). Selenium concentrations in the tissues usually reflect the level of dietary selenium intake over a wide range. In tissues, selenium is found partly bound to proteins in a manner incompletely understood, partly incorporated into proteins as selenium analogues of sulphur amino acid and as a component of the enzyme glutathione peroxidase.

The blood level of selenium in animals is highly responsive to changes in selenium level in their diet. The blood level of selenium in healthy citizen of United States

of America has been reported (60) to vary from 0.10 to 0.34 ug/ml of blood. These values may be different for other people in different geographical locations as regional differences occur in selenium status of human subjects. However, most of the selenium in the erythrocyte of man is known (19, 23) to occur as component of the seleno-enzyme, glutathione peroxidase.

Dietary intake and requirments: The requirment for selenium by animals varies with the form of selenium ingested in the diet and the nature of the diet (60). However, a dietary intake of 0.1 ppm of selenium provides a satisfactory margin of safety against dietary variables or enviromental stress. Similar amounts of selenium were shown (33) to prevent muscular dystrophy in cattle and sheep grazing on selenium deficient soil. Liver necrosis in rats can also be prevented by the same amount.

Compounds of selenium such as sodium selenite and other inorganic selenium compounds are more effective than organic compounds of selenium in preventing symptons of selenium deficiency. Thus as little as 0.1 ppm of inorganic selenium compounds is required in the body to perform same function as

selenium itself (33).

Seleno-enzyme in Erythrocyte: The active role played by selenium in the body, is as a result of it being a component of an enzyme, glutathione peroxidase (EC 1.11.1.9) which catalyses the peroxidation of glutathione in the cell as shown in the equation below.

$$2GSH + H_2O_2 \xrightarrow[\text{Peroxidase}]{\text{Glutathione}} GSSG + 2H_2O$$

(Reduced glutathione) (Oxidized glutathione)

Glutathione peroxidase is a seleno-enzyme and contains 4g atom of selenium per mole of the protein (3, 47). The activity of the enzyme depends on its content of selenium (3, 10, 46, 47). This enzyme is present in a variety of body components in different amounts in different tissues with differences in species. In the blood glutathione peroxidase activity is mostly found in the erythrocyte and only 1-2 percent in plasma (50). Among the tissues, the enzyme has the highest activity in the liver (23), although some activity also occur in heart, kidney, muscle and lung tissues. The activity in the intestine and skeletal muscle has been reported (34) to be very low.

The glutathione peroxidase found in erythrocyte has the same properties as that found in the tissues. Levander and Coworkers (29) have observed that the rat liver mitochondria contains glutathione peroxidase which has similar chromatographic behaviour as the erythrocyte enzyme. It was also observed that the response of the liver enzyme to dialysis against glutathione or EDTA was similar to that of the erythrocyte.

Properties of glutathione peroxidase: The molecular weight of the enzyme ranges between 76 and 92 $x10^3$ and it contains eight equivalents of sulphydryl groups per mole of the protein (57). The enzyme has no flavin, heme, or any other prosthetic group. It has been reported to be stable at pH 7.0 and $4^{\circ}C$. Human erythrocyte glutathione peroxidase loses only 15-20 percent of it's activity when kept under the above conditions for four months (35). At lower pH such as 4 the enzyme is very unstable and loses all it's activity in 20 minutes (3).

Purified form of glutathione peroxidase is less stable than the crude form and infact the former releases its selenium when treated with potassium cyanide or other inhibitor (47).

At physiological concentrations of its substrates (glutathione and peroxides), it is in the reduced form and hence cannot be inhibited by it's usual inhibitors like cyanide. The optimum pH for peroxidase activity is 8.5 and its Michaelis constant (km) for glutathione is 4.1 milli molar (3).

Selenium, Glutathione Peroxidase and the cell membranes:

As indicated earlier, selenium as a component of the enzyme glutathione peroxidase play an active role in the protection of cell membranes against oxidative damage by hydrogen peroxide. Glutathione peroxidase catalyses the peroxidation of glutathione with hydrogen peroxide, the latter being reduced to water. Glutathione itself is oxidized to form a dimer (oxidized glutathione) with a disulphide link. The acceptor of the hydrogen from the glutathione is NADP. The equation is given below.

$$2GSH + H_2O_2 \xrightarrow[\text{Peroxidase}]{\text{Glutathione}} GSSG + 2H_2O$$
$$+ NADP^+ \qquad\qquad + NADPH.$$

The oxidized glutathione formed (GSSG) is reduced back to reduced glutathione by NADPH and the reaction is catalysed

by an enzyme, glutathione reductase, which is also widely distributed in the cell.

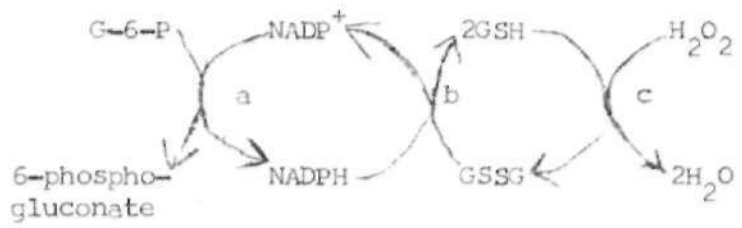
$$2NADPH + GSSG \xrightarrow[\text{reductase}]{\text{Glutathione}} 2GSH + 2NADP^{+}$$

The continual availability of NADPH for the reactions is ensured by the reaction of another enzyme, Glucose-6-phosphate dehydrogenase linked to the system.

$$\text{Glucose-6-phosphate} \xrightarrow[\text{hate dehydrogenase}]{\text{Glucose-6-phosp-}} \text{6-phosphoglu-conate}$$

$NADP^{+}$ $\qquad$ $NADPH^{+}$

This enzyme is also found in the cell and the reaction occurs in the phosphate pentose pathway. A general scheme involving the three reactions above is given below.



(a) Glucose-6-phosphate dehydrogenase.

(b) Glutathione reductase.

(c) Glutathione peroxidase.

The above reactions ensure that any hydrogen peroxide produced in

the cell, especially from auto-oxidation of certain drugs and other enzyme systems, is destroyed. Thus the integrity of the cell membrane is maintained, because if the hydrogen peroxide is not destroyed, it can attack the unsaturated bonds of the fatty acids leading to the disruption of the cell membrane. The series of reactions is particularly important in the maintenance of the erythrocyte membrane in animals.

The hydrogen peroxide produced in the cell, if not destroyed, can also cause excessive oxidation of funtioning hemoglobin Fe^{2+} in erythrocyte to the Fe^{3+} methemoglobin which is ineffective in transporting oxygen in the body.

Selenium deficiency: A deficiency of selenium in diet would obviously result in reduced activity of glutathione peroxidase in the tissues. The symptons of selenium deficiency have been observed in many animals. Nutritional muscular dystrophy, a degenerative disease of the striated muscules occur in animals such as sheep and rat on diet deficient in selenium (33). Cows and sheeps grazing on soil deficient in selenium develop white muscle disease with-in a short time (19). The white muscle disease is characterised biochemically, by subnormal selenium and glutathione peroxidase in blood and tissues (19).

Other diseases reported (60) to be caused by either low selenium intake or it's deficiency include Exudative diathesis and pancreatic Fibrosis in chicks, Hepatosis dietetica in pigs and reproductive disorders in many other animals. In man selenium deficiency can lead to muscular pain in quadriceps and hamstring muscles. Van rij and coworkers (61) reported the alleviation of such pains in a surgical patient by selenium given as selenomethione.

Selenium and Heart diseases: Recent reports (41) have indicated the involvement of selenium in heart function in Man. A fatal disease of the human heart called cardiomyopathy is said to be related to deficiency of selenium in diet of subjects having such disease. The disease is caused by bacterial and vival infections, a variety of toxic chemicals, amyloidosis and other infiltrative disease, nutritional disorders such as beriberi and idiopathic muscular dystrophy (41).

Young growing animals are more susceptible to selenium deficiency, hence the disease is more common among them. The symptons of caridomyopathy include shortness of breath, enlarged heart (and liver), congestive heart, gallop rythm

and of course high blood pressure. The activity of glutathione peroxidase in tissues of subjects having the disease is low compared to that of normal people (41). This may evidently be due to the low selenium intake or increase in need of selenium of the subjects.

Sodium selenite has been found to be effective in reducing the incedence of Keshan cardiomyopathy in China (41). A significant decrease in number of children with Keshan cardiomyopathy was observed in China in 1974 (41) when the children were given sodium selenite (500 mg per week) orally before the out break of the disease. Much work still needed to be done to ascertain how selenium is involved in the heart muscle disease.

Selenium toxicity: Although selenium is very essential and plays an active role in the body physiology, it is toxic at a high dose level. The toxicity of selenium in animals varies with the amounts and chemical forms of the element ingested. It also depends on the duration and continuity of the selenium intake and also on the nature of the rest of the diet. Generally, signs of selenium toxicity start showing at 3 to 20 ppm dietary levels. Such signs in animals,

include dullness and lack of vitality, emaciation and loss of hair from the tail. These signs are commonly found in animals grazing on soil with high level of selenium (60). In some animals, atropy of the heart and cirrhosis of the liver occur. Rats and dogs on diet containing about 20 ppm of selenium were observed to die within a short time (60). Thus selenium can be considered as a nutritional essential and at the same time a deadly poision.

Assessment of selenium status: The dependence of glutathione peroxidase activity of animal tissues on dietary selenium intake has been useful in assessing selenium status of animals. Scott (56), Rotruck (52), Godwin (19), Hafeman and Coworkers (23) working with different animals, have all observed that selenium status of animals is sensitively reflected in glutathione peroxidase activity. They also observed that low level of selenium in diet of animals caused low activity of the enzyme and adequate selenium intake reversed the effect.

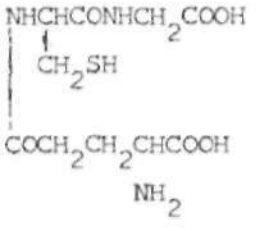
A significant direct correlation between plasma selemiun concentration and peroxidase activity in the erythrocyte have been reported (46). Thus measurement of peroxidase activity in

blood has been used as a means of identifying selenium deficiency (57). Furthermore experiments (50) have shown that the enzyme activity in whole blood is always related to the erythrocyte selenium concentration. These findings clearly demonstrate the sensitivity of glutathione peroxidase as an indicator of selenium status in human subjects and therefore used in the present study.

GLUTATHIONE

Glutathione is a tripeptide and the chief non-protein thiol in the cell. It accounts for about 98 percent of the total non-protein thiol found in the red blood cell of animals (28). Animals, plants and bacteria have enzymes which promotes the continual symthesis and breakdown of glutathione.

The thiol is made up of the amino acids glycine, cysteine and glutamic acid residues linked together by a peptide linkage. The sturcture is shown below.



Glutathione contains sulphydryl group (SH) and it is present mostly in the reduced form in the cell. Only very small amount of the oxidized form of glutathione is detectable in animal cell. Glutathione is kept in the reduced form by NADPH and enzyme glutathione reductase which is widely distributed in animal tissues. In animals including man, glutathione reduced form (GSH) is predominantly found in the liver, brain, intestine and human lens. It does not occur in measurable quantities in extracellular fluids such as lymph and plasma (28). In the muscle of animals (heart and skeletal) glutathione is very low except in cases of muscular dystrophy when it is elevated.

Blood level: The blood level of glutathione of human subjects reflects the tissues levels. The level varies widely from one individual to another, over the range of 36 to 96 mg per 100 ml of blood (15). Geographical location affects the blood level of glutathione in human subjects (7). The method of assay of blood glutathione most used is that of Beutler et al (4) and the nitroprusside method.

Functions: The biological function of glutathione depends to a large extent, on it's sulphydryl group (SH) that is readily oxidized to form a disulphide bridge linking two glutathione molecules.

i.e. $2GSH \xrightarrow[\text{oxidation}]{\text{Acceptor}} G\text{-}S\text{-}S\text{-}G + \text{acceptor, } 2H$

Glutathione thus acts as an intracellular reducing agent in the body whose primary function is to protect the SH group. Several enzymes which catalyse reduction of disulphides, peroxides, dehydroascorbate, in the cell, utilize glutathione as a reductant. The thiol also serves as a coenzyme for some enzymes including glyoxalase, maleylacetoacetic insomerase and also for oxidation of formaldehyde to formate.

As indicated earlier, the special ability of glutathione to reduce hydrogen peroxide in the cell serves as one of the protective measures against disruption of the membrane of the cells.

In some individuals, glutathione is absent completely from their erythrocyte, conegenitally due to lack of one of the enzymes that synthesize it in the body. The red blood cells of such individuals are fragile and easily-hemolyse when

exposed to hydrogen peroxide generating drugs. The cells also usually show reduced survival time, from 100 to 30 days life span (28). This, of course, leads to hemolytic anemia. Deficiency of Glucoe-6-phosphate dehydrogenase (in-born error) in some human subjects, leads to low blood glutathione level in such subjects. Erythrocyte of these subjects show drug induced hemolysis both in vitro and in vivo and are also deficient in -SH enzymes. This is probably because the oxidized glutathione once formed is not reduced back to it's reduced form due to NADPH deficiency.

Elevated glutathione level may be found in liver erythrocyte and muscle usually in individuals with alpha-tocopherol deficiency (53). This may be a physiological response to compensate for the antioxidant effects of the missing alpha tocopherol.

Relationship between Vitamin E, Selenium and Glutathione in the cell: The prevention of disruption of cell membrane by physiological oxidants (such as hydrogen peroxide and oxygen) is one single function that vitamin E, selenium and glutathione partake. The scheme below (26) illustrates how the three interact to protect the cell membrane.

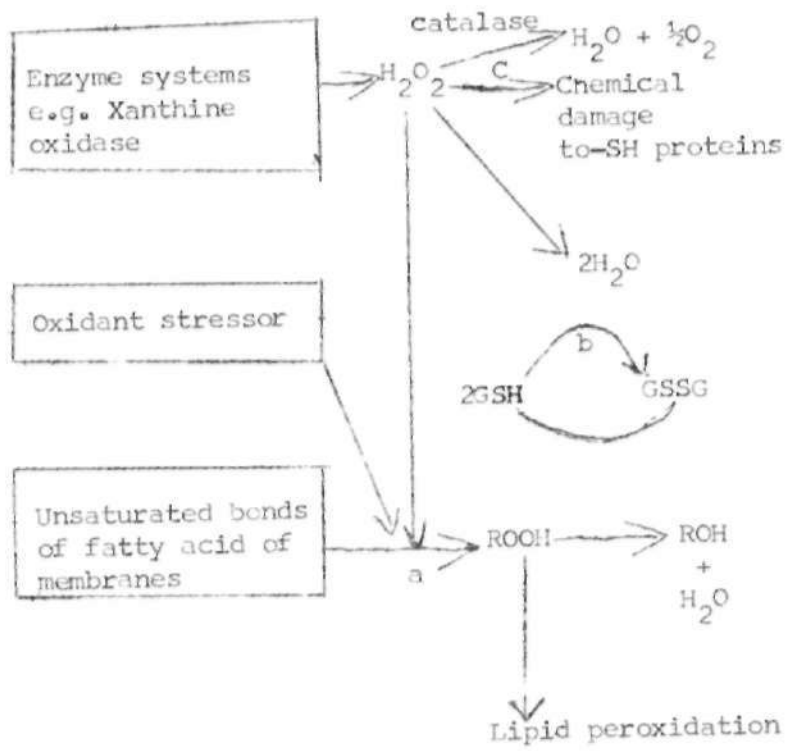


Fig 2. Interaction between vitamin E, selenium and glutathione in the cell.

Vitamin E blocks reaction (a) and prevents reaction (c) from taking place.

Selenoenzyme, glutathione peroxidase catalyses reaction (b).

A relationship between glutathione and vitamin E levels exist in animal tissues. Liver, erythrocyte and muscle glutathione levels are elevated in vitamin E deficient animals. Muscle and Erythrocyte glutathione are increased by 54 and 33 percent respectively when rabbits are fed on vitamin E deficient diet for 19 days (53). As indicated earlier the increase may be due to a physiological compensating mechanism for the antioxidant effects of the missing vitamin E. Selenium level has no effect on glutathione level in the body (33). Supplementation of diet with selenium also does not have any effect on the glutathione level in animals (33). A relationship between nutritional need for selenium and vitamin E has also been observed in several studies (33). Vitamin E deficiency in animals increases tissue glutathione peroxidase activity (60) and this may be to prevent greater susceptibility of the tissue to peroxidation in the abscence of vitamin E. It has also been suggested (33) that vitamin E may protect reduced selenium against oxidation in the cell.

AIM OF THE STUDY

There has not been any assessment of vitamin E and selenium status in human subjects in the Northern part of Nigeria.

1. This study aims to assess the vitamin E and selenium status in bothy healthy and unhealth subjects living in the Zaria region. The findings may be useful to clinicians, as also may add to the fundamental literature on this population.

2. There has been an increase in the incidence of cardiovascular diseases in Nigeria and a recent report (38) indicates that about ten million Nigerians suffer from heart diseases at the present time. The second objective of this study is to find out if blood level of either vitamin E or selenium could be implicated in these diseased states in Nigeria. Selenium content in local dietaries are not known, but it's deficiency has been implicated in a form of heart muscle disease called cardiomyopathy (41). It would therefore be worthwhile to explore the status of selenium in this population.

3. Thirdly an attempt will also be made to assess the glutathione level of this population which has never been reported in Nigeria.

4. The fourth objective of the study is to assess the ascorbic acid status in relation to vitamin E and selenium status in the subjects to be used.

MATERIALS AND METHODS

Abbreviations used, grades and sources of special chemicals

1. L-Ascorbic acid. Analar grade, BDH Chemicals Ltd., Poole England.
2. Bovine Serum Albumin (Crystalline), Sigma Biochemicals.
3. DNPH (2,4-Dinitrophenyl hydrazine)-Reagent for organic synthesis, Hopkins and Williams Essex England.
4. Disodium hydrogen phosphate-Laboratory reagent, May and Baker Ltd., Dagehan England.
5. DTNB (5,5' Dithiobis-(2 nitrobenzoic acid)- BDH Biochemicals.
6. EDTA (Ethylenediaminetetra-acetic acid (disodium salt)- Hopkins and Williams.
7. GSH (Glutathione (reduced)- BDH Biochemicals.
8. Hydrogen Peroxide - Laboratory reagent, BDH Chemicals Ltd., 30%.
9. Metaphosphoric acid - Laboratory reagent, Hopkins and Williams Essex England.
10. Potassium dihydrogen phosphate-Laboratory reagent, May and Baker Ltd., Dagehan England.
11. Sodium Azide - BDH Chemicals Ltd.,
12. Sodium Chloride - Analar, BDH Chemicals

13. Sodium citrate - General purpose reagent, Hopkins and Williams England.

14. Thiourea - BDH Chemicals Ltd.

15. Trichloroacetic acid - Analar BDH Chemicals Ltd.

2. Apparatus

Colorimetric measurements were made with SP6-400u.v. spectrophotometer (Pye Unicam).

Blood centrifugations were carried out with bench centrifuge (M.SE England). Centrifugations involving rat tissues were done with Beckman high speed centrifuge (Model JP21).

Corning pH meter (model 7) was used for pH determinations through out the study.

3. Human subjects

Healthy student and staff (both junior and senior) volunteers from Ahmadu Bello University Zaria and other volunteers who are resident around Samaru, Zaria and Kaduna were used as healthy subjects.

Both in and out patients of Ahmadu Bello University Teaching Hospital at Kaduna and Zaria, University Health centre and Wusasa Hospital, were used as unhealthy subjects. The unhealthy

subjects were grouped according to their diagonised diseases (as reported by the specialist consultant). The groups are as follows. Heart muscle diseases, Hytertensive or diabetic, kidney disease, liver disease, malnutrition (Marasmu & Kwashiolor).

Data such as sex, age and tribe of each subject was collected as shown in the protocol shown in appendix A.

4. Collection of Samples

Random Venous blood samples (about 3.0 ml) was drawn from each subject with a disposable hypodermic synringe. The sample was collected into sample bottles containing EDTA as anticoagulant to prevent clotting. The samples were brought to the laboratory for processing and analysis, at most within two hours of collection.

5. Preparation of Hemolysate

Each blood sample was centrifuge at 3000 r.p.m. in a clinical bench centrifuge for 10 minutes. The plasma was decanted off from the sedimented red blood cell and placed in a clean dry sample tube. The red blood cell suspension was then washed twice with five times its volume of ice cold normal saline. The cells were centrifuged out at 200 r.p.m. after each washing and were finally suspended in clean saline solution.

Red blood cell suspension (0.5ml) was used for erythrocyte hemolysis test. The remaining red blood cell suspension was kept refrigirated at 4°C until it was used (at most 24 hours) for the assay of glutathione peroxidase activity.

The plasma was used for ascorbic acid and glutathione analysis.

Erythrocyte hemolysis test (54)

Proceedure: The reagents used in this test include the following:

(a) Normal saline (0.9% w/v sodium chloride).

(b) Phosphate buffer (0.1M) pH 7.4 - prepared by adding 50 ml of 0.2M potassium dihydrogen phosphate to 40 ml of 0.2M potassium hydroxide. The pH of the mixture was adjusted to 7.4 and diluted to 100 ml.

(c) Hydrogen peroxide (2%).

(d) Saline buffer solution - 1:1 mixture of phosphate buffer and normal saline.

The reagents and erythrocyte suspension were added to three test tubes as shown in the table below.

TABLE 1. Protocol of addition of reagents for erythrocyte hemolysis test

Test Tube	Erythrocyte suspension(ml)	Reagents (ml)		
		Saline buffer	Water	Hydrogen peroxide
A	0.1	4.75	0	0
B	0.1	0	4.75	0
C	0.1	4.25	0	0.5

The contents of each tube were mixed well by shaking and incubated at 37°C for 1 hour. The tubes were then removed from water bath and allowed to stay for another 1 hour at room temperature. Contents of each were further mixed and centrifuged at 4000 rpm for 10 minutes in a clinical centrifuge. The resultant coloured supernatant from each tube was decanted into cuvette and the optical density was measured at 415 nm against a blank (mixture of water and normal saline).

Calculation: The percentage hemolysis of erythrocyte of each sample was calculated using the formula below.

$$\text{hemolysis (\%)} = \frac{\text{Absorbance of C} - \text{Absorbance of A} \times 100}{\text{Absorbance of B} - \text{Absorbance of A}}$$

Assay of Glutathione Peroxidase Activity

The method of Gross et al (21) and as adapted by Emerson et al (16) was used to assay for the enzyme activity in the erythrocyte of the human subject.

Principle of the method: The amount of residual reduced glutathione left after exposure of glutathione peroxidase activity, in presence of hydrogen peroxide for a fixed time, is measured colorimetrically. The equation of the reaction is given as:

$$2GSH + H_2O_2 \xrightarrow[\text{Peroxidase}]{\text{Glutathione}} GSSG + 2H_2O$$

Glutathione oxidation comences on addition of hydrogen peroxide and continues with a steady decreasing rate for at least 15 minutes. The reaction is first order with respect to glutathione and the amount of the latter oxidized between first and third minute is determined colorimetrically.

Proceedure (21, 16): The following solutions were used for the assay.

(a) Glutathione reduced (0.008M).

(b) Sodium azide (0.03M).

(c) EDTA disodium salt (0.009M).

(d) Hydrogen peroxide (0.0018M).

Hemolysate of each blood sample was prepared by adding 2.0 ml of water to 1.0 ml of the erythrocyte. Hemlysate was then freezed and thawed and phosphate buffer saline (0.5 ml) was added to 0.5 ml of the thawed hemolysate. The final incubation

mixture contained diluted hemolysate (0.5 ml), GSH (0.5 ml), Sodium azide (0.5 ml), EDTA disodium salt (0.5 ml) and water (3.0 ml). After equilibration at 37°C the reaction was iniated by the addition of hydrogen peroxide (1.0 ml) and samples were removed at 1 and 3 minutes. The reaction was stopped by precipitation with metaphosphoric acid and residual GSH was determined by the method of Beutler and Coworkers (4).

The non-enzymatic oxidation of GSH was determined by substituting water (0.5 ml) in place of the hemolysate, in the final incubation mixture.

Unit of activity: One unit of activity of glutathione peroxidase is the amount of the enzyme required to catalyse the oxidation of one micromole of glutathione in one minute per one ml of the erythrocyte (red blood cell)(16).

Assay of Glutathione in blood: The method of Beutler et al (4) is still one of the most reliable and most used (28) for assaying reduced glutathione in blood. This was used in this study to assay for reduced glutathione in blood of human subjects.

Principle of the method: The method is based upon the development of a relatively stable yellow colour complex, when an aromatic compound 5,5' - dithiobis-(2 nitrobenzoic acid) (DTNB), is added to sulphydryl compounds such as reduced glutathione. The colour developed in the reaction is highly reproducible and related to the amount of reduced glutathione present in a constant fashion (28). The coloured complex formed absorbs maximally at 412 nm.

Proceedure: Reagents used for this assay include the following.

(a) pricipitating solution: Glacial metaphosphoric acid (1.67 g), disodium salt of EDTA (0.2 g) and sodium chloride (30 g) were mixed in 100 ml of distilled water. The solution was kept in fridge at $4^{\circ}C$.

(b) phosphate solution: Disodium hydrogen phosphate solution (0.3 M) was prepared in water and stored at $4^{\circ}C$.

(c) DTNB solution: DTNB (40 mg) was dissolved in 100 ml of 1 percent sodium citrate solution.

(d) Glutathione (reduced) standard solution (200 ug per ml).

One mg of reduced glutathione was dissolved in 5.0 ml of EDTA solution.

The final mixture in the test tube contained, blood (0.2 ml), water (1.8 ml) and precipitating solution (3.0 ml). The contents of the tube were mixed well and allowed to stand for 5 minutes before centrifugation and filtration. To 2.0 ml of the filtrate 8.0 ml of phosphate solution and 1.0 ml of DTNB solution were added. The optical density of the resulting yellow solution was then measured at 412 nm against a blank containing 8.0 ml phosphate solution, 2.0 ml diluted precipitating solution and 1.0 ml of DTNB solution.

Standard curve: The amount of glutathione in the blood sample was obtained from the standard curve (Fig. 3) of absorbance against various amounts of standard glutathione solution (200 ug/ml) treated in same manner as the blood sample.

Standardization of the methods used

Random blood samples (5.0 ml each) were collected from 10 healthy donors and were assessed for vitamin E, selenium, and glutathione status by the methods described in the text. For each sample, six determinations of each parameter were carried out. The mean, standard error, and coefficient of variation of the results obtained were calculated as shown in the table which follows:

Parameters	RESULTS		
	Mean*	Standard Error	Coefficient of variation
Erythrocyte hemolysis (%)	8.2	0.25	3.0
Glutathione Peroxidase activity units x 10^2	3.5	0.15	4.2
Blood glutathione (mg/100 ml)	40	2.0	5.0

*Mean of six determinations.

These results were used as a measure of the reliability of the methods used in the study. From the standard errors and coefficients of variation obtained, it is clear that the methods used for the assessment of these nutrients in the subjects are fairly reliable. Hence these methods were used through-out the study.

TABLE II: Age group, sex and Tribe of controls and subjects of the study

HEALTH STATUS	AGE (Yrs)					SEX		TRIBE			
	<2	2-10	11-19	20-40	740	MALE	FEMALE	IBO,	HAUSA,	YORUBA	OTHERS
Controls	0	3	8	32	2	32	13	7	16	13	9
Heart Muscle Disease	0	0	2	11	17	18	12	0	15	1	14
Hypertensive or Diabtic	0	0	2	99	4	10	5	2	2	2	9
Pregnant or Lactating	0	0	0	15	0	0	15	0	0	5	10
Malnutrition[a]	11	4	0	0	0	15	0	0	11	0	4
Kidney Disease	0	0	0	15	0	15	0	5	5	0	5
Liver Disease	3	0	0	12	0	15	0	0	6	3	6
TOTAL	14	7	12	94	23	105	45	14	55	24	57

Key

*Other tribes: Fulani, Gwari, Kaje, Birom, Nupe, Idoma

a malnutrition: Subjects sith kwashiokor or Marasmus.

Table III: Erythrocyte hemolysis, glutathione peroxidase activity and blood glutathione in Male and Female healthy subjects

PARAMETERS	MALE (32)			FEMALE (13)			COMMENT
	Mean	S.E.	95% Confidence interval	Mean	S.E.	95% Confidence interval	($P > 0.05$)
Erythrocyte hemolysis (%)	4.8	0.54	3.72-5.88	5.1	0.77	3.56-6.64	Not significant
Glutathione Peroxidase activity units x 10^{-2}	3.7	0.49	2.72-4.68	2.5	0.26	1.98-3.02	Not significant
Blood glutathione mg/100 ml	39.4	4.90	29.6-49.2	40.0	7.66	24.7-55.30	Not significant

( ) Number in parenthesis indicate number of subjects.

S.E. standard error of mean ($P < 0.05$).

Result and Discussion

There was no significant difference ($p \geq 0.05$) between healthy male and female subjects with regards to erythrocyte hemolysis (%), erythrocyte glutathione peroxidase activity and blood glutathione. These results indicate that sex difference may not affect the vitamin E and selenium status of healthy individuals. These also agree with earlier observation by Weimo and Drapper (63) that difference in tocopherol levels of individuals can-not be attributed to sex difference. However because of the limited number of human samples available for the study, it is not possible to conclude from this study that sex difference may not play a role in vitamin E and selenium status of human subjects. Both the mean erythrocyte hemolysis (%) and mean glutathione peroxidase activity for both sexes were within the normal range for this study, indicating adequate vitamin E and selenium in the subjects.

As regards age, no significant difference was noticed in erythrocyte hemolysis (%) and glutathione peroxidase activity values between the age groups studied. Haris and Coworkers (24) in their study on tocopherol values in normal human subjects, observed that plasma tocopherol level does not change significantly between 6 and 17 years of age, but a very small steady

increase may be noticed after maturity indicating that the erythrocyte hemolysis (%) value for normal adults (above 17 years of age) may be lower than that for children under this age. Such a difference was not observed in this study.

TABLE IV: Erythrocyte Hemolysis (%), Erythrocyte Glutathione Peroxidase Activity and Blood reduced Glutathione in controls and subjects of the Study

Health Status	No of Subjects	ERYTHROCYTE HEMOLYSIS (%) Mean	Standard Error	95% Confidence Interval	Significance (P<0.05)	ERYTHROCYTE GLUTATHIONE PEROXIDASE ACTIVITY UNITS* x 10^{-2} Mean	Standard Error	95% Confidence Interval	Significance (P<0.05)	GLUTATHIONE (mg/100 ml blood Mean	Standard Error	95% Confidence Interval	Significance (P<0.05)
Control	45	5.4	0.59	4.2-6.6		3.8	0.34	3.1-4.5		38.4	4.03	30.3-46.5	
Heart Disease	30	13.1	0.93	11.2-15.0	S	2.5	0.31	1.8-3.1	S	42.4	4.31	33.8-51.0	Not Significant
Hypertensive or Diabetic	15	8.1	2.89	2.3-13.9	Not Significant	4.0	0.58	2.8-5.1	Not Significant	30.0	6.17	17.7-42.3	do
Pregnant or Lactating	15	4.8	1.24	2.3-7.3	do	4.0	0.56	2.9-5.1	do	34.1	1.94	30.2-38.0	do
(a) Malnutrition	15	9.4	3.08	3.2-15.5	do	4.0	0.75	2.5-5.5	do	31.1	3.22	24.7-37.6	do
Kidney Disease	15	5.7	0.17	5.4-6.0	do	3.0	1.26	0.5-5.5	do	46.2	4.35	37.5-54.9	do
Liver Disease	15	4.1	0.80	2.5-5.7	do	4.6	1.05	2.5-6.7	do	35.7	3.18	29.3-42.1	do
TOTAL	150												

*μmoles of GSH oxidized/min/ml of erythrocyte.

S = Significant.

(a) Malnutrition: Subjects with Kwashiokor or Marasmus.

Result and Discussion

The mean erythrocyte hemolysis (%) values of all groups of subjects studied except those with heart muscle diseases, are within the acceptable (6) normal range, indicating adequacy of vitamin E in the body of the subjects. The mean erythrocyte hemolysis (%) value of heart muscle diseased subjects was 13.1 and is significantly higher ($P < 0.05$) than that of the control subjects, indicating a low vitamin E status in such subjects. The heart muscle diseased subjects can thus be classified as having a subclinical or marginal level of vitamin E, using the method of classification of Binder and Spiro (6). It is not clear from this study whether the low level of vitamin E status observed in the heart muscle diseased subjects is the relevant cause of the disease or incedental due to poor dietary intake. Patients with heart problems in the hospital are usually adviged not to take much fatty food. The low fat intake by this subjects could also lead to low vitamin E intake, as absorption of the latter is usually determined by corresponding fatty acid absorption especially those of poyunsaturated fatty acid (11). It has been observed (43) that for every gram of polyunsaturated fatty acid taken by normal adult man about 0.6 mg of alpha tocopherol

is also absorbed. This is equivalent to about14 mg a day when polyunsaturated fatty acid intake is high. Also adults require about 5 mg of alpha tocopherol a day when the polyunsaturated fatty acid in-take is low.

The mean value of glutathione peroxidase activity in blood of the subjects analysed, except those with heart muscle disease, indicate adequate selenium in the body. Heart muscle diseased subjects have a mean glutathione peroxidase activity of 2.5×10^{-2} units which is significantly lower ($p < 0.05$) than that of the healthy subjects. This indicates a low level of selenium status in the heart subjects, since glutathione peroxidase activity is an indicator of selenium status. The result agree with the recent finding of group of chinese researches that some form of heart muscle disease could be associated with selenium deficiency (41).

It is not possible from this study, to confirm that selenium deficiency or it's low level was responsible for the heart muscle diseases, since the diet and other drugs being given to such patients were not analysed for selenium. It could be that the low selenium level observed by this assessment was due to the poor amount in their diet or poor absorption. More investigations need to be done to confirm

if low selenium in the tissue could affect the cardiac muscles.

Although the blood glutathione levels of all the groups of subjects irrespective of the state of health, are within the normal range of the study the mean level is higher in heart muscle diseased subjects when compared to others. Since glutathione in the cell together with glutathione peroxidase act to destroy the physiological oxidant hydrogen peroxide, and vitamin E also prevents the action of hydrogen peroxide on the membrane lipids, the high level of glutathione observed in heart muscle diseased subjects, could be to compensate for the antioxidant effect of missing vitamin E.

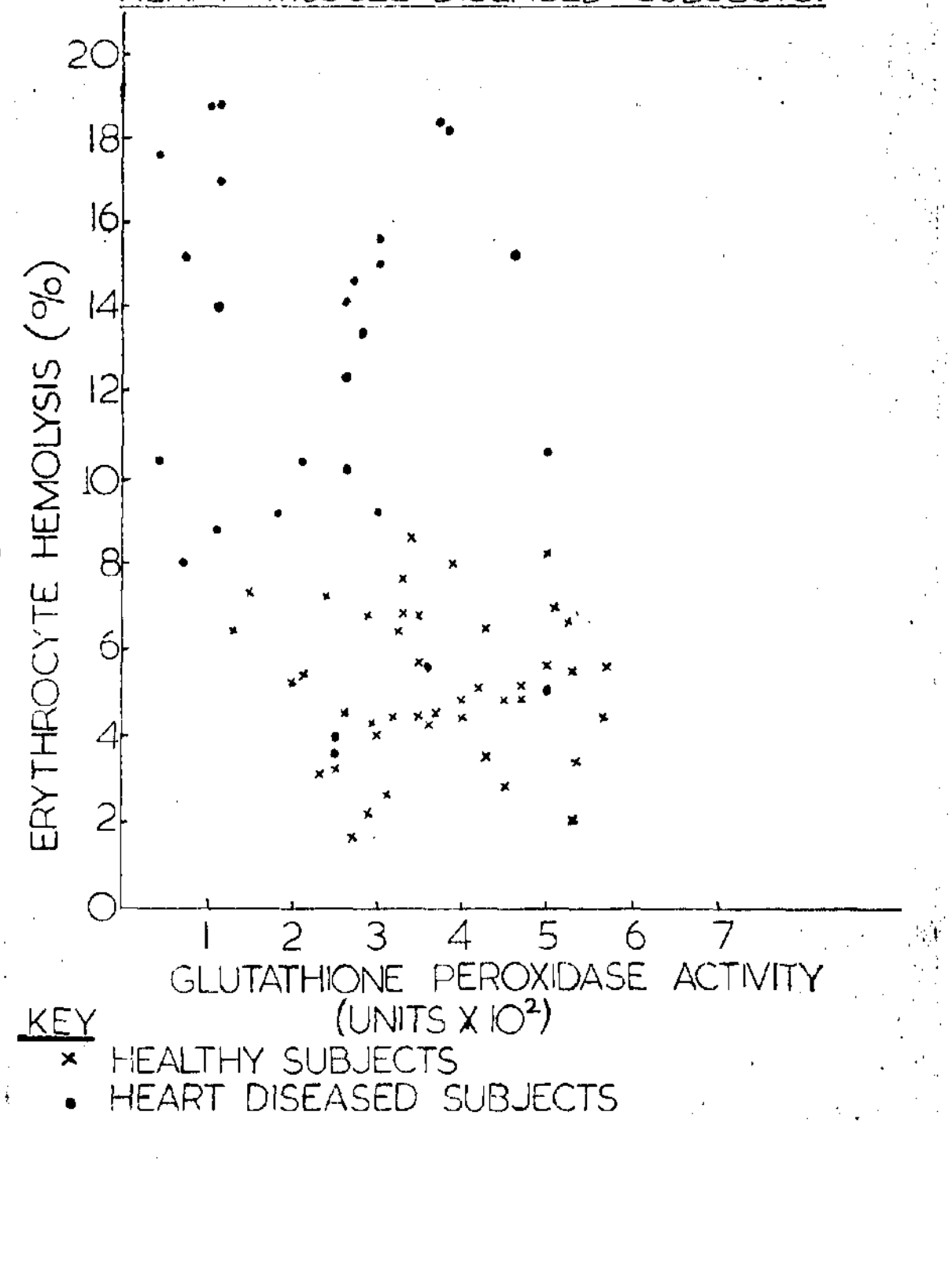
Relationship between Vitamin E and Selenium Status in controls and Heart muscle diseased subjects

It is evident from the scatter diagram in fig.5 that control subjects have significantly higher levels of blood glutathione peroxidase activity and significantly lower level of erythrocyte hemolysis (%) values than the heart muscle diseased subjects. These indicate a higher selenium and vitamin E status in control subjects compared to the heart muscle diseased ones. The blood peroxidase activity and erythrocyte hemolysis (%) of about 82 percent of the controls lie between 2 and 5 x 10^{-2} units and 2 and 8.3 respectively. About 63 percent of the subjects with heart muscle diseases have peroxidase activity of less than 3 x 10^{-2} units while 85 percent of such subjects have erythrocyte hemolysis (%) greater than 8.0.

As regards subjects with heart muscle disease, no correlation or relationship ($r \simeq 0$) was found between their erythrocyte hemolysis (%) and glutathione peroxidase activity values. Thus indicating that no relationship between selenium and vitamin E status of such subjects studied.

There was a small degree of positive correlation ($r = +0.11$) between erythrocyte hemolysis (%) and glutathione peroxidase

FIG. 5. RELATIONSHIP BETWEEN ERYTHROCYTE HEMOLYSIS (%) AND GLUTATHIONE PEROXIDASE ACTIVITY OF HEALTHY AND HEART MUSCLE DISEASED SUBJECTS.

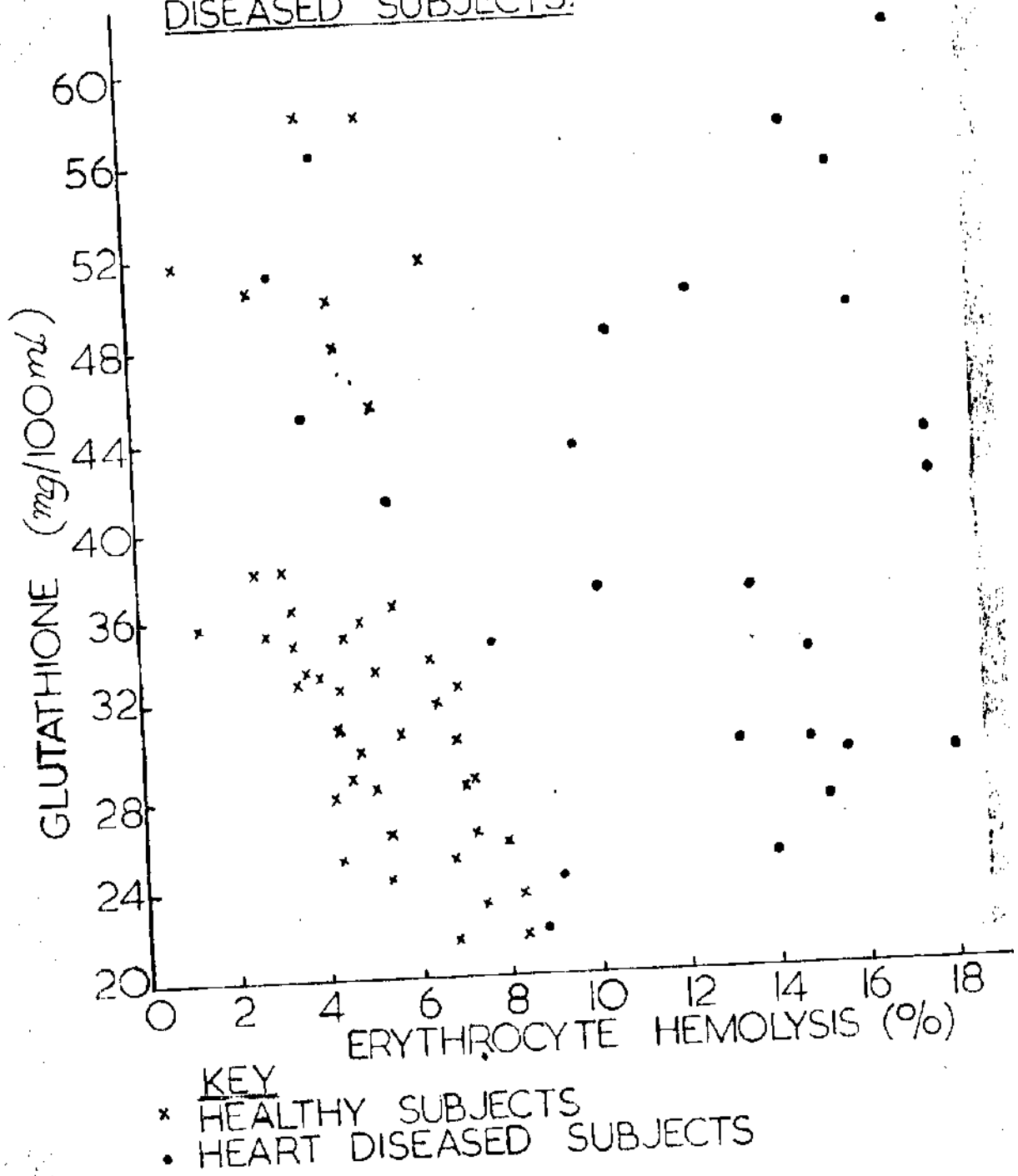


activity of the controls. Thus an increase in erythrocyte hemolysis (%) of such subjects is likely to be accompanied by an increase in glutathione peroxidase activity. Hence an increase of glutathione peroxidase activity would be expected when the vitamin E status of such subjects is lowered. This agree with earlier report (60) that vitamin E deficiency in animals and man increases erythrocyte peroxidase activity.

Relationship between blood glutathione and vitamin E status of controls and subjects with heart muscle diseases

The scatter diagram in Fig. 4 indicates that a band appears to be formed at low values of blood glutathione ($\leq$40 mg per 100 ml) and erythrocyte hemolysis (%) ($\leq$8) of the controls. About 80 percent of the control subjects analysed have their values within this band. There was no relationship (correlation coefficient r = 0) between the values of blood glutathione and erythrocyte hemolysis (%) of control and subjects with heart muscle diseases studied, when plotted on the scatter diagram (Fig. 4). This indicates that no relationship occurs between vitamin E. status and glutathione level of the subjects used. Although Ryerson and

FIG. 4. RELATIONSHIP BETWEEN BLOOD GLUTATHIONE AND ERYTHROCYTE HEMOLYSIS (%) OF HEALTHY AND HEART DISEASED SUBJECTS.

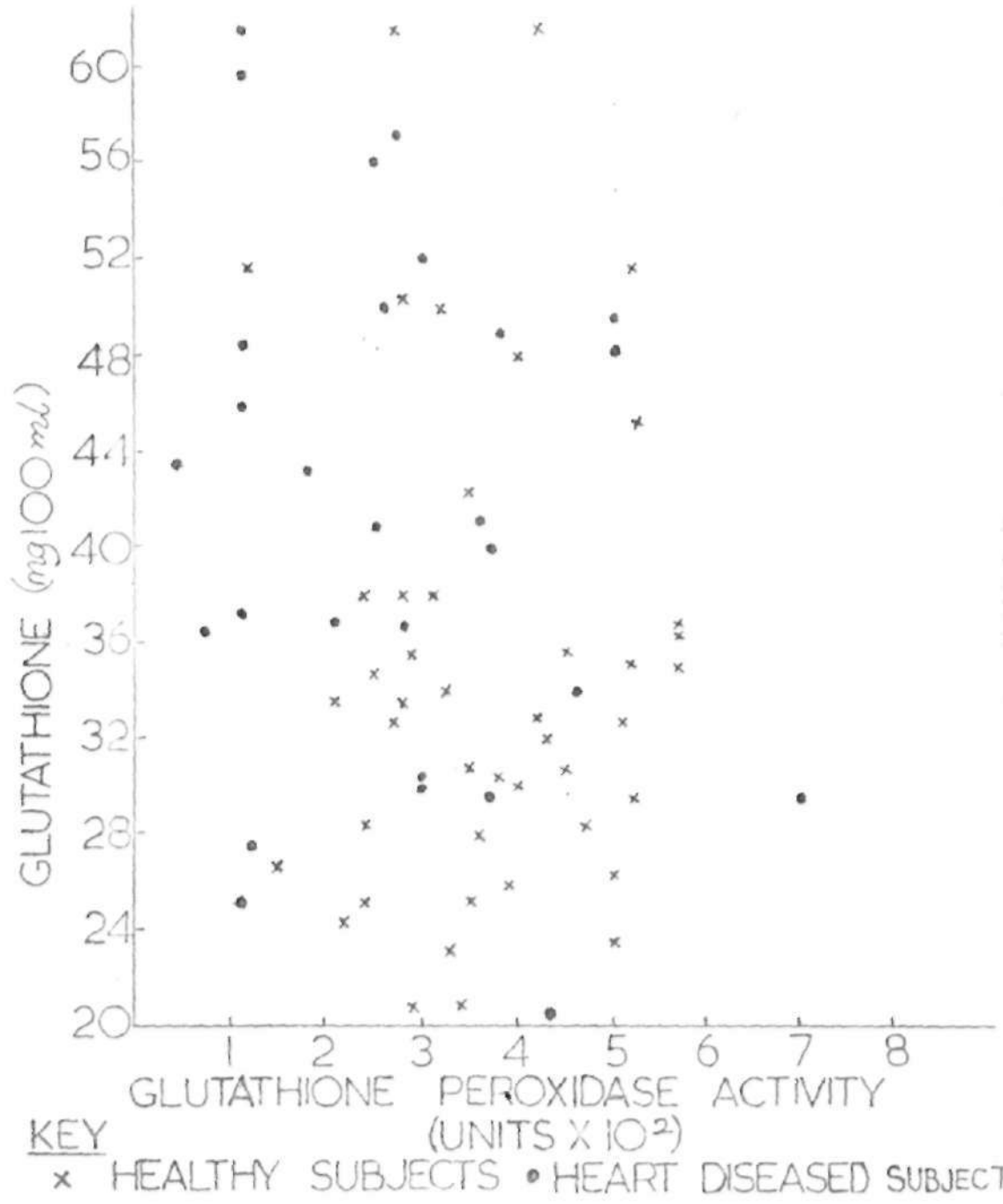


Coworkers (53) have reported that erythrocyte glutathione increases in vitamin E deficiency in man and animals, such a relationship was not observed in this study. However the glutathione and hemolysis values of subjects with heart muscle disease were significantly higher than those of the controls. About 74 percent of the heart diseased subjects have higher erythrocyte hemolysis (%) values than the controls. This indicates that subjects with the heart muscle diseases have lower vitamin E status than the controls or healthy ones.

Relationship between blood glutathione and selenium status of controls and heart muscle diseased subjects

As indicated in Fig. 6, the heart muscle diseased subjects have on the average higher blood glutathione than the control subjects. Although no definite pattern on the distribution of subjects according to blood glutathione is evident from the graph, 80 percent of the control subjects have a blood glutathione level of less than 38 mg per 100 ml while 60 percent of subjects with heart muscle disease have theirs above the 38 mg per 100 ml mark.

FIG.6 RELATIONSHIP BETWEEN BLOOD GLUTATHIONE AND GLUTATHIONE PEROXIDASE ACTIVITY OF HEALTHY AND SUBJECTS WITH HEART MUSCLE DISEASES.



The erythrocyte peroxidase activity distribution between the subjects also does not show any definite pattern. However, a high percentage of both the control and heart muscle diseased subjects have a peroxidase activity of between 2 and 5 x 10^{-2} units. Also no definite relationship ($r = 0$) between glutathione and selenium status in blood of both control and heart diseased subjects could be found in this study. This, however, agrees with earlier work on these parameters in animals (33) that selenium level is not affected by glutathione level of the body in animals (and supplementation of diet with selenium does not affect glutathione level either).

Effect of Vitamin E Supplement on Vitamin E and Selenium Status of a Subject Deficient in Vitamin E - A CASE STUDY

A particular male subject had a mean erythrocyte hemolysis (%) of 18.5 when determined over a period of 5 weeks. This indicates a deficiency of vitamin E in his blood. The subject also had a mean glutathione peroxidase activity of 3.4×10^{-2} units which is within the normal range found in this study. The subject was on weekly antimalarial prior to this investigation and was also free from heart muscle disease symptons.

An oral supplement of vitamin E (60 mg d-alpha-tocopherol per day) was then given to the subject for 3 days. It was observed that both the erythrocyte hemolysis (%) and glutathione peroxidase activity of the subject fell significantly by 78 and 51 percent respectively. These results indicate an improvement in the vitamin E status of the subject, indicating that vitamin E supplements have caused a reduction in erythrocyte hemolysis and has improved the erythrocyte membrane stability in this subject.

The erythrocyte glutathione peroxidase activity was also lowered by vitamin E supplement given to the subject. This

suggests that a relationship between vitamin E and seleno-enzyme synthesis does exist in human subjects. Hence the activity of glutathione peroxidase in human may be regulated by the adequacy of vitamin E in the body. This needs further investigation and confirmation.

Effect of starvation on enzyme systems

The term starvation is used to describe prolonged intake of insufficient amount of food to balance energy expenditure. Starvation is one of the nutritional problems which affect the world's population especially in the developing countries.

During starvation, there is reduction of physical movement and less energy is required to maintain the reduced activity. The tissues proteins are depleted to provide the necessary energy. Enzyme activity changes during starvation, depending on the enzyme type and the tissue where it occurs (64). A few enzymes such as alkaline phophatase have their activities enhanced in tissues of starving animals. However most other enzymes have reduced enzyme activity during starvation. As a result of advancement in biological and medical fields, effects of starvation on some diseases are now being studied clinically through enzymic studies during starvation.

All organs and tissues in the body are not equally affected by starvation. Brain and spinal cord, for example, suffer only little loss of weight during starvation whereas drastic reduction in weight and metabolism take place in liver, intestine and muscles. The cardiac muscle atropy during starvation and a decreased cardiac work has been observed. The decrease was shown (64) to be due to a reduced cardiac out-put associated with a minor fall of blood pressure. The size of the heart of a starved animal also decreases.

In cardiomyopathy, a disease of the cardiac muscle, the glutathione peroxidase activity in the erythrocyte has been shown (41) to be lower than normal values, indicating that affected selenium metabolism could be associated with the heart muscle disease. Results reported earlier in this study (Table IV) show that the mean activity of glutathione peroxidase in subjects with heart muscle diseases is $2.5 \times 10^{-2} \pm 0.6$ units which is significantly lower than those of the healthy subjects.

Both cardiomyopathy and starvation produce an overall effect on the function of the heart. Hence it was decided to carry out investigations on the activity of the selenoenzyme in

heart tissues affected by starvation. There was no practical method for producing heart muscle disease in human subjects. Therefore, an animal model was constructed and the effect of starvation on the activity of glutathione peroxidase in the heart and other tissues of the rat was studied. The distribution of the enzyme activity between the cytosol and mitochondria of the rat heart and the effect of starvation on this distribution were also studied.

Determination of glutathione peroxidase activity in rat tissues.

METHODOLOGY

Female albino white rats, 8 to 12 weeks old, supplied by animal colony of the Faculty of Pharmaceutical Sciences, Ahmadu Bello University Zaria, were used. The rats were fed on animal feed from PFizer, Nigeria Limited. Each rat was weighed at the start of starvation and prior to sacrifice. All rats were starved one hour before the sacrifice.

Proceedure

A. Preparation of tissues for homogenisation - The rat to be sacrificed was placed inside a beaker containing absorbant cotton soaked with diethyl ether and covered with a glass plate. Within 2 to 3 minutes the rat became anaesthesized. It was taken out and placed on a tray. The following operations were quickly done all in 2 to 3 minutes. The abdomen was quickly opened with a pair of surgical scissors. The Liver, Heart, Kidney, Lungs and intestine were removed and chilled immediately in ice cold Saline-EDTA solution. All these organs except intestine were cut into small pieces while still in the saline-EDTA solution to allow them to bleed and chill well. The chilling solution was repeatdly changed until freed from visible blood.

The intestine removed was placed on cold metallic plate, open up longitidinally and washed with cold saline-EDTA to remove the undigested food materials. The mucosal cells were scrapped with a clean cold microscopic slide, and were collected in ice cold Saline-EDTA solution.

B. Homogenisation - Each of the tissues was then homegenised for about 60 to 90 seconds in a glass homogeniser[a] in about

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[a]Potter Elvelyvein glass homogeniser with teflon pestle, Arthur Thomas Co. Philadelphia, Penn. U.S.A.

20 volumes of Saline-EDTA solution. The homogeniser tube was kept in ice cold water with floating ice blocks, during the homogenisation proceedure.

The homogenates, about 30 ml each, were subjected to centrifugation at 750 g for 15 minutes using the high speed centrifuge[b] to remove debris, nuclei and connective tissues. The supernatants from above were then centrifuged at 10 for 1 hour. The resulting supernatant were collected and used for glutathione peroxidase activity assay by the method of Gross et al (21) as adapted by Emerson et al (16). Activity was calculated as micromoles of GSH oxidized per minute per milligram tissue protein.

C. Estimation of Protein in tissue preparations - The Biuret method of protein estimation was used (48). Reagents used for the estimation include the following:-

(a) Crystalline bovine serum albumin, sigma standard solution 5 mg/ml.

(b) Biuret reagent. Prepared by dissolving 3 g of hydrated copper sulphate ($CuSO_4 \cdot 7H_2O$), and 9 g sodium potassium tratrate in 1 litre of 0.02N sodium hydroxide.

Standard curve - Various standard albumin solutions were put in test tubes numbered 1 to 6 to contain 0, 2.5, 5, 7.5 10 and 15 mg protein per tube respectively. Water was added to the tubes to make a total volume of 2.0 ml in each. Biuret reagent (3.0 ml) was added to each tube and contents were mixed well by gentle shaking. The tubes were incubated in a water bath at 37°C for 10 minutes. The absorbance of the resulting bluish green solution was measured at 540 nm using a spectrophotometer (SP6-400).

A standard curve of absorbance against protein concentrations mg per tube (Fig. 7) was plotted. Supernatant preparations (2.0 ml) from each tissue were treated in same way as standard albumin solutions and the amounts of protein in each tissue preparation was calculated from the standard curve.

TABLE V: Glutathione Peroxidase Activity in Rat Tissues Supernatants

TISSUE	SPECIFIC ACTIVITY* UNITS x 10^2	
	MEAN(a)	RANGE
Liver	0.25 ± 0.04	0.19 - 0.28
Heart	0.14 ± 0.04	0.10 - 0.17
Kidney	0.13 ± 0.03	0.11 - 0.14
Lung	0.14 ± 0.03	0.12 - 0.16
Intestinal Mucose	0.07 ± 0.04	0.04 - 0.10

(a) Eight female rats with body weight ranging from 185 - 227 g were used.

* Specific activity: µmoles of GSH oxidized per minutes per mg tissue protein.

Result and Discussion

Glutathione peroxidase activity was present in all the tissues analysed. This indicates that the peroxidase system whose primary function is to destroy hydrogen peroxide, occurs in tissues other than the erythrocytes. The results also show that, compared to the other tissues, rat liver contains more

glutathione peroxidase activity. Heart, Kidney and Lung have about the same glutathione peroxidase activity value which is lower than that of the liver. These observations agree with that reported by Mills (34) in 1960. They also indicate that the rat liver may be much more protected from oxidant damage than other tissues. This is probably due to the importance of liver in the body, more so with the various activities occuring in the organ.

The activity of glutatione peroxidase in the intestinal mucosal cells were only 28 percent of that in the liver, indicating a very low level of activity of the selenoenzyme in this tissues. Thus the result obtained in the present study also agrees with earlier observation by Rotruck and Coworkers (52) on the activity of the enzyme in intestine of animals. They observed that the intestine in animals is inherently low in glutathione peroxidase even with adequate selenium diet.

Glutathione peroxidase activity in tissues of animals depends on the type of animal, method of assay of the enzyme and definition of the enzyme units (23). Thus the values obtained for rat tissues in this study, may not be the same

with what obtains in Man and separate studies with tissues in man may therefore be necessary to determine the actual peroxidase activity in the tissues of Man.

TABLE VI: Effect of sta

Rats: Sex and Number	Dietary State	Mean (at o Star
Female-8	FED	196.5
Female-8	Starved (48 hr)	192.3

*Specific act

Result and Discussion

It is evident from Table VI that starvation for 48 hours had no significant effect on the activity of glutathione peroxidase in kidney, lung, liver and intestinal mucosa cells of the albino rats used. A significant increase ($P < 0.05$) of about 43 percent in peroxidase activity of the rat heart was observed. Thus the effect of starvation on the activity of this selenoenzyme in rat varies with the type of tissues or organs under study. During starvation, some protein is lost in some tissues in the body of animals, especially the muscles. This loss could result in an apparent increase in specific activity of enzymes in these tissues. However an increase of 43 percent in specific activity observed in this study in rat heart within 48 hours seem more than what can be accounted for by loss of tissue protein.

In cardiomyopathy, the peroxidase activity, has been shown (41) to be low in the erythrocyte. Thus a decrease in peroxidase activity would be expected in heart tissue of starved rat since both cardiomyopathy and starvation affect the heart. Instead an increase in the activity of glutathione peroxidase was observed in this study. Further investigations

need to be carried out to find out why there is increase in the activity of this enzyme in the rat heart during starvation. Such investigations should include the effect of starvation on the demand and synthesis rate of the enzyme at the molecular level. The increase in peroxidase activity of starved rat heart could also be due to lower turnover rate of the enzyme molecules as a result of the dietary restriction.

TABLE VII: Effect of Starvation on glutathione peroxidase activity in mitochondria and cytosol of heart tissue of albino rat

Number of Rats	Mean Heart Weight(g)	Dietary State	Glutathione Peroxidase, Specific Activity x 10^{-2}				
			Total	Cytosol	Percentage of total	Mitochondria	Percentage of total
4	0.91 ± 0.02	FED	0.12 ± 0.02	0.08	68.20	0.037	31.80
4	0.09 ± 0.01	Starved	0.17 ± 0.03	0.12	70.58	0.05	29.40

Result and Discussion

The results obtained are shown in Table VII. In the fed rat, 68.2 percent of the total glutathione peroxidase activity was found in the cytosol while 31.8 percent was associated with the mitochondrial fraction. This agrees with observation made by Holmes and Coworkers (27) that glutathione peroxidase predominantly occur in the cytosol of cells of animals.

However starvation increased significantly the total specific activity by 41.6 percent. Although the total activity of glutathione peroxidase in the rat heart is increased during starvation, it appears that it's distribution (%) between the cytosol and mitochondria is not affected by starvation. The increase may be due to some hormonal regulation, since it is now believed (39) that changes in enzymes activities during starvation could be regulated by hormonal changes. Further work is needed to explore this interaction. A reduction in heart weight of the starved rat compared to the fed one was also observed.

CONCLUSION AND RECOMMENDATIONS

Vitamin E and selenium status of human subjects of different health states, leaving in Zaria area, were assessed. The vitamin E status was assessed as erythrocyte hemolysis (%) and selenium status as erythrocyte glutathione peroxidase activity.

The mean erythrocyte hemolysis (%) (5.4 ± 0.59) and mean glutathione peroxidase activity (3.8×10^{-2} units) of the healthy subjects assessed indicate that the subjects have adequate vitamin E and selenium in their body. These values were not influenced significantly by differences in sex, age or tribe of the subjects.

As regards the unhealthy subjects assessed, the vitamin E and selenium status of subjects with diabetis, hypertension, kidney and liver diseases were not significantly different from those of the healthy ones. This indicate that the vitamin E and selenium status may not be changed due to the development of these diseases in human subjects. Pregnant and lactating women were also assessed and no significant difference was observed between their vitamin E and selenium status when compared to the healthy subjects. However the selenium and vitamin E status (as assessed by the methods used in this study)

of subjects with heart muscle diseases, indicate that such subjects have low levels of the nutrients in their blood when compared to the healthy ones. It was not clear from this study whether the lowered vitamin E and selenium status found in such subjects was either due to restricted dietary intake or caused by the occurence of the diseases. Further investigations would be necessary to throw more light on this finding. The type of heart muscle diseases refered to in this study are given in appendix A.

The finding that selenium status of the heart muscle diseased subjects was lower than that of the normals agrees with the recent finding by chinese researchers that selenium deficiency might be implicated in some form of heart muscle disease(41) This study could not further prove this as it was not possible to give selenium supplement to diet of the heart diseased patients and possibly see if the heart disease symptons would be removed. This is because the subjects were under strict medical observation. Observations made on a particular male,vitamin E deficient subject in this study, indicate that there might be a metabolic interrelationship in man between vitamin E and the selenoenzyme glutathione peroxidase.

The vitamin E status of the subject was noticed to be improved by oral administration of d-x-tocopherol for 3 days, but the selenium status was however lowered significantly, as indicated by glutathione peroxidase activity. The subject had no signs of heart muscle disease symptons but was on weekly antimalarial drug. These observations further indicate that symptons of vitamin E deficiency may be alleviated by administration of oral vitamin E tables and further that glutathione peroxidase activity may be regulated by the adequacy of vitamin E in human subjects.

Vitamin E and selenium act as reducing agents preventing oxidants like hydrogen peroxide from attacking cell membranes. Since ascorbic acid and glutathione also act physiologically as reducing agents, the human subjects used in this study were also assessed for these reducing agents. The healthy subjects had a mean of 38.4 ± 4.03 and 0.70 ± 0.14 mg per 100 ml of erythrocyte glutathione and plasma ascorbic acid respectively. There was, however, no significant difference between these values and those obtained for the unhealthy subjects. Thus indicating that glutathione and plasma ascorbic acid of human subjects may not be affected significantly by vitamin E and selenium status as also by the diseased states studied.

Since there was no practical method of producing heart disease in human, an animal model, using the rat, was constructed to throw more light on the involvment of selenium as glutathione peroxidase in the heart. The study of effect of starvation on the activity of the enzyme in some tissues of the rat indicates that the activity was significantly increased in heart tissue of starved rats as compared to the fed ones. The activity of the enzyme in liver, kidney, lung and intestinal mucosa was not affected by starvation. These observations could not be explained by this study but the increase in activity of glutathione peroxidase in heart may be due to increase in synthesis rate of the enzyme at molecular level or lower turnover rate of the enzyme molecules due to dietary restrictions. Further studies such as effect of starvation on demand and synthesis rate of the enzyme at molecular level might help to explain these observations.

The animal study also show that the selenoenzyme activity in rat heart was located more with cytosol than with the mitochondria. This agrees with earlier work by Holmes and Coworkers (27) on the glutathione peroxidase activity. However complete starvation for 48 hours was also observed to increase

significantly, the activity of the selenoenzyme in cytosol (by 62.5 percent) without any significant effect on its mitochondrial activity. This shows that the overall increase noticed in rat heart during starvation was due to increase cytosol activity of the enzyme. This again could not be explained but may be connected with hormonal regulation of the enzyme activity. However, this is opened to further investigations but suggests that the selenoenzyme (therefore selenium) is involved in the normal functioning of the heart in rat. It is difficult to assume that these observations in rat are what obtains in Man as they may differ from animal to animal until further investigations proof otherwise.

The following recommendations may however throw more light on the involvment of vitamin E and selenium in healthy and diseased conditions (especially heart muscle diseases) in man.

Firstly, more subjects, both healthy and those with heart muscle diseases from different localities should be assessed for selenium and vitamin E status. The results obtained from such a wide study will be more acceptable and throw much light on importance of these nutrients in the body.

Secondly, the diet which the heart muscle diseased patients are being fed on in hospitals should be analysed for vitamin E and selenium in such further studies on the involvement of the nutrients in heart muscle disease. This would give a clue as to whether adequate selenium and vitamin E are being provided for such subjects. Thus indicating whether the low level of these nutrients in the subjects was due to restricted dietary intake.

A third important recommendation for further research on this work, is to collect data on the amount of these nutrients in the local food being eaten by the subjects in their respective areas of abode before being caught with the disease. This will show whether in the first case, enough of these nutrients is available to the people of the area.

Fourthly, efforts should be made to see if oral supplementation of vitamin E and very small amount of selenium compound, to heart muscle disease patients in hospitals will remove or alleviate symptons of heart diseases. This, of course, will require the co-operation of the medical staff in the hospitals.

Finally, if positive results are obtained from all these studies then we can start to think of extending such studies to involve other cardio vascular disease.

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APPENDIX A

TABLE VIII: Erythrocyte Hemolysis (%) and Erythrocyte Glutathione Peroxidase Activity in individual subjects with Heart Muscle Diseases.

Sample Number	Soure of Patients*1	Diagnosis*2	Age (Yr) Sex*3	Erythrocyte Hemolysis (%)*5	Glutathione Peroxidase Activity*6 Units*4 x 10^2
60	THK	Heart failure	45, M	22.6	4.3
61	"	"	50, M	10.7	5.0
62	"	"	32, M	5.1	5.0
74	THZ	"	55, M	14.8	4.6
90	THK	"	30, M	3.8	2.5
93	"	"	43, M	10.3	2.1
98	"	"	28, F	5.6	3.6
101	"	"	45, M	18.1	3.8
115	"	"	35, F	15.2	2.6

Table VIII contd.

67	THZ	Congestive Heart failure	55, F	15.5	7.0
69	"	"	50, F	18.2	3.7
71	"	"	43, M	9.3	0.4
91	THK	"	26, M	4.1	2.5
96	"	"	55, M	15.5	3.0
92	"	"	35, M	12.3	2.6
64	THZ	Hypertensive Heart failure	56, M	18.9	1.1
68	"	"	50, F	14.0	1.1
73	"	"	57, M	17.7	0.4
88	THK	"	40, F	9.2	3.0
94	"	"	51, M	15.2	1.2
97	"	"	30, F	8.8	1.1
70	THZ	Post Partum Heart failure	30, F	17,6	1.1
75	"	"	30, F	17.7	1.1

Table VIII contd.

89	THK	"	40, F	7.9	0.7
95	"	"	32, F	9.4	1.8
65	THZ	Constructive Pericarditis	22, M	19.9	3.7
102	THK	"	53, M	14.6	2.7
63	THZ	Valvular Heart disease	15, M	13.5	2.8
66	"	Rheumatic Heart disease	15, F	17.0	1.1

*[1] THK Teaching Hospital Kaduna (Ahmadu Bello University).
THZ Teaching Hospital Zaria (Ahmadu Bello University).

*[2] As reported by specialist consultant.

*[3] M-Male, F- Female.

*[4] Unit of activity is Umoles of GSH oxidized per minute per ml of erythrocyte.

*[5] Normal range - 0 - 10%

*[6] Normal range - 3.0 - 5.7

APPENDIX B

Ascorbic acid: Vitamin E and selenium have been described in the text to act as reducing agents preventing oxidants like hydrogen peroxide from attacking cell membrane. Since ascorbic acid also acts physiologically as a reducing agent, plasma level of this water soluble vitamin was also analysed in the human subjects used in this study, to explore any possible metabolic interactions of these nutrients. The method of Roe and Kuether (51), as adapted by Lowry and Coworkers (31) was used in which 2, 4 dinitrophenyl hydrazine is allowed to form a stable coloured complex measurable at 515 nm with dehydroascorbic acid in presence of sulphuric acid (concentrated).

A standard curve (Fig. 8) of ascorbic acid concentration versus absorbance was used to evaluate the sample of blood plasma, deprotenised with 5% trichlroacetic acid and subsequently treated alike. The results obtained are shown in the folloing Table IX.

TABLE IX: Plasma ascorbic acid in control and Unhealthy subjects used.

State of Health	Number of subjects	Plasma Ascorbic Acid (mg/100 ml)			
		Mean	Standard Error	95% confidence Interval	Difference
Control	45	0.70	0.07	0.56-0.84	NOT Significant
Heart Muscle Disease	30	0.79	0.07	0.65-0.93	do
Hypertensive or Diabetic	15	0.71	0.08	0.55-0.87	do
Pregnant or Lactating	15	0.67	0.09	0.49-0.85	do
Liver disease	15	0.72	0.11	0.51-0.93	do
Malnutrition	15	1.15	0.25	0.66-1.64	do
Kidney disease	15	0.63	0.13	0.36-0.89	do

a Kwashiokor and Marsmus.

Comments: All the subjects, irrespective of their health state had mean plasma ascorbic acid level in the acceptable normal range (31) i.e. (0.5 to 1.5 mg/100 ml). Even in case of patients with heart muscle disease, the level was 0.79, while the group revealed reduced vitamin E and selenium status. Data on individual subjects (not shown above) indicated normal plasma ascorbic level even when erythrocyte hemolysis (%) was as high as 22.6. Within the limited scope of this study, ascorbic acid status did not seem to influence vitamin E and selenium status in the human subjects studied.

Fig 3 Standard curve for the estimation of glutathione

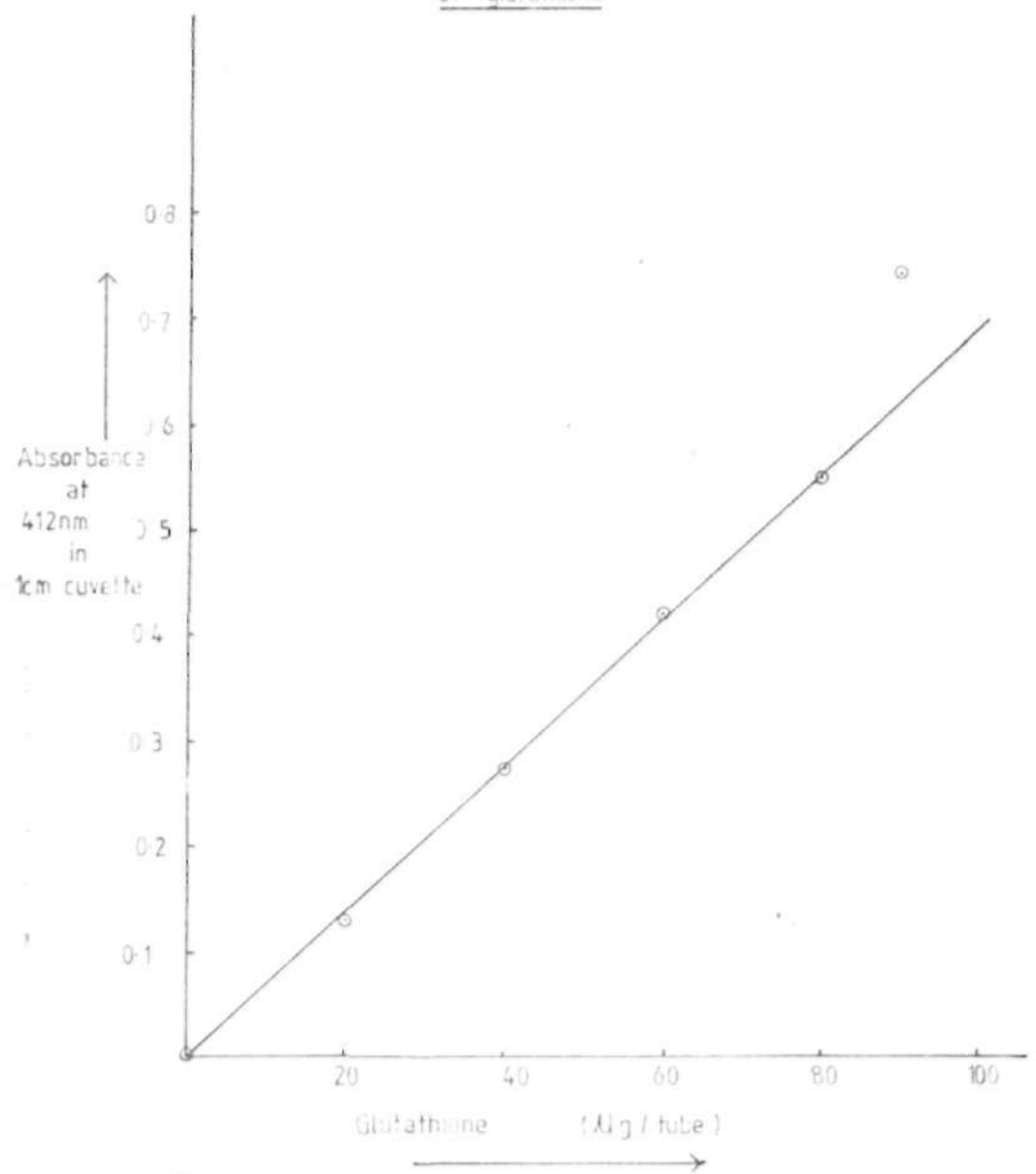

Fig 7 Standard curve for estimation of protein.

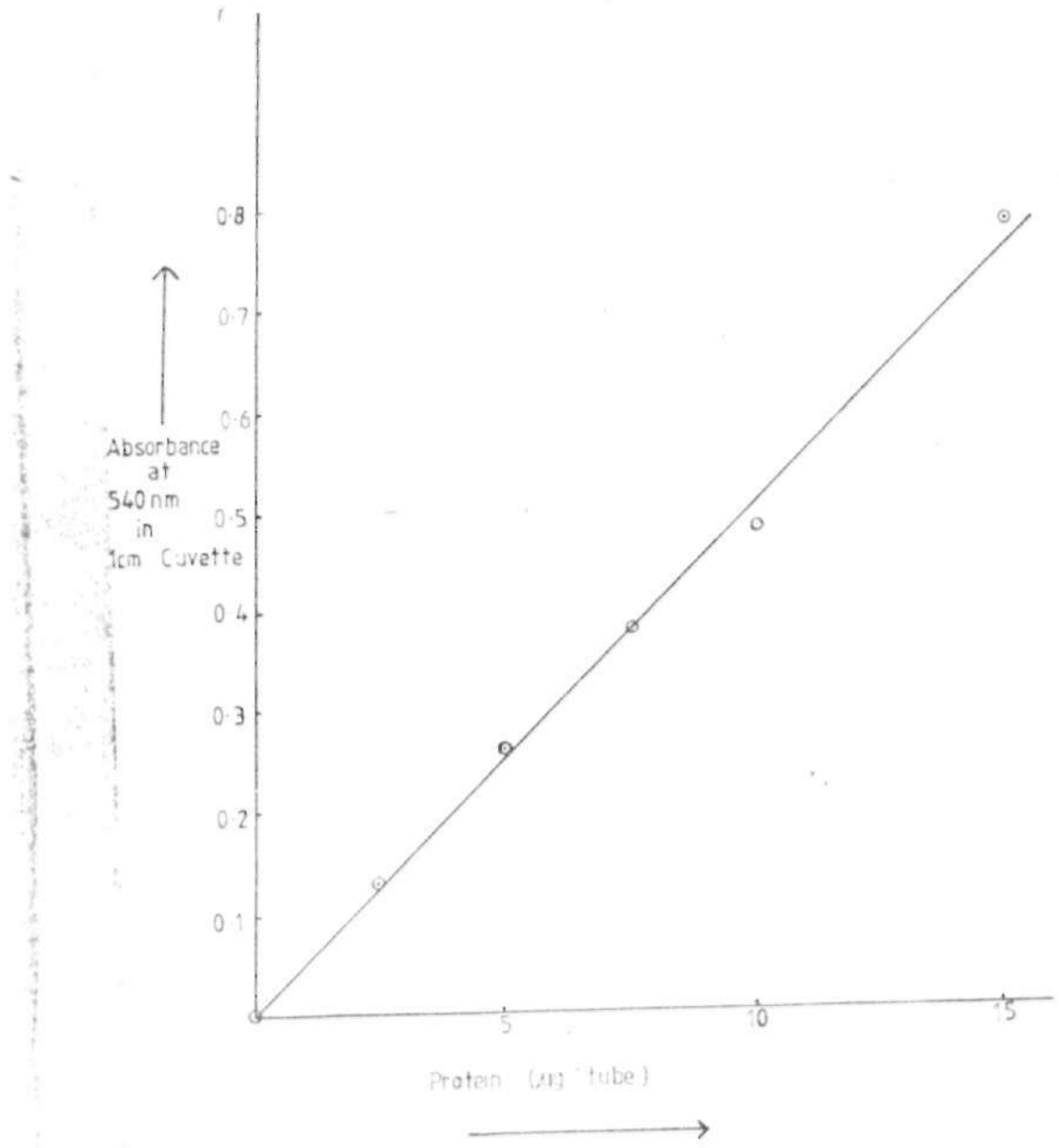

Fig 8 Standard curve for the estimation of ascorbic acid