BEHAVIOUR OF GLUTATHIONE IN PRESERVED HUMAN BLOOD

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science in Biochemistry.

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August, 1966.

This thesis is affectionately

dedicated to my parents.

PREFACE

The present study represents part of a larger research on blood preservation which has been carried on continuously under the direction of Professor O.F. Denstedt of the Department of Biochemistry at McGill University since 1939 at the beginning of the Second World War. The accumulated evidence, although circumstantial, indicates beyond doubt that the loss of viability of stored erythrocytes is due to the depletion of ATP as a result of progressive failure in glycolysis (1, 2). The particular facet of this research programme assigned to the present author is the investigation of the role, if any, of glutathione in the metabolism of the mature erythrocyte and its relationship to energy metabolism or to the maintenance of the structural integrity of the erythrocytes during storage. The investigation was prompted by the knowledge that glutathione is a cofactor bound to the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (3). Some investigators have reported that the activity of this enzyme as well as that of glucose-6-phosphate dehydrogenase decreases with aging of the erythrocytes (4). Both these enzymes are known to be associated with glutathione metabolism in the red cell. Glutathione is also a specific cofactor of the enzyme glyoxalase which catalyzes the interconversion of methylglyoxal (pyruvic aldehyde) and DL-lactic

acid. While this enzyme in the red cell is stable, its function, if any, in cell metabolism is not yet known. The relatively high concentration and the remarkable constancy of glutathione in the erythrocyte suggest its possible involvement in a regulatory mechanism in cell metabolism. This view is strengthened by the evidence that the GSH level in the blood is changed in various diseases and on administration of the adrenocortical, thyroid, pituitary, pancreatic, and parathyroid hormones (5). The lowered level and the increased instability of glutathione in certain hemolytic disorders (6) further suggest the involvement of glutathione in the metabolic processes of the cell. Alivisatos (7) in our laboratory observed that the glyoxalase activity of erythrocytes preserved in isotonic citric medium at $5^{o}C$ decreased rapidly when the endogenous glucose became depleted during storage of blood. He suggested that the decrease in enzyme activity may be attributable to the decreased content of reduced glutathione in the preserved blood. Fessler (8) incubated preserved human erythrocytes with glycine-C^{14}, cysteine, and glutamic acid and demonstrated the synthesis of radioactive glutathione by the cells. Francoeur (9) has demonstrated that both NADPH and NADH can serve as cofactors of glutathione reductase in human erythrocytes. Brownstone (10) provided further evidence that the pentose phosphate pathway, which is responsible for the reduction of NADP in

the mature human erythrocyte remains active during storage of the blood in ACD medium. Many authorities (11, 12, 13, 14, 15) consider NADPH to be important in maintaining the integrity of the red cell primarily because it is required to keep glutathione and hemoglobin in the reduced state and essential for the stability of glucose-6-phosphate dehydrogenase. The presence of glutathione reductase and the pentose phosphate shunt suggests that glutathione plays some kind of protective role in the red cell. Mathai (16) has noted a decrease of total glutathione (GSH + GSSG) and the appearance of oxidized glutathione (GSSG) in citrated human blood when the endogenous glucose became depleted during storage.

The present thesis concerns an investigation undertaken to follow up Mathai's study on the behaviour of glutathione in preserved human blood stored at $5^{o}C$. Much of the earlier work reported in the literature concerning the estimation of glutathione in the blood and other tissues has become open to criticism owing to the lack of specificity of the methods used. The author has endeavoured to refine the methods and to extend Mathai's study of the changes that occurred in glutathione in the erythrocytes during preservation of blood in the cold and to ascertain the fate of glutathione. A detailed account of experimental procedure is included. The study of the methods

used and their application to follow up previous findings by Mathai form the subject of this thesis. The role of glutathione in cell metabolism during blood storage is discussed in the light of the present results.

ACKNOWLEDGEMENTS

I should like first to express my gratitude to my research director, Dr. O.F. Denstedt, Gilman Cheney Professor of Biochemistry, for his constant encouragement, inspiring and catalytic guidance and other help given to me all the way throughout the period of my graduate work. It has been mainly through his encouragement and support that this study has been accomplished. It has been a privilege and my good fortune to have known and learned from Professor Denstedt.

I am deeply grateful also to the Honourable Guy Favreau, the Honourable John N. Turner, the Rev. Fr. A. Sillery, and the Rev. C. Miller, for their kind help through a difficult period at the beginning of my graduate study.

To Dr. R. Johnstone, Dr. D. Rubinstein, Dr. H.S. Tan, and my laboratory colleagues and friends, I should like to express sincere thanks for advice and constructive criticism on various occasions during our happy association in the past three years.

My sincere appreciations are also acknowledged to Mrs. Gertrude Hartmann for her interest and care in typing this thesis, and to Mr. Joseph Cassoff for technical assistance

during the past summer.

Finally, I am grateful to the Defence Research Board of Canada for financial support during my graduate study, from a grant to Professor Denstedt, and to the Blood Depot of the Canadian Red Cross Society in Montreal, for materials supplied from time to time during the study.

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LIST OF ABBREVIATIONS

ACD	acidified citrate dextrose
ATP	adenosine triphosphate
pCMB	para-chloromercuribenzoate
DDT	dichlorodiphenyltrichloroethane-1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane
2,3-DPG	2,3-diphosphoglycerate
DPN, DPNH	diphosphopyridine nucleotide and reduced DPN
FAD	flavin adenine dinucleotide
G-6-P	glucose-6-phosphate
GSH	reduced glutathione
GSSG	oxidized glutathione
H_2O_2	hydrogen peroxide
HPO_3	metaphosphoric acid
Hb	hemoglobin
Km	Michaelis constant
M., M	molar concentration, mole
R.P.M.	revolutions per minute
R.C.F.	relative centrifugal force
NAD,NADH	nicotinamide adenine dinucleotide, reduced NAD
NADP, NADPH	nicotinamide adenine dinucleotide phosphate, reduced NADP
6-PGl	6-phosphogluconic acid
RBC	red blood cell

SH	sulfhydryl
SFH	stroma free hemolyzate
TCA	trichloroacetic acid
TPN,TPNH	triphosphopyridine nucleotide, reduced TPN
Tris	trihydroxymethylaminomethane
BAL	British Anti-Lewisite

INTRODUCTION

The widespread interest in the metabolism and enzymology of the erythrocyte in the past 25 years has been stimulated by the need for efficient methods for the preservation of blood. To achieve this, it is necessary to know the nature of the biochemical and physical changes that are responsible for the loss of viability and ultimate breakdown of the cells during storage. In the field of blood preservation the interest in glutathione arises out of evidence that it appears to play an important role in the maintenance of stability and viability of the erythrocytes. Thus Fegler (17) obtained evidence that the degree of hemolysis induced by exposure to oxygen appears to be related to the decrease in the reduced glutathione (GSH) content and that the latter protects the cells against oxidative damage. Evidence that glutathione, added to hemolyzate preparations, prevented methemoglobin formation was reported by Morrison and Williams (18) as long ago as 1938. The finding has been confirmed by various authors (19, 20, 21, 22). In 1957, Klebanoff (23) showed that incubation of red cells with adenosine or inosine, both of which were found to protect the erythrocytes against hemolysis also led to an increase in the concentration of glutathione in the cells. The influence of

the intracellular SH-groups in the erythrocyte on the integrity of the cells has further been demonstrated by Benesch and Benesch (24). These workers reported that the hemolytic action of organic mercurials such as p-chloromercuribenzoate (pCMB) is not due to the denaturation of intracorpuscular hemoglobin but to the blockage of the thiol groups of the cellular glutathione.

The discovery (25) in 1926 of the hemolytic action of certain antimalarial drugs such as primaquine and the sulfonamides in susceptible individuals led to the discovery of an intrinsic biochemical defect in the erythrocytes of these persons in the form of a deficiency of glucose-6-phosphate dehydrogenase in the cells (26,27, 28, 29, 30, 31). The enzyme deficiency results in an impaired capacity of the erythrocytes to generate NADPH which is important for the maintenance of glutathione in the reduced form and the reduction of methemoglobin. When the defective red cells are challenged, as on exposure to the drugs, reduced glutathione (GSH) is oxidized and the oxidized glutathione (GSSG) no longer is readily reduced (or perhaps very slowly if NADH is available) (32, 33, 34). Also the reduction of methemoglobin is impaired and methemoglobin tends to accumulate (35, 36, 37). It is known further that the

deficient cells are low also in the reduced pyridine nucleotides, NADPH and NADH (38, 39), and contain a demonstrable concentration of the oxidized forms of these coenzymes (38,40). The administration of hemolytic drugs, or the feeding of fava beans to these susceptible individuals, results in a markedly decreased GSH concentration just prior to severe hymolytic episodes (41). There is now ample evidence that a decrease in intracellular GSH and concomitant oxidation of hemoglobin to methemoglobin are significant changes leading to the final lysis of the red cells (42, 43, 44). Thus it is of importance, in the interest of improving methods of blood preservation, to investigate the mechanisms of the changes and the manner in which GSH is involved.

Contrary to the afore-mentioned evidence and the hope of workers to discover an important role for GSH, recent studies have indicated that the inability of the erythrocytes to maintain the normal concentration of GSH apparently is not the critical condition that predisposes to hemolysis (45, 52). Nor is there convincing evidence that methemoglobin formation , or its accumulation, is an essential and important prelude to the degradation of hemoglobin or destruction of the red cells (46, 47). Notwithstanding reports of increased formation of methemoglobin in erythrocytes on long storage (48), the presence of methemoglobin in itself has not been shown to

alter the life span of erythrocytes (49). A normal erythrocyte survival time has been demonstrated for the cells of patients with hereditary methemoglobinemia whether the cells be in vivo or be transfused into a normal or a sick person (49, 50).

The demonstration of typical Heinz body hemolytic anemia, induced by injection of sulfonamides or nitrofurantoin (51) into normal subjects, indicates that other factors also play an important part in the development of drug-induced hemolysis. Evidence against the widely held view that lowering of the GSH concentration in the red cell in itself predisposes the cell to hemolysis was first demonstrated by Tsen and Collier (52). They showed that pCMB, a SH-binding agent, in relatively low concentration, could cause hemolysis by its effect directly upon the stroma with little decrease in the concentration of GSH in the erythrocyte. Subsequently, Jacob and Jandl (53) were able to show that erythrocyte destruction, both in vitro and in vivo, can be effected by blockage of membrane SH-groups alone. It would thus appear that cell stability is largely dependent upon the integrity of the membrane thiol groups rather than on the intracellular GSH.

However, whether the structural stability of the red cell during aging in vitro under conditions of storage is dependent

upon the integrity of the membrane thiol groups is not yet established. Some investigators have found that a marked decline in the activity of glucose-6-phosphate dehydrogenase and other SH-enzymes occurs during the aging of the red cells (4). Several studies have been published also on the important role of the intracellular thiols in the activity of the glycolytic enzymes in the erythrocytes (54, 55, 56, 57, 58). It is with a view to clarifying some of the unsettled points that workers in our laboratory, including the present author, have endeavoured to investigate the relationship between GSH and cellular metabolism in preserved blood during storage at 5°C.

CHAPTER ONE

BIOLOGICAL SIGNIFICANCE OF GLUTATHIONE

The presence of glutathione in all living cells whether plant, animal, or bacteria (59) has roused the curiosity of many investigators to concern themselves at one time or another with its biological significance. It is not within the scope of this thesis to review the voluminous literature on the biochemistry of GSH. The subject has been well reviewed by Colowick et al. (60), Crook (61), Knox (62), Boyer (64), and Toennies (65). The discussion which follows will be confined mainly to the literature related to the role and behaviour of glutathione in the erythrocyte.

I. A Brief Recounting of General Biological Functions of Glutathione.

Ever since the discovery of glutathione by Hopkins (66), investigators have endeavoured to ascertain the function of glutathione in cellular metabolism. Knox observed: "A glance at the indices of Chemical Abstracts, under 'GSH, effects on -' will disclose its myriads of salutary effects, most of which are nonspecific, indirect, or ill-defined. No attempt will be made to analyze these effects on biological systems. Gluta-

thione is commonly used, like a gimmick in an advertised product, as an additive to make a system better."

Out of such observations, there are, however, generally acknowledged, though poorly-understood, prototypes of glutathione function such as follows:-

(a) A specific cofactor for several enzymes (62): glyoxalase, formaldehyde dehydrogenase, maleylacetoacetate isomerase, glyceraldehyde-3-phosphate dehydrogenase, maleic acid isomerase, DDT-dehydrocholinase, certain of the enzymes in folic acid degradation, and indolypyruvic acid enol-keto tautomerase.

(b) A protective agent against alloxan diabetes (5).

(c) Its role in protecting living organisms against radiation damage (67).

(d) A promotor of cell division and cell growth (68).

(e) A protector and reactivator of SH-enzymes and hence its role in the regulation of cellular metabolism (64).

(f) A role in amino acid transfer and protein synthesis by transpeptidation (69, 70).

However, not all these functions are peculiar to glutathione. Lazarow (5) has shown that the protective effect of glutathione against experimental alloxan diabetes is a property common also

to cysteine, BAL, and thioglycolic acid and other thiol compounds. Free sulfur, sulfides, and many sulfur-containing compounds, have proven to be protective agents against radiation damage (67). In addition, in irradiated lens, SH-enzymes, for example, glyceraldehyde-phosphate and acetaldehyde dehydrogenases, glyoxalase, and GSH-reductase were affected earlier than enzymes not dependent on the thiol group. But surprisingly, hexokinase which is also SH-dependent is not affected (71). On the other hand, the role of GSH-dependent enzymes such as formaldehyde dehydrogenase and glyoxalase, has not yet been established. In former years, workers tried to find a place for glyoxalase in the glycolytic system, but without success. The view that glutathione may play a role in protein synthesis when coupled with transpeptidation has now been discredited (62, 72).

II. Enzymatic Reduction and Oxidation of GSH.

Biochemists have long tried to discover the biochemical role of the redox properties of glutathione in cell metabolism. The relatively low redox potential of GSH makes it oxidizable and reducible *in vivo* and *in vitro*. As yet the exact values of the redox potential have not been established. Measurement of the potential is beset with difficulties. In the first place, unlike thiols ascorbic acid, cytochrome c, and the pyridine nucleo-

tides, are "electromotively" sluggish systems (59). Second, the thiol compounds have the peculiar capability of forming compounds with noble metals and thus erroneous values are obtained with noble metal electrodes (59). Thus the E_o' value for the thiol group has variously been reported as -0.35 (73), -0.23 (74), and -0.14 v (75).

The ease of interconversion of the sulfhydryl and the disulfide form of glutathione led Hopkins et al. (66, 76) to surmise that glutathione probably functioned as a carrier in the respiratory chain. Other workers (59) have linked it to the activity of the sulfhydryl enzymes.

(1) Glutathione Reductase.

The disulfide form of glutathione (GSSG) can be reduced to GSH by NADH or NADPH (particularly with the latter) in the presence of glutathione reductase. This enzyme has been isolated from animal and plant tissues, yeast and bacteria. Kohman and Sanborn (77) and Ganapathy (78) were the earliest to demonstrate the reduction of GSSG by plant tissues. Later, Firket and Camhaire (79) showed that a rapid increase in the concentration of chemically detectable sulfhydryl groups occurred in dry pea seeds after hydration. Mapson and Goddard (80,81) and Conn and Vennesland (83, 84) identified the enzyme as glutathione reductase. Subsequently, Rall and Lehninger (85) isolated

a similar enzyme from yeast and liver.

The enzymes from different biological sources have somewhat different properties. Thus the reductase from E.coli is inhibited by sulfhydryl reagents (86), whereas Collier (87) observed that the enzyme from the human erythrocyte is only slightly inhibited by these reagents. The enzyme from animal tissues originally reported by Rall and Lehninger was said to have a specific requirement for TPNH (85). Francoeur and Denstedt (33) found that the reductase in rabbit erythrocytes was considerably more active with TPNH than with DPNH.

The reductase in plants is said to be specific for the hydrogen donor TPNH. There is no reaction with DPNH. From yeast and liver Krimsky and Racker (3) isolated an enzyme reaction with both DPNH and TPNH. They found furthermore that at high enzyme concentrations TPNH and DPNH are equally reactive while at low levels the enzyme was 100 times more active with TPNH. So far convincing evidence has been obtained for the existence of distinct TPNH- and DPNH-dependent enzymes. The glutathione reductases from E. coli, peas, yeast and rat tissues have been shown to be FAD-conjugated proteins (88).

A recent report by Proskuryakov and Menshikh (89) describes the formation of an extracellular glutathione reductase by

E. coli 3995 grown on protein hydrolysates. The view has been advanced that this enzyme may play a role in digestive process, perhaps in the reduction of disulfide containing peptides to a more readily utilizable form. Lately, Arrigoni and Rossi (90) have described a procedure by which glutathione reductase can be isolated from the pea plant. The enzyme can reduce S-sulfoglutathione to GSH according to the reaction:

$$TPNH + GSSO_3^- \longrightarrow TPN^+ + GSH + SO_3^-$$

This reduction doubtless will receive increasing interest in view of Waley's (91) demonstration of the natural occurrence of sulfoglutathione in the lens of the eye.

(2) Glutathione transhydrogenase.

In 1955 Racker (92, 93) described a newly discovered enzyme from liver that catalyzes the reduction of disulfides other than GSSG. The enzyme, called GSH-homocystine transhydrogenase, was considered to be specific for the reduction of homocystine, but has been shown to act also on certain other disulfides. It is not known whether there are numerous enzymes of this type, each specific for a particular disulfide, or whether most of the enzymes are capable of reacting with several substrates. Thompson et al. (94) found that yeast preparation catalyzes the reduction of a number of low molecular weight disulfides. Phil, Eldjarn

et al.(95, 96) doubt the existence of the enzyme in view of the rapidity of the spontaneous, nonenzymatic reduction of disulfides by GSH itself. At the present time these are open questions.

Perhaps the most interesting GSH transhydrogenase studied thus far is the insulin-degrading enzyme from beef liver. Narahara and Williams (97) suggested that this inactivating process involves reduction of disulfide bonds and is due to the action of a transhydrogenase coupled with glutathione reductase according to the following reactions:

$$2\ GSH + (RS)_2 \longrightarrow GSSG + 2\ RSH$$

$$2\ TPNH + GSSG \longrightarrow 2TPN^{+} + 2GSH$$

$$\text{Overall: } 2\ TPNH + (RS)_2 \xrightarrow{GSH} 2TPN^{+} + 2\ RSH$$

This particular transhydrogenase was purified to a high degree by Tomiazawa and Halsey (98) and the purified enzyme studied by Katzen and Stetten (99, 100). The latter workers established that the reaction is directed to the reduction of the disulfide. They demonstrated that all three of these linkages in the insulin molecule were cleaved with the release of the A and B polypeptide chains and with opening of the disulfide ring in the A chain (101). Oxytocin and vasopressin also were found to be substrates whereas lipoic acid,

homocystine or cystine and ribonuclease were not. Katzen et al.(100) speculated also on the possible role of this reductive cleavage of insulin in a "feed back" mechanism to control the level of insulin in the tissue.

(3) Enzymatic oxidation of GSH in plant and animal tissues.

Several enzymes capable of catalyzing the oxidation of GSH have been reported. Mapson (102) has demonstrated the presence in plants of an enzyme which catalyzes oxidation of GSH and other thiols by dehydroascorbic acid. In 1959, Mills (227) reported the presence in animal tissues of a peroxidase which catalyzes the oxidation of GSH by H_2O_2. Catalase (103), nitroglycerin reductase (104) and cytochrome-cytochrome oxidase (105) are known to catalyze oxidation of GSH. Francoeur (33) has reviewed the oxidative action of catalase on glutathione.

(4) Oxidation of GSH in human plasma.

Since 1912 a considerable number of papers have appeared in the literature reporting the presence of oxidases and peroxidases in human blood plasma. The results and interpretations by different authors are contradictory and confusing. Batelli and Stern (106) found no evidence for the presence of an oxidase in the plasma. They refuted the report that plasma can catalyze the oxidation of paraphenylene diamine.

In 1913 Neumann (107) demonstrated that plasma gave a

negative guaiac reaction with or without addition of hydrogen peroxide. He found that the reactions with paraphenylene-diamine and the Nadi reagent were indefinite and doubtful.

Hizume (108) was able to confirm Neumann's results with dialyzed plasma. He showed also that dopa and cathecol were oxidized at the physiological (pH) hydrogen ion concentration. Brocq-Rousseu and Roussel (109, 110), on the other hand, denied the existence of an oxidase in plasma.

Kwasniewski and Henning (111) demonstrated that the rate of oxidation of benzidine varied with different specimens of plasma in the presence of hydrogen peroxide. They concluded that the reaction was caused by a plasma peroxidase, which was observed to be present in an exceptionally high concentration in the plasma of certain patients with leukemia.

Agner (112) pointed out that the oxidase activity of the plasma of the horse increased during the course of immunization with diphtheria toxin. Shacter (113, 114) found a catecholase in plasma which was relatively insensitive to cyanide.

The controversy over the existence of oxidase in the plasma has prompted attempts to demonstrate glutathione oxidase in the plasma. The latest work along this line came from our laboratory. Mathai (16) in 1960, found that the oxidation of GSH

by human plasma apparently is a nonspecific and nonenzymatic reaction but is catalyzed by many proteins. Mathai (16) showed that the reaction is catalyzed almost equally strongly by plasma, albumin, globulins and purified globin. The only protein tested which did not have a catalytic action was purified gelatin. While Mathai's work was under way a similar study was carried out by Zimmerman and coworkers (115) who consider also that the oxidation reaction is nonenzymatic.

III. Glutathione in Cerebral Tissues.

(1) Occurrence, breakdown, oxidation and reduction.

Glutathione is present in the brain in concentration of 1 to 2 μMole/gm (116). The values for its content there depend on the manner of removing the tissue for analysis. According to Thomson and Martin (117), total glutathione in the cerebral tissues of the rat and the guinea pig, rapidly fixed, in situ, approximated to 3 μMoles per gram tissue. However, if the tissues were removed rapidly at room temperature, a fall occurred in the total glutathione and accompanied by an increase in the oxidized form. When the excised tissues were incubated for 2 hours the fall of total glutathione in the tissue could be prevented by the addition of a small quantity of GSH to the incubation medium; under these conditions the level of GSSG did not rise. Without the addition of GSH there

by human plasma apparently is a nonspecific and nonenzymatic reaction but is catalyzed by many proteins. Mathai (16) showed that the reaction is catalyzed almost equally strongly by plasma, albumin, globulins and purified globin. The only protein tested which did not have a catalytic action was purified gelatin. While Mathai's work was under way a similar study was carried out by Zimmerman and coworkers (115) who consider also that the oxidation reaction is nonenzymatic.

III. Glutathione in Cerebral Tissues.

(1) Occurrence, breakdown, oxidation and reduction.

Glutathione is present in the brain in concentration of 1 to 2 μMole/gm (116). The values for its content there depend on the manner of removing the tissue for analysis. According to Thomson and Martin (117), total glutathione in the cerebral tissues of the rat and the guinea pig, rapidly fixed, in situ, approximated to 3 μMoles per gram tissue. However, if the tissues were removed rapidly at room temperature, a fall occurred in the total glutathione and accompanied by an increase in the oxidized form. When the excised tissues were incubated for 2 hours the fall of total glutathione in the tissue could be prevented by the addition of a small quantity of GSH to the incubation medium; under these conditions the level of GSSG did not rise. Without the addition of GSH there

was a decrease in the total glutathione concentration during the period of incubation even under anaerobic conditions. These workers (117) attributed the disappearance of GSH to its breakdown through the action of γ-glutamyl transferase in the tissue.

In brain tissue, in vivo, glutathione exists predominantly in the reduced form. This indicates the action of the patent glutathione reductase in brain tissue. Rall and Lehninger (85) were the first to demonstrate the reduction of GSSG by brain tissues. McIlwain and Tresize (118) found the rate of reduction of GSSG by the reductase of brain to be 250-300 μMoles of glutathione per gram tissue per hour.

Correlation of the changes in the total concentration of GSG in the brain with change in the cerebral activity has been studied by different investigators. Kudryavtseva and Kudryavtseva (119) found that during electro-shock treatment the concentration of total glutathione in the brain of the rabbit increased markedly and that the proportion of the oxidized and the reduced forms also changed. Helbig (120) found in the dog that a lethal dose of Pentothal lowered the concentration of glutathione in the brain.

(2) GSH and glucose metabolism in cerebral tissues.

The metabolism of glutathione in cerebral tissue is assoc-

iated also with carbohydrate metabolism. Since glucose in the brain is generally believed to be metabolized almost entirely by way of the Embden-Meyerhof pathway, the discussion here will be confined to this aspect.

When cerebral tissues are incubated in the presence of glucose, the addition of glutathione produces an increase in the rate of aerobic glycolysis. Studies on glycolysis by Balazs and Lagnado (121) showed that glycolysis in brain mitochondria is inhibited in the presence of oxygen as a result of inactivation of the triosephosphate dehydrogenase. This observation has been considered to indicate that glutathione plays a part in the Pasteur effect (122, 123, 124). In addition, it is believed to play a role in regulating intermediary metabolism (59, 125) and in maintenance of the SH-groups on the enzymes in the reduced state and thus the maintenance of the enzymes in the potentially active form (59, 126).

McIlwain and coworkers (127) in the course of their studies on brain tissue found that there is a pronounced difference between the results obtained with cell-free and cell-containing systems. Thus, the rate of anaerobic glycolysis in a partly cell-free preparation from brain was influenced by the balance between the ratio of the oxidized to the reduced forms of glutathione. With the cell-free system, on the other hand,

the added glutathione whether in the reduced or the oxidized form, was without effect. Aldridge (128) found that the respiration of cerebral mitochondria was not influenced by the addition of reduced glutathione. His view is supported by the findings of a number of workers (129, 130, 131) who have shown that the respiratory activity in cell-free or cell-containing preparations was virtually independent of the presence of glutathione in concentrations from 0.3 to 3 mMoles.

Aerobic glycolysis has also been found to be relatively unaffected by the presence of glutathione except at relatively high concentrations. Bumm and Appel (132) and Weil-Malherbe (124) found that aerobic glycolysis, in the presence of 10 mM glutathione, was increased from the normal value of 10 to 20 μMoles of lactate/g of tissue/hr. to 40 μMoles. McIlwain (127) showed that the changes in respiration and glycolysis with high concentrations of glutathione in the tissue were associated with changes in the concentration of the inorganic and creatine phosphates which products are regarded as the main factors in regulating glucose metabolism in the tissue. How glutathione influences the change in these phosphates is not known. Furthermore, the changes can occur without appreciable change in the balance of GSSG and GSH in the tissue. Conversely, this balance can be disturbed without producing any marked effect on glucose metabolism.

IV. Glutathione and Human Diseases.

Glutathione is primarily an intracellular substance. Synthesis of GSH from the component amino acids has been demonstrated in the liver, the intestine, the adrenals, and erythrocytes (142, 134, 135, 136, 137). An enzyme system which catalyzes the transfer of the γ-glutamyl radical from GSH to other amino acids or peptides was discovered by Hanes and associates (69) and has been found isolated from sheep brain, liver, kidney and the pancreas (138). It would appear that the level of GSH in blood and other tissues reflects a dynamic equilibrium between synthesis and hydrolysis, and that there exists in the body a homeostatic mechanism which maintains a constant level of GSH in the various tissues. Variation in the glutathione level in blood and other tissues, however, is said to occur under various physiological con - ditions and in certain diseases.

Grunert and Phillips (139) found that when rats were fed a sodium-deficient diet the blood GSH was decreased by 43 per cent with an increased susceptibility to alloxan diabetes. A marked decrease in the concentration of GSH in the liver and kidney (140, 141) also has been observed in rats fed on a low-protein diet. Vitamin-C deficient guinea pigs showed a drop of 46 per cent in the blood GSH concentration and 48 per

cent in the pancreas (142). On the other hand, the GSH concentration is increased in the liver and the kidney in the rat after thyroidectomy (143) and the feeding of a diet with added thiouracil (144) or cysteine (143, 140).

These observations, however, have not been consistently observed in various tissues or in various species of animals. For instance, Waelsch and Rittenberg (133) found that guinea pigs on a diet deficient of vitamin-C showed a decrease in GSH level in the pancreas, blood, adrenals, and intestine, but not in the liver and the kidney. Houssay et al. (144) showed that feeding of a diet containing thiouracil to rats increased the GSH level in the liver and kidney, but did not alter the total glutathione in the blood. The feeding of a protein-free diet, on the other hand, has been shown by Leaf and Neuberger (140), and Edwards et al. (141) to lower the GSH content of the liver and the kidney, and raised the content of the blood. Furthermore, according to Grunert (145), induced sodium deficiency in the chicken or the guinea pig did not change blood GSH level, but lowered the concentration in the blood of the rat by 43 per cent, while that in the liver was increased by 32 per cent.

Yaoi (146), in 1927, observed that no change occurred in GSH content in the muscle of the pigeon on feeding a diet deficient in the vitamin-B complex. Arimoto et al. (147) and

Kawahara (148) in the same year observed a decrease in the total GSH content in the liver, kidney, and spleen of animals on a vitamin-B deficient diet. Similarly, various investigators have obtained inconsistent results for the action of insulin, adrenalin, anemia and diabetes mellitus on blood GSH level (5). It is believed that severe ketonuria is a complicating factor in diabetes.

In many chronic states of metabolic derangement the blood GSH level is greatly decreased. This has been observed in Addision's disease, Cushing's syndrome, hyper- and hypothyroidism, severe diabetes, liver disease, pernicious anemia, hemolytic anemia associated with glucose-6-phosphate dehydrogenase deficiency, mental disease and all forms of cataract. In acute states, on the other hand, the level in the blood tends to be increased, as for example, after insulin injection, electric shock, fever, and methionine deficiency (150). More recently, Hess (231) reported evidence of increased activity of both the NADPH and the NADH-linked glutathione reductases in the plasma in liver diseases. In 1961, Carson et al. (151) reported a case of hemolytic disorder associated with GSH reductase deficiency.

CHAPTER TWO

THE ROLE OF REDUCED GLUTATHIONE (GSH) IN THE ERYTHROCYTE

I. Biological Aspects of Red Cell Stability.

The red cell, *in vivo*, has a normal life span of approximately 120 days. This represents the interval between a cell's delivery from the bone marrow and its destruction by reticuloendothelial cells. It is believed that the senile erythrocytes disintegrate in the blood stream and that only erythrocyte fragments are disposed of by the reticuloendothelial cells. Destruction of red cells by hemolysis, *in vivo*, prior to this length of time is considered abnormal. Anemia develops when the rate of cell destruction is not compensated by replacement of the cells by erythropoiesis. Though little is known about the intimate details of destruction of the normal red cells, it is generally accepted that normal aging or the abnormal rate of destruction in hemolytic disorders is brought about by physical and chemical deterioration of the cells so that they become nonviable and unstable.

Biochemically, the mature human red cell can be considered to be composed of three components: the cell membrane, the hemoglobin, and other soluble elements including enzymes, coenzymes, and glycolytic substrates. It is evident that a single defect,

whether genetic or acquired, in anyone of these three units could alter the stability or function of the cell and predispose them to premature hemolysis, in vivo. The biophysical chemistry of hemolysis has been a subject of extensive study by many investigators. The following is a brief survey of the literature relevant to this author's study.

(1) Cellular metabolism and red cell hemolysis.

The red cell contains a large number of enzymes but some of them are known to be vestigial from the more active nucleated precursor forms and perform no essential function in the mature erythrocyte. For instance, it is known also that the mature erythrocyte does not possess an active tricarboxylic and cytochrome system for aerobic energy production by oxidative phosphorylation. Authorities agree that glycolysis and the pentose metabolic pathway are the only sources of energy production in the normocyte. The energy is stored in the form of ATP and the pyridine nucleotides. Of these pathways, glycolysis is by far the more important. About 90 per cent of the glucose is metabolized by way of the Embden-Meyerhof pathway in the normal erythrocyte and only about 10 per cent through the shunt (149).

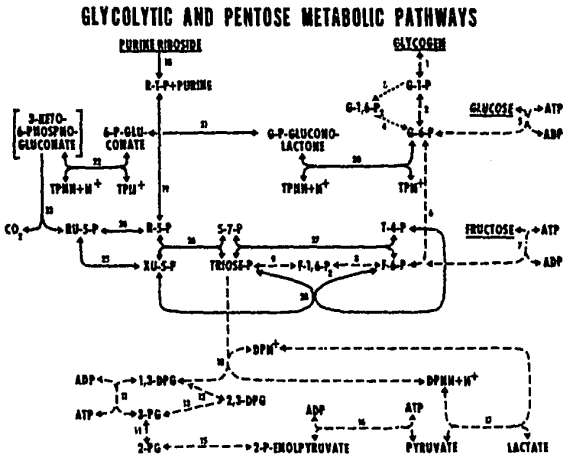
Several hemolytic disorders are known to be associated with enzyme deficiencies in the erythrocyte (Fig.1). How these defects lead to instability of the cell is not yet understood.

Legend to Fig.1.

Enzyme Deficiency in Some Hemolytic Anemias.

Hemolytic disorder	Enzyme deficiency	Metabolic impairment (Reaction in Fig.1)
Hereditary spherocytosis	Phosphofructokinase	8
Hereditary spherocytosis	Enolase deficiency	15
Congenital non-spherocytic hemolytic anemia	2,3-diphosphoglycerate mutase	14
" " "	Pyruvate kinase	16
Congenital non-spherocytic hemolytic anemia, favism, primaquine sensitivity	Glucose-6-phosphate dehydrogenase	20

FIGURE 1



Pentose metabolic pathway represented by solid lines and glycolytic pathway by broken lines.

However, the view is generally held that biochemical and biophysical changes leading to the impairment of metabolic efficiency during aging are the main factors that determine how long the cells can survive in the circulation.

(a) Red cell aging.

There is ample evidence that a general decrease in metabolic capacity does occur in the erythrocyte during aging. As indicated in Table I, Prankerd has listed some of the differences that have been observed in the chemistry of old and young cells. Lohr and Waller (4) have studied the changes that occur in the enzymes with aging in vitro and in vivo. Contu and Lenzerine (63) have reviewed the physiological and pathological alterations in erythrocyte metabolism. Kellermeyer et al. (30) claimed that hereditary deficiency of glucose-6-phosphate dehydrogenase is associated with increased sensitivity of the red cells of certain individuals to primaquine, and that the life span of the red cells varies inversely with the dose of primaquine administered. The activity of this enzyme has been found to decrease with aging of the normal cells in the circulation.

(b) Hemolytic disorders associated with red cell enzyme defects.

The following hemolytic diseases are known to be associated with intrinsic enzyme defects in the erythrocytes. These deficiencies, in turn, may arise from deficiencies or abnormalities

TABLE I

Changes Recorded in Old and Young Red Cells

Enzyme changes	
G-6-P dehydrogenase	Increased activity in young cells
6-PGl dehydrogenase	" " " " "
Triose-P dehydrogenase	" " " " "
Aldolase	" " " " "
Methemoglobin reductase	" " " " "
Glyoxalase	" " " " "
Metabolic changes	
Glycolysis	" " " " "
Oxygen utilization	Decreased " " " "
Nucleoside utilization	Increased " " " "
Components altered	
Phosphate esters	No difference
Glutathione (GSH)	No difference
Total SH	Increased activity in young cells
Lipids	" " " " "
Methemoglobin	Increased in old cells
Changes during blood storage	
Phosphate esters	Decreased breakdown of ATP and 2,3-DPG in young cells
Electrolytes	Decreased loss of potassium from young cells

Table adapted from J.W. Harris, "The Red Cell" (302).

in the hemopoietic mechanism.

(i) Hereditary spherocytosis.

This is a congenital hemolytic disorder characterized by the presence of spherocytes in the peripheral blood. The condition was first described by Valaris and Masius as long ago as 1871 (152). The premature cell destruction appears to take place largely in the spleen and as a result of an intrinsic metabolic defect which impairs the stability of the red cells . It is known furthermore that the utilization of glucose in the red cells is impaired. Prankerd et al. (153) have observed also, impairment in the ability of the red cells to phosphorylate certain intermediates, mainly connected with the formation of adenosine triphosphate and 2,3-diphosphoglycerate. They concluded (154) that the metabolic block apparently involves phosphofructokinase or enolase. Allison's hypothesis (157) that the primary defect lies in phosphatide metabolism has been criticized by Prankerd (155). Allison's findings were further investigated and discredited by Phillips and Roome (156).

(ii) Glucose-6-phosphate dehydrogenase deficiency.

This disorder is recognized as a heterogeneously ex - pressed, sex-linked enzyme defect (158, 159, 160) which has been observed among most of the human races. The subject has been reviewed by authorities in Italy (161), Germany (39) and

the United States (163). Individuals with the abnormality may be subdivided into a number of groups on the basis of clinical features and physico-chemical characteristics of the red cells. For instance, individuals with the red cell defect may be classified on the basis of both the electrophoretic pattern and physico-chemical differences such as pH optima curves, K_m value for G-6-P and NADP (Table II). The abnormality is seen in individuals with Primaquine sensitivity, favism, or congenital non-spherocytic hemolytic anemia (164, 165). The metabolic defect has now been identified as deficiency of glucose-6-phosphate dehydrogenase. In the case of hereditary nonspherocytic hemolytic anemia it is now known that most of the patients have a major deficiency of glucose-6-phosphate dehydrogenase, accompanied by lesser deficiency in pyruvate kinase (166), triosephosphate isomerase (168), 2,3-diphosphoglycerate mutase (167, 169), glutathione reductase (151, 162) and adenosine triosephosphatase (176). It would appear, therefore, that the impairment of the cells arises from a multiple rather than a single deficiency.

(iii) Hereditary elliptocytosis.

This disorder usually presents a symptomless hematological curiosity. Dacie et al. (171) reported that about 12 per

TABLE II*

Heterogeneity of Properties of G-6-P Dehydrogenase Among Different Population Groups

Ethnic or geographic group	Enzyme activity [a] % of normal		Substrate affinities (Km)		pH Optima	Stability[b]	Electrophoretic[c] mobility
	Red cells	White cells	G-6-P	TPN			
Negro males	10-15	Normal or slightly decreased	Normal	Normal	Normal	Decreased	A-
Barbieri, males (Northern Italian)	50	Normal	Slightly increased	Increased	Normal	Normal	Fast
Sicilian	1-4	Decreased	Slightly increased	Increased	Normal	Decreased	Unknown
Sardinian	1	Decreased	Unknown	Unknown	Unknown	Decreased markedly	Fast
CNSH(Caucasian)	3-9	Unknown	Increased	Increased	"Symmetrically narrow"	Decreased markedly	Unknown
CNSH(Caucasian)	1	Decreased	Increased	Normal	Higher	Decreased markedly	Unknown
Sephardic Jew	3-6	Decreased	Slightly decreased	Slightly decreased	"Broad"	Decreased	Normal

* Table according to P.A. Marks (301)

Abbreviations

CNHS - Chronic, nonspherocytic hemolytic anemia

Km - Michaelis constant

G-6-P - Glucose-6-phosphate

TPN - Triphosphopyridine nucleotide

a Enzyme activity is expressed in per cent of the mean normal level in red cells or white cells.

b Stability of G-6-P dehydrogenase during incubation of hemolyzates in the presence of stroma.

c Electrophoretic mobility in starch gel.

A- Refers to type of G-6-P dehydrogenase and follows nomenclature of Boyer.

cent of the individuals show mild hemolysis without anemia. De Gruchy (172) divided the cases into three groups: (1) those with no sign of hemolysis, (2) those with hemolysis but without anemia, and (3), those with hemolytic diseases along with anemia. They found also that in most of the individuals the cells showed impairment in glycolytic activity.

(iv) NADH diaphorase deficiency (Hereditary methemoglobinemia).

Methemoglobinemia may be of two kinds: hereditary and acquired. The hereditary type may be further divided into two groups: one, associated with the presence in the cells of abnormal methemoglobin, Hb-M, and the other, with inability of the erythrocytes to maintain hemoglobin in the reduced (ferro) state.

Gibson (173) has shown that methemoglobin in the normal erythrocyte can be reduced by two pathways which are dependent respectively, on NADPH and NADH for enzyme function. It is obvious that in erythrocytes deficient of glucose-6-phosphate dehydrogenase one of the consequences is impaired reduction of methemoglobin via the NADPH pathway. In fact Brewer et al.(37) have utilized this abnormality of methemoglobin reduction as a basis for a test of glucose-6-phosphate dehydrogenase deficiency. However, an occasional individual with glucose-6-phosphate de-

hydrogenase deficient cells shows no aberration of methemoglobin reduction (177). The NADPH-linked pathway remains dormant unless an agent such as methylene blue is present to oxidize the reduced coenzyme. Gibson (173) suggested that hereditary methemoglobinemia represents a deficiency of the NADPH-dependent diaphorase. This view was confirmed directly by means of enzyme assays. However, Eder et al. (174) reported normal values for plain adenine dinucleotide which is an essential component of diaphorase. Recently, Scott (175, 176) has purified the NADH diaphorase from the red cells of a patient with hereditary methemoglobinemia. He showed that the enzyme involved, in its heat stability and pH optimum differed from that in normal erythrocytes. He concluded that the NADH diaphorase from normal cells is a mixture of two enzymes and that in hereditary methemoglobinemia one is lacking.

(v) Congenital Heinz-body anemia.

Gasser (178) described congenital Heinz-body anemia in premature infants who developed hemolytic jaundice in the first few days of life. There is experimental evidence that lowered catalase activity is associated with increased Heinz-body formation.

(2) Interrelation between abnormal hemoglobin and hemolysis.

(a) Hemoglobinopathy.

Hereditary abnormalities in the type of intracorpuscular hemoglobin are invariably associated with a shortened red cell life span. To what extent, if any, the peculiarity in the hemoglobin is directly responsible is not yet known. As a rule the abnormality in hemoglobin is accompanied also by other anomalies. The literature on the subject has been reviewed by numerous authorities (179, 180, 181, 182, 183, 184). Reviews of the physicochemical, genetic and clinical effects of hemoglobinopathies also are available (185, 186, 187, 188). From the genetic aspect, the determinant for the abnormal type of hemoglobin appears probably to be an allele of the genes for hemoglobin-A (189). The abnormal hemoglobins differ in relation to solubility and electrophoretic mobility in the Tiselius apparatus and on paper.

Among the hemolytic disorders associated with abnormal hemoglobin are: sickle cell anemia, methemoglobinemia (Hb-M), hemoglobin-C disease, and the thalassemias. These may be complicated by associated disorders. Some of these are listed in Table III.

(b) Methemoglobin formation in relation to aging of the red cells.

There is evidence that the concentration of methemoglobin

TABLE III *

Genotypes and Phenotypes of Various Hemoglobinopathies and Traits.

Disease or Trait	Genotypes	Hemoglobins detected
Sickle-cell diseases		
Classical sickle-cell anemia	SS	S+F
Sickle-cell thalassemia	AS Th th	S+(A)+F
Sickle-cell Hb C disease	SC	S+C+(F)
Sickle-cell Hb D disease	SD	S+D
Sickle-cell Hb E disease	SE	S+E
Sickle-cell Hb J disease	SJ	S+J
Sickle-cell Hb N disease	SN	S+N
Thalassaemias		
Thalassaemia Major	AA Th Th	A+F+A_2
Thalassaemia Minor	AA Th th	A+(F)+A_2
Sickle-cell Thalassaemia	AS Th th	S+(A)+F
Hemoglobin C Thalassaemia	AC Th th	C+(A)+F
Hemoglobin D Thalassaemia	AD Th th	D+(A)+F
Hemoglobin E Thalassaemia	AE Th th	E+F
Possible thalassaemias		
Hemoglobin H disease	not clarified	A+H+(F)
Hemoglobin QH disease	not clarified	A+Q+H
Lepore trait	A Lepore	A+Lepore+F
Non-sickling, non-thalassaemic diseases		
Hemoglobin C disease	CC	C+(F)
Hemoglobin D disease	DD	D+(F)
Hemoglobin E disease	EE	E+(F)
Hemoglobin M disease	AM	A+M
Asymptomatic traits		
Sickle-cell trait	AS	A+S
Sickle-cell Hb P trait	SP	A+S+P
Hemoglobin C trait	AC	A+C
Hemoglobin D trait	AD	A+D
Hemoglobin E trait	AE	A+E
Hemoglobin G trait	AG	A+G
etc., etc.		

* According to C.J.C. Britton (303)

increases during aging of the red cells in the circulation. Waller et al. (48) in his study of transfused red cells reported that a progressive increase in methemoglobin concentration in these cells occurred after 40 days in the circulation and amounted to a level of 8 per cent after 80 days. These authors attribute the increase to a progressive decline in the metabolic activity of the transfused cells during aging in the circulation and particularly to the decrease in ability of the cells to maintain hemoglobin in the reduced state. Bonsignore et al. (190) and Jaffe (191) observed a deficiency of methemoglobin reductase activity in glucose-6-phosphate deficient cells.

Lemberg and Legge (192) suggested that methemoglobin is an intermediate in the pathway leading from hemoglobin to sulfhemoglobin or choleglobin. Webster (193) observed that increased methemoglobin formation is associated with the drug induced hemolytic anemias. Harley and Mauer (194) observed that the destruction of hemoglobin invariably is preceded by methemoglobin formation when human erythrocytes are incubated with mild oxidizing agents. Janal et al. (195) also considered that methemoglobin formation is a necessary step in the destruction of hemoglobin. More recent studies by Beutler and his associates (46, 47) do not support this view. Jaffe (196) observed that

the concentration of methemoglobin in the red cells did not increase during spontaneous hemolysis of human erythrocytes in vivo, thus contradicting the claim of Harley and Mauer (194). Hurley et al. (50) also considered methemoglobin formation to be an incidental occurrence and that the presence of methemoglobin in the cell does not prejudice its life span in the circulation. This conclusion was based on the further observations that the erythrocytes of patients with hereditary methemoglobinemia, attributable to an enzyme defect, had a normal life span in the patients' circulation and also after transfusion into a normal recipient (50).

Jung (197), in 1949, had observed a higher concentration of methemoglobin in osmotically more fragile, and presumably the older erythrocytes than in the younger red cells. Beutler and Mikus (198), on the contrary, failed to demonstrate a difference in methemoglobin in cells of various ages. These workers, furthermore, and contrary to the observation of Löhr (199), found no difference between young and old cells in the capacity to reduce methemoglobin. Hence the question whether methemoglobin is an essential step in the degradation of hemoglobin under normal conditions or just an incidental occurrence remains open.

(3) Changes in the cell stroma in relation to hemolysis.

The stroma in the red cell forms the structural framework (200) within and on the inner surface of the plasma membrane. The evidence to date indicates that the stroma is lipoprotein in character (201, 202, 203) and is associated with a number of enzymes. The ATPase activity of the cell has been shown by several workers (205, 206, 207) to be associated with the stroma. Likewise, aldolase activity of the cells is mainly in the stroma (208). The ATPase of the cell membrane is believed to control the transport of Na^+ and K^+ across the membrane (209). Other factors in, or properties of, the membrane have been shown capable of changing the activities of red cell enzyme known to be important in hemolysis. Ramot and coworkers (210) reported an activator of glucose-6-phosphate dehydrogenase in normal stroma but not in defective cells. They subsequently suggested that deficiency of glucose-6-phosphate dehydrogenase in primaquine sensitive cells is attributable to the lack of an activator component.

Thus it appears evident that certain stromal components play a role in cellular metabolism of the red blood cell. In the following the correlation of stromal shape and membrane SH-groups to hemolysis is discussed.

(a) Apparent relationship between cell shape and stability.

The determination of osmotic fragility curves as a means

of characterizing various hemolytic anemias is a commonly used hematological procedure to distinguish whether cell fragility in a given blood disorder is attributable to an abnormal state of the cell membrane or to other factors. That the shape of the corpuscles may be influenced by (or, at least, is often associated with the state of) a cellular component is evidenced in the distortion in shape seen in blood disorders associated with various abnormal hemoglobins. There are, however, some types of hereditary hemolytic anemia in which no abnormal cellular component has yet been identified, the shape of the red cells being the only demonstrable abnormality. The spherocytes of congenital spherocytosis (212) and the elliptocytes of hereditary elliptocytosis, fall into this category. In the "immune" types of hemolytic anemia, which is the least understood, the primary defect appears to be in the cell membrane (213).

(b) Role of membrane SH-groups in the maintenance of cell integrity.

Jandl and Jacob (53) considered the membrane SH-groups to play an important role in the maintenance of integrity and stability of the erythrocyte. They treated red cells with a non-hemolytic, non-sphering concentration of p-chloromercuribenzoate (pCMB). This reagent is an SH-inhibitor to which the cell mem-

brane is impermeable. They observed a consequent loss of K^+ from the cell accompanied by gain of Na^+ and water and increase in cell volume, finally resulting in hemolysis. These effects occurred unaccompanied by any change in the concen- t ration of the intracellular glutathione or in the glycolytic activity of the cells up to the time of dissolution of the cells.

Likewise, Weed, Eber and Rothstein (214) demonstrated that exposure of red cells to a small amount of primaquine caused membrane injury and resulted in impaired cation gradient across the membrane without interference with the metabolic activity of the cells. The same results were obtained with glucose-6-phosphosphate dehydrogenase deficient and with normal red cells. Tsen and Collier (52) also have shown that the hemolytic action of various SH-binding agents can be attributed to their action on the SG-groups of the stromal proteins rather than on the free cellular GSH.

(4) Interrelationship among the three cellular components.

In the human erythrocyte, the three components (hemoglobin, stroma, and the soluble fraction containing many of the enzymes) are intimately related. Warburg and his coworkers (229, 230) have shown that many of the metabolic processes - glucose utilization, methemoglobin reduction, and others - of

the red cell depend on the integrity of the cell.

The stromal membrane is considered to be continuous with the cytoplasm of the cell in that it contains cytoplasm and many of the enzymes present in it. These materials can be removed from the stroma by repeated washing with saline. There is a large body of evidence that the permeability of the cell membrane to ions and various substances is controlled by the metabolic potential and activity of the cell. Blostein and Rubinstein in our laboratory (232) showed that the metabolic control of red cell permeability to ions is virtually lost at $5^{o}C$.

Anderson and Turner (215) consider on the basis of their work that hemoglobin is present also in small concentration in the red cell membrane, although contradictory evidence has been reported by others (216, 217).

(5) The involvement of GSH in red cell stability.

In the human red cell, GSH metabolism, glycolysis and the pentose metabolism form a single system in several respects. First, GSH is the substrate of GSH reductase which requires NADP or NAD in the reduced form for activation. These reduced cofactors are generated respectively in the pentose and the glycolytic pathway. During the reduction of GSSG, NADPH is oxidized to NADP which is the required cofactor of the G-6-P

dehydrogenase and 6-phosphogluconic dehydrogenase. The NAD regenerated in the reduction of GSSG can be utilized in the glycolytic system. Thus the three systems are coordinated. Second, Rappoport and Scheuch (218) and Scheuch et al. (219) have reported that several of the enzymes in the erythrocyte require the protection of GSH. Among these are glyceraldehyde -3-phosphate dehydrogenase, pyrophosphatase, hexokinase, glutathione reductase. Thus, GSH is associated with triose-phosphate-dehydrogenase. Disulfides are known to inhibit hexokinase. Since hexokinase is the rate-limiting enzyme in the Embden-Meyerhof and the shunt pathways, any accumulation of GSSG could seriously inhibit the glucose utilization and energy metabolism of the cell. GSH is an essential cofactor of the enzyme glyoxalase which catalyzes the conversion of pyruvic aldehyde to lactic acid. While this enzyme system is potentially active in the erythrocyte there is no evidence of its involvement in the metabolism of the cell.

The involvement of glutathione in red cell metabolism is evident from the circumstance that many of the hemolytic disorders are associated with specific defects in the GSH and carbohydrate metabolism of the cells. Thus in G-6-P dehydrogenase deficiency the concentration of GSH in the red cells is low and the GSH is unstable (6). Furthermore, the GSH reductase

activity is increased (220). The presence of an increased concentration of GSSG in primaquine-sensitive negroes has been observed by Szeinberg and Chari-Birton (221). Oort, Loos and Prins (222) in 1960 described four cases of hereditary absence of GSH in the erythrocytes associated with chronic non-spherocytic hemolytic anemia. In the same year Carson, Brewer and Ickes (151) described a condition of glutathione reductase deficiency in a Caucasian patient whose red cells showed a pronounced susceptibility to hemolysis. Later, a similar case was described by Löhr and Waller (169). Apparently, however, in this patient's red cells both the glycolytic and the pentose phosphate pathways remained active.

There is evidence also that GSH protects hemoglobin from oxidation in the red cell. A type of methemoglobinemia associated with glutathione reductase deficiency and a low concentration of GSH in the red cells has been reported by Townes and his coworkers (223, 224). Jandl et al. (20, 195) have investigated the protective role of GSH in preventing the oxidation of hemoglobin, in vitro, under the influence of certain drugs. They showed that oxidation of GSH preceded the formation of methemoglobin and sulfhemoglobin by the action of acetylphenylhydrazine. Increasing the amount of GSH in the system was found to protect the hemoglobin against oxidation. Harley and Mauer

(225, 194) confirmed the findings of Jandl. In the same connection the isolation of an enzyme, GSSG peroxidase, from erythrocytes by Mills (226, 227, 13) is of particular interest. Cohen and Hochstein (228) suggest that H_2O_2 plays a critical role in drug-induced hemolysis and that GSH, GSH peroxidase, coupled with G-6-P dehydrogenase and NADPH constitute a system for the destruction of H_2O_2. They conclude that the GSH peroxidase in the erythrocyte plays a more important role than catalase in protecting hemoglobin and other compounds or groups against oxidation by H_2O_2. Mathai in our laboratory (16) has studied the stability, behaviour and fate of GSH in human blood (red cells) during preservation in the cold. Her observations are discussed in the following sections.

II. Behaviour of Glutathione in Preserved Citrated Blood During Storage.

Until recently there has been little evidence of the change in the concentration of glutathione in blood during preservation. In contrast to the observation of Alivisatos (7) and Szeinberg et al. (233), Blanchaer and coworkers in 1955 (234), Palek and Volker (235), in 1964, observed a decline of the concentration of GSH in ACD-preserved blood during storage. More recently, Guntherberg and Rademacher (236) have reported the

observation of a decrease in the content of GSH accompanied by an increase in the GSSG level in preserved blood during storage. The fall in GSH content was not compensated quantitatively by the increase in the concentration of GSSG. The total glutathione content (GSH + GSSG) decreased to about 50 per cent on storage for 8 weeks. Mathai (16), in our laboratory, observed a decline in the concentration of total glutathione in preserved citrated blood during storage in the cold. In view of the possible role of GSH in the protection of sulfhydryl-dependent enzymes such as hexokinase, glucose-6-phosphate dehydrogenase, and glyceraldehyde dehydrogenase, and hence its possible importance in the maintenance of the glycolytic mechanism in the preserved red cell, this topic has been studied further.

Mathai preserved human (venous) blood in 1.8% sodium citrate medium at 5°C and observed the following changes in blood glutathione. First, the total concentration of glutathione (reduced and oxidized) in the preserved blood remained almost unchanged until the endogenous glucose became depleted. Thereafter, it decreased rapidly both in the whole blood and the red cells. Second, in the beginning of storage, concurrent with the progressive fall of glucose content, the concentration of oxidized glutathione (GSSG) in the whole blood increased slow-

ly, but more rapidly after the depletion of glucose, reaching a maximum by about the 18th to 22nd day. Thereafter, it steadily decreased. Third, oxidized glutathione was detected only in the whole blood. None of the oxidized form was found in the specimens of washed red blood cells prepared periodically from the stored sample during the storage period of approximately 50 days. The behaviour of the glutathione in the experiment is indicated in fig. 2.

It is known that changes in the stability and permeability of cell membrane can occur when the energy metabolism of t he red cell is impaired, or retarded by lowering the temperature. The presence of GSSG in the whole blood and its absence from the red cells led Mathai to assume that after glycolysis in the stored blood ceased, glutathione escaped into the plasma either as a result of alteration in the permeability of the cell membrane or progressive hemolysis of cells. She found that GSH is readily oxidized in contact with plasma or other proteins. Mathai suggested also that the decrease of total glutathione could be attributed to "hydrolysis" by the action of γ-glutamyl transferase in the erythrocyte.

Mathai's observations have raised certain points of in-

Figure 2

Behaviour of Glutathione in Citrated Blood During Storage at 5°C.

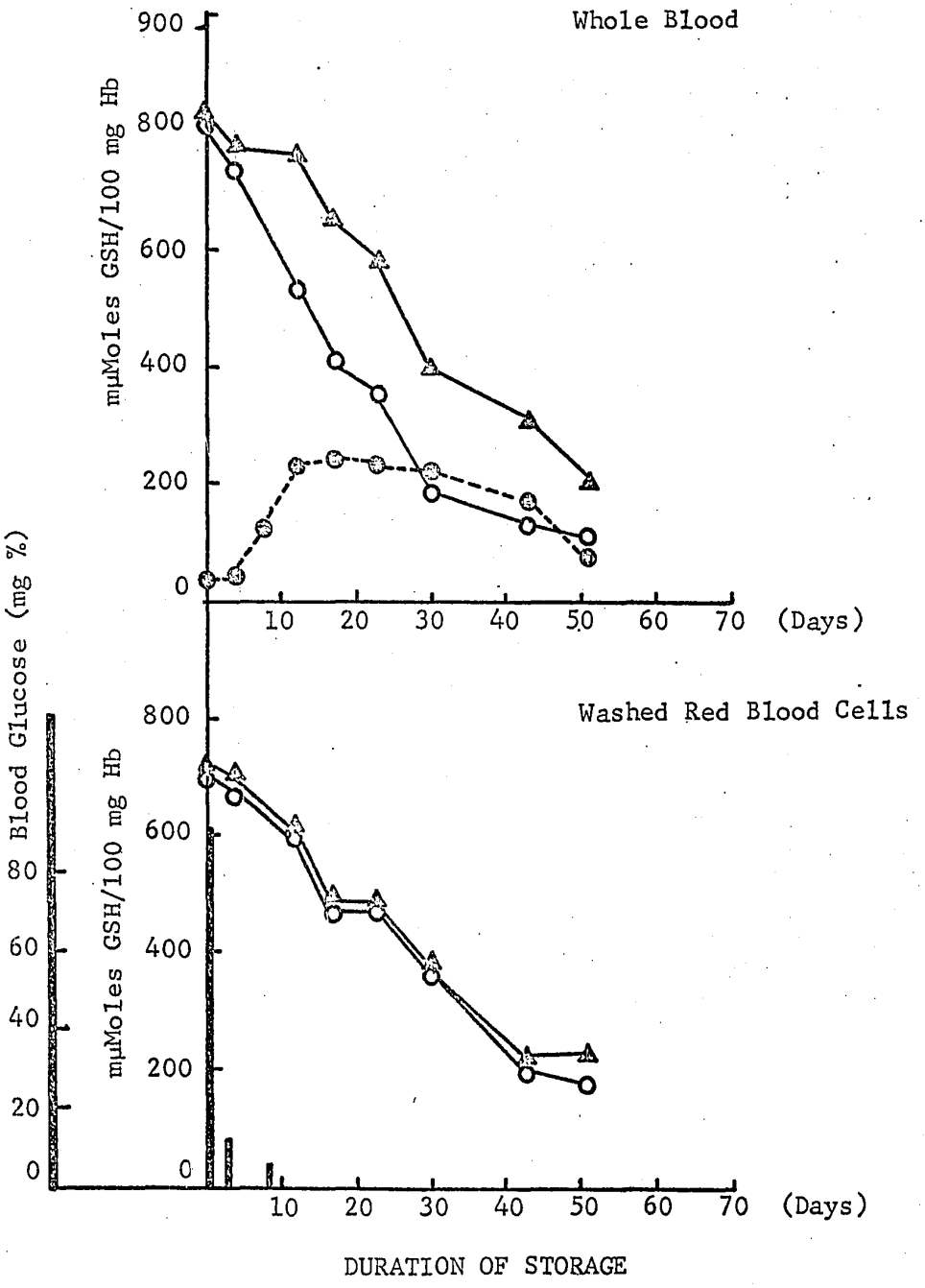
* Taken from Mathai's thesis (16).

Total glutathione

Reduced glutathione

Oxidized glutathione

Figure 2


Whole Blood
mμMoles GSH/100 mg Hb
900
800
600
400
200
0
10
20
30
40
50
60
70
(Days)
Washed Red Blood Cells
mμMoles GSH/100 mg Hb
800
600
400
200
0
10
20
30
40
50
60
70
(Days)
Blood Glucose (mg %)
80
60
40
20
0
DURATION OF STORAGE

terest. In the first place, there is as yet no evidence that the stroma of the erythrocyte is permeable to glutathione. Jowett and Quastel (237) demonstrated that GSH cannot enter the red cell. Confirmation of their results has been obtained by a group of Polish workers (238). It is possible, however, that in Mathai's work the change of membrane permeability of the erythrocyte might occur in the absence of glucose and in the cold. Furthermore, swelling of the erythrocytes due to the hypotonicity of the anticoagulant, trisodium citrate solution (1.8%) might result in increased permeability of the stroma thus facilitating the escape of GSH. On the other hand, swelling of the red cell does not always increase the cell permeability. Thus Ponder (239) reported that the surface area of the erythrocyte does not increase during small (9%) increases in cell volume. Solomon (240) claimed that swelling of the membrane itself under such conditions would result in compression of the pores to a smaller diameter and thus decrease permeability of the membrane. When the cells are swollen to the limit, however, the pore size may be enlarged. It is unfortunate that Mathai did not measure the amount of hemoglobin in the plasma of the samples taken from the preserved specimen during storage, since this would have indicated whether the GSH

was liberated from the red cell by hemolysis. In the experimental section of this thesis further comments on Mathai's findings are given in the light of the writer's study.

In 1957, Fegler (17) reported that spontaneous hemolysis of erythrocytes is related to the presence of oxidized GSSG in the cell. He found that hemolysis occurred when 40 per cent of the intracellular GSH became oxidized. It is reasonable to assume that the absence of GSSG in saline-washed red cells, as studied by Mathai, was probably explainable by hemolysis during the washing of the cells containing GSSG. Since hexokinase has been suggested as the rate-limiting step of glycolysis and since it is said to be inhibited by disulfide (219), the question arises whether GSSG may be formed in the erythrocytes under Mathai's conditions of storage.

Having been unsuccessful in accounting for the gradual disappearance of GSSG in older specimens of preserved blood Mathai has offered the suggestion that the observed progressive disappearance of glutathione content of the stored cell may be attributable to the action of γ-glutamyl transferase. The presence of this enzyme in the erythrocyte was described by Rouser *et al.* (82) in 1956. Whether this enzyme is present also in the plasma has not been reported but it would appear unlikely. There is also no evidence whether this enzyme will

act on the γ-glutamyl groups in oxidized glutathione. Thus the progressive disappearance of GSSG, as reported by Mathai, and further confirmed in our laboratory, remains to be explained.

While there is general agreement among workers as to the level of reduced glutathione in blood, there is disagreement about the occurrence of oxidized GSSG in erythrocytes. The disagreement appears to have arisen owing to the lack of a specific and reliable method for the determination of oxidized glutathione. In Mathai's work, oxidized glutathione was estimated by the use of two methods, namely, the method of Dohan and Woodard which involves electrolytic reduction of the GSSG, and the alloxan 305 method. The electrolytic method has been criticized by various workers (see Introduction to methods).

In the following pages the author will describe efforts to check and to extend Mathai's findings and to improve the reliability of the method for the estimation of oxidized glutathione. Attempts have been made to investigate especially the decrease of GSH content and the production of GSSG in the stored blood.

E X P E R I M E N T A L

CHAPTER ONE

GENERAL METHODS

I. Analytical.

(1) Estimation of glucose.

Glucose was determined with the glucose oxidase method using the commercial reagent, Glucostat kit, obtained from the Worthington Biochemical Corporation, Freehold, New Jersey, and according to the procedure described in the pamphlet provided with the reagent. Glucose standards were prepared in 0.25 per cent benzoic acid and kept in the refrigerator.

(2) Estimation of hemoglobin.

(a) Cyanmethemoglobin method.

For whole blood and washed red cell suspensions, hemoglobin was determined with the cyanmethemoglobin method as described by Miale (241). A solution of cyanmethemoglobin standard was used as reference. This standard was provided by courtesy of the Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada.

(b) Benzidine micromethod for the estimation of hemoglobin.

Small amounts of hemoglobin in the plasma were estimated with the benzidine method as modified by Crosby and Furth (242). Care was taken that all the glassware was free of sulfate. The benzidine reagent was stored in a brown bottle to safeguard against deterioration. In the procedure, blood was added to the benzidine reagent and the hydrogen peroxide added last. Wu (243) who originated this method stressed that low values were obtained when the sequence was changed. A colour change through green to purple is regarded as positive result whereas a direct change to the purple colour without first turning green is taken as a negative test (244). A good review concerning the sensitivity of various methods for the estimation of minute amounts of hemoglobin has been published by Hanks et al. (274).

(3) Estimation of methemoglobin and sulfhemoglobin.

Methemoglobin and sulfhemoglobin were estimated according to the method of Miale (241).

II. Chromatographic procedures.

(1) Chromatographic separation of cysteine, glycine, glutamic acid, reduced and oxidized glutathione.

Standard solutions (10 mg per cent) of reduced and oxidized glutathione and glycine, cysteine and glutamic acid in

0.2 M NH_4HCO_3 buffer solution at pH 7.4 containing 1/15 M N-ethylmaleimide (NEM), were prepared. A solution containing a mixture of 10 mg of each of the above mentioned substances on 100 ml of the same medium also was prepared. The solutions were then applied to Whatman No.1 paper (11x11 inch sheets) and chromatographed in air-tight chambers by means of the two-dimensional ascending technique and with the solvent mixtures described below. The chromatograms were run for 12 hours in each direction. Finally, the sheets were dried in air and the chromatograms developed by spraying with 0.5 per cent ninhydrin in acetone solution.

The composition of the solvents finally adopted after trials with several systems, were as follows:

System No.1: Mixture of isopropanol, formic acid, and water in the proportion of 65 : 1 : 34 (v/v).

System No.2: Mixture of n-butanol, formic acid, water, and 95 per cent ethanol in the proportion of 47 : 1 : 29 : 23 (v/v).

(2) Separation of "products of hydrolysis" of oxidized or reduced glutathione after incubation with plasma or hemolyzate of washed red blood cells.

Specimens of plasma prepared from ACD-preserved blood and of the stroma-free hemolyzate, prepared from KCl-washed red

cells, were incubated with added quantities (0.4 mg per ml incubation medium) of oxidized or reduced glutathione at 37°C for 3 hours. At the end of the period the proteins were precipitated with 10 per cent trichloroacetic acid (TCA) and removed. The filtrate was evaporated to dryness by lyophilization. The residue was then taken up in a small amount of 1/15 M N-ethylmaleimide solution. The resulting solution was chromatographed according to the procedure described above.

III. Preparations.

(1) Collection of blood.

Blood was collected without the aid of reduced pressure into isotonic (3.2 per cent) solution of trisodium citrate, and in special experiments, into hypotonic (1.8 per cent) trisodium citrate in the proportion of four volumes of blood to one volume of the anticoagulant medium. In the majority of instances glucose was not included in the anticoagulant medium, but in others, the ordinary ACD medium was used. ACD blood of various storage ages was kindly supplied from time to time by the Canadian Red Cross Society Blood Depot in Montreal. When blood samples were required, the red cells were resuspended carefully by slowly repeated inversion of the

bottle for twenty times and samples were then withdrawn aseptically with a syringe and a No.18 needle.

(2) Hemolyzates.

(a) Hemolyzed whole blood: Whole blood was diluted with three times its volume of distilled water and hemolyzed by freezing and thawing twice.

(b) Stroma-free hemolyzate: The hemolyzate of saline-washed red cells was centrifuged in the cold for 30 minutes at 10,000xg. in an International refrigerated centrifuge. The upper clear supernatant was removed with a Pasteur pipette and constituted the stroma-free hemolyzate (SFH).

(c) Stroma: The remaining residue (stroma) in the preparation of SFH was washed several times with isotonic (0.154 M) potassium chloride until the washings were free of hemoglobin by visual observation. The washed stroma was then suspended in potassium solution to the original volume.

(3) Red blood cell suspensions.

Citrated whole blood, in 50-ml. siliconized heavy-walled centrifuge tubes was centrifuged at 3000 r.p.m. in the cold ($5^{\circ}C$) for 15 minutes. This was carried out in a Model PR-I International refrigerated centrifuge with head No.269 (radius 21 cm.). It is known that erythrocytes can be packed to a

constant volume at various speeds and that the packed volume and the trapped plasma are not the same for each speed. The ideal centrifugal force for the sedimentation of red cells is considered to be about 2260xg. The speed (in r.p.m.) required to give this gravitational force with a rotor of a given diameter may be calculated according to the following equation:

$$\text{R.C.F. (in g.)} = 1118 \times 10^{-8} \times R_1 \times N^2$$

$$= 284 \times 10^{-7} \times R_2 \times N^2$$

where

R_1 = radius of centrifugation in cm.
= distance from shaft to bottom of centrifuge tube.

R_2 = radius of centrifugation in inches

N^2 = r.p.m. (revolutions per minute)

After centrifugation, the plasma including the white-cell layer (the buffy coat) was removed by aspiration. The packed cells were resuspended in cold isotonic KCl or NaCl to the original volume and the sample was again centrifuged at the same speed, the process being repeated twice. After the final washing the cells were then resuspended in isotonic (0.154 M)

constant volume at various speeds and that the packed volume and the trapped plasma are not the same for each speed. The ideal centrifugal force for the sedimentation of red cells is considered to be about 2260xg. The speed (in r.p.m.) required to give this gravitational force with a rotor of a given diameter may be calculated according to the following equation:

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KCl or NaCl solution to give the original volume. By using siliconized centrifuge tubes hemolysis of erythrocytes during the washing treatment was reduced to a minimum.

CHAPTER TWO

SPECIAL METHODS AND RESULTS

I. Study on the Estimation of Reduced Glutathione (GSH) in Blood.

(1) Introduction - Review of existing methods.

Patterson and Lazarow (245, 246) have surveyed the commonly used techniques for the estimation of reduced glutathione. Among the methods at present available are: the iodometric method (247, 248, 249), the silver-nitrate titration method (250), and others involving the use of nitroprusside (251, 252), arsenophosphotungstic acid (253), phospho-18 -tungstic acid (254), potassium ferricyanide (255), naphthoquinone sulfonate (258, 259), alloxan ("305" method) (256), the glyoxalase procedure (257), and the more recently introduced bis-(p-nitrophenyl) disulfite method (271). However, all these methods are satisfactory only for the estimation of reduced glutathione in aqueous solution.

With biological materials, only the alloxan and the glyoxalase methods are regarded satisfactorily specific and reliable since the biological systems frequently contain interfering substances such as ascorbic acid, cysteine, ergothioneine or others (260). More recently, Thomson and Martin (117) have estimated GSH in animal tissues with the iodometric titration method, the nitroprusside method, and the glyoxalase method and found these procedures equally suitable. Other workers also have used various methods for the estimation of GSH in blood and with good agreement. On the other hand, in the estimation of the oxidized or the total glutathione in erythrocytes by various workers there are large discrepancies. Oxidized glutathione cannot be detected with any of the aforementioned reagents. Usually, GSSG is estimated from the difference between the GSH measurements before and after application of a suitable reduction procedure. The reduction of the GSSG has been carried out with zinc dust (261, 248, 262, 263), magnesium (264, 265), sodium amalgam (266), H_2S (249), sodium cyanide (267), hydrogen gas at 38°C(268), electrolytically (269) and enzymatically with glutathione reductase (83, 80, 85, 270). All these methods, however, are subject to error, in most cases because of the incomplete reduction of GSSG.

In the estimation of glutathione in the red cell, since nearly all of the glutathione is present in the reduced form, the oxidized glutathione, if present, is only a small fraction of the total. Consequently, even under favourable conditions, the error in the determination of oxidized glutathione can be very large. For instance, if the GSH estimation, before and after reduction, has an error of two per cent, and if the true GSSG content is 4 per cent of the total, the value for the GSSG will have an average relative error of 70% according to this calculation:

$$\frac{\sqrt{2^2 + 2^2}}{4} \times 100 = 70\%$$

Another source of error arises from the spontaneous re-oxidation of reduced glutathione by oxygen in the air during the assay procedure. It is well known that GSH undergoes autoxidation in neutral, alkaline, or in slightly acid medium. Other discrepancies may arise from the various methods of preparation of the protein-free filtrate. This, along with the difference in the methods of estimation can lead to wide differences in the results. Thus the accurate estimation of GSSG is beset with many difficulties.

The writer's predecessor, Mathai, estimated the oxidized glutathione by first reducing the GSSG by electrolytic reduct-

ion according to the method of Dohan and Woodward (269) and then estimated the total GSH with the alloxan "305" method as modified by Kay and Murfitt (276). Mathai,who used both methods for the estimation of GSSG,had established the specificity and reliability of the modified alloxan method by comparison with the glyoxalase method. However, she did not test the reliability of the combined procedures by performing recovery experiments with added known quantities of GSSG.

Furthermore, Kay and Murfitt reported that their modified alloxan "305" method gave from 78 to 93 per cent, with a mean of 86 $\pm$ 4%, recovery of GSH added to a hemolyzate of whole blood. They offer no explanation for the low recovery. In their work, they used 10 per cent trichloroacetic acid (TCA) solution for preparation of the protein-free filtrate. The use of TCA has been criticized by Gabbe (272), Gulland and Peters (273) and Schelling (265) on the ground that it gives incomplete extraction of GSH. Bierich and Kelle (275) noted that TCA favours the oxidation of a small amount of GSH. Pirie (277), on the other hand, in 1930 found that mixtures of serum proteins and GSSG gave TCA filtrates which contained GSH.

Likewise, various workers have obtained variable results also with the electrolytic method for reduction of GSSG. Dohan

and Woodward (269), the authors of this method, claimed recoveries of 98 to 104 per cent of GSSG added to tissues. Bhattachonya and Stewart (278) obtained recoveries of 78-98 per cent under one condition and 97 to 103 per cent under another. Flanagan et al. (42) obtained recoveries of only 83 to 87 per cent and suggested, as an explanation, that the commercial sample of GSSG from Schwartz Laboratory was not pure. Variable recoveries of 88 to 98 per cent were obtained also by Miller and Horiuchi (279). Benesch and Benesch (280), who used a desalter for the electrolytic reduction procedure, attributed the discrepancy to the presence in the GSSG preparation of 2 moles of ethanol. Thomson and Martin (117) found that the electrolytic method gave quantitative reduction of GSSG with consistent recoveries of 91.4 ± 4.9 per cent.

In view of the high variability in the results obtained by the various workers the writer found it necessary to re-examine both the electrolytic method and the alloxan method modified by Kay and Murfitt to ascertain the causes of the variability of these assay methods in the estimation of small amounts of GSSG.

(2) Critical examination of the method of Kay and Murfitt.

Reduced glutathione in blood was estimated according to the method described by Kay and Murfitt as modified by Mathai, who found that 4.5 per cent metaphosphoric acid is preferable to 10 per cent trichloroacetic acid as the reagent for deproteinization.

(a) Reagents.

(i) 4.5% metaphosphoric acid.

This reagent, in the form of pellets, was obtained from Fisher Scientific Company. This 'Reagent Grade' material is a mixture of HPO_3 and $NaHPO_3$.

(ii) 0.24 M phosphate buffer pH 7.6 at 25°C, was prepared by mixing 0.24 M Na_2HPO_4 and 0.24 M KH_2PO_4 in the ratio of 88 ; 12 (v/v). The solution was stable at room temperature.

(iii) Standard GSH solution.

GSH was commercially obtained from Nutritional Biochemicals Corp., Cleveland, and Sigma Chemicals Inc., St. Louis. A stock solution was prepared by dissolving 10 mg GSH in 10 ml HPO_3 (2.25%). From this stock, standard solutions of various concentrations were prepared by dilution with the same acid. The stock solution, if frozen, remained stable

for at least two days.

(iv) 0.1 M glycine.

(v) "Neutralizing" NaOH series.

This reagent was prepared by mixing 0.24 M phosphate buffer prepared above with 1 N. NaOH (219 : 21 v/v). From this stock, solutions of various pH values between pH 10.3 and pH 11.8 were prepared.

(vi) Alloxan reagent.

10 mg of alloxan monohydrate were dissolved in 10 ml of dilute HCl (1 drop conc. HCl per 100 ml of H_2O). The reagent was prepared fresh for each run and was used promptly.

(3) Preliminary experiments with Kay and Murfitt Method. (276)

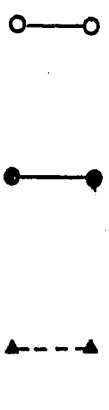
In the following experiments the alloxan method, modified by Kay and Murfitt, and further by Mathai,(16) was studied with standard solutions of GSH. The validity of the method was later established by recovery experiments. The details of the procedures as used by the writer are given in the following paragraphs.

(a) Influence of the concentration of hydrogen ion and salt.

Aqueous solutions of GSH undergo slow spontaneous oxidation in air (oxygen). Oxidation can be retarded by keeping the solution at a low temperature and at an acid pH. Fig.3 indicates the influence of the hydrogen ion and the salt con-

Figure 3

Influence of pH and Salt Concentration on Estimation of Reduced Glutathione.

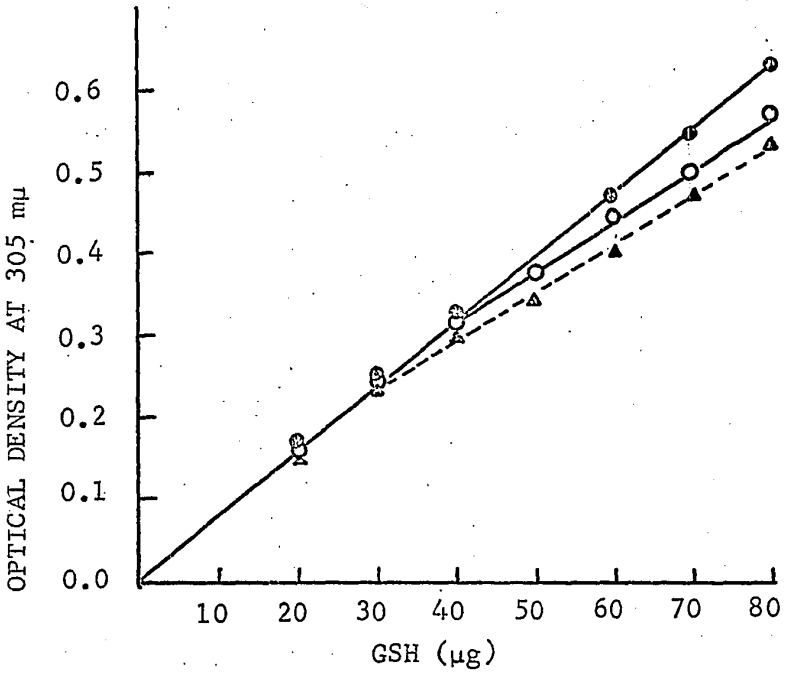


Standard solution of GSH prepared in distilled water.

Standard solution of GSH prepared in 2.25 per cent metaphosphoric acid.

Standard solution of GSH prepared in 2.25 per cent metaphosphoric acid saturated with NaCl.

Figure 3


OPTICAL DENSITY AT 305 mμ
0.0
0.1
0.2
0.3
0.4
0.5
0.6
10
20
30
40
50
60
70
80
GSH (μg)

centration on the calibration curves obtained with standard solutions of GSH. Only with standard solutions prepared with metaphosphoric acid was it possible to obtain good proportionality between the glutathione concentration and the optical density of the solution at 305 mμ. It was found further that the solution remained stable for at least two hours, whether kept at room temperature or at 37°C. The low results obtained with simple aqueous solutions were presumably attributable to oxidation of GSH by oxygen (air). With the standard solutions of GSH saturated with NaCl, the absorption peak at 305 mμ (shown in fig. 4) was found to be lower with concentrations of GSH larger than 40 μg in 6 ml of the assay system. In the procedure for the estimation of blood glutathione, NaCl was used with acid precipitant to ensure complete protein precipitation. By using only the linear portion of the curve, accurately reproducible results were obtained with the procedure.

(b) Influence of the concentration of NaCl.

The tendency of the presence of NaCl to lower the optical density of the solution was studied over a range of concentrations. As indicated in Table IV, the depressant effect of the NaCl on the reaction of GSH with alloxan as indicated by the decrease in the optical density became significant only

Figure 4

Absorption Curves of the GSH-Alloxan Complex

Curve (a) : In 2.25 per cent metaphosphoric acid.

Curve (b) : In 2.25 per cent metaphosphoric acid saturated with NaCl.

Figure 4

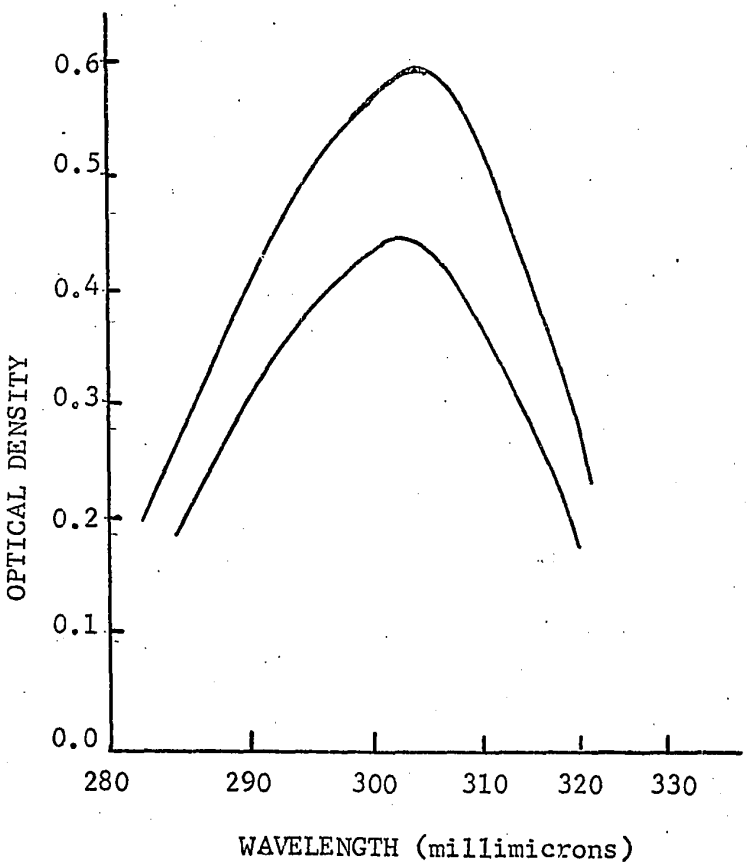

0.6
0.5
0.4
0.3
0.2
0.1
0.0
OPTICAL DENSITY
280
290
300
310
320
330
WAVELENGTH (millimicrons)

TABLE IV

Influence of NaCl Concentration on the Optical Density of the Medium.

Concentration of NaCl (mg/ml)	Trial Number 1	2	3	4	5	Mean
	(Optical density at 305 mμ)*					
0	.60	.60	.65	.63	.63	.62
100	.60	.63	.63	.62	.63	.62
200	-	-	.60	.58	.60	.62
300	-	-	.56	.58	.56	.57
400	.54	.54	.57	.56	.56	.55
800	.53	.55	.55	.55	.56	.55
1000	-	-	.55	.56	.56	.55

* Quantity of GSH used for determination, 80 μg in HPO_3 solution.

at concentrations higher than 0.3 g NaCl per ml of solution.

(c) Influence of the hydrogen ion concentration and the presence of oxygen on the reaction in the presence of NaCl.

Table V indicates also that the depressant effect of NaCl on the optical density was not attributable to oxidation of the GSH, since reduction of the solution by the electrolytic method of Dohan and woodward did not result in increased absorption. Nor did saturation of the solution with NaCl affect the rate of reaction between GSH and alloxan (Table VI). Further, the effect could not be attributed to change in the hydrogen ion concentration of the assay system, since this had been carefully adjusted to pH 7.6.

(d) Recovery experiment.

Even though the suppressant effect of NaCl was not understood, GSH added to whole blood could be almost quantitatively recovered (97%), provided that the precipitation step was carried out promptly after the addition of GSH. Table VII shows the influence of delay in estimation after the addition of GSH.

(4) Procedure finally adopted for estimation of GSH in Blood.

Four (4) ml of whole blood or washed red blood cell suspension were laked with an equal volume of distilled water

TABLE V

Estimation of a Given Quantity of GSH* Before and After Exposure to Electrolytic Reduction

Trial number	Without reduction treatment	After reduction treatment
	(Optical density at 305 mμ)	
1	.53	.53
2	.53	.52
3	.54	.53
4	.53	.52

* Quantity of GSH used for estimation,

80 μg in HPO_3 saturated with NaCl.

TABLE VI

Influence of the Presence of NaCl on the Rate of Reaction Between GSH and Alloxan

Time of reading (minutes)	Without NaCl* (Optical density at 305 mμ)	With NaCl**
5	.61	.47
10	.64	.55
15	.64	.55
20	.63	.54
25	.62	.53
30	.61	-
35	.60	-
40	.58	.49

* 80 μg GSH in HPO_3

** GSH in HPO_3 and saturated with NaCl

TABLE VII

Influence of Delay on Estimation of GSH Added to Whole Blood

Time (in minutes)	GSH recovered (%)
0	97
10	80
20	78
90	63

Reduced glutathione was added to specimens of whole blood. After standing at 25°C for various periods an aliquot was removed for estimation of GSH.

in a 50 ml Erlenmeyer and further hemolyzed by freezing in a bath of CO_2 ice and alcohol mixture, and then thawing by immersion of the flask in lukewarm water, repeating the process once. Then the hemolyzate was immediately deproteinized with 8 ml of 4.5% HPO_3. The mixture was swirled for 2 minutes to obtain an even precipitate. Complete deproteinization was achieved by adding approximately 6 grams of NaCl to the mixture which was then shaken vigorously for another 2 minutes. It was often impossible to obtain a perfectly clear filtrate without the addition of sodium chloride to saturate the medium (hemolyzate and metaphosphoric acid), an observation also made by Henry (282). The mixture was then filtered through a Whatman No. 1 filter paper. Since the commercial metaphosphoric acid was a mixture of HPO_3 and $NaPO_3$ it was a considerable buffer system by itself. According to the Henderson-Hasselbach equation the pH value is affected by the pK: $pH = pK + \log \frac{B}{A}$. But the value of pK varies with the ionic strength of the buffer system since according to the Debye-Hückel equation:

$$pK = pK_o + (Z-1) \times \frac{-i}{1+ i}$$

where:

pK_o = pK at infinite dilution

pK_e = corrected pK

Z = change of the acid anion

i = ionic strength of the solution

A , B = actual concentrations of the acid and conjugate base in the solution

Thus the saturation of the acid with NaCl alters the pK value of the system and consequently lowers the pH. The presence of NaCl in high concentration thus promotes more complete precipitation of the protein. The quantity of filtrate so obtained was sufficient for the estimation of reduced and total glutathione. When GSH alone was to be estimated, half the amount of filtrate was prepared.

A portion (0.4 ml) of the filtrate was used for estimation of GSH according to the procedure described. A solution of "neutralizing" NaOH of suitable pH value was used for adjustment of the pH to 7.6.

Spectrophotometric readings were made in duplicate using matched silica cuvettes and a Beckman DU spectrophotometer. The readings were taken at wavelength of 305 mμ and at a slit width of 0.46 mm.

II. Estimation of Oxidized Glutathione (GSSG) In Blood.

(1) Introduction.

Theoretically, oxidized glutathione (GSSG), when converted to GSH and estimated with the alloxan method, should give approximately the same analytical value as an equal weight of GSH, since, according to the equation given below, 1 gram of GSH and 1 gram of GSSG give about the same number of moles of GSH., to react with alloxan:

$$GSSG + 2H^{+} + 2e \xrightarrow{red'n} 2GSH \xrightarrow{alloxan} \text{"305-comp'd"}$$

Thus, one mole of GSSG gives 2 moles GSH. Hence, 1 gram GSSG (mol.wt. = 612.6) will give 1/612.6 (or 1.632) μmoles of GSSG or 3.264 (2 x 1.632) μmoles of GSH.

On the other hand, 1 gram GSH (mol.wt. = 307.33) is equivalent to $\frac{1}{307.33}$ or 3.254 μmoles of GSH. Nevertheless, the results obtained indicated that when commercial GSSG was determined by this method, it yielded only 81.1 per cent of the theoretical quantity of an equal amount of GSH (See Table VIII and fig. 5 below).

(2) Reagents.

(i) sulfosalicylic acid 4 per cent solution

(ii) KCl salt bridges (34% KCl in a 4% solution of agar) made of glass tubes 6 to 8 mm in diameter.

(iii) Mercury. Pure mercury was obtained commercially or by distillation. The mercury used in our procedure periodically was cleaned by washing with dilute nitric acid and then with distilled water according to the procedure described in the Precautions (see page 91).

(iv) GSH and GSSG were obtained from the Sigma Chemicals Inc. or the Nutritional Biochemical Corp. Standard solutions were prepared with 2.25 per cent HPO_3. In the frozen state the reagent was stable for at least 5 weeks.

(3) Apparatus and operational procedure.

The apparatus for the electrolytic reduction of GSSG was patterned after that described by Dohan and Woodward (269). Five (5) ml of GSSG solution in 2.25 per cent HPO_3 were reduced over a mercury cathode in a 50 ml beaker (4.6 cm diameter) at 30 ma. for 15 minutes. The reduction was complete in 9 minutes. The D.C. current was supplied from a power supply. The GSG produced by reduction of the oxidized glutathione was determined with the alloxan method previously described.

(4) Preliminary experiments with the Dohan-Woodward electrolytic method for reduction of GSSG.

(a) Results of preliminary analysis.

In a trial with the electrolytic reduction method, it was found that commercial GSSG when estimated by this method yielded only 81.1 per cent of the theoretical value. The actual values obtained are recorded in Table VIII and represented in fig. 5. One would suspect, therefore, either that the commercial samples of oxidized glutathione were impure, as has been suggested by other workers (280, 42), or that the reduction procedure does not give complete reduction of the GSSG. In the writer's study, attention was given to various precautions stressed by other workers concerning the operation of the method but these were found unnecessary and to have no actual virtue. Some of these aspects are discussed in the following.

(b) Study of influence of various conditions and factors on estimation of GSSG.

(i) Influence of different protein precipitation agents.

Numata (283) reported that some of the reduced GSH in blood undergoes oxidation during deproteinization with metaphorphoric

TABLE VIII

Analytical Values of Standard Solutions of Commercial Oxidized Glutathione (GSSG)

Trial No.	Conc. of GSSG sol'n	Amount of GSSG for assay	Optical density reading at 305 mμ				Mean
1	5 mg%	20 μg	0.14	0.15	0.15	0.15	0.15
2	"	"	0.13	0.13	0.11	0.13	0.12
3	"	"	0.14	0.14	0.14	0.14	0.14
4	"	"	0.12	0.14	0.13	0.10	0.12
5	"	"	0.14	0.14	0.14	0.14	0.14
6	"	"	0.13	0.13			0.13
7	"	"	0.14	0.16	0.15		0.15
8	"	"	0.13	0.13	0.13		0.13
9	"	"	0.14	0.14	0.14		0.14
10	"	"	0.14	0.14	0.14		0.14
11	"	"	0.11	0.13			0.12
12	"	"	0.15	0.15	0.16		0.15
13	"	"	0.14	0.15			0.14
14	"	"	0.15	0.16	0.15		0.15
15	"	"	0.13	0.12			0.12
16	"	"	0.14	0.14	0.13		0.14
17	"	"	0.14	0.13	0.15	0.13	0.14
18	"	"	0.13	0.12			0.12
19	"	"	0.16	0.17			0.16
20	"	"	0.16	0.18	0.15		0.16
21	"	"	0.16	0.17			0.16
22	"	"	0.14	0.13	0.14		0.14
23	"	"	0.15	0.16			0.15
24	"	"	0.14	0.15			0.14
25	"	"	0.14	0.14	0.15	0.15	0.14
26	"	"	0.14	0.15			0.14
							mean of means =0.14 (.13-.16)
1	10 mg%	40 μg	0.28	0.28			0.28
2	"	"	0.23	0.25			0.24
3	"	"	0.24	0.26			0.25
4	"	"	0.23	0.25			0.24
5	"	"	0.27	0.27			0.27
6	"	"	0.27	0.27			0.27
7	"	"	0.23	0.24			0.23
8	"	"	0.23	0.24			0.23
9	"	"	0.25	0.28			0.26
							mean of means =0.24 (range:.23-.28)

Standard solutions of GSSG prepared in 2.25% HPO_3 and estimated with alloxan method after treatment of electrolytic reduction.

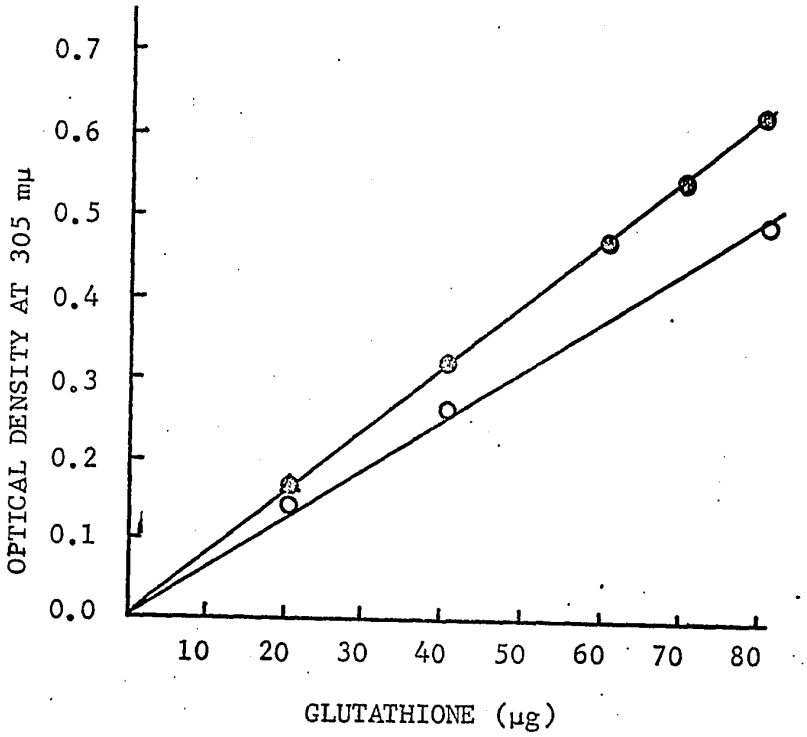
Figure 5

Standard Curves for Reduced and Oxidized Glutathione

Reduced glutathione (GSH) in metaphosphoric acid.

Oxidized glutathione (GSSG) in metaphosphoric acid.

Figure 5


OPTICAL DENSITY AT 305 mμ
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
10
20
30
40
50
60
70
80
GLUTATHIONE (μg)

acid. Consequently the writer has tried several common protein precipitants, among them only metaphosphoric acid was found to be satisfactory. The results are indicated in Table IX. Evidence (Table X) is also available that metaphosphoric acid did not oxidize GSH, since reduction under anaerobic conditions did not increase the accountable amount of GSH.

(ii) Influence of the purity of the mercury used as the cathode.

The mercuric ion is known to form mercaptide with glutathione. Both Mathai (16) and Flanagan et al. (42) have emphasized that prior to the reduction of GSSG solution, the electric current should be passed through the mercury cathode to reduce any mercuric ion that might be present ($Hg^{++} + 2e \rightarrow Hg$). On the strength of the results cited in Table XI, obtained in the present experiments, this precaution was found to be unncessary.

(iii) Recovery of GSSG added to blood.

The reliability of the electrolytic reduction method was finally established to our satisfaction by success in accounting for the quantity of GSSG added to whole blood and for the quantity of GSH oxidized on addition to plasma. This is indicated by the results in Tables XVIII and XIX. Thus, when GSH is added to plasma, it quickly and completely becomes oxidized to GSSG.

TABLE IX

Influence of Different Protein Precipitation Agents

Experiment No.	Protein precipitant	Results
1	10% TCA	No reduction of GSSG
2	formic acid	Not suitable as a protein precipitant
3	tungstic acid	Solution turned dark blue during reduction and interfered with method of determination
4	4% sulfosalycilic acid	Gave an infinite background absorption at 305 mμ; hence not suitable
5	$Ba(OH)_2$ and $ZnSO_4$	Gave zero value
6	2.25% HPO_3	Gave reproducible analytical values

TABLE X

Electrolytic Reduction of Commercial GSSG in Nitrogen Atmosphere

Medium	Concentration of GSSG solution	Gaseous atmosphere	Optical density at 305 mμ for 80 μg GSSG	Mean
	(mg %)			
2.25% HPO_3 *	20	O_2 (air)	.47 .47 .47 .46 .48	.47
		N_2	.46 .46 .46 .44 .47 .46	.46

* HPO_3 gassed with nitrogen for 20 minutes before being used for preparation of GSSG solution.

Reduction carried over mercury cathode for 15 minutes at 30 ma. Solution after reduction promptly assayed with the alloxan method.

TABLE XI

Influence of the Purity of the Mercury Used as the Cathode

Expt. No.	Concentration of GSSG solution	Condition of mercury cathode	Optical density at 305 mμ	Mean
1	5 mg%	clean with diluted HNO_3	.16 .17	0.16
	"	reduced at 35 ma. for 20 minutes	.16 .16 .16 .17	0.16
	"	reduced at 39 ma.for 30 minutes	.15 .16 .15 .15	0.15
	"	reduced at 35 ma. for 90 minutes	.14 .14 .14 .15	0.14
2	20 mg%	Newly distilled sample	.46 .46 .46 -	0.46
	"	reduced at 35 ma. for 60 minutes	.47 .47 .48 .46	0.47
	"	clean with dil. HNO_3 and filtered through gold-foil filter	.46 .46 .46 .46	0.46

TABLE XI

Influence of the Purity of the Mercury Used as the Cathode

Expt. No.	Concentration of GSSG solution	Condition of mercury cathode	Optical density at 305 mμ	Mean
1	5 mg%	clean with diluted HNO_3	.16 .17	0.16
	"	reduced at 35 ma. for 20 minutes	.16 .16 .16 .17	0.16
	"	reduced at 39 ma.for 30 minutes	.15 .16 .15 .15	0.15
	"	reduced at 35 ma. for 90 minutes	.14 .14 .14 .15	0.14
2	20 mg%	Newly distilled sample	.46 .46 .46 -	0.46
	"	reduced at 35 ma. for 60 minutes	.47 .47 .48 .46	0.47
	"	clean with dil.HNO_3 and filtered through gold-foil filter	.46 .46 .46 .46	0.46

On reduction and estimation of the GSH, the recovery of the original quantity is virtually complete. This attests the reliability of the reduction method. The incomplete recovery in the case of the commercial GSSG is considered to indicate impurity of the material.

(c) Procedure finally adopted for the estimation of total glutathione (GSH + GSSG) in blood.

Filtrates of blood fractions for analysis were prepared as described in the procedure adopted for estimation of GSH in blood. The concentration of reduced glutathione (GSH) in the filtrate was estimated with the alloxan method. For estimation of the total glutathione (GSH + GSSG), five ml of the filtrate were reduced with the mercury cathode in a 50 ml beaker (4.6 cm diameter) at 30 ma. for 15 minutes. Promptly after the reduction treatment, 0.4 ml of the reduced filtrate was used for determination of GSH with the alloxan method. The value obtained represents the total glutathione in the filtrate. The oxidized glutathione was estimated by subtracting the value obtained initially for the reduced glutathione from that of the total glutathione.

(d) Procedure for the assay of GSSG in the plasma.

The estimation of oxidized glutathione in plasma is beset

with certain difficulties. The use of paper chromatography for isolating the GSSG proved unsatisfactory because of the time and manipulations required. In considering alternative methods, the first problem encountered was the choice of a protein precipitating agent which would give complete extraction of the small amount of GSSG and would not interfere with the later application of electrolytic reduction. Among the acids, trichloroacetic, tungstic, sulfosalicylic and metaphosphoric, commonly used for precipitation of proteins, only metaphosphoric acid was found not to interfere with the electrolytic reduction or the alloxan method. Even the use of metaphosphoric acid presented some difficulty. To obtain satisfactorily clear filtrate with this acid it was necessary to saturate the acid solution with NaCl. This requirement, in turn, presented a further problem. However, the presence of NaCl in the filtrate is not desirable in a succeeding step where it is necessary to concentrate the solution to a small volume for reduction and to bring the concentration of GSH within the range of the alloxan method. The procedure finally evolved for the estimation of oxidized glutathione is described below.

The specimen of plasma was deproteinized by the heat coagulation method described by Hunter (284). Twenty (20) ml of

plasma, diluted with 80 ml of distilled water and acidified by further addition of 20 ml 0.05 N acetic acid, were heated in a boiling water bath for ten minutes. The sample was cooled and filtered through Whatman No.42 paper under reduced pressure. A measured volume (80 ml) of the supernatant was desiccated by lyophilization. The residue, taken up in 10 ml of 4.5 per cent HPO_3, was saturated with NaCl and filtered. Five (5.0) ml of the clear filtrate were then subjected to electrolytic reduction and estimation of GSSG according to the described procedures.

The method gave accurately reproducible results in the recovery of small amounts of GSSG added to buffer or to plasma. Added to buffer, the recovery of GSSG was practically quantitative (97%), but when added to serum or plasma it was incomplete, consistently ranging between 50-53 per cent with an average of 52.2. An explanation for this partial recovery is suggested later, but has not yet been definitely established. However, it was possible to detect the presence of 0.5 mg GSSG in 20 ml of plasma.

III. Technical Precautions in the Estimation of Oxidized Glutathione.

The following technical precautions are recommended in the use of Kay and Murfitt and the Electrolytic reduction

method for the estimation of GSSG by itself or in mixtures with GSH.

(a) Kay and Murfitt method. (276)

(i) The reaction between GSH and alloxan is sensitive to changes in the H^+-ion concentration. Care should be taken to maintain the pH between 7.45 and 7.75, and preferably near the optimum pH 7.6.

(ii) GSH is readily oxidized by oxygen in the air in the presence of traces of metallic ions. Standard solutions of GSH gave reproducible values only when prepared in meta-phosphoric acid. GSH, in aqueous solution, is unstable in the presence of oxygen (air) and, on standing, gives lower values.

(iii) The optical density for the control tube, against the phosphate blank should be read immediately, since the value is liable to be increased on standing.

(iv) The maximum optical density reading usually was attained between 10 to 20 minutes after the addition of the alloxan reagent. Thereafter, it tends rapidly to fall. The readings, therefore, were taken within 15 to 20 minutes instead of 20 to 40 minutes as recommended by Kay and Murfitt.

(b) Precautions with the electrolytic method.

(i) It is important to use only pure mercury for the cathode. Mercury that tarnishes readily or shows a scum on the surface should be cleaned or replaced. Purification by distillation is the most satisfactory method, but there are simpler methods that are almost as satisfactory. One is to filter the mercury through a gold-foil filter. A more commonly used procedure (291) is to pass the mercury in finely divided droplet form through a column of dilute nitric acid, repeating the washing two or three times. It should then be washed by similar treatment with distilled water. Finally, it may be 'dried' in a vacuum oven at about 110°C for a short time to remove any traces of nitric acid.

(ii) The degree of reduction is influenced by the volume of the solution and by the current density which, in turn, is dependent on the surface of the cathode and the magnitude of the current applied. These conditions, therefore, should be standardized.

(iii) Efficient, but not too rapid stirring of the solution during reduction is important for complete reduction. The glass-rod stirrer should be immersed in the solution just above the mercury surface and should not disturb the mercury.

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(iii) Efficient, but not too rapid stirring of the solution during reduction is important for complete reduction. The glass-rod stirrer should be immersed in the solution just above the mercury surface and should not disturb the mercury.

(iv) Sulfosalicylic acid, recommended by some workers as the anodic electrolyte, was found to interfere with the alloxan method. Care must be taken not to contaminate the solution with this acid, as may happen, for example, if the anode end of a previously used agar-KCl bridge is inadvertently inserted into the solution.

(v) After the reduction treatment, the solution should be assayed immediately to prevent re-oxidation of glutathione.

(vi) The agar-KCl bridge should be examined frequently for the presence of gas bubbles at the ends, and changed, if necessary. The accumulation of bubbles reduces the efficiency of reduction and gives low results.

CHAPTER THREE

STUDIES ON THE TOTAL GLUTATHIONE CONTENT OF NORMAL FRESH AND PRESERVED BLOOD SPECIMENS

I. Introduction.

Having succeeded in controlling the main factors responsible for the variability previously experienced in our laboratory and apparently also by other workers, particularly in the estimation of GSSG in blood, the writer proceeded to examine the behaviour of glutathione in fresh and preserved human blood specimens. The blood specimens were obtained from student donors and also specimens of various storage ages were kindly provided from time to time by the Canadian Red Cross Society Blood Depot in Montreal.

Among the special points to be studied were: (1) to what extent GSH undergoes oxidation in the red cells under various conditions of temperature, conditions of preservation (for example, with and without the presence of glucose), (2) whether glutathione escapes by diffusion from preserved erythrocytes during storage for prolonged periods, or whether it is released only by hemolysis of the cells, (3) the presence of oxidized glutathione in normal fresh and citrated blood in storage, and

(4) to investigate the disappearance of glutathione in preserved citrated blood.

II. Glutathione in Fresh Normal Blood.

(1) Values in literature.

Most investigators believe that glutathione in the blood is confined to the red cell. Values reported in the literature are presented in Table XII.

(2) Values obtained.

By application of the described technique, determinations were carried out on the glutathione level in a number of specimens of ACD preserved blood of various storage ages, kindly donated by the Canadian Red Cross Blood Depot in Montreal. The results are given in Table XIII. The average value of total glutathione in the blood of healthy donors was about 700 (711) μmoles per 100 mg hemoglobin , (range 540-881 μmoles per 100 mg hemoglobin). Very little oxidized glutathione was found in the blood samples analyzed.

III. Behaviour of Glutathione in the Hemolyzate from ACD Blood on Incubation at 25°C for 2 Hours.

It is generally accepted that glucose has a protective effect against the alteration of glutathione in blood during incubation or storage. Alivisatos (7) many years ago in our laboratory, and Henry (260) reported that the reduced glutathione in the

TABLE XII

Reported Concentration of GSH in Normal Human Blood

Blood Preparation	Value reported	Unit	Reference
Erythrocytes	79	mg/100 ml RBC	(304)
	220±41	µmoles/100 ml packed cells	(289)
	150-210	µmoles/100 ml packed cells	(305)
	58-95	mg/100 ml RBC	(42)
Whole blood	33.9±4.39	mg/100 ml blood	(306)
	26.7±31.9	mg/100 ml blood	(307)
	35	mg/100 ml blood	(248)
	18±5	mg/100 ml blood	(308)

TABLE XIII

Glutathione Content of Normal Human Erythrocytes

Donor	Total Glutathione	GSH	GSSG
	(mμMoles GSH/100 mg Hb)		
1	540	540	0
2	683	683	0
3	820	798	22
4	792	790	2
5	715	710	5
6	590	593	0
7	553	553	0
8	810	800	10
9	750	745	5
10	700	685	15
11	881	875	6
12	700	690	10
Mean value	711.1	705.1	6.0
Range	540-881	540-875	0-22
S.D.	±107.31	±93.14	±7.0

Each value is the average of duplicate estimations.

erythrocyte becomes more susceptible to oxidation and further alteration when the concentration of glucose in the blood approaches depletion. Henry (260) found this to occur when the concentration falls to less than 35 mgm per 100 ml of blood. Mortensen (288) and others (285, 287, 289) also observed that the decrease in the glutathione parallels the progressive fall in the glucose in the red cells during storage at 5°C or incubation at 25° or 37°C.

To study the protective effect of glucose, the writer followed the change of concentration of total, reduced and oxidized glutathione in a 4-week old specimen of blood that had been collected in the ACD medium and stored at 5°C for 4 weeks. The specimen showed some hemolysis. A sample was withdrawn aseptically and was incubated at 25°C. The results obtained were in agreement with the observations of other investigators. During incubation the glucose content remained unchanged owing to the failure inthe ability to utilize the sugar. There was no change in the content of glutathione in the sample during the incubation period of two hours in the presence of glucose. The results are given in Table XIV.

IV. Behaviour of Glutathione in Preserved Citrated Blood During Storage at 5°C.

The results obtained in the foregoing experiment indicate

TABLE XIV

Behaviour of Glutathione in ACD Blood During Incubation at 25°C

Duration of incubation	Glucose	GSH	GSSG	Total
(minutes)	(mg %)	(mμMoles GSH/100 mg Hb)		
0	180	492	191	683
30	180	492	191	683
90	172	492	191	683
120	180	492	191	683

Values are average of duplicate estimations.

Blood collected into ACD medium and incubated at 25°C for two hours.

that the presence of glucose is essential for maintenance of the glutathione in the reduced state even in hemolyzed blood specimens. It appears therefore that the decrease in the total glutathione in citrated blood observed by Mathai (16) in our laboratory may have occurred because of depletion of glucose. An experiment therefore was carried out to follow the state of glutathione in blood specimens preserved in citrate alone during storage at $5^{o}C$.

Venous blood from a single donor was collected into two concentrations respectively, 1.8 per cent (hypotonic) and 3.2 percent (isotonic) solutions of trisodium citrate. The proportion of blood to citrate was 4 : 1 (v/v). The specimens were labeled A and B, respectively. The purpose of using the hypotonic citrate solution in one sample was to examine whether hypotonicity of the medium would produce any change in membrane permeability owing to swelling of the red cells, thus possibly enabling the intracellular or the membrane GSH to escape by diffusion during storage.

On the day of collection and on the 14th and the 15th day of storage, samples were removed aseptically from the containers with a syringe and size 18 needle to avoid hemolysis of the less stable cells. In emptying the syringes the needles

were first removed. The degree of hemolysis was determined by measurement of the concentration of free hemoglobin in the plasma.

The content of total glutathione, reduced glutathione and oxidized glutathione in the whole blood, the corresponding saline-washed erythrocytes and in the plasma were estimated. Each estimation was done in duplicate. The results are given in Table XV.

It is noteworthy that on the 15th day of storage when the endogenous glucose became depleted, the total glutathione in the whole blood as well as in the saline washed cells, also was decreased. No oxidized glutathione was detectable in the washed cells nor in the plasma. Very little oxidized glutathione was found in the whole blood. It amounted to less than 10 per cent of the total glutathione in contrast to Mathai's (16) finding of 37.5 per cent in one sample and 76.8 per cent in another around the 17th and the 18th day of storage.

V. Permeability of Erythrocyte Stroma to Glutathione.

(a) Effect of washing.

Erythrocytes prepared from ACD-preserved blood of various storage ages were washed with 0.9 per cent of potassium chlor-

TABLE XV

Relation between the Changes in the Concentration of Glucose and of the Total, Reduced and Oxidized Glutathione in the Whole Blood and Components During Storage at 5°C.

Duration of storage	Components	Hemoglobin content	Blood glucose	Total glutathione (GSH + GSSG)	Reduced (GSH)	Oxidized (GSSG)
(Days)		g %	mg %	(mμmoles GSH/100 mg Hb)		
0	Whole Blood					
	A	13	89	535	535	0
	B	13	89	535	535	0
	Washed RBC					
	A	-	-	580	580	0
	B	-	-	554	554	0
	Plasma					
	A	6.8*	-	0	0	0
	B	6.8*	-	0	0	0
15	Whole Blood					
	A	13	0	380	345	35
	B	13	0	388	352	36
	Washed RBC					
	A	-	-	355	355	0
	B	-	-	380	380	0
	Plasma					
	A	77.7*	-	0	0	0
	B	68.5*	-	0	0	0

Specimen A - Blood collected into hypotonic (1.8%) citrate sol'n.
" B - Blood collected into isotonic (3.2%) citrate sol'n.
Values are average of duplicate closely agreeing estimations.

* In mg %

ide solution or with isotonic solution of potassium chloride containing glucose. In accord with the findings reported in the literature (237, 297), the washing treatment did not alter the glutathione content of the erythrocytes. Table XVI gives the analytical data obtained.

(b) Glutathione content of washed erythrocytes during preservation in the cold.

Saline-washed erythrocytes were prepared from 5-day-old ACD-preserved blood. The cells were suspended in various media and stored in the cold. On the 6th and the 16th days of storage samples were removed aseptically and the cells were separated from the suspension medium by centrifugation in the cold, washed with cold isotonic KCl solution, and finally resuspended in phosphate buffer solution. The concentration of glutathione, hemoglobin, methemoglobin and sulfhemoglobin was estimated. Estimations for these substances were carried out also on the corresponding suspension media. A comparison between the values obtained before and after storage showed t hat there was decrease in total glutathione in the washed erythrocytes stored in the absence of glucose. However, no glutathione was detected in the suspension medium thus indicating that glutathione had not diffused from the cells during

TABLE XVI

The Effect of Washing on the Glutathione Content of the Erythrocyte

Blood Specimens	Duration of storage (days)	Total glutathione (mμMoles GSH/100 mg Hb)	GSH	GSSG
1.Whole blood	27	563	563	0
RBC-glucose	27	563	563	0
RBC-KCl	27	-	563	0
2.Whole blood	28	563	563	0
RBC-glucose	28	563	563	0
RBC-KCl	28	563	484	79
3.Whole blood	29	563	563	0
RBC-glucose	29	563	595	0
RBC-KCl	29	563	563	0

Each value is the average of duplicate estimations.

RBC-glucose represents cells washed with isotonic solution of KCl containing glucose (0.134 M. KCl plus 0.02 M. glucose in one litre of solution).

RBC-KCl indicates erythrocytes washed with isotonic (0.154 M) KCl solution.

After washing, the cells were resuspended in the corresponding saline solution for assay of glutathione.

storage. The protective influence of added glucose against the loss of glutathione during storage is evident from the results. No methemoglobin or sulfhemoglobin was found in the cells during the period of preservation. The results are given in Table XVII.

VI. Disappearance of Glutathione in Citrated Blood During Storage.

Since glutathione cannot diffuse through the stroma, it is reasonable to suppose that the observed disappearance of glutathione in citrated blood during cold storage was probably attributable to its destruction within the erythrocyte. On the other hand, if it were to escape from the cells it might be destroyed also in the plasma. Mathai (16) noted a gradual loss of the oxidized glutathione which loss apparently occurred in the plasma of preserved blood.

The occurrence of enzymic hydrolysis of glutathione in animal tissues with the liberation of component amino acids has been reported by Neubeck and Smythe (259) and by Hanes et al. (69). These authors suggest that the enzyme, γ-glutamyl transferase, a weak "hydrolytic" enzyme, controls the tissue glutathione level. They isolated the enzyme from sheep brain, liver, kidney and pancreas. The loss of glutathione was thought

TABLE XVII

Glutathione Content of Washed Erythrocytes in Cold Storage

Specimen	Duration of storage	Total glutathione	Reduced Hb	Met-Hb.*	SHb**
	(days)	(mμM GSH / 100 mg Hb)	(mg%)	(mg%)	(mg%)
1.Washed RBC	0 (fresh)				
Sample A		520	-	0	0
" B		507	-	0	0
" C		510	-	0	0
2.Medium	6				
Sample A		0	10	-	-
" B		0	9	-	-
" C		0	8	-	-
Washed RBC					
Sample A		358	-	0	0
" B		390	-	0	0
" C		526	-	0	0
3.Medium	16				
Sample A		0	-	-	-
" B		0	-	-	-
" C		0	-	-	-
Washed RBC					
Sample A		323	0	0	0
" B		323	0	0	0
" C		485	0	0	0

Suspension medium: A = 0.1 M phosphate buffer, pH 7.4
B = mixture of 0.1 M phosphate buffer pH 7.4 and 1.8% sodium citrate dihydrate in ratio of 4:1 (v/v)
C = 0.1 M phosphate buffer pH 7.4 containing 352 mg glucose/100 ml.
Solutions A and B were autoclaved. Solution C was prepared by mixing sterilized phosphate buffer and glucose solution.

* Met-Hb = methemoglobin
** SHb = sulfhemoglobin

to be attributable to the "hydrolytic action" of γ-glutamyl transferase. Supporting this view is the demonstration by Rouser et al. (82) of the presence of the enzyme in erythrocytes, leucocytes, platelets and lymphocytes. This would imply further that a dynamic equilibrium exists in the erythrocyte between the synthesis of glutathione and its breakdown by the enzyme γ-glutamyl transferase. The writer has endeavoured to demonstrate the presence of the enzyme in preserved blood specimens.

Specimens of an hemolyzate of packed red cells, the plasma, stroma-free hemolyzate (SFH), and of hemolyzed whole blood were prepared from preserved ACD blood. The specimens of blood fractions were then incubated for various periods at 37°C with added reduced or oxidized glutathione. The results are given in Tables XVIII and XIX. No evidence was obtained that the glutathione in any of the preparations underwent hydrolysis. Both the reduced and oxidized glutathione added to whole blood, plasma, or stroma was almost quantitively recovered before or after the incubation. However, low recovery values were obtained when hemolyzates were subjected to the same treatment. The low results in this experiment do not necessarily imply that the glutathione had undergone hydrolysis since approximately the low re-

TABLE XVIII

Analytical Recovery of GSH Added to Preparations of Blood Fractions

Blood preparations	Recovery of Added Glutathione	
	Before incubation	After incubation
	(%)	(%)
Plasma	95.4	92.6
Whole blood	97	-
SFH	86	87
Stroma	100	100
Hemolyzate of whole blood	88	90

Total GSH accounted for after incubation

Conditions: 2 ml of the blood preparation, 2 mg GSH, in total volume of 5 ml incubated at 37°C for 2 hours.

TABLE XIX

Recovery of GSSG Added to Preparations of Blood Fractions

Blood fraction	Recovery of Added GSSG *	
	Before incubation	After incubation
	(%)	(%)
Plasma	94.6	95.4
Whole blood	95.7	-
Hemolyzate of whole blood	77.7	77.7
Hemolyzate of washed RBC	74.4	78.6
SFH	86.9	84.7
Stroma	-	95.3

*GSSG recovered as GSH after electrolytic reduction.

Conditions: 2 ml of blood preparation incubated with 2 mg GSSG in a total volume of 5 ml at 37°C for 2 hours in 0.2 M Tris buffer, pH = 7.8.

covery values were obtained before and after incubation. One reasonable supposition is that the added glutathione in the hemolyzates may have become bound to the blood proteins and thus would be removed in the precipitation process that was applied after the incubation treatment. The addition of glycylglycine, leucine, phenylalanine, glutamine, which are known activators of γ-glutamyl transferase, did not alter the results (Table XX). The very low recovery obtained with cysteine probably is attributable to the formation of mixed disulphide during the incubation. It is known that cysteine is more readily oxidized than glutathione in air. If cystine were to be formed it would react with glutathione to form a mixed disulphide which would more readily combine with blood proteins. In the presence of cysteine, therefore, one would expect to obtain very poor recovery of the added glutathione.

To establish that glutathione does not undergo hydrolysis into its component amino acids, a deproteinized filtrate prepared from the incubation mixture was subjected to two-dimensional paper chromatography. Only one spot was obtained which was identified as GSSG. No trace of any of the products of hydrolysis was obtained. A diagrammatical representation of the chromatogram is shown in fig. 6.

TABLE XX

Influence of Added Transferase Activators on the Recovery of GSH Incubated with Hemolyzate

Activators of γ-glutamyl transferase	Recovery of Added GSH	
	Before incubation	After incubation
	(%)	(%)
Without activator	100	-
Glycylglycine	93.3	97
Glutamine	93.3	90
Phenylalanine	80	80
Leucine	80	80
Cysteine	40	40

GSH is determined as total glutathione after electrolytic reduction.

Percentage recovery is based on the pre-incubation content of total glutathione added to hemolyzate without the addition of amino acid.

Conditions: 2 ml SFH from packed erythrocytes incubated with 2 mg GSH and amino acid (13 μmoles/ml incubation mixture) 0.1 M phosphate buffer, pH 7.6, 37°C for 1 hour.

TABLE XX

Influence of Added Transferase Activators on the Recovery of GSH Incubated with Hemolyzate

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GSH is determined as total glutathione after electrolytic reduction.

Percentage recovery is based on the pre-incubation content of total glutathione added to hemolyzate without the addition of amino acid.

Conditions: 2 ml SFH from packed erythrocytes incubated with 2 mg GSH and amino acid (13 μmoles/ml incubation mixture) 0.1 M phosphate buffer, pH 7.6, 37°C for 1 hour.

Figure 6

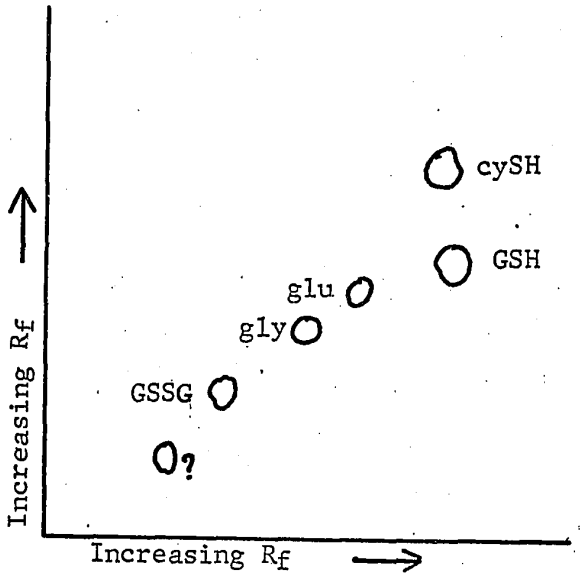
Chromatogram of Reduced and Oxidized Glutathione Added to and Incubated with Blood Plasma or Red Cell Hemolyzate

(a) Reference chromatogram of a mixture of GSH, GSSG, cySH, gly, and glu. An unidentified additional spot was obtained representing probably a disulphide formed by reaction of a thiol with the GSSG in the mixture.

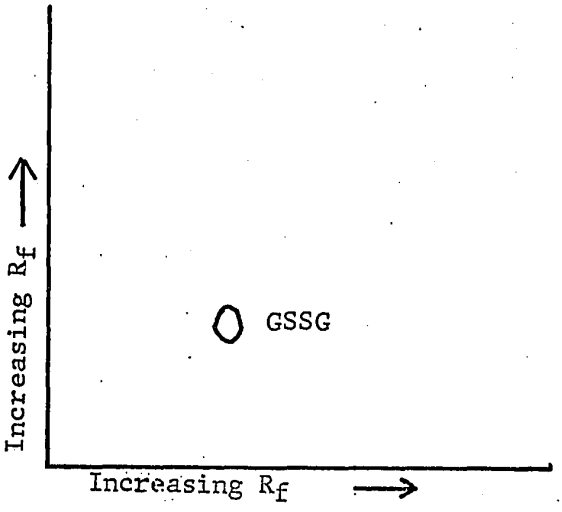
(b) Typical chromatogram of filtrate of deproteinized incubation mixture containing plasma or erythrocyte hemolyzate with added GSH or GSSG.

Figure 6

(a)


cySH
GSH
glu
gly
GSSG
O?
Increasing R_f
Increasing R_f

(b)


GSSG
Increasing R_f
Increasing R_f

From the evidence given, it is reasonable to conclude that γ-glutamyl transferase apparently does not occur in the erythrocyte, the plasma,nor in the stroma . Hence the disappearance of glutathione in preserved citrated blood during storage cannot be attributed to hydrolysis by the transferase. It appears more probable that it becomes bound to proteins in the erythrocyte and was removed during the deproteinization applied in the assay procedure. Since the contents of the red cell are composed almost entirely of hemoglobin, it is reasonable to surmise that hemoglobin is the protein to which binding of the glutathione may occur.

D I S C U S S I O N

(1) Methods of assay.

In the trial assays of GSH and GSSG with the modified alloxan method of Kay and Murfitt, the addition of the prescribed sodium chloride was found to suppress the absorption peak at 305 mμ. Sodium chloride has been also reported by Hanks and coworkers (274) to decrease the absorption values in their benzidine method for the determination of hemoglobin. In the writer's experience the suppressant effect of sodium chloride continues to present a problem. However, under standardized conditions reduced glutathione, added to whole blood, or blood plasma, could be quantitatively accounted for. The results for recovery are in substantial agreement with the findings of other workers who used a different assay method (Table XXI). When GSH was added to hemolyzates a low recovery was obtained. This result also is in agreement with those obtained by Kay and Murfitt (276), and by Jocelyn (289).

Oxidized glutathione, when reduced by electrolytic reduction and then estimated with alloxan, can be accounted for only to the extent of about 81% of the theoretical value. This has

TABLE XXI

Values Reported in Literature for the Recovery of Reduced Glutathione Added to Whole Blood or Plasma.

Specimen	Methods for Assay	GSH recovered (%)	Reference
Plasma	Electrolytic reduction and glyoxalase method	97-101	(269)
"	"	96-100	(278)
Blood	Glyoxalase method	70	(211)
Blood (saturated with CO)	"	98	(211)
Blood	"	93-104	(278)
Hemolyzed blood	Alloxan method	86	(276)
Hemolyzed blood (with addition of EDTA)	Nitroprusside method	95	(289)
Hemolyzed blood(without added EDTA)	"	Irregular results	(289)

been the experience also of other workers. Our suspicion that re-oxidation of the GSH in the acid medium may occur was ruled out by the observation that GSH prepared in 2.25 per cent metaphosphoric acid remained unchanged at 37°C for at least 2 hours. The precaution stressed by Flanagan et al. (42) and by Mathai (16) that electric current should be passed through the mercury cathode to remove mercuric ions before being used for reduction of oxidized glutathione was found to be of little effect in surmounting the difficulty. Nevertheless, under standardized experimental conditions, this procedure usually gave about a 95 per cent recovery of oxidized glutathione added to whole blood or plasma. Furthermore, reduced glutathione, after complete spontaneous oxidation on addition to blood plasma and then estimated by electrolytic reduction and with alloxan, was quantitatively recovered. The suspicion that the commercial glutathione, particularly the GSSG, was impure was lessened by the finding that on subjection to paper chromatographic analysis the commercial GSH or GSSG gave only one spot. Therefore there seems little ground for doubting that the reduction of GSSG by the electrolytic method is quantitative. However, other workers (280) have suspected that the commercial GSSG may be contaminated with non-amino compounds such as H_2O or ethanol. We have

further considered, without any good evidence, that oxidized glutathione may form an intermolecular complex with itself, which may be resistant to reduction.

(2) Presence of oxidized glutathione in normal and preserved citrated blood.

Most workers consider that glutathione in the blood is confined to the cellular elements, and it is known that by far the greater proportion of it is found in the red cells. Whether the red cells or individuals in health ever contain any of the glutathione in the oxidized form is not yet possible to say definitely because of the errors inherent in the methods for the estimation of GSSG. Whether the oxidized form occurs in the plasma in healthy individuals also is a disputed question. A list of the values reported for GSSG in blood by various investigators is given in Table XXII. Since we and others have shown that glutathione, in either form, cannot pass into or out of the red cell by diffusion through the membrane one is obliged to conclude that any GSSG found by analysis in the plasma must have been released by hemolysis.

The presence of oxidized glutathione in the erythrocyte in the circulation also is doubtful. Since glucose is always present in the cells, in vivo, accumulation of GSSG cannot normally

occur since any that may be formed will be rapidly reduced by the glutathione reductase system in the cell. Evidence has been obtained in the present study that only negligible amounts of oxidized glutathione were found in washed red cells prepared from preserved ACD blood.

Mathai (16) demonstrated with citrated blood stored in the cold that glutathione reductase of the preserved erythrocyte remained active even after the endogenous glucose was depleted. She found, however, that the concentration of oxidized glutathione in the preserved specimens during storage increased to as high as 60 per cent of the total. Her finding suggested that the GSSG was confined to the plasma. The writer found, to the contrary, that under similar conditions less than 10 per cent of total glutathione in the preserved specimen was in the oxidized form and that there was no GSSG in the plasma. The mechanical fragility of the preserved cells, however, was found to be greatly increased. It appears, therefore, that the increase in oxidized glutathione, observed by Mathai, in citrated blood during storage at $5^{o}C$ and which began when the glucose in the specimen became depleted, must have been attributable to hemolysis. While the formation of GSSG in preserved blood apparently is inhibited as long as glucose is present, it is not possible to say whether the de-

TABLE XXII

Oxidized Glutathione in Normal Human Blood

Specimen	Value	Unit	Literature
Erythrocyte	265	µmoles/litre cells	(309)
	149	"	(")
	353	"	(")
	176	"	(")
	258	"	(")
	211	"	(")
	102	"	(")
	50	"	(")
	250	"	(")
	45	"	(")
	0-15	µmoles/100 ml packed cells	289
Whole blood	0		269

pletion of glucose is the only factor that determines whether oxidation of GSH can or cannot occur.

(3) Disappearance of glutathione in blood on incubation of the red cells or during preservation in the cold.

The gradual decline in the content of glutathione in blood, or red cell suspensions, or the hemolyzate of erythrocytes during incubation, has been observed by various workers (21, 287, 288, 290). Fegler (17) has shown that intracellular GSH becomes oxidized in red cells upon exposure to oxygen. Jocelyn (289) demonstrated further the loss of glutathione in the oxygenated red cells, but under anaerobic conditions, for example, in the presence of nitrogen there was no de - crease in the GSH concentration.

It appears that in the absence of glucose metabolism, whether by depletion of the sugar or by failure of the mechanism by which it normally is utilized in the cell as in hemolyzates, oxidation of the intracellular GSH occurs and some of the glutathione, on oxidation, disappears, as is indicated by the decrease in the total glutathione content. Beutler et al. (285) have observed that both glucose and inosine added to suspensions of erythrocytes prevented the fall of glutathione during incubation, while added glucose-1

-phosphate, glucose-6-phosphate, 6-phosphogluconate, fructose-6-phosphate, inosine, ribose-5-phosphate, fructose-1-6-diphosphate, ATP, NADPH, NADH retarded the rate of decrease of the GSH in the hemolyzate on incubation. No synthesis of GSH occurs in the preserved erythrocytes owing to the absence of GSH precursors in the medium. As observed by Mathai (16) and the present author, the glutathione in the preserved citrated blood begins to fall when the endogenous blood glucose becomes depleted. We have confirmed also the observation of Alzona *et al.* (292) that the total glutathione (GSH and GSSG) content of citrated or heparinized blood falls to 50 or 60 per cent of the initial value by the 21st day of storage at 4°C. Although the fate of glutathione that disappears has not been ascertained in our study the writer is reasonably certain of the following conclusions: (a) that the loss of glutathione in the erythrocytes during preservation is not attributable to its diffusion from the cells, (b) that the destruction by the action of γ-glutamyl transferase does not occur since the enzyme is not present in the red cell. The results obtained in these experiments show that GSH or GSSG, upon incubation with hemolyzate of erythrocytes, does not undergo hydrolysis. GSH is known to be resistant to the action of

all the known peptidases with the exception of carboxypeptidase which splits off the glycine component from the γ-glutamylcysteinyl residue (300). This enzyme, however, is not active in the red cell.

In the writer's opinion the more likely explanation is that when the glucose becomes depleted the intracellular GSH gradually undergoes oxidation in the presence of the oxygen dissolved in the medium. The oxidized glutathione thus formed then combines with something, possibly the globin of the hemoglobin. Evidence was obtained in the present study that recovery of GSH or GSSG added to hemolyzates of washed red cells was incomplete compared with that obtained on addition to unhemolyzed whole blood. In further support of this view, Allen and Jandl (20) studying the oxidative destruction of hemoglobin, showed that the action of glutathione in protecting hemoglobin against oxidation involves some kind of binding of the glutathione to the hemoglobin. Thus, when S^{35}- or C^{14}-labelled GSH was mixed with hemoglobin and hydrazine or hydrazide-like compounds a portion of the radioactive GSH became precipitable with the hemoglobin and travelled with the hemo-

globin on electrophoresis. Huisman et al. (293) in their studies on the heterogenity of hemoglobin obtained evidence of the formation of a hemoglobin A - glutathione complex produced, in vitro, on incubation of Hb-A with GSSG.

By the same reasoning the disappearance of oxidized glutathione, if any, in the preserved red cells on hemolysis of the cells and exposure to the nonspecific catalytic action of the plasma proteins (16, 115) undergoes oxidation to GSSG which then probably reacts with the thiol groups of proteins to yield mixed disulphide derivatives according to this equation:

$$GSSG + Pr - SH \longrightarrow GSS - Pr + GSH$$

This view is strengthened by the writer's observation (see Experimental page 89) that a small amount (1 or 2 mg) of GSSG, on addition to a relatively large volume (.e.g 20 ml) of plasma could be analytically accounted for to the extent of only about 50 per cent, whereas addition of the GSSG to buffer solutions could be quantitatively "recovered". Further strong support for the writer's interpretation is afforded by the observations of many authors of the inter-reaction between protein SH-groups and disulfide compounds such as cystamine (294) or diphenyl disulfide (295).

The writer concludes therefore that the observed mysterious loss of glutathione from blood (red cells) in preserved citrated blood is probably attributable to its oxidation and subsequent combination with the SG-groups of proteins in the erythrocyte or plasma with formation of a mixed disulphide complex which, in the analytical procedure, would be precipitated with the proteins and thus be removed.

(4) <u>The role of reduced glutathione in the erythrocyte and its importance in cellular metabolism during storage</u>.

The maintenance of the level of glutathione in the erythrocyte and its maintenance in the reduced state appear to depend on the metabolic activity of the cell. It appears also to be dependent indirectly on the maintenance of the ATP. The reduction of oxidized glutathione through the glutathione reductase system, and the biosynthesis of glutathione as with the maintenance of ATP in the red cell are known to be linked with the utilization of glucose. The literature on the organization and metabolism of the red cell has been reviewed recently in an excellent book by Bishop (296).

As for the role of glutathione in metabolism and maintenance of viability in the erythrocyte many authorities (17, 24,

43, 54) consider that the intracellular thiols, particularly reduced GSH, play an important role in the maintenance of the stability of the red cell. This view might reasonably be extended to include that the action of glucose and nucleosides such as adenosine and inosine, on the maintenance of viability in erythrocytes during preservation may be responsible also for the maintenance of the level of glutathione and for keeping it in the reduced state in the cells.

As mentioned in the introductory section of this thesis, there is considerable indirect evidence also for the involvement of GSH in the stabilization of the red cell membrane and protection of the cell against hemolysis (see Introduction). Thus, an abnormally reduced level of reduced glutathione, usually associated with a deficiency of glucose-6-phosphate dehydrogenase or glutathione reductase, is accompanied by abnormal susceptibility to hemolysis. It is not yet established, however, to what degree the decreased stability of the cells is connected directly with the low level of reduced glutathione. Investigators have studied the behaviour of GSH deficient erythrocytes toward SG-reagents such as p-chloromercuribenzoate (p-CMB) of N-ethylmaleimide (NEM). It is to be expected that

reaction of the membrane with 'thiol' reagents must adversely influence cell stability. Some information of interest has been obtained from these experiments. Jacob and Jandl (53) have reported that a very small amount of p-CMB has a pronounced adverse effect on the viability of the erythrocyte even though the level of GSH in the cells remains normal. These workers consider that the thiol-groups of the membrane are of greater importance than the metabolic role of GSH in the stabilization of the membrane. On the contrary side of the picture there is evidence that glutathione is not absolutely essential for maintenance of the integrity of the red cell and that an abnormally low concentration of GSH in the cells has no direct relation to increased susceptibility to hemolysis (222). Considering the conflicting views that currently prevail as to the function of GSH in the red cell the matter still may be considered as being unresolved.

Apart from consideration of the role of glutathione in the erythrocyte there is more positive information on the behaviour of glutathione in the red cell during preservation of citrated blood in the cold. Some authors have studied this subject and have found that the GSH level remains unchanged in

the red cells in blood specimens preserved in ACD medium (233, 298). Alivisatos (7) in our laboratory many years ago demonstrated the protective action of glucose on the maintenance of GSH level in specimens preserved in citrate-dextrose medium at 5°C. He found that the GSH remained unchanged over a period of 60 days. It is evident from this finding that the reduced glutathione has no bearing upon the gradual failure of the glycolytic system in the preserved specimens during storage. The mechanism by which glucose exerts a protective action on the maintenance of the GSH is not known. The action may be incidental since glucose added to a simple solution of GSH in buffer solution can protect the thiol from spontaneous oxidation by oxygen (286). The development of the acidic reaction in preserved blood specimens, as a result of the formation of lactic acid in glycolysis, also favors the stabilization of GSH.

Recently, Prins et al. (299) have reported a case of congenital nonspherocytic hemolytic anemia associated with an almost total absence of GSH in the red cells. It was shown that these abnormal red cells had a low glyoxalase activityowing to the deficiency of GSH. The GSH-reducing and the glycolytic capacity, and the ATP level of the cells

were found to be virtually normal. Harris et al. (281) have provided evidence also that GSH is not an essential cofactor or component of glyceraldehyde-3-phosphate de - hydrogenase. In view of these considerations, it is doubtful if GSH plays any important role in the metabolism or the stabilization of the red cell during storage.

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SUMMARY AND CONCLUSION

The alloxan method of Kay and Murfitt (276) for the estimation of reduced glutathione and the electrolytic reduction method of Dohan and Woodward (269) for the estimation of oxidized glutathione (GSSG) and Mathai's modification of these procedures have been re-examined and further adapted for the estimation of small amounts of both forms of glutathione in preserved erythrocytes. The technical precautions reported by Flanagan et al. (42) and by Numata (283) have been found to be of insignificant importance and unnecessary. Other precautions that have proved valuable in the present author's experience, are described on page 91. In agreement with the findings of Flanagan et al.(42) and Benesch and Benesch (280) the writer found that the oxidized glutathione available commercially cannot be completely reduced to GSH by the electrolytic method. As the materials from different companies were found to be pure (at least in chromatographic tests) the resistance of a certain proportion of it to reduction appears to be attributable to the formation of some sort of intermolecular complex by the oxidized glutathione itself. However, by standardizing the analytical

procedures and observing the recommended precautions the reliability and precision of the methods have been considerably improved. GSH, added to whole blood, can be accounted for to the extent of 95-97 per cent, and GSSG, to the extent of 95-96.5 per cent.

The precise estimation of GSSG in blood plasma by the procedures is beset with special difficulties as described in the thesis. However, with a modification of the procedure the writer has been able to estimate amounts of GSSG down to 2.5 mg per cent.

With the modified procedures the writer obtained values with 2 specimens of normal blood ranging from 540 to 880 mμ Moles GSH per 100 mg hemoglobin or an average value of about 700 mμ Moles/100 mg hemoglobin. This value is in close agreement with that given by Jocelyn (289), namely, an average for 10 blood specimens of 220 μmoles per 100 ml packed cells. This is about the equivalent of 691 μmoles/100 mg hemoglobin, on the basis of a normal hemoglobin of 14 grams per cent and a hematocrit of 44.

The content of glutathione in citrated blood was found to be decreased to about 70 per cent of the original value at the end of the second week of storage. During this interval

the endogenous glucose had become depleted. Little, if any, oxidized glutathione was found in the blood sample and none in the saline-washed erythrocytes. No oxidized glutathione was found in the plasma of the preserved specimens. Thus there was no evidence of the escape of GSH from the cells by diffusion through the membrane during storage of simply citrated specimens up to 3 weeks at 5°C. Even when the blood was preserved in hypotonic sodium citrate (1.8%) the membrane impermeability to GSH was maintained at least to the end of the 3rd week. Similar results were obtained with washed red cells suspended in phosphate buffer and preserved in the cold in the absence of glucose. Mathai's finding of oxidized glutathione in blood specimens preserved in 1.8 per cent citrate in the cold must have been attributable to hemolysis of some of the cells.

The action of glucose in maintaining the level of GSH in blood during incubation at ordinary temperatures and during storage for more than 30 days in the cold (5°C) has been noted, in the literature, by several workers, but the mechanism of the protective action is still not understood. However, it is now definitely known that glucose does not act by preventing

the GSH from escaping from the cells by diffusion.

The fate of the portion of the GSH which disappears from the red cells on prolonged storage of citrated blood specimens has not yet been elucidated. There is no evidence of its destruction by hydrolysis. There is convincing indirect support, however, for the writer's view that in the older specimens some of the GSH undergoes oxidation, and that the GSSG reacts and combines with the thiol groups of hemoglobin, and hence is removed during the deproteinization procedure.

Considering that GSH in the red cells in blood specimens preserved in citrate with added glucose, remains virtually unaltered and constant for many weeks, and long after most of the red cells have become nonviable, it is difficult to find reason for considering GSH to be of any metabolic or other significance in the preservation of red cell viability during storage.

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