OBSERVATIONS ON THE METABOLISM OF ASCORBIC ACID IN RELATION TO GLUTATHIONE

A Thesis

submitted by

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GENERAL INTRODUCTION

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Ascorbic Acid

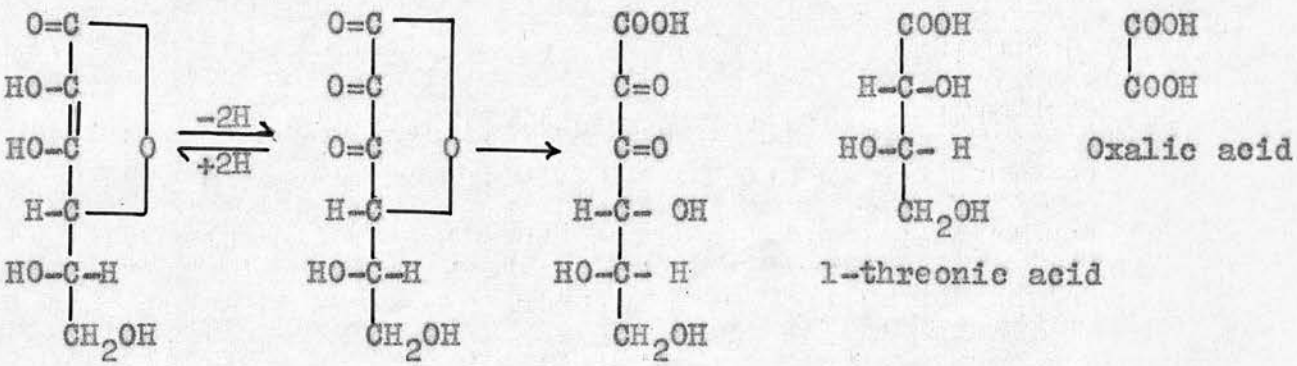
Long before the isolation and crystallization of vitamin C the deficiency disease caused by its lack was known. Scurvy was the scourge of sea-faring men ever since they relied on stored food during long voyages. The disease was by no means restricted to sailors and it took a heavy toll of human lives in time of war and famine when fresh food was scarce. People like Hawkins (1) and Lind (2) rightly appreciated the usefulness of lemon juice and fresh vegetables in preventing and curing this malady. In the early twentieth century while the concept of " accessory food factors " to be termed later "Vitamine " by Funk (3,4) was in the air, Holst (5) was studying the etiology of ship-beri-beri by keeping chickens and pigeons on bread, groats, and unpeeled grains. He thought that convincing evidence regarding the etiology of ship-beri-beri could not be obtained from experiments on poultry so he experimented with mammalia. Holst and Frolich (6) observed that when guinea pigs were kept on a similar diet they developed a disease similar to scurvy in man. They considered the disease to result from the lack of a " special nutrient ". The disease was curable by supplementing the diet with fresh cabbage, potato, apple, etc. The work of the aforesaid authors was of great significance not only because of its

conformity with the vitamin hypothesis but also because of the fact that for the first time an experimental animal which required ascorbic acid had been found. This discovery was of great assistance to the workers of later date, because, during the isolation and the determination of the chemical nature of vitamin C, biological assays using the guinea pig had necessarily to be done frequently.

Twenty-six years ago Szent-Gyorgyi (7a) while working in Cambridge isolated a reducing substance from adrenal cortex, cabbage, and orange. The reducing substance was a highly active carbohydrate derivative, isomeric with glycuronic acid; and after losing water formed a reversible crystalline lactone anhydride with a formula $C_6H_8O_6$. The exact constitution was not known, but he proposed the name " hexuronic acid ". Szent-Gyorgyi at that time did not realize the identity of his substance with vitamin C. Five years later King and Waugh (8) isolated the antiscorbutic vitamin in crystalline form and identified their substance with that of Szent-Gyorgyi's " hexuronic acid ". Later in the same year Svirbely and Szent-Gyorgyi (9,10) definitely established the antiscorbutic potency of " hexuronic acid " and the identity of the latter substance with vitamin C. Szent-Gyorgyi and Haworth (11) proposed the name ascorbic acid (AsA). Harris and Ray (12) followed by other workers confirmed the work of King and Waugh, Svirbely and Szent-Gyorgyi. The structural formula and chemical properties have been worked out by Hirst and his colleagues (13,

14,15). Other workers also contributed to the solution of this important problem. Synthesis was effected by Reichstein and his associates (16,17).

Hirst and his coworkers (13,14), Borsook, Davenport, Jeffreys, and Warner (21) demonstrated that, in vitro, ascorbic acid is converted to dehydroascorbic acid, diketogulonic acid, threonic acid, and oxalic acid (see below).

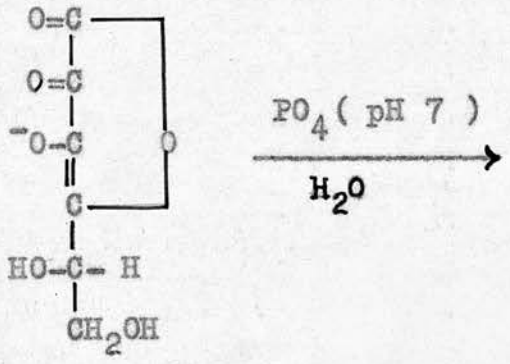


The immediate reversible oxidation product of ascorbic acid is dehydroascorbic acid (DHA). Numerous workers (18-22) have reported that dehydrogenated ascorbic acid is reduced to ascorbic acid in vivo and is completely utilized as such, i.e. dehydro-ascorbic acid has the same antiscorbutic potency as ascorbic acid. There are certain findings, which lend credence to the view that glutathione is responsible for this reduction, and will be discussed later. However, dehydroascorbic acid suffers an irreversible non-oxidative change, both in vitro and in vivo, and is converted to diketogulonic acid (DKA) (13,14,21,23,24). Hirst et al. (14) have shown that the lactone ring once broken

is not reconstructed easily in vitro, except by drastic treatment with HI. In vivo there is no such mechanism for the reconstruction of the lactone ring, therefore, DKA is not antiscorbutically potent (21,23). Penney and Zilva (23) who have extensively studied the in vitro and in vivo conversion of DHA to DKA suggested that in the body ascorbic acid is probably destroyed as follows:

$$\text{AsA} \rightleftharpoons \text{DHA} \longrightarrow \text{DKA}$$

In the same year Rosenfeld (26) published a paper in which he questioned the existence of DKA as a normal metabolic intermediate in the catabolism of ascorbic acid. According to the latter author the reversible oxidation product of ascorbic acid undergoes an intramolecular stabilization resulting in, probably the formation of enolized DKA lactone; catalytic amount of phosphate induces hydrolytic splitting of the 6-carbon chain of the latter compound and as a result of which oxalic acid is formed quantitatively and possibly oxalyl threose. (See below).



Enol-lactone diketogulonic acid

Oxalic acid

Recently Damron, Monier, and Roe (24) suggested that ascorbic acid is not converted to DHA or DKA in the course of its normal metabolic destruction, although, when large doses of AsA or DHA are administered then these compounds are destroyed via the pathway proposed by Penney and Zilva (23). At the present moment, the significance of DKA as a metabolic intermediate in the normal catabolism of AsA is polemical, however, the former compound as a chemical entity and the product of irreversible biological transformation has gained acceptance in the literature (13-15, 21,23,27).

Although Rosenfeld (26) suggested from the results of his in vitro experiments that oxalate is the end-product of ascorbic acid metabolism, it was not until very recently that convincing experimental proof for such conversion in vivo has been furnished. Burns, Burch, and King (25) administered ascorbic acid labeled with C^{14} to guinea pigs and measured the amount of C^{14} eliminated in respiratory CO_2 and in urine. They found that in 24 hrs. on the average 28% of the C^{14} appeared in the respiratory CO_2 and 5% in the urine; out of the latter 30-60% was present in the urinary oxalate. The above experiment is strongly suggestive that the conversion of AsA to CO_2 is a main route of its metabolism and that oxalate is an end-product of ascorbic acid metabolism.

Very recently King and his group (28-31) working with glucose-^{14}C have shown that D-glucose is almost certainly a precursor of ascorbic acid in animals, and since the yield of labeled ascorbic

acid from glucuronic acid is high, it indicates that glucuronic acid is an intermediate in the normal route of ascorbic acid formation. Isherwood, Chen, and Mapson (32) have provided evidence which suggests that a similar conversion takes place in plants.

The ease with which the reduced form of the vitamin reacts with numerous oxygen carriers and undergoes reversible oxidation-reduction that biochemists have been trying to associate it with various enzyme systems taking part in cellular respiration. Unfortunately however, to date, most of the attempts to link ascorbic acid with any definite enzyme system have ended in failure.

The known facts about ascorbic acid have been summarized recently by Harris (33) and are cited below:

(1) In ascorbic acid deficiency there is functional derangement of the formative cells of the body (odontoblasts, ameloblasts, cementoblasts, osteoblasts) and they stop producing normal type of new tissues (dentine, enamel, cement, bone). Normal production of collagen is also hampered in ascorbic acid deficiency.

(2) It has been proved almost certainly that ascorbic acid takes part in two well defined chemical reactions (a) conversion of folic acid to folinic acid, (b) the metabolism of tyrosine and related amino-acids.

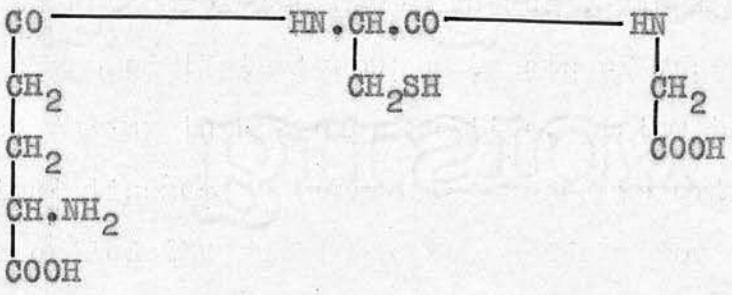
A new aspect of chemical role of ascorbic acid has been suggested

quite recently by Becker, Burch, Solomon, Venkitasubramanian, and King (34). They have shown that cholesterol metabolism is deranged in scorbutic guinea pigs.

Glutathione

The discovery of glutathione as a chemical substance dates back further than that of the ascorbic acid. In 1888 de Rey Pailhade (35,36) showed that aqueous extracts of yeast have the power to reduce sulfur to hydrogen sulfide. Later he observed that many animal tissues possessed the same property. de Rey Pailhade called the substance responsible for this effect " Philothion ". However, it was forgotten for a long time, until Hopkins (37) isolated this substance from yeast, muscle, liver etc. He thought the substance to be a dipeptide containing glutamic acid and cysteine, and suggested the present name to maintain a link with de Rey Pailhade's " Philothion ". Hopkins suggested that glutathione (GSH) plays a role in the oxidation-reduction system in animals. Stewart and Tunnicliffe (38) synthesized glutaminyl-cysteine and regarded it as identical with Hopkins's material. Hunter and Eagles (39) prepared glutathione according to Hopkins's method and they could not corroborate their finding with that of Stewart and Tunnicliffe; their analytical results indicated that glutathione was not a simple dipeptide of cysteine and glutamic acid and that there was a third amino-acid present, possibly, serine in ester linkage; although, they had no experimental proof to

support their contention. Hopkins (40) reinvestigated the problem and found that, in addition to glutamic acid and cysteine, glycine was also present. Through the work of Hopkins (40) Pirie and Pinhey (41), Kendall, MacKenzie, and Mason (42), Harrington and Mead (43) the structure of glutathione was established finally as gamma-glutamyl-cysteinyl-glycine.



gamma-glutamyl-cysteinyl-glycine

The universal distribution of glutathione has been recognized. It is present almost exclusively in the intracellular fluid. Rapkine (44-46) first formulated the role of glutathione in cell division and cell growth. He showed that in sea urchin eggs the concentration of glutathione falls soon after fertilization and that subsequently there is a considerable increase about 45 minutes after fertilization. However, the mechanism of action of thiols in cell division or growth is not known as yet.

Lohmann (47) showed for the first time that glutathione acts as a coenzyme in the glyoxalase system and the activity of which is dependent upon the amount of glutathione present.

Recently Racker (48) has worked out the details of the mechanism of this action of glutathione. No other enzyme system has been discovered where glutathione acts as a coenzyme. Hopkins and Morgan (49) found wide distribution of glyoxalase in living organisms (vertebrates, invertebrates, and higher plants). The metabolic significance of glutathione-glyoxalase system, however, is not clear at the present time.

Glutathione plays a protective role in animal organisms. There are a number of enzymes which need the presence of -SH groups in their protein moiety for activity, a number of these enzymes are connected with the oxidation of aminoacids, carbohydrates, and fats. If the -SH groups of these enzymes are attacked by substances which destroy the -SH groups, glutathione restores the sulfhydryl groups either by withdrawing the destroying agent or reducing the -SH groups if the latter are oxidized. Thus glutathione is a protective or reactivating agent (50). Within this protective role, probably, falls the action of glutathione against the induction of alloxan and dehydroascorbic acid diabetes (51). Soluble thiols of which glutathione is perhaps the most representative example has been suggested as responsible for the maintenance of the " steady state ", and the regulation of the energetic processes of the cells (50).

Numerous other papers have been published on the possible biological function of glutathione but, to date, nothing has been definitely established.

The metabolism of glutathione has been studied quite extensively

in recent years with the aid of isotopic components of the tripeptide. Waelsch and Rittenberg (52,53) demonstrated the rapid incorporation of isotopic glycine and glutamic acid into the glutathione of liver and intestine of rabbits and rats. The rate of incorporation of the two aminoacids into glutathione is far quicker than into the proteins of the same organs. According to the aforesaid authors the half-lifetime of glutathione in vivo is 2-4 hours. Other observers have since confirmed the rapid turnover of glutathione both in vivo and in vitro (54,55). Bloch and his associates (54,56-58) have reported the enzymic synthesis of labeled glutathione from C^{14}glycine and N^{15}glutamic acid in liver slices and homogenates. Their studies strongly indicate that adenosinetriphosphate (ATP) participates in the synthesis of glutathione.

The enzymic splitting of glutathione into its component aminoacids has been reported by a number of workers (59-63). Recently Olson and Binkley (63) have separated the enzyme responsible for the hydrolytic cleavage of gamma-glutamyl linkage from the one responsible for the hydrolysis of cysteinylglycine.

In a recent review Barron (50) has concluded that glutathione may be oxidized in the cells by cupric ions, cytochrome c-cytochrome oxidase, and H_2O_2-peroxidase. Ames and Elvehjem (64,65) purported that cytochrome c is mainly responsible for the oxidation of glutathione; in the absence of the aforesaid enzyme the oxidation of glutathione is effected, possibly, by

a coenzyme I-linked enzyme system. The influence of coenzyme I on the oxidation of glutathione, in the presence of cytochrome c, is only secondary in nature. They observed that ascorbic acid increased the rate of oxidation of glutathione by tissue homogenates both in the presence and in the absence of cytochrome c. However, since on further addition of ascorbic acid the oxidation rate of glutathione did not increase, they regarded the stimulating action of ascorbic acid on the rate of oxidation of glutathione as catalytic. Conn and Vennesland (66) have demonstrated the presence of an enzyme in wheat germ which catalyzes the reduction of oxidized glutathione (GSSG) in the presence of triphosphopyridine nucleotide (TPN). Mapson and Goddard (67) have also expounded the existence of such an enzyme in a number of plant tissues. Rall and Lehninger (68) have shown that a TPN-linked glutathione reductase exists in kidney, liver, spleen, heart muscle, brain, skeletal muscle, blood cells, and blood serum. The specific activity of the enzyme decreases in the order listed above. According to Rall and Lehninger the aforesaid enzyme shows specificity for both GSSG and $TPNH_2$ and effects the reduction of the former as follows:

$$TPNH_2 + GSSG \longrightarrow 2GSH + TPN$$

At the present moment there is no unanimity of opinion regarding the state of glutathione in blood and tissues, although the foregoing literatures show that there exist, in vivo, mechanisms for the oxidation and reduction of glutathione.

Glutathione and Ascorbic Acid

Glutathione and ascorbic acid have been closely associated in the minds of the biochemists because of the ubiquitous distribution of both, the close similarity of their properties with respect to the ease with which both are oxidized and reduced, and the tremendous concentration of both in embryonic tissues where synthetic processes occur at a great speed etc. Szent-Gyorgyi (7a) in the earliest communication on " hexuronic acid " observed that oxidized " hexuronic acid " is reduced by animal tissues and concurrent with the reduction of the former a progressive diminution of the nitroprusside reaction takes place. He concluded that oxidized " hexuronic acid " is reduced by glutathione, fixed thiol groups, and other unidentified factors. In a later publication Szent-Gyorgyi (7b) purported the presence of an enzyme " hexoxidase " in plant tissues which catalyzed the oxidation of " hexuronic acid ". He reported that the former, in the absence of the latter, is without any effect on glutathione. On the other hand, if " hexuronic acid " is present then glutathione is vicariously oxidized. Hopkins and Morgan (69) amplified the idea and demonstrated that, when ascorbic acid and glutathione are present together in the presence of ascorbic acid oxidase, ascorbic acid is completely protected from oxidation by this specific oxidase, and glutathione is directly oxidized. In the above system when reduced glutathione disappears almost

completely then ascorbic acid is oxidized. They explained the above protective action of glutathione on the basis of active hydrogen transfer from two molecules of glutathione to each activated molecule of ascorbic acid, and thus keeping the latter in the reduced form. Their observations also include such facts that glutathione protects ascorbic acid from catalytic oxidation by copper, and that dehydroascorbic acid is reduced by glutathione. According to the aforesaid authors glutathione inhibits copper induced oxidation of ascorbic acid by forming a compound with copper and thereby preventing the effective contact between copper and ascorbic acid. In the above paper it was further suggested that glutathione in very high concentrations might protect ascorbic acid from oxidation in the liver tissues. Kertesz (97) could not confirm the work of the above authors. Crook and Hopkins (71) repeated and extended the work of Hopkins and Morgan (69) and fully confirmed the original work. At a later date Crook (98), Crook and Morgan (72) further substantiated the original claim by partial separation of ascorbic acid reductase from oxidase and demonstrated the fact that the former was responsible for catalyzing the reduction of dehydroascorbic acid by glutathione.

Roe and Barnum (73) observed that whole blood, blood plasma, and washed red cells of different species when incubated with dehydrogenated ascorbic acid reduced the latter to ascorbic acid. On the other hand, Na-fluoride treated or heated plasma

and tungstic acid filtrate of whole blood lost the power to reduce dehydroascorbic acid to ascorbic acid. They concluded that dehydroascorbic acid is reduced by an enzyme present in the blood of humans, guinea pigs, and rats. Borsook, Davenport, Jeffreys, and Warner (21) suggested on the basis of their in vitro experimental findings that glutathione is perhaps mainly responsible for the reduction of dehydroascorbic acid in some animal tissues. Schultze, Stotz, and King (74) could not confirm the work of Roe and Barnum (73) and they concluded that dehydroascorbic acid is reduced by a reversible reaction with glutathione and fixed -SH compounds of the tissues. Barron (50) believes that the reduction of dehydroascorbic acid by glutathione as suggested by Borsook et al. does not take place under physiological conditions. Recently in human subjects Prunty and Vass (70) have shown statistically significant negative correlation between the values of plasma ascorbic acid and reduced glutathione levels in the red cells after the administration of large doses of ascorbic acid. Furthermore, they have pointed out that a fall in the reduced glutathione of blood is accompanied by a rise in the oxidized glutathione in the above cases.

To date, no enzyme system which catalyzes the oxidation-reduction of ascorbic acid, such as has been found in plants, has been definitely established in animal tissues. Although, glutathione has been suggested as the principal reducing agent for the reduction of dehydroascorbic acid in animal tissues,

the case is far from being decisively proved.

Oxidized ascorbic acid and glutathione have been related in an entirely different biochemical role in recent years, i.e. in relation to the production of experimental diabetes and its prevention (75).

The gradual unfolding of our knowledge about ascorbic acid and glutathione is fascinating and it has been very briefly summarized in this review. In the subsequent sections literatures, which have a direct bearing on the problems studied, will be discussed more fully.

PART I

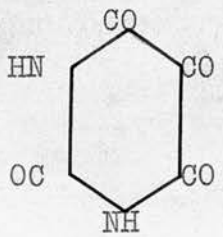
INVESTIGATIONS ON THE POSSIBLE EXISTENCE OF DEHYDROASCORBIC ACID IN RABBIT PLASMA AND ITS ALTERATION BY INJECTION OF ALLOXAN

PART I

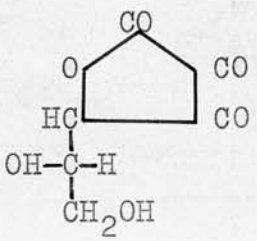
Investigations on the Possible Existence of Dehydroascorbic Acid in Rabbit Plasma and its Alteration by Injection of Alloxan:

INTRODUCTION

Since the discovery by Dunn, Sheehan, and McLetchie (76) that alloxan causes a selective necrotic degeneration of the beta-cells in the islets of Langerhans of the pancreas, numerous papers have been published on the possible role of alloxan in the etiology of human diabetes. To date, it has not been proved decisively that alloxan exists normally in the body (77), or that it appears in significant amount under abnormal metabolic conditions, notwithstanding that there are some suggestions to that effect (78). Nevertheless, alloxan has been a very helpful tool to the biologists as a means of producing experimental diabetes and for following many of the complicated metabolic derangements associated with that condition. Very recently Patterson (79,80) has shown that the intravenous injection of dehydroascorbic acid into rats results in the production of diabetes. He pointed out the structural resemblance of the dehydroascorbic acid molecule to that of alloxan. (see below):



Alloxan



Dehydroascorbic acid

Principotto (81) has extended Patterson's observation by making rabbits diabetic with dehydroascorbic acid. Unlike alloxan, ascorbic acid is definitely known to be a normal constituent of the body tissues and one of the chemical properties of ascorbic acid is that it is fairly easily oxidized reversibly to dehydroascorbic acid (13-15). However, till the recent publication of the paper by Stewart, Horn, and Robson (83), ascorbic acid has been generally thought to exist mainly in the reduced form in the body (21,84,85). Stewart et al. reinvestigated the problem and suggested that dehydroascorbic acid exists in the human plasma and that diketogulonic acid does not exist. They further found confirmatory evidences in their experiments with ACTH, Cortisone, and Salicylate administration which altered the ratio of ascorbic acid to dehydroascorbic acid (86).

It has been suggested that alloxan combines with or oxidizes reduced glutathione (82). If, reduced glutathione is responsible for maintaining ascorbic acid in the reduced form in animals, as has been suggested in plants by Hopkins and Morgan (69), then, there is a possibility that alloxan by combining with or oxidizing reduced glutathione may indirectly cause the formation of dehydroascorbic acid.

The immediate purpose of the work in this section is, therefore, to investigate whether dehydroascorbic acid exists in the plasma

of other animals besides human and whether the diabetogenic action of alloxan is due to or associated with the formation of dehydroascorbic acid.

EXPERIMENTAL

Rabbits purchased from recognized dealers and subsequently inbred in this laboratory were used throughout the work of this thesis. During this experiment they were maintained on a daily ration of bran, oats, hay, and water ad libitum. In a few preliminary experiments it was observed that the ascorbic acid level in the plasma of such animals was very low. Since the purpose of the experiment was to observe whether dehydroascorbic acid was present in significant amount in the plasma of rabbits, and whether the concentration of the aforesaid substance altered by alloxan injection, the vitamin C concentration in the plasma of the experimental animals was raised by supplementing the diet with cabbage. In this way the estimation of ascorbic acid in the plasma was facilitated and experimental errors minimized.

Technique of Blood Collection:

The apex beat of the heart was palpated and as close to it as possible a hypodermic needle was pushed into the thoracic cavity towards the throb at an angle of 45° from the long

axis of the rabbit's body till the blood came out in spurts, which indicates that the left ventricle is pierced. If the right ventricle is pierced then the flow is much slower and when the auricles are punctured the flow is of even type. The outflowing blood was collected in a heparinized syringe. In one bleeding approximately 20-40 ml. of blood were collected. The same animal was used again sometimes, provided it received no other form of treatment and at least seven weeks elapsed between two bleedings.

Blood obtained by the above procedure was centrifuged for 10-15 minutes; ascorbic acid and total ascorbic acid determinations were made in the same sample of non-hemolyzed plasma.

Methods

Ascorbic acid was estimated by the modified indophenol method of Stewart, Horn, and Robson (83). Total ascorbic acid was estimated by the method of Roe and Kuether (87), and Mapson and Ingram (88).

Recoveries of the pure substance added to plasma by the indophenol method ranged from 95-100% and by the Roe and Kuether's phenylhydrazine method from 98-101%. When 12 determinations were made by the indophenol and phenylhydrazine methods on the same sample of plasma containing no ascorbic acid and to which 1 mg.% of ascorbic acid was added the recoveries were as follows:

Methods	Mean	Range	Standard Deviation
Indophenol	0.954 mg. per 100 ml.	0.925-1.0 mg. per 100 ml.	±0.0297
Phenylhydrazine	0.981 mg. per 100 ml.	0.95-1.02 mg. per 100 ml.	±0.034

The principles of the above methods may be recalled briefly:

A. Indophenol method:

The reduction of the dye 2 : 6 - dichlorophenolindophenol by ascorbic acid is complete within 30 seconds and other reducing substances normally present in the plasma do not react with indophenol within that short time and under the specified conditions. Hence the indophenol method measures the amount of reduced ascorbic acid present in the plasma.

B. Roe and Kuether's method for total ascorbic acid:

All the reduced ascorbic acid is converted by activated charcoal to dehydroascorbic acid. When the latter substance is incubated with 2:4-dinitrophenylhydrazine it is converted to diketogulonic acid (23,89) and couples with 2:4-dinitrophenylhydrazine. The resulting osazones are dehydrated with 85% H_2SO_4 and a red color is produced which is measured photometrically. It should be noted that any preformed dehydroascorbic

acid and diketogulonic acid also react with phenylhydrazine to form osazones. Therefore, this method measures ascorbic acid plus dehydroascorbic acid and diketogulonic acid.

C. Mapson and Ingram's method for total ascorbic acid: Esch. coli reduces dehydroascorbic acid to ascorbic acid under anaerobic conditions, at pH 6.2 and 35°. Hence any increase in indophenol reducing power of plasma after Esch. coli treatment is believed to be due to the reduction of dehydroascorbic acid to ascorbic acid.

RESULTS AND DISCUSSION

The results obtained by the indophenol and phenylhydrazine methods are spoken of as indophenol and phenylhydrazine values in the course of this discussion. In the Tables the column DHA ? shows the difference between the phenylhydrazine and indophenol values; a plus sign is used when the former is higher than the corresponding value of the latter and a minus sign indicates the reverse.

The analyses for ascorbic acid and total ascorbic acid in the plasma of rabbits whose diets were supplemented with cabbage are presented in Table 1a. It can be seen that in most cases the phenylhydrazine values are lower than the indophenol values and that the differences are often greater than would be expected as a result of experimental error. These results are quite different from those obtained

TABLE 1a

Concentrations of Ascorbic Acid and Total Ascorbic Acid in the Plasma of Rabbits Maintained on a Diet Supplemented with Cabbage

AsA---- Ascorbic acid
DHA---- Dehydroascorbic acid
Very high cabbage---- Cabbage ad libitum every day of the week
Medium cabbage------- Cabbage ad libitum thrice a week

Rabbit No.	Body Weight	Indophenol method AsA	DHA ?	Phenyl-hydrazine method Total AsA	Treatment
	g.	mg. per cent	mg. per cent	mg. per cent	
5	1450	2.12	+0.13	2.25	
6	2000	1.65	-0.05	1.6	
7	1600	1.55	-0.2	1.35	Very high cabbage
8	1500	1.87	-0.2	1.67	
9	1500	0.95	-0.125	0.825	
10	2100	0.925	-0.175	0.75	
11	1550	0.92	-0.07	0.85	
12	1320	0.7	-0.075	0.625	
17	-	0.775	-0.15	0.625	Medium cabbage
18	-	0.575	-0.1	0.475	
5	1640	1.22	-0.17	1.05	
6	2150	1.82	-0.02	1.8	
19	-	0.95	+0.02	0.97	

in the human plasma by Stewart, Horn, and Robson (83). It seemed likely that some substance from cabbage, which when assimilated and circulated in the plasma of these rabbits, was responsible for giving such falsely high indophenol values. Long, Miles, and Perry (90) suggested that cabbage probably contains a factor of -SH nature. It was argued, therefore, that, if cabbage contains a factor of sulfhydryl nature the latter substance would react with indophenol but not with phenylhydrazine, and as a result of this a falsely high indophenol value would be obtained and the true difference, if any, between the phenylhydrazine value and the indophenol value would be masked. On the other hand, if ascorbic acid and total ascorbic acid were measured by the same method, then, interference would be present in both cases and if dehydroascorbic acid existed then the total ascorbic acid value would be higher than the ascorbic acid value. It was, therefore, decided to reduce the dehydroascorbic acid by a suitable reducing agent and to estimate the total ascorbic acid by the indophenol method. The first choice of reducing agent was H_2S as originally applied by Stewart et al. (83). In the course of this investigation it was found that when dehydroascorbic+ acid was added to plasma to give a concentration of 0.5 mg.% and the metaphosphoric acid filtrate was submitted to H_2S reduction under the conditions mentioned by the aforesaid authors, only 30% of the dehydroascorbic acid

+ Dehydroascorbic acid was prepared either by aeration in presence of copper or according to Patterson (79).

could be recovered as ascorbic acid by the indophenol method. The reason for such low recovery is that the pH of the metaphosphoric acid filtrate is approximately 1.5 and at that pH H_2S does not reduce dehydroascorbic acid quantitatively. Levenson, Rosen, and Hitchings (91) also reported such low recoveries of DHA at pH 1.5. At this stage, the recoveries of known amounts of DHA, added to plasma, were determined in metaphosphoric acid filtrates at pH 3.5 and 37° according to Levenson et al. (91). At the level of 1.0-1.5 mg. of DHA per 100 ml. plasma the recoveries were 100-102%. Stewart et al. (83) have reported that human plasma contains 0.07-0.3 mg.% of DHA. When H_2S recoveries were determined at the latter levels, under the conditions of Levenson et al., consistently higher recoveries were obtained. Since H_2S reduces many other substances besides DHA (92) such anomalous results may be expected, and small amounts of DHA cannot be estimated accurately in the presence of biological materials by its use. The next choice of reducing agent was Esch. coli as applied by Mapson and Ingram (88) and Stewart et al. (86). In Table 1b are presented the analyses for ascorbic acid and total ascorbic acid by the indophenol, phenylhydrazine, and Esch. coli methods in the plasma of rabbits whose diets were supplemented with cabbage. The results are almost similar to those in Table 1a and need no further comment. At this point attempts were made to reduce dehydroascorbic acid, added to rabbit plasma, by Esch. coli. It was observed that the same culture of Esch. coli which reduced DHA quantitatively in

TABLE 1b

Concentrations of Ascorbic Acid and Total Ascorbic Acid in the Plasma of Rabbits Maintained on a Diet Supplemented with Cabbage

AsA----- Ascorbic acid
DHA----- Dehydroascorbic acid
Very high cabbage----- Cabbage ad libitum every day of the week
Medium cabbage-------- Cabbage ad libitum thrice a week

Rabbit No.	Body Weight	Indophenol method AsA	DHA ?	Phenyl-hydrazine method Total AsA	Esch. coli method Total AsA	Treatment
	g.	mg. per cent	mg. per cent	mg. per cent	mg. per cent	
14	1940	1.07	-0.09	0.98	0.662	Medium cabbage
15	1730	0.6	-0.14	0.46	0.426	
21	1980	1.2	+0.02	1.22	1.0	
22	2000	1.1	-0.1	1.0	0.925	
11	2050	1.07	-0.02	1.05	1.1	
13	1850	0.8	+0.02	0.82	0.75	Very high cabbage
19	2500	0.65	0	0.65	0.6	
17	1100	3.25	-0.4	2.85	3.05	
18	2200	2.25	-0.05	2.2	2.0	

aqueous solutions failed to do so in the presence of plasma. In view of the latter finding no further attempt was made to estimate DHA by the difference between the indophenol and Esch. coli values.

Since the above experiment failed to show whether there was any interference due to the cabbage factor, another attempt was made to analyze the plasma of rabbits, which were deprived of cabbage for different lengths of time, for ascorbic acid and total ascorbic acid. The data are shown in Table 2. It is clear that the results show the same trend as those in Table 1a and Table 1b. This experiment partially, but not wholly, excludes the possibility of interference by the cabbage factor. To exclude such a possibility completely, it was deemed necessary to analyze the cabbage for ascorbic acid and total ascorbic acid by the indophenol and phenylhydrazine methods. The results were as follows:

Indophenol method : : : 40.4 mg. AsA per 100 g.

Phenylhydrazine method : : 42.5 mg. AsA per 100 g.

The above experiment shows that the phenylhydrazine value is higher than the indophenol value in the cabbage. These results in common with those in Table 2 negate the idea that the higher indophenol values than the phenylhydrazine values in the plasma of rabbits, whose diets were supplemented with cabbage, (Tables 1a and 1b), were due to interference by some substance from cabbage.

TABLE 2

Concentrations of Ascorbic Acid and Total Ascorbic Acid in the Plasma of Rabbits Deprived of Cabbage

AsA---- Ascorbic acid
DHA---- Dehydroascorbic acid

Rabbit No.	Body Weight	Indophenol method AsA	DHA ?	Phenyl-hydrazine method Total AsA	Treatment
	g.	mg. per cent	mg. per cent	mg. per cent	
13	1330	0.475	+0.025	0.5	Complete deprivation of cabbage for 5 days
14	1500	0.525	0	0.525	Same
15	1300	0.375	-0.125	0.25	Complete deprivation of cabbage for 7 days
16	2000	1.02	-0.02	1.0	Same
20	2000	0.225	+0.125	0.35	Complete deprivation of cabbage for 15 days

It was considered important to know whether results similar to those in Tables 1a, 1b, and 2 could be obtained in the plasma of rabbits whose diets were supplemented with synthetic ascorbic acid instead of cabbage. The data are shown in Table 3. These results show that the phenylhydrazine values are higher than the corresponding indophenol values in all cases but one. It should be pointed out in this context that the results of this experiment do not necessarily suggest that since cabbage was excluded from the diet of these animals and there was no interference from the cabbage factor, therefore, the phenylhydrazine values were higher than the indophenol values. If there was any interference with the indophenol method by the cabbage factor then it would have been detected when ascorbic acid and total ascorbic acid estimations were done in the cabbage by the indophenol and phenylhydrazine methods. Lack of specificity of one or both the methods of ascorbic acid estimation appears to be the explanation for the observed difference between the results of ascorbic acid estimation in the plasma of rabbits fed on cabbage and those fed on synthetic ascorbic acid. Since the phenylhydrazine method measures both dehydroascorbic acid and diketogulonic acid it is difficult to decide whether the higher phenylhydrazine values than the indophenol values (Table 3) are due to dehydroascorbic acid or diketogulonic acid. Because of the lack of specificity of the H_2S reduction it cannot be applied to differentiate between the two substances (when present in such small amounts). Further along the text this

TABLE 3

Concentrations of Ascorbic Acid and Total Ascorbic Acid in the Plasma of Rabbits Maintained on a Diet Supplemented with Synthetic Ascorbic Acid

AsA----- Ascorbic acid
DHA----- Dehydroascorbic acid

Rabbit No.	Body Weight	Indophenol method AsA	DHA ?	Phenyl-hydrazine method Total AsA	Treatment
	g.	mg. per cent	mg. per cent	mg. per cent	
20	-	0	+0.25	0.25	10 mg. AsA/day for 7 days
21	-	0.4	+0.35	0.75	10 mg. AsA/day for 7 days
23	-	0.4	+0.175	0.575	10 mg. AsA/day for 5 days 75 mg. AsA/day for 2 days 50 mg. AsA/day for 5 days
24	1400	1.15	+0.175	1.325	50 mg. AsA/day for 15 days
54	-	0.35	+0.15	0.5	50 mg. AsA/day for 17 days
55	-	0.5	0	0.5	Same
56	-	0.125	+0.195	0.32	Same
48	-	0.95	+0.18	1.13	Same

difference between the phenylhydrazine and indophenol values will be referred to as " apparent dehydroascorbic acid ".

There is no specific direct method available for the accurate estimation of small amounts of dehydroascorbic acid in biological materials. Hence it is almost impossible to supply an incontrovertible proof for the existence of small amounts of dehydroascorbic acid in biological materials. The preceding experiments have pointed out the limitations of indirect procedures for the measurement of small amounts of dehydroascorbic acid. Nevertheless, if consistently higher values had been obtained by the phenylhydrazine method than by the indophenol method then it could have been stated, with some reserve, that the differences were due to dehydroascorbic acid. Since the phenylhydrazine values are not significantly higher than the indophenol values in the majority of cases, except those reported in Table 3, it cannot be concluded that dehydroascorbic acid exists in rabbit plasma.

At this stage, it was decided to investigate the effect of alloxan injection, if any, on the level of " apparent dehydroascorbic acid " in the plasma of rabbits whose diets were supplemented with synthetic ascorbic acid. The results of such estimations are presented in Table 4. Since alloxan disappears from the circulation within a few minutes (93,94), it was thought that any change in the ascorbic acid concentration in the plasma would be manifested within a short time after alloxan injection. Bearing this in mind, the first experiment

TABLE 4

Concentrations of Ascorbic Acid and Total Ascorbic Acid in the Plasma of Rabbits Injected with Alloxan and Maintained on a Diet Supplemented with Synthetic Ascorbic Acid

AsA---- Ascorbic acid DHA---- Dehydroascorbic acid

Rabbit No.	Body Weight	Indophenol method AsA	DHA ?	Phenyl-hydrazine method Total AsA	Blood Sugar	Treatment
	g.	mg.%	mg.%	mg.%	mg.%	
12	1680	1.03	-0.38	0.65	-	50 mg. AsA/day for 15 days. Received 200 mg/Kg. alloxan in one inj. Blood taken 15 min. after inj.
8	1550	1.0	0	1.0	414	50 mg. AsA/day for 15 days. Received 400 mg. alloxan in 3 inj. over a period of 7 days. Blood taken on 4th day after last inj.
7	1650	0.487	+0.063	0.55	358	50 mg. AsA/day for 15 days. Received 660 mg. alloxan in 4 inj. over a period of 11 days. Blood taken on 5th day after last inj.
4	1790	0.725	-0.225	0.5	416	50 mg. AsA/day for 23 days. Received 200 mg./Kg. alloxan in one inj. Blood taken on 4th day after last inj.
3	1140	0.412	-0.137	0.275	459	50 mg. AsA/day for 25 days. Received 200 mg./Kg. alloxan in one inj. Blood taken on 6th day after last inj.

was done on rabbit No. 12 which received 200 mg./Kg. of alloxan in a single injection and blood was drawn by cardiac puncture 15 minutes after the completion of the injection for ascorbic acid analyses. When indophenol was added to the plasma filtrate of the above rabbit the spectrophotometer indicated a very rapid fading of the residual color of the indophenol within 30 seconds and afterwards. The rate of fading in the above case was far quicker than the rate of fading of indophenol when standard ascorbic acid was added to the latter. Also the filtrate when incubated with phenylhydrazine formed a precipitate which was insoluble in 85% H_2SO_4. As a result of which the phenylhydrazine method gave very low value (see Table 4). On subsequent investigation it was found that when alloxan (an amount that would be present after injection of 200 mg./Kg., assuming no loss and the blood volume to be 100 ml. per 1000 g. body weight) was added to pure ascorbic acid solution (1 mg.%) and the same solution was estimated by the indophenol and the phenylhydrazine methods, 35% higher ascorbic acid was recovered by the indophenol method; 43% lower ascorbic acid was recovered by the phenylhydrazine method. In view of this it is surprising that Siliprandi (95) using the indophenol method of Mindlin and Butler (96) could observe a fall in the ascorbic acid concentration immediately after alloxan injection. No mention has been made by the above author of any interference with the indophenol method under the above condition. Another very curious thing that does not escape notice is that

Siliprandi could observe the presence of alloxan in blood for more than an hour. The latter finding is quite contrary to the well established fact that alloxan is destroyed within a few minutes after injection (93,94). In view of this interference with the indophenol and phenylhydrazine methods by some reduction product of alloxan, possibly dialuric acid (82,94), no further attempt was made to measure ascorbic acid within a short period after alloxan injection.

However, it was of interest to observe the level of " apparent dehydroascorbic acid " in the plasma of diabetic rabbits. Ascorbic acid and total ascorbic acid analyses in the plasma of rabbits No. 8, 7, 4, and 3 (Table 4) were done after the rabbits were diabetic for different lengths of time. In contrast to the results in Table 3, those in Table 4 show that although the rabbits are maintained on synthetic ascorbic acid there is no " apparent dehydroascorbic acid " in their plasma. Often the indophenol values are significantly higher than the corresponding phenylhydrazine values. These figures could be interpreted as indicating a possible removal of dehydroascorbic acid accompanying the diabetes produced by previous alloxan injection, and such an interpretation would have a number of interesting consequences. However, if alloxan exists in significant amount in the blood of diabetic animals as has been suggested by Loubatiers (78) the above type of results may be expected on that account and this seems a more probable explanation. Certainly there is nothing to suggest a

production of dehydroascorbic acid in permanently diabetic rabbits and this is really the significant point.

PART II

STUDIES ON THE IODOMETRIC AND GLYOXALASE METHODS FOR THE ESTIMATION OF GLUTATHIONE IN BLOOD AND TISSUES

PART II

Studies on the Iodometric and Glyoxalase Methods for the Estimation of Glutathione in Blood and Tissues:

INTRODUCTION

The methods for the estimation of glutathione in blood and tissues can be classified, according to the types of reagents used, as follows: (a) Iodine (99-106), (b) Nitroprusside (107-111), (c) Ferricyanide (112, 113), (d) Arsenophosphotungstic acid (114, 115), (e) Phospho-18-tungstic acid (116), (f) Glyoxalase (117, 118), and (g) Silver nitrate (119, 120).

Although glutathione can be estimated accurately, in pure solutions, by most of the above methods, only the glyoxalase method is reliable when applied to biological materials. Substances which usually interfere with the glutathione estimation, in biological materials, by one or the other methods mentioned above are uric acid, ascorbic acid, cysteine, ergothioneine, and phenols. The glyoxalase method is claimed to be entirely free from interference by any of the above mentioned substances (47, 117). The specificity of glutathione in the glyoxalase system depends on the reduced form of the intact tripeptide (60, 117, 134). In the 1930's Lohmann (47) showed that glutathione acts as a coenzyme in the glyoxalase system and the enzyme activation is dependent, within a certain range, upon the

glutathione concentration. Woodward (117) exploited the idea and developed a manometric procedure for the estimation of glutathione (GSH) in blood and tissues. At that time she could not employ this method for the estimation of oxidized glutathione (GSSG), after reduction of the latter by metals, because of the toxicity of most of the metals towards glyoxalase or because of incomplete reduction of the oxidized glutathione by the metals. However, in 1939 Dohan and Woodward (118) successfully reduced GSSG by electrolytic reduction and showed by recovery experiments that the GSSG can be estimated quantitatively in biological materials by the combined use of electrolytic reduction and the manometric method. In that paper they reported that the GSH content of blood and tissue filtrates of rats and rabbits remained the same before and after electrolytic reduction and it was concluded that blood and tissues do not contain any oxidized glutathione. It is interesting to note that a similar suggestion had been made previously by Tunnicliffe (99). However, Thompson and Voegtlin (100) who used Tunnicliffe's iodometric method of glutathione estimation and employed metallic magnesium for the reduction of GSSG in blood and tissue filtrates, observed a 5% increase in the titration values after Mg reduction and concluded that most of the glutathione exists in the reduced form and less than 10% is present in the oxidized form. At a still later date, Schelling (113) used the more specific ferricyanide method of Mason (112) for the estimation of glutathione. He subjected 1:5 Folin-Wu blood filtrates to nascent hydrogen reduction with Mg powder and observed

a definite increase in+ thiol values (sometimes 100%) after such reduction; but he was not convinced that the increase was due to the reduction of GSSG. Schelling did not make any definite comment on the presence or absence of the oxidized glutathione but ascribed the higher thiol values after Mg. treatment to the reduction of some other substances besides glutathione which were interfering with the method. Fujita and Numata (105, 110) applied H_2S for the reduction of GSSG in blood and tissue filtrates and estimated the total glutathione by the iodometric and nitroprusside methods. They reported the presence of considerable amounts of GSSG in blood and tissues. Dohan and Woodward (118) criticized the work of Fujita and Numata (105), on the basis of their finding that H_2S is retained in the dilute (1:20) filtrates and such traces of H_2S can consume sufficient iodine to lead to considerable errors when the results are calculated as GSSG per 100 g. of tissue. However, a number of other papers have appeared on the glutathione contents of tissues and blood (121-128, 133, 179) since the publication of Dohan and Woodward's paper; but there is no general agreement on the question of the existence of oxidized glutathione in tissues and blood. This warranted a reinvestigation of the problem.

+ The word "thiol" is used rather loosely in this text to denote thiols and other compounds measured by the non-specific iodometric method.

EXPERIMENTAL

Methods

Glyoxalase method for the estimation of GSH:

The method of Woodward (117) was used with minor modifications.

Reagents:

A. Acetone dried yeast:

Fresh baker's yeast (Distillers Co. Ltd., Edinburgh) was dried according to Albert, Buchner and Rapp (129) and stored in the ice box. The yeast was rendered glutathione-free before use by suspending 2-3 g. of acetone dried yeast in 80-90 ml. of distilled water and centrifuging. The procedure was repeated three times. Finally the washed yeast was made up as 33-36% suspension in water (the concentration of yeast was chosen such that with 0.5 ml. of 20 mg.% standard glutathione solution it produced approximately 200 microliters of CO_2 in 20 minutes).

B. 1% Methyl glyoxal (pyruvic aldehyde) (L. Light and Co. Ltd., Bucks, England). Made by diluting a 30% aqueous stock solution.

C. 0.2 M sodium bicarbonate. (A.R.) (B.D.H.).

D. 0.8 M sodium bicarbonate. (A.R.) (B.D.H.).

E. 3% (w/v) sulfosalicylic acid (B.D.H.).

F. Glutathione. (Yeast Research Outstation, Distillers Co. Ltd., Glenochil, Menstrie, Clackmannanshire; and Distillers Co. (Biochemicals) Ltd., Fleming Road, Speke, Liverpool).

A fresh 20 mg.% (w/v) stock glutathione (purity 99.8%) solution

was prepared every day. The stock solution was diluted to make 5 and 10 mg.% solutions.

The glyoxalase activity was measured at 25° in a circular Warburg machine. Warburg flasks with a single sidearm (capacity 0.8-1.0 ml.) and a total volume of 15-16 ml. were used. The following were pipetted into the main chamber of each flask: 0.5 ml. of yeast suspension, 0.2 ml. of methyl glyoxal, 0.4 ml. of 0.2 M sodium bicarbonate, and water to make the total fluid volume, including the measurements in the sidearm, to 2 ml. 0.5 ml. of standard solutions containing 5, 10, 20 mg. of glutathione per 100 ml. were measured into the sidearms respectively. The standard glutathione solutions were not neutralized. 0.5 ml. of 3% sulfosalicylic acid (SSA) blood or tissue filtrate was neutralized in the sidearm with 0.8 M sodium bicarbonate (the requisite amount being determined by a separate titration using methyl orange as an indicator). The standards and the unknowns were run in duplicate.

In running a typical estimation the following procedure was adopted: All the reagents for the main chamber were pipetted first. The standard solutions of glutathione and the requisite amounts of 0.8 M $NaHCO_3$ (for the neutralization of the sulfosalicyclic acid filtrates) were measured into the sidearms. The flasks containing the standard solutions were fitted to the manometers. The unknown filtrates were pipetted into the sidebulbs and the flasks were immediately fitted to the manometers. The manometers and the vessels were flushed with a mixture of 95% N_2 and 5% CO_2 for 15 minutes at 25°. After gassing, the

manometers and the vessels were shaken for 1 minute. At the end of which the contents of the side-bulbs were tipped in and the shaking continued for 4 minutes with the stop-cocks of the manometers open. The shaking mechanism was stopped, the stop-cocks were closed and the fluid levels of the manometers were adjusted such that the left hand columns read between 0-20 mm. Shaking was resumed again and readings were taken at 5 minute intervals for 25 minutes without stopping the shaking mechanism. The first five minutes readings were discarded. A calibration curve was constructed by plotting the microliters of CO_2 evolved in 20 minutes against the glutathione concentrations. The unknowns were read off from the curve and the glutathione concentrations calculated according to the dilutions of the filtrates. The concentration of glutathione in the unknowns was kept limited to 10 mg. of glutathione per 100 ml.

A yeast blank with no glutathione was included in each estimation and the readings of the standards and the unknowns were corrected for blank gas evolution. When the number of unknowns were greater than that could be accommodated with the standards, then a separate yeast blank was repeated with the unknowns.

Iodometric estimation of GSH and GSSG:

The method of Woodward and Fry (101) was followed.

Electrolytic reduction for the estimation of oxidized glutathione:

3% sulfosalicylic acid (SSA) blood and tissue filtrates were subjected to electrolytic reduction as described by Dohan and Woodward (118). After electrolytic reduction the total glutathione estimations were done by the glyoxalase and iodometric methods.

The details of the electrolytic reduction will be described in the appropriate sections.

Choice of Sulfosalicylic Acid Concentration for the Electrolytic Reduction of Blood Filtrates:

In a few preliminary experiments it was observed that when 5 ml. of 1:5,2% sulfosalicylic acid blood filtrate were reduced for 10 minutes with a current density of 4.26 milliamperes per sq. cm., the filtrate became alkaline to methyl orange. If glutathione estimation was done in such a filtrate by the glyoxalase method, the GSH value was considerably lower after than before reduction (Table 5a).

TABLE 5a

GSH mg.%

Before	After
38.2	33.6
58.1	45.0
42.2	17.5
22.5	20.0

Unfortunately, the exact pH values of the above filtrates after the reduction were not measured; since the filtrates were alkaline to methyl orange the pH values must have been above 4.0. At this stage, it was decided to determine the exact pH values, of blood filtrates before and after reduction, using various acid concentrations and reduction conditions. The results of such experiments are presented in Table 5b. It can be seen from the

TABLE 5b

pH of Sulfosalicylic Acid Filtrates before and after Electrolytic Reduction

Filtrates	Acid concentration	pH		Conditions of reduction					
		Before	After	Volume reduced	Cathode vessel diameter	Salt bridge bore	Current	Current density $\frac{\text{current}}{\text{area}}$	Time of reduction
	%			ml.	cm.	mm.	milli-amperes	milli-amperes per sq.cm.	min.
Blood filtrate 1:5	2.0	1.84	3.36	10	3.5	4.5	24	2.49	20
"	2.3	1.8	3.22	10	3.5	4.5	24	2.49	20
"	2.3	1.8	5.68	5	3.6	4.5	44	4.32	10
"	2.3	1.8	4.4	5	3.6	4.5	44	4.32	10
"	2.3	1.6	3.5	5	3.6	4.5	44	4.32	10
"	3.0	1.4	1.78	10	3.5	4.5	24	2.49	20
"	3.0	1.36	1.74	10	3.5	4.5	24	2.49	20
"	3.0	1.47	1.9	10	3.5	4.5	24	2.49	20
"	3.0	1.29	1.98	5	3.6	4.5	44	4.32	10
"	3.0	1.4	2.36	5	3.6	4.5	44	4.32	10
"	3.0	1.52	2.35	5	3.6	4.5	44	4.32	10
"	3.0	1.32	2.14	5	3.6	4.5	44	4.32	10
Liver filtrate	3.0	1.09	1.34	5	3.6	4.5	44	4.32	10
"	3.0	1.02	1.22	5	3.6	4.5	44	4.32	10

above table that the pH values of 2% and 2.3% SSA blood filtrates rise above 3.0 even after reduction with a low current density of 2.49 milliamperes per sq. cm. and with a high current density of 4.32 milliamperes per sq. cm. the pH values go near or above 4.0. It should also be noted that both the initial and the final pH values show wide variation. Such variations may be expected because of the fact that different blood samples have varying buffering capacities, which influence both the initial pH and the pH after reduction. Woodward and Fry (101) showed that glutathione undergoes considerable autoxidation within an hour at pH values over 3.0. Table 5b shows that the pH values of 2.3% SSA blood filtrates rise above 3.0 under all conditions of reduction; specially when the filtrates were reduced with a high current density the pH values ranged from 3.5-5.68. Taking the above factors into consideration it is not surprising that some loss of GSH was observed after electrolytic reduction with a high current density (Table 5a). Furthermore, it should be noted in the latter table that the loss of GSH is not the same in all the cases. This is in harmony with the fact that filtrates show wide variation of pH values after reduction (Table 5b), therefore, the loss of GSH shall not be the same in all cases. It will be shown later, where reduced and total glutathione (after electrolytic reduction with a low current density) estimations were done by the glyoxalase method, on the same sample of blood deproteinized by 2.3% or 3% SSA, the oxidized glutathione value was lower in the former than in the latter.

During an experiment in which several reductions are done, the filtrates stand for an hour or more after the electrolytic reduction, before the total glutathione contents are estimated manometrically. Since the above results indicate that loss of GSH occurs, after electrolytic reduction, in 2% or 2.3% SSA blood filtrate, it is not safe to submit such filtrates to the electrolytic reduction if the total GSH is to be estimated by the glyoxalase method. In view of the fact that 3% SSA blood filtrates maintain the pH around 2.0 at high or low current densities (Table 5b) and at that pH no loss of GSH occurs for a considerable length of time (101), 3% SSA is preferable to 2% or 2.3% SSA. During the course of this work numerous 3% SSA blood filtrates were reduced with high and low current densities, and although the exact pH values were not measured in each case, none of the filtrates became alkaline to methyl orange.

Effect of pH on the Electrolytic Reduction:

Portions of 3% SSA were brought to pH 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0 by means of dilute HCl and Na_2HPO_4. Oxidized glutathione was added to these solutions to make a final concentration of 20 mg. of GSSG per 100 ml. 5 ml. of each solution were reduced for 10 minutes with a current density of 4.32 milliamperes per sq. cm. and the degree of reduction was determined by the iodometric method. 100% reduction was observed in all cases. When the above solutions were left after the electrolytic reduction for 12 hours, 13-17% loss of GSH was observed at pH 2

(pH 3.5 after reduction), while in the others only 2-3% loss of GSH occurred. This experiment shows that there is no optimum pH for the electrolytic reduction; it proceeds equally well at all pH values. This is in substantial agreement with Dohan and Woodward (118); they found the same degree of reduction in 1.8-3.4% sulfosalicylic acid concentrations.

Recoveries of Reduced and Oxidized Glutathione from Different Media by the Glyoxalase Method:

The recoveries of reduced glutathione, when the latter was added to plasma or whole blood, by the glyoxalase method, are presented in Table 6. The average recovery from the plasma is 98.6% (range 95-100%) and from the whole blood is 97.4% (range 93-102%). Taking into consideration the various factors which influence the glutathione estimation by the above method the recoveries are quite good. Table 7 presents the recoveries of oxidized glutathione, when the latter was added to plasma, by the combined use of electrolytic reduction and the glyoxalase method of estimation. Quantitative recoveries are obtained with current densities of 2.49, 4.26, and 4.32 milliamperes per sq. cm. It can be seen from the latter table that similar recoveries are obtained from both 3% and 2.4% sulfosalicylic acid filtrates. This is due to the fact that 1:5 plasma filtrates of 2.4% SSA have lower pH than 1:5 blood filtrates of the same acid, and consequently plasma filtrates do not become as alkaline as blood filtrates under similar conditions of reduction. When H_2O_2 was used as an oxidizing agent for the GSH (130) sometimes very low recoveries (88-90%) of GSSG were obtained, possibly

TABLE 6

Recoveries of GSH from Whole Blood and Plasma by the Glyoxalase Method

GSH --- Reduced Glutathione

Media	Acid concentration	GSH added	GSH calculated	GSH estimated	Recovery
	mg.%	mg.%	mg.%	mg.%	%
Blood Plasma	3	30	30	30	100
"	3	50	50	49	98
"	3	50	50	50	100
"	3	25	25	25	100
"	3	25	25	23.7	95
Whole Blood	3	10	31.5	31.2	99
"	3	10	31.5	30.0	95
"	3	40	53.8	53.1	98.6
"	3	40	53.8	50.0	93.0
"	3	40	52.0	49.0	94.2
"	3	20	56.2	57.5	102
"	3	20	56.2	56.2	100

TABLE 7

Recoveries of Oxidized Glutathione from Plasma by the Glyoxalase Method and Electrolytic Reduction

GSH - Reduced glutathione GSSG - Oxidized glutathione

Media	Oxidizing agent	GSSG added	GSSG calculated	Estimated as GSH	Recovery	Conditions of reduction						
						Acid concentration	Volume reduced	Cathode vessel diameter	Salt bridge bore	Current	Current density $\frac{\text{current}}{\text{area}}$	Time of reduction
		mg.%	mg.%	mg.%	per cent	per cent	ml.	cm.	mm.	milliamperes	milliamperes per sq.cm.	min.
Blood Plasma 1:5	H_2O_2	25	25	23.75	95	3	5	3.5	4.5	41	4.26	10
"	H_2O_2	50	50	49.3	98.7	3	5	3.5	4.5	41	4.26	10
"	H_2O_2	50	50	48.7	97.5	3	5	3.5	4.5	41	4.26	10
"	H_2O_2	30	30	28.7	95.6	2.4	5	3.5	4.5	41	4.26	10
"	H_2O_2	30	30	29.0	96.6	2.4	5	3.5	4.5	41	4.26	10
"	H_2O_2	25	25	22.5	90.0	3	5	3.5	4.5	41	4.26	10
"	H_2O_2	25	25	23.1	92.4	3	5	3.5	4.5	41	4.26	10
"	I_2	50	50	50	100	3	5	3.5	4.5	41	4.26	10
"	I_2	30	30	28.8	96	3	10	3.5	4.5	24	2.49	20
"	I_2	30	30	30	100	3	10	3.5	4.5	24	2.49	20
"	I_2	30	30	31	103	3	10	3.5	4.5	24	2.49	20
"	I_2	50	50	50	100	3	10	3.5	4.5	24	2.49	25
"	I_2	50	50	50.5	101	3	10	3.5	4.5	24	2.49	25
"	I_2	50	50	49.0	98	3	5	3.5	4.5	44	4.32	10
"	I_2	50	50	50	100	3	5	3.5	4.5	44	4.32	10
"	I_2	50	50	47.5	95	3	5	3.5	4.5	44	4.32	10

due to the oxidation of GSH beyond the disulfide stage. However, when GSH was oxidized with the theoretical amount of N/100 iodine solution, uniformly good recoveries were obtained. The presence of HI does not interfere with the glyoxalase method.

Recoveries of Oxidized Glutathione from Blood, Plasma, and Aqueous Solutions after Zinc Reduction by the Iodometric Method

GSSG was added to plasma or whole blood to give oxidized glutathione concentrations of 10, 15, 30 mg. per 100 ml. and the solutions were made protein-free by 3% SSA. 10 ml. of the 1:5 SSA filtrates were submitted to Zn reduction for 30 minutes. The amount of reduction was checked through iodate titration. It was observed that only 69% of the added GSSG could be recovered as GSH from the blood filtrates and 52% from the plasma filtrates. Such inability of zinc to reduce GSSG quantitatively in the presence of plasma or serum filtrate has been observed by Dohan and Woodward (118). In common with them, it has been observed in the course of this work that zinc can bring about 100% reduction of GSSG in aqueous 3% SSA solution; because of the low recoveries from blood or plasma and since zinc reduced samples cannot be used for the estimation of total glutathione by the glyoxalase method (zinc reduced samples are toxic to the glyoxalase), electrolytic reduction, which gave complete recovery, was ultimately adopted as the standard procedure.

Investigations on the Presence of Oxidized Glutathione in Blood:

Human Blood:

Dohan and Woodward (118) mentioned in their communication that the oxidized glutathione can be reduced by electrolytic reduction to GSH and the latter can be estimated by the glyoxalase method as well as by the iodometric method (101). Furthermore, they recovered added GSSG quantitatively from red blood cells and red blood cell filtrates by the combined use of electrolytic reduction and the iodometric method of estimation. It is rather surprising that no attempt was made by the above authors to estimate any oxidized glutathione that might normally be present in blood by the latter procedure. In some pilot experiments 3% sulfosalicylic acid blood filtrates were subjected to electrolytic reduction and the glutathione values before and after reduction were estimated by the glyoxalase method. Consistently higher GSH values were observed after than before reduction. It was of interest, therefore, to make a comparative study on various blood samples deproteinized by 3% SSA, reduced electrolytically or by zinc, and estimating the reduced and total glutathione values by the iodometric and glyoxalase methods.

The following experimental procedure was adopted: Venous blood samples from normal subjects and hospital patients were drawn in heparinized syringes and immediately upon collection the samples were deproteinized. For deproteinization 1 volume of non-hemolyzed blood was added very slowly with agitation to 4 volumes of 3%

sulfosalicylic acid and the mixture was thoroughly shaken. Since the blood was not hemolyzed prior to deproteinization, thorough shaking was essential to ensure complete extraction of the glutathione from the disintegrated corpuscles. Glutathione and total glutathione (reduced plus oxidized glutathione) estimations were done on aliquots of the same filtrate as follows: GSH was estimated by the iodometric (101) or glyoxalase method. A 10 ml. portion of the filtrate was reduced by 30-40 mg. of zinc dust and the total glutathione was estimated by the iodometric method. Another 10 ml. of the filtrate were subjected to electrolytic reduction with a current density of 2.49 milliamperes per sq. cm. for 20 minutes. 5 ml. of the reduced sample were titrated with iodate and in the remaining filtrate total glutathione was estimated by the glyoxalase method.

In order to clarify the arguments in this section, several observed facts have been recalled here. The iodometric method is non-specific for GSH estimation and zinc does not reduce GSSG quantitatively in blood or plasma filtrate; on the other hand, the glyoxalase method is specific for the estimation of glutathione and the GSSG is quantitatively reduced by the electrolytic reduction in the presence of biological materials. Table 8 presents the analyses for reduced and total glutathione, in different blood filtrates, by various methods. It can be seen from the latter table that the thiol values increase after electrolytic reduction, irrespective of the method of estimation. It might be argued that the increases in thiol values after Zn

TABLE 8

Glutathione Analyses of Human Blood by Various Methods

GSH - Reduced glutathione GSSG - Oxidized glutathione
The results are expressed in mg. per cent

Sample No. and Diagnosis	Iodometric method zinc reduction			Iodometric method electrolytic reduction			Glyoxalase method electrolytic reduction		
	GSH	GSSG	Total GSH	GSH	GSSG	Total GSH	GSH	GSSG	Total GSH
No.1 Normal	30.3	6.8	37.1	30.3	8.0	38.3	36.8	6.3	43.1
" 2 Diabetic	16.8	6.5	23.3	16.8	6.8	23.6	17.5	6.2	23.7
" 3 Duodenal ulcer	27.3	10.4	37.7	27.3	10.4	37.7	31.8	6.3	38.1
" 4 Sciatica	26.6	7.1	33.7	26.6	7.7	34.3	31.0	6.5	37.5
" 5 Duodenal ulcer	28.8	4.9	33.7	28.8	6.4	35.2	26.2	4.8	31.0
" 6 Normal	37.4	4.3	41.7	37.4	5.2	42.7	41.2	5.3	46.5
" 7 Normal	21.7	4.9	26.6	21.7	7.4	29.1	19.0	1.0	20.0
" 8 Normal	28.5	8.3	36.8	28.5	9.2	37.7	24.7	6.3	31.0
" 9 Neuritis	26.6	8.6	35.2	26.6	11.4	38.0	26.8	7.2	34.0
"10 Softening of skull	30.9	7.4	38.3	30.9	9.5	40.4	32.0	7.7	39.7
"11 Normal	28.8	7.7	36.5	28.8	8.0	36.8	26.0	5.0	31.0
"12 Normal	32.8	8.6	41.4	32.8	8.6	41.4	29.0	7.0	36.0
"13 Diabetic	26.0	10.5	36.5	26.0	10.8	36.8	22.7	5.4	28.1
"14 Normal	36.8	9.5	46.3	-	-	-	35.0	3.7	38.7
"15 Epileptic	-	-	-	30.6	6.0	36.6	29.0	-	28.7
"16 Normal	-	-	-	34.5	4.1	38.6	41.2	-	40.5

or electrolytic reduction, as estimated by the iodometric method, may be due to substances other than glutathione. However, reference to Table 8 shows that out of the sixteen cases studied only in two cases (No. 15 and 16) the glutathione values, as obtained by the glyoxalase method, remained the same before and after electrolytic reduction. On the other hand, in the same two cases the thiol values obtained by the iodometric method are higher after than before electrolytic reduction. In those two cases in question, therefore, the increases in thiol values obtained after electrolytic reduction by the iodometric method are in all probability due to substances other than glutathione. In the rest of the cases, however, the glutathione values obtained by the iodometric and manometric methods are higher after than before reduction. Since the glyoxalase method is extremely specific for the estimation of glutathione, it can be assumed that the increases in thiol values, as estimated after electrolytic reduction by the manometric method, are due to the reduction of oxidized glutathione. Hence the increases in thiol values, estimated in common with the glyoxalase method by the iodometric method, are also partly, but not wholly, due to the reduction of oxidized glutathione. The latter statement can be verified by comparing the results obtained after Zn or electrolytic reduction by the iodometric method with those obtained after electrolytic reduction by the manometric method. Such comparisons are shown in Fig. 2-5, each point in those figures represents the result of one estimation, on the same filtrate, by each method. If the two methods give

identical values then the points should fall on the line with slope 1; if one gives higher results than the other then the points would shift towards the method giving the higher results. A similar comparative study has been made in Fig. 1 between the reduced glutathione values obtained by the iodometric method and those obtained by the manometric method. In the latter figure the points are scattered almost equally on both sides of the middle line, which implies that the methods agree well and probably measure the same substance. Taking into account the extreme specificity of the glyoxalase method, it may be assumed that the substance measured by both the methods is glutathione. The total and oxidized glutathione values given by the iodometric method are higher than those obtained by the glyoxalase method (Fig. 2-5). The latter findings indicate that probably during electrolytic and zinc reductions of blood filtrates, substances other than oxidized glutathione are also reduced, and when such filtrates are analyzed by the non-specific iodometric method these interfering substances consume iodine thereby giving falsely high glutathione values, but do not activate the glyoxalase system. Since GSSG in the presence of protein is 100% reducible by the electrolytic reduction but is only 70% reducible by the Zn reduction, and interfering substances are formed during both the reductions it might be expected that the total and oxidized glutathione values obtained by the iodometric method after electrolytic reduction of blood filtrates would be higher than the corresponding values obtained by the same method after Zn reduction; Fig. 6 and 7 show that such is the case.

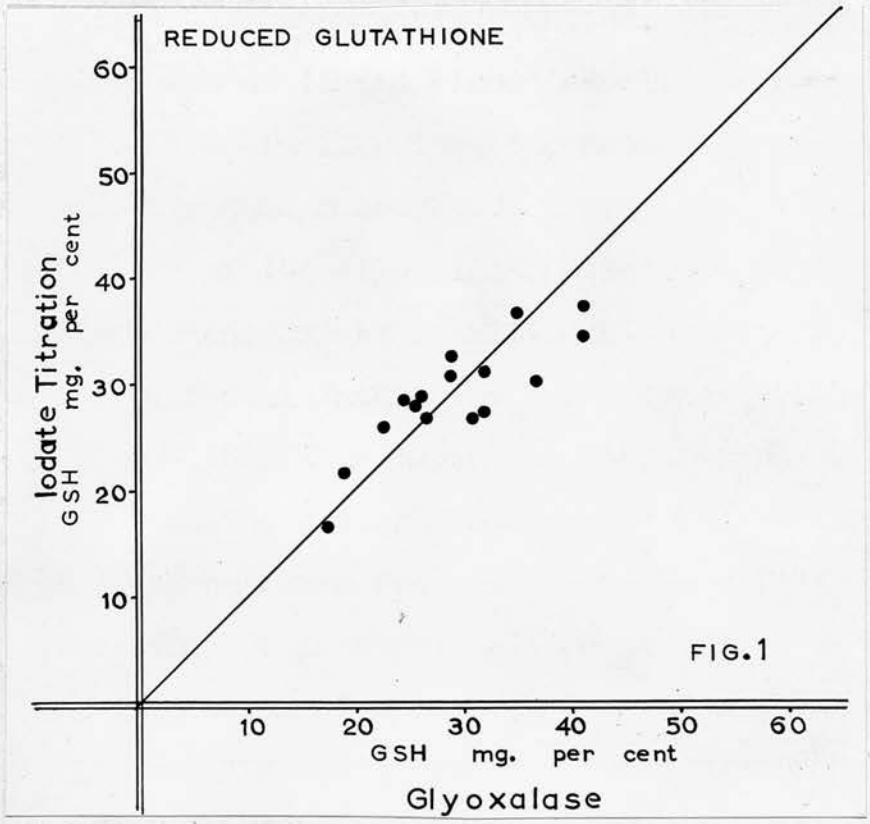


FIG. I. A comparison between the reduced glutathione values of blood obtained by the glyoxalase method and those obtained by the iodometric method.

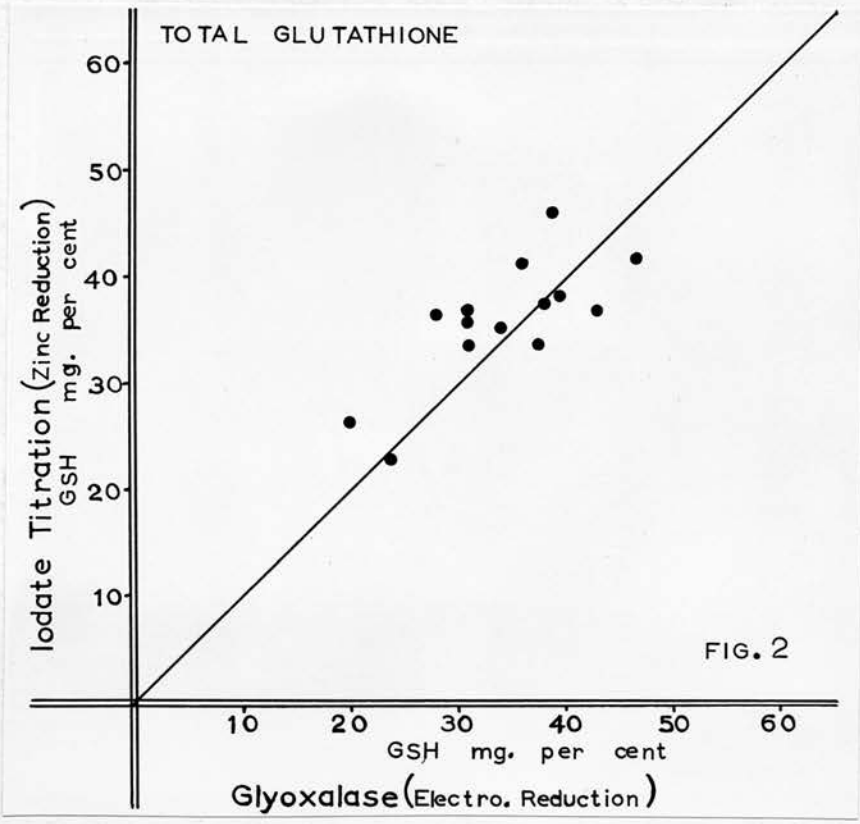


FIG. 2. A comparison between the total glutathione values of blood obtained after electrolytic reduction by the glyoxalase method and those obtained after zinc reduction by the iodometric method.

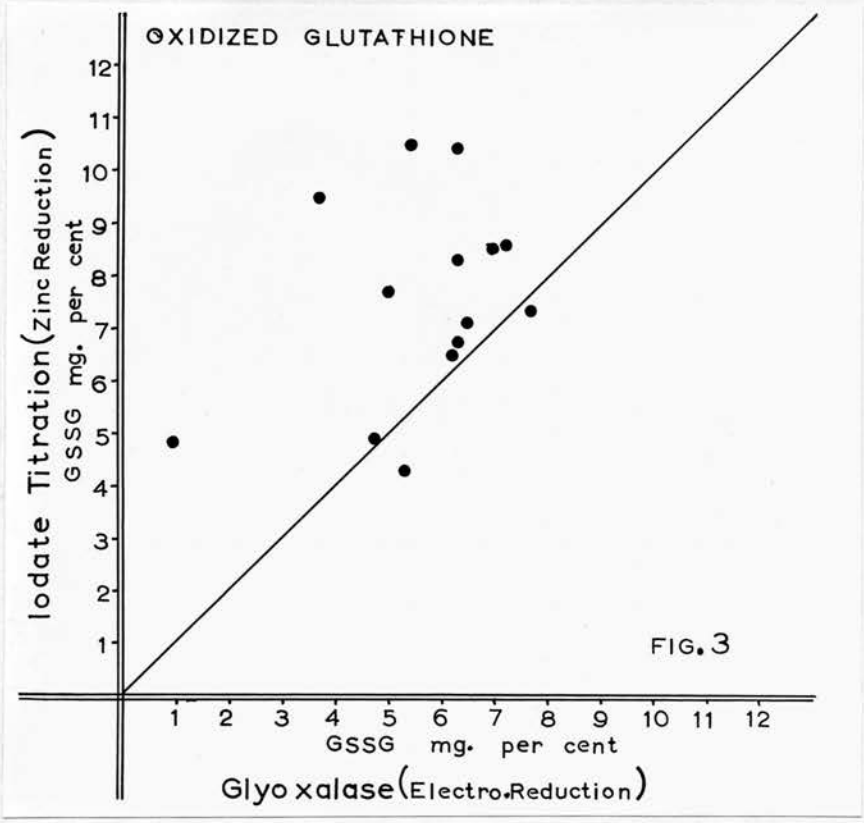


FIG. 3. A comparison between the oxidized glutathione values of blood obtained after electrolytic reduction by the glyoxalase method and those obtained after zinc reduction by the iodometric method.

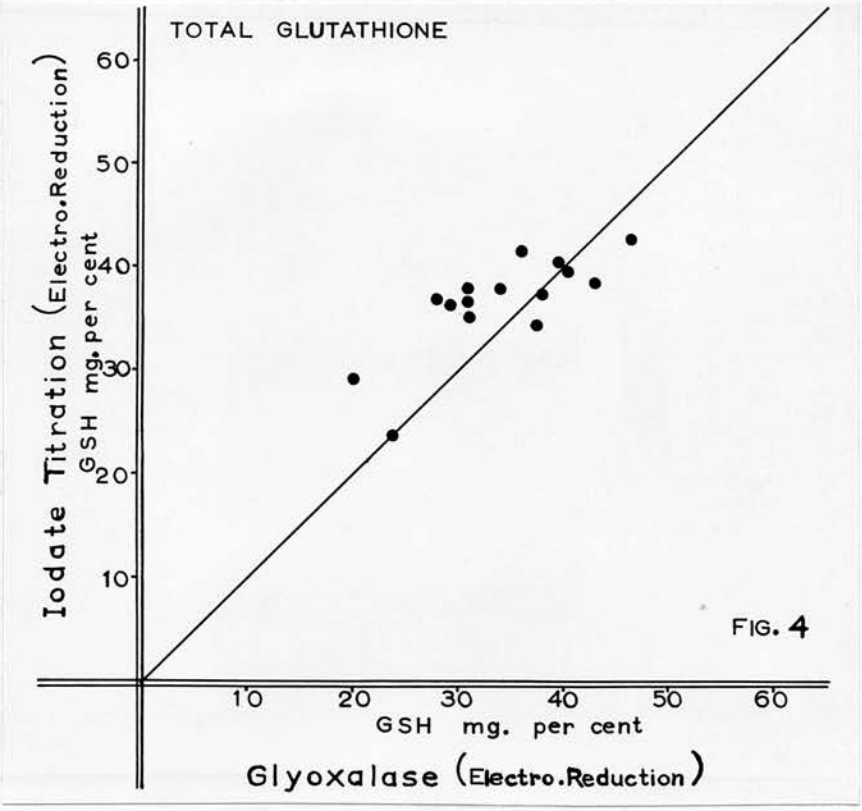


FIG. 4. A comparison between the total glutathione values of blood obtained after electrolytic reduction by the glyoxalase method and those obtained after electrolytic reduction by the iodometric method.

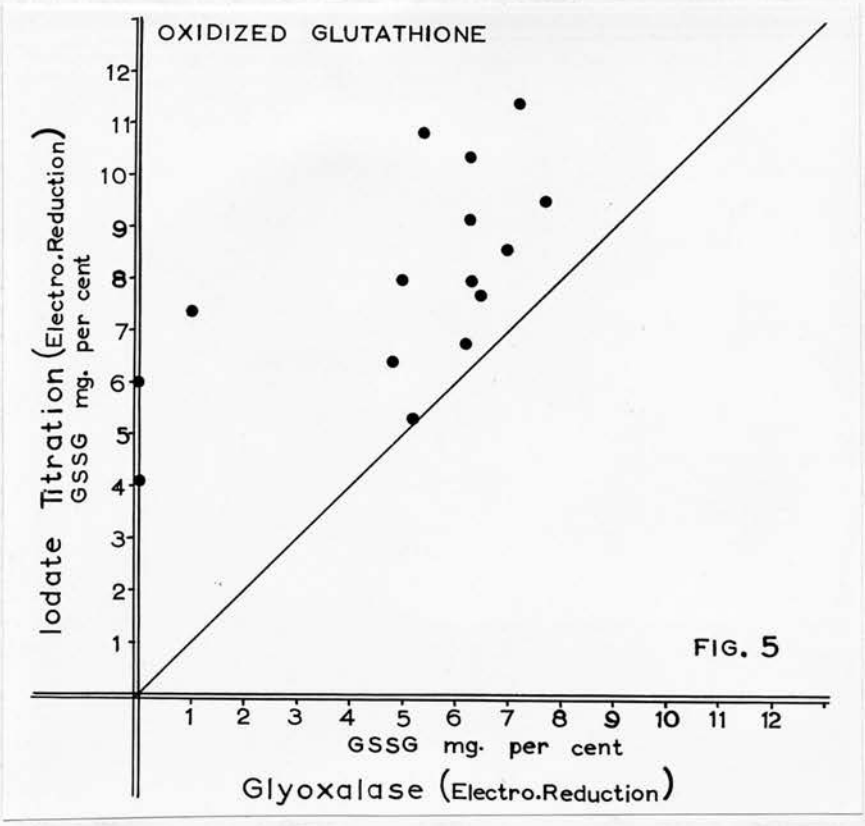


FIG. 5. A comparison between the oxidised glutathione values of blood obtained after electrolytic reduction by the glyoxalase method and those obtained after electrolytic reduction by the iodometric method.

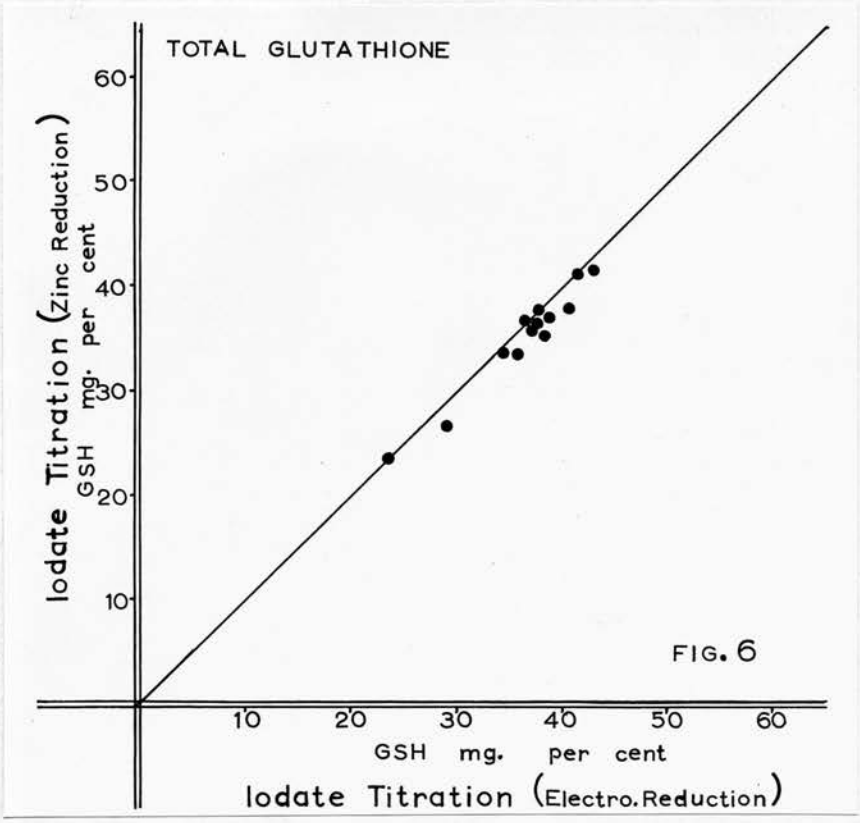


FIG. 6. A comparison between the total glutathione values of blood obtained after electrolytic reduction by the iodometric method and those obtained after zinc reduction by the iodometric method.

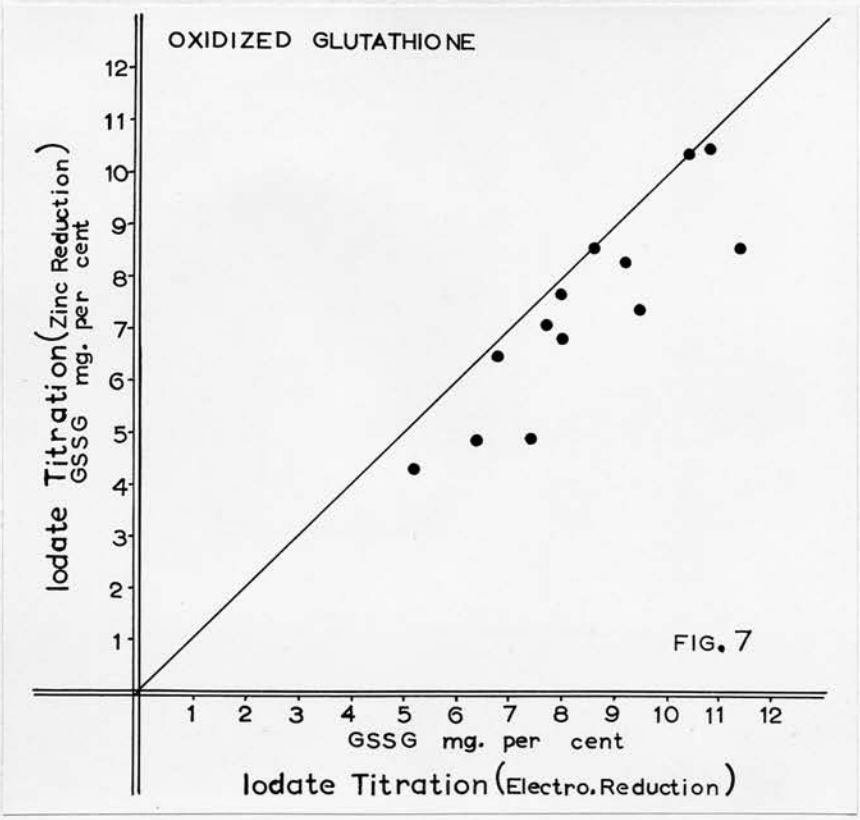


FIG. 7. A comparison between the oxidized glutathione values of blood obtained after electrolytic reduction by the iodometric method and those obtained after zinc reduction by the iodometric method.

It can be concluded, therefore, that the iodometric method is reliable for the estimation of GSH in blood filtrates, but is unsuitable for the accurate estimation of total glutathione in blood filtrates.

Rabbit and Rat Blood:

Blood samples were collected in heparinized syringes from the abdominal aorta of rats and by cardiac puncture from rabbits. As soon as possible after collection the samples were deproteinized with 3% sulfosalicylic acid as described for the human blood. GSH and total glutathione (after electrolytic reduction) estimations were done by the glyoxalase method. The electrolytic reduction was carried out as follows: 5 ml. of the filtrates were reduced for 10 minutes with a current density of 4.26 or 4.32 milliamperes per sq. cm. The results of this experiment are presented in Table 9. It is evident from the latter table that considerable amounts of GSSG are present in all the samples and the rat blood contains significantly higher amount of GSSG than the rabbit blood. The difference may be attributable to the variation in the GSH content of the arterial and venous blood (101, 131, 132). This explanation is not quite tenable in the above cases, because blood samples were collected from the rabbits by a technique, described in Part I, which ensures puncture of the left ventricle, therefore, the blood samples were arterial. However, there is another possibility that the blood from different regions of the body may contain varying

TABLE 9

Glutathione Analyses of Rabbit and Rat Blood by the Glyoxalase Method

GSH -- Reduced glutathione GSSG -- Oxidized glutathione
The results are expressed in mg. per cent

Animal No.	GSH	GSSG	Total GSH
Rat 1	16.3	21.2	37.5
" 2	16.3	23.4	39.7
" 3	22.5	25.0	47.5
" 4	16.9	20.6	37.5
" 5	18.8	19.9	38.7
" 6	17.5	20.5	38.0
Rabbit 1	22.5	6.8	29.3
" 2	35.0	13.2	48.2
" 3	30.0	13.0	43.0
" 4	25.0	21.3	46.3
" 5	24.0	12.0	36.0
" 6	34.3	11.9	46.2
" 7	22.4	7.3	29.7
" 8	43.1	10.9	54.0
" 9	24.0	16.0	40.0
" 10	38.7	7.5	46.2
Rabbit blood before CO	27.5	17.5	45.0
Same after CO	33.7	16.3	50.0
Rabbit blood before CO	24.0	16.0	40.0
Same after CO	30.6	16.9	47.5

amounts of GSSG. In the absence of any experimental evidence to support the later contention the difference is suitably explained as due to the species variation.

Effect of Carbon Monoxide on the GSSG Content of Blood:

Numata (132) observed that GSH is oxidized during the deproteinization of blood with HPO_3. He suggested that the protein precipitation of blood or organs with high hemoglobin contents must be done in an atmosphere of carbon monoxide to prevent such oxidation. The recovery experiments of added GSH from the whole blood (Table 6) prove that no oxidation of GSH occurs during the protein precipitation of blood with 3% SSA. However, to be absolutely sure that such is the case it was decided to estimate the GSH and total glutathione in the same blood sample, treated and non-treated with carbon monoxide before deproteinization, by the glyoxalase method. The same experimental procedure was adopted for the deproteinization and electrolytic reduction as reported for the rabbit and rat blood. The results of two such experiments with rabbit blood are presented in Table 9. It is interesting to note that both the GSH and total GSH values are 5-6 mg.% higher in the carbon monoxide treated samples than in the non-treated samples; but the GSSG values in the carbon monoxide treated and non-treated samples are practically the same. Incidentally it might be mentioned that aqueous solutions of GSH, when estimated by the glyoxalase method, show the same GSH contents before and after CO treatment. Experiments have

not been done to investigate the nature of the interference by CO; because such an unphysiological level of CO (100% saturation) is not ordinarily encountered whereby the specificity of the manometric method may be jeopardized. However, the significant point of this experiment is that the oxidized glutathione values remain the same whether blood samples are treated or not treated with CO before the deproteinization.

Miscellaneous Experiments:

These experiments were done with the hope that it might be possible to find some explanation for the discrepancies between the results of Dohan and Woodward (118) and the results reported here.

Glutathione and total glutathione estimations were done in freshly drawn human blood samples by the combined use of electrolytic reduction and the glyoxalase method. Heparin was used as an anticoagulant. The samples were deproteinized with 2.3% or 3% SSA as previously described for the human blood. 10 ml. of 1:5, 2.3% or 3% blood filtrate were submitted to electrolytic reduction with a current density of 2.49 milliamperes per sq. cm. for 20 minutes; the total glutathione estimations were done as quickly as possible (loss of GSH occurs if 2.3% SSA filtrates are allowed to stand after the electrolytic reduction). Three such experiments were done and the following, 2.3, 4.2, and 1.6 mg.% GSSG were observed in 2.3% SSA filtrates; the corresponding GSSG values in 3% SSA filtrates were 5, 5.3, and 5.3 mg.%.

These experiments support the previous contention that 2.3% SSA blood filtrates are unsuitable for the electrolytic reduction; but they do not explain the inability of Dohan and Woodward (118) to find any increase in GSH values of blood filtrates after electrolytic reduction by the glyoxalase method. It is most unfortunate that they concluded on the basis of only two determinations that oxidized glutathione does not exist in blood. In this context it should be pointed out, of course, that Dohan et al. submitted 5 ml. of 1:5, 2.3% SSA blood filtrates to electrolytic reduction with a current density of 4.2 milliamperes per sq. cm. for 10 minutes; in our experience such reduction conditions take the pH of these filtrates to near 4 or above 4. As has been stated already, we found considerable loss of GSH at the latter pH; it is curious that Dohan et al. found no such loss. In the absence of any information in the paper of the above authors about how soon the estimations were done after electrolytic reduction, it is difficult to draw any conclusion.

In the course of this investigation other factors were also checked which might be responsible for the oxidation of GSH during the deproteinization of blood. Since the mixture of blood and 3% SSA was shaken for the complete extraction of GSH from the corpuscles, it was decided to check if such shaking caused any oxidation of GSH. Samples of blood were deproteinized with and without shaking and the GSH estimations were done by the manometric and iodometric methods. It was observed that thorough shaking was essential for the complete

extraction of GSH from the disintegrated corpuscles and no oxidation of the GSH occurs under such conditions.

Experiments were also done to find out if any oxidation of GSH occurred between the collection and deproteinization of blood samples. It has been found that the amounts of GSH, GSSG, and total glutathione remained the same no matter whether the blood samples were taken directly into 3% SSA (cooled to 0° or at room temperature) or into a test-tube and then pipetted into 3% SSA.

The preceding experiments seem to exclude any possibility of the formation of oxidized glutathione during the technical manipulations of blood samples. Therefore, the GSSG, which is measured, may be considered to exist preformed in the blood of human, rabbits and rats.

Question of the Existence of Oxidized Glutathione in Tissues:

A suitable amount of fresh tissue (0.5-0.8 g. of liver or 0.4-0.7 g. of pancreas) was taken in 5-7 ml. of 3% sulfosalicylic acid and weighed. The sample was ground with acid washed white sand in an all-glass mortar and pestle. The extraction was repeated a few times and finally the whole coagulum was transferred to a volumetric flask and made up to volume (25 ml. for liver and 10 ml. for pancreas) with 3% sulfosalicylic acid. The extract was centrifuged and filtered. Glutathione and total glutathione estimations were done by the glyoxalase method. For the electrolytic reduction 5 ml. of the filtrate

were reduced for 10 minutes with a current density of 4.32 milliamperes per sq. cm. The pH of the filtrate, after electrolytic reduction, under the above conditions, was below 1.5.

The analyses for reduced and total glutathione in liver and pancreas of rats and rabbits are presented in Table 10. For the sake of comparison a few results obtained by the iodometric method after Zn or electrolytic reduction of the above filtrates are also presented. It is evident from the above table that the GSH values obtained by the iodometric method are higher than the corresponding values obtained by the manometric method. From these results it is concluded, that the degree of interference by substances other than glutathione is greater in tissue filtrates than in blood filtrates when glutathione is estimated by the iodometric method. It is surprising, however, that the thiol values show no rise after Zn or electrolytic reduction when estimated by the iodometric method; because the iodometric method is vulnerable to interference by substances other than glutathione which might be reduced in the course of Zn or electrolytic reduction. However, taking into account that not very many estimations were done, any attempt to draw any conclusion regarding the above issue will be abortive. In common with Woodward (117) these results indicate that the iodometric method gives erroneously high glutathione values in tissue filtrates. In agreement with Dohan and Woodward (118) no consistent rise in the GSH values

TABLE 10

Glutathione Analyses of Tissues by Various Methods

GSH - Reduced glutathione GSSG - Oxidized glutathione

The results are expressed in mg. per 100 g. of tissue

Tissues	Glyoxalase method electrolytic reduction			Iodometric method zinc reduction			Iodometric method electrolytic reduction		
	GSH	GSSG	Total GSH	GSH	GSSG	Total GSH	GSH	GSSG	Total GSH
Rat Liver	165	-	-	288	0	288	-	-	-
" "	133.7	16.3	150	258.2	-	-	-	-	-
" "	221.3	-	209.4	-	-	-	-	-	-
" "	213.0	-	210.0	-	-	-	-	-	-
" "	297.2	-	260.8	412.5	-	400.7	412.5	-	407.5
" "	214.7	0	214.7	-	-	-	-	-	-
" "	229.5	-	228.3	-	-	-	-	-	-
Rabbit liver	242.7	8.3	251.0	-	-	-	-	-	-
" "	283.6	0	283.6	-	-	-	-	-	-
Rat pancreas	45.8	7.9	53.7	87.3	0	87.3	-	-	-
" "	60.0	-	59.4	-	-	-	-	-	-
" "	50.8	-	49.3	-	-	-	-	-	-
" "	48.3	-	47.1	-	-	-	-	-	-
Rabbit "	31.2	9.6	40.8	-	-	-	-	-	-
" "	50	0	50	-	-	-	-	-	-

of the liver and pancreas filtrates after electrolytic reduction could be observed in this experiment. It might be mentioned in this context that the pH values of the above tissue filtrates were well below 2.0 even after electrolytic reduction with a high current density of 4.32 milliamperes per sq. cm. Lower concentrations of sulfosalicylic acid may be used safely for making tissue extracts, without going into the risk of taking the pH above 3.0 after electrolytic reduction.

It is concluded, therefore, that oxidized glutathione does not exist in measurable amounts in the tissues studied in the course of this investigation.

DISCUSSION

A comparative study on the iodometric and manometric methods, for the estimation of glutathione in blood and tissues, has been done. Both the above methods agree fairly well on the values of GSH in blood. However, for the accurate estimation of total glutathione in blood the method of Dohan and Woodward (118) requires certain modifications. In the course of this investigation it has been observed that 2.3% sulfosalicylic acid blood filtrate is unsuitable for the electrolytic reduction; because the pH of the above filtrate rises above 3 and 4 when submitted to electrolytic reduction with current densities of 2.49 and 4.32 milliamperes per sq. cm. respectively. Since glutathione is autoxidizable at pH 3.0 (101), 2.3% sulfosalicylic

acid blood filtrates are best avoided for the electrolytic reduction. On the other hand, if 3% sulfosalicylic acid blood filtrate is used for the electrolytic reduction, the pH remains below 2.0 with a current density of 2.49 milliamperes per sq. cm. and goes little above 2.0 with a current density of 4.32 milliamperes per sq. cm. At pH 2.0 glutathione is stable for a considerable length of time (101). Hence 3% sulfosalicylic acid is preferable to 2.3% sulfosalicylic acid for making blood filtrates if the total glutathione values are to be estimated after electrolytic reduction by the manometric method. Using this modification considerable increases in the GSH contents of blood filtrates were observed. In view of the specificity of glutathione in the glyoxalase system it is reasonable to assume that glutathione is responsible for the increases in the thiol values.

Although Dohan and Woodward (118) could recover added GSSG quantitatively from red blood cell filtrates and red blood cells by the combined use of electrolytic reduction and the iodometric method of estimation, no attempt to estimate any oxidized glutathione that might normally be present in blood, by the above method was made by the aforesaid authors. In the course of this work confirmatory evidences, regarding the presence of oxidized glutathione, were sought by submitting blood filtrates to electrolytic or Zn reduction and estimating the thiol values by the iodate titration. Increases in thiol values of blood filtrates were observed consistently after Zn or

electrolytic reduction, irrespective of the method of estimation. Since the iodometric method is not specific for the estimation of glutathione, the results given by the latter method were compared with those given by the manometric method (Fig. 2-5). If the ratio of the total glutathione value obtained by the iodometric method after Zn or electrolytic reduction to that obtained by the manometric method after electrolytic reduction had been unity, then it could be concluded that in all probability the substance which was measured by both the methods was oxidized glutathione. Fig. 2-5 show that such is not the case. The total glutathione values obtained by the iodometric method after Zn or electrolytic reduction are higher than the corresponding values obtained by the glyoxalase method after electrolytic reduction, which suggests that when blood filtrate is reduced by Zn or electrolysis, other substances are reduced along with the oxidized glutathione and those interfering substances consume iodine, thus falsely high total glutathione value is obtained. It can be concluded, therefore, that the increases in thiol values of blood filtrates as estimated by the iodometric method after Zn or electrolytic reduction are partly, but not wholly, due to the oxidized glutathione. Hence the iodometric method is unreliable for the accurate estimation of oxidized glutathione in blood.

Experiments of different sorts were done to exclude the possibility that the oxidized glutathione as measured by the manometric method was formed during the technical manipulation of blood

samples. All those experiments negatived such a possibility. These experiments strongly suggest that oxidized glutathione exists preformed in human, rat, and rabbit blood.

In contrast to blood, however, in tissues no measurable amount of oxidized glutathione could be detected. This finding confirms that of Dohan and Woodward (118) and Herrmann and Moses (179). In agreement with Woodward (117) these experiments show that the iodometric method gives erroneously high glutathione values in tissue filtrates. Ennor (133) on the other hand, found close agreement between the GSH values of tissue extracts obtained by the manometric method and those obtained by the iodometric method. He considered that Woodward (117) obtained low GSH values in the tissue filtrates by the glyoxalase method, because of the toxicity of sulfosalicylic acid towards glyoxalase. However, his experimental results showed that glutathione can be measured accurately in the presence of sulfosalicylic acid. In the course of the present work it has been observed that neutralized sulfosalicylic acid does not in any way affect the glyoxalase system. The higher iodine consumption by the tissue filtrates is most probably due to the interference by ascorbic acid, as has been suggested by Woodward (117), and possibly by other thiol compounds.

PART III

INVESTIGATIONS ON THE BLOOD AND TISSUE GLUTATHIONE CHANGES AFTER DEHYDROASCORBIC ACID INJECTION

PART III

Investigations on the Blood and Tissue Glutathione Changes after Dehydroascorbic Acid Injection:

INTRODUCTION

Almost every year, since the discovery of the diabetogenic action of alloxan, numerous attempts to explain the mechanism of the action of the latter substance have been published. Leech and Bailey (93) observed a precipitous drop in the blood glutathione of rabbits immediately after the injection of alloxan. Since then, other observers have noted a similar fall in the blood glutathione of rats and guinea pigs after the injection of alloxan (135-137). Lazarow (138) claimed that the diabetogenic action of alloxan can be prevented by a prior injection of either glutathione or cysteine, but these substances when injected after alloxan do not afford any protection against its diabetogenic action. On the basis of other experimental evidences Lazarow (82) finally postulated, that alloxan forms an addition compound with the sulfhydryl groups of the essential enzymes of the beta-cells in the islets of Langerhans of the pancreas, as a result of which the enzymes are inhibited and death of the beta-cells ensues. The protective action of glutathione and other thiol compounds against the toxic action of alloxan has been explained as due to the transformation of the latter to a non-diabetogenic compound by the

former. Lazarow pointed out that alloxan reacts initially with the blood glutathione, and the concentration of the latter in the blood falls abruptly. Since the tissues are the secondary sites of the reaction the fall in the tissue glutathione is not so marked. According to Lazarow, the concentration of glutathione in the blood and in the beta cells is of crucial importance in determining the vulnerability of animals to the diabetogenic action of alloxan.

It is of interest in this context to review some of the recent publications, which lend credence to the sulfhydryl theory of Lazarow, in relation to the production of diabetes with substances other than alloxan. Conn and his group (139-141) observed a fall in the glutathione content of blood during the induction of diabetes in human subjects with the adrenocorticotrophic hormone (ACTH). Hess, Kyle, and Doolan (142) confirmed the work of the aforesaid authors. Lazarow and Berman (143) reported that cortisone injection into rats, with the object of producing a diabetic state, is associated with a drop in the blood glutathione; the decrease of the latter appears to correlate with the degree of cortisone-induced glycosuria. Nath et al. (144,145) claimed that the subcutaneous injection of acetoacetic acid causes hyperglycemia, reduction of glucose tolerance, rise in blood lactic acid, inactivation of insulin, and a drop in blood glutathione. The blood glutathione fall is not manifested immediately following the injection of acetoacetate but is observed some 90 min.

after the injection of the diabetogenic substance. Grunert and Phillips (146) have shown that weanling rats on a diet deficient in sodium are more susceptible to the diabetogenic action of alloxan than on a diet adequate in sodium. They traced the increased susceptibility of the Na-deficient animals to the low glutathione concentration in the blood of these animals. Illing, Gray, and Lawrence (147), Planchart and Villalba (148) reported that the glutathione concentration in the blood of diabetic patients is lower than that of normal persons. Conn, Louis, and Johnston (149) investigated the role of glutathione in relation to the alleviation of ACTH-induced diabetes in man. They found that when GSH was injected intravenously into such persons the intensity of hyperglycemia was temporarily reduced. However, the sulfhydryl theory of Lazarow (82) has not found universal support. Joiner (150) could not confirm the work of Conn et al. (139-141). Grunert and Phillips (151), Ingbar, Otto, and Kass (152) did not observe any change in the blood glutathione following the injection of ACTH into experimental animals. Lazarow (153) himself observed, that the injection of glutathione into cortisone-diabetic rats resulted in a severe increase of glycosuria. He argues that the potentiation of the diabetogenic action of cortisone by glutathione may be due to the protection of the former from destruction by the latter. Stock, Currence, and Swanson (154), Caren and Carne (155) found no abnormal value of blood glutathione in diabetic patients.

The close similarity of the structure and properties of dehydroascorbic acid to those of alloxan stimulated Patterson to search for the diabetogenic action of the former compound. He observed that the injection of dehydroascorbic acid and related substances into rats produced hyperglycemia and glycosuria (79,80), but that diketogulonic acid was not diabetogenic (156). Like alloxan-diabetic animals, dehydroascorbic acid-diabetic rats developed cataract (157,158) and the diabetic symptoms were ameliorated by adrenalectomy (159). Patterson and Lazarow (75) reported that the diabetogenic action of dehydroascorbic acid can be prevented by a prior injection of either glutathione, or cysteine. The diabetogenic action of dehydroascorbic acid can also be prevented by previous injection of atropine (160,161). From indirect experimental evidence it has been suggested that the mechanism of the action of alloxan and dehydroascorbic acid is similar (51,75).

The present work was undertaken to investigate the nature of blood and tissue glutathione changes following the injection of dehydroascorbic acid, with a view to gaining some more evidence on the possible mechanism of the action of this substance.

EXPERIMENTAL

Before describing the actual experimental procedures, it is felt desirable to mention the dietary history of the animals, since diet considerably influences the production of experimental

diabetes (146,162). Rabbits were maintained on pellets of the following composition: Bruce and Parkes (163). Bran 15%, Barley meal 20%, Ground-nut meal 15%, Linseed cake 10%, Dried meat and bone meal 8%, Dried grass meal 30%, $CaCO_3$ 1%, NaCl 1%. The theoretical analysis of this diet is as follows: Crude digestible protein 16.5%, Fat 4.6%, Soluble carbohydrate 33.7%, and Fiber 6.7%. Each rabbit received approximately 100 g. of the pellets per day, about 15 g. of hay per day and water ad libitum. Twice a week the rabbits received additional supplements of cabbage.

Wistar albino Glaxo rats were used. They were maintained on rat cubes of the following composition and water ad libitum: Wheat offal bran 17.7%, Wheat ground 17.7%, Sussex ground oats 17.7%, Maize ground 8.8%, Meat and bone meal 8.8%, Barley ground 8.8%, Fish meal 4.5%, Dried skimmed milk powder 14%, NaCl 0.4%, Cod-liver oil 0.4%, Dried yeast 1.2%.

Blood was collected by cardiac puncture from rabbits and from the abdominal aorta of rats. Heparin was used as an anti-coagulant. Injections were given into the marginal ear vein of rabbits and through the tail vein of rats; before each injection blood was drawn into the syringe to make sure that the needle was in the vein. In a few preliminary experiments the rabbits were sedated with nembutal but later this was found unnecessary; the use of nembutal does not affect the results. Since blood samples were collected from the abdominal aorta of rats, the animals were anesthetized by nembutal before

laparotomy. Dehydroascorbic acid solution in 0.9% NaCl (100 mg./ml.) was prepared according to Patterson (79); this solution contains traces of hydroquinone as indicated by a positive test for the latter with Chloramine-T. The control animals, therefore, were injected with an ether-extracted solution of pure hydroquinone in normal saline to simulate the conditions, with respect to hydroquinone, in the DHA-injected animals. To prepare the above solution 0.66g. of pure hydroquinone were dissolved in 10 ml. of ether and shaken for 15 minutes with 10 ml. of saline. The saline solution was removed and extracted five times with pure ether. Finally the excess of ether was removed by suction and the solution was used without any further treatment. It has been observed in this work that the DHA solution prepared according to Patterson (79) is reducible (95-98%) to ascorbic acid by H_2S at pH 3.5 and 37° (91). Unless otherwise stated, the glutathione and total glutathione estimations in tissues and blood were done throughout this work by the combined use of electrolytic reduction and the glyoxalase method. Blood and tissue filtrates were prepared with 3% sulfosalicylic acid as described in the last section. For the electrolytic reduction 5 ml. of 3% sulfosalicylic acid filtrate were reduced for 10 minutes with a current density of 4.32 milliamperes per sq. cm. During the course of this work blood samples were taken at varying intervals of time after dehydroascorbic acid injections for glutathione analyses, and therefore, it was expected that such blood samples would contain varying amounts

of DHA; since GSH donates its hydrogen to DHA (69) and it also forms an addition compound with the latter (166), it was, therefore, necessary to test the validity of the glyoxalase method in the presence of varying amounts of DHA. It was observed that, when 0.125, 0.25, 0.5, and 1.0 mg. of DHA was added to Warburg flasks containing 0.05 mg. of pure GSH, the glyoxalase reaction remained unaffected. It should be noted that the highest concentration of DHA used in this experiment corresponds to the theoretical amount that would be present in 0.5 ml. of 1:5 blood filtrate after the injection of 1 g. of DHA per 1000 g. body weight (assuming no loss of DHA and the blood volume to be 100 ml. per 1000 g. of body weight). Blood sugar estimations were done by the method of Hagedorn and Jensen (164). It was observed that the glucose values in blood samples taken immediately or sometime after the injections of dehydroascorbic acid were high; on subsequent investigation it was found that dehydroascorbic acid solution gives a very high blank value in this method of sugar estimation. Blood sugar analyses, therefore, were done 24 hours after the injection of dehydroascorbic acid.

Effect of Dehydroascorbic Acid Injection on Blood Glutathione Contents of Rabbits:

Rabbits (♂ or ♀) weighing between 1500-2800 g. were given an initial desensitizing dose (D.D.) and 15 minutes later a final dose (F.D.) of dehydroascorbic acid. The amounts of DHA that were

injected are shown in the Tables 11 and 12. In some animals the final dose was not injected in one injection, but in parts at 10-15 minute intervals (indicated by spaces in the tables). This procedure was adopted because these animals are extremely susceptible to the injection of dehydroascorbic acid; a fact which is a little surprising in view of the higher GSH content of rabbit blood, as compared with rat blood. Sometimes in the course of injections the animals showed signs of respiratory failure, and in these the injections were discontinued, the animals being revived by artificial respiration before the injections were continued again. Blood from the experimental animals was collected, before any injection and then at varying intervals of time upon completion of the injection, and analyzed for glutathione and total glutathione.

In a few preliminary experiments the glutathione and total glutathione estimations were done by the iodometric method of Woodward and Fry (101). The data are presented in Table 11.+ It is apparent from this table that no significant fall in the blood glutathione (rabbits No. 1, 10, 24, 13 and 14) within 15 minutes to 1 hour after dehydroascorbic acid injection has taken place. However, Banerjee, Belavady, and Mukherjee (165) observed a fall in the blood glutathione of rabbits, after the dehydroascorbic acid injection, using the same method of glutathione estimation. Their observations are a little surprising

+ In Tables 11 and 12 those rabbits marked "dead" under the column heading Treatment died within 24 hours after the injection of dehydroascorbic acid, and their blood sugar values were not determined.

TABLE 11

Concentration of Glutathione (Iodometric Estimation) in Rabbit Blood before and after Dehydroascorbic Acid Injection

GSH - Reduced glutathione GSSG - Oxidized glutathione DHA - Dehydroascorbic acid
D.D. - Desensitizing dose F.D. - Final dose

Rabbit No. and Weight in g.	Time	GSH	GSSG	Total GSH	Treatment
		mg.%	mg.%	mg.%	
No. 1, 2100	Contr.	50.3	4.9	55.2	D.D. 500 mg. DHA
	15 min.	46.6	7.3	53.9	F.D. 1000 mg. DHA
	40 min.	49.0	6.2	55.2	
	70 min.	42.9	10.4	53.3	
	100 min.	48.4	7.4	55.8	
	24 hrs.	38.6	7.4	46.0	
	72 hrs.	34.3	6.1	40.4	
No. 10, 1800	Contr.	55.2	6.1	61.3	D.D. 200 mg. DHA
	15 mins.	57.6	7.4	65.0	F.D. 800 mg. DHA
	45 min.	57.6	7.4	65.0	Dead
	75 min.	57.6	7.4	65.0	
No. 24, 1500	Contr.	49.7	4.2	53.9	D.D. 500 mg. DHA
	10 mins.	45.3	8.6	53.9	F.D. 1000 mg. DHA
					Dead
No. 13, 1800	Contr.	42.9	3.7	46.6	D.D. 500 mg. DHA
	15 min.	44.7	10.5	55.2	F.D. 500-800-500 mg. DHA
No. 14, 1800	Contr.	46.3	4.0	50.3	D.D. 300 mg. DHA
	15 min.	48.4	6.8	55.2	F.D. 300-600-700-500 mg. DHA

TABLE II (Contd.)

Rabbit No. and Weight in g.	Time	GSH	GSSG	Total GSH	Treatment
		mg.%	mg.%	mg.%	
No. 20, 2500	Contr.	47.2	13.5	60.7	D.D. 500 mg. DHA Received 2 g. of DHA for 3 days
	15 min. after 3rd inj.	64.4	22.7	87.1	
No. 23, 2300	Contr.	40.4	6.5	46.9	Received 20 ml. of ether-extracted hydroquinone soln. for 3 days
	15 min. after 3rd inj.	33.7	9.2	42.9	
No. 6, 2400	Contr.	33.1	4.8	37.9	Received single inj. of 20 ml. 0.9% NaCl
	15 min.	33.1	4.8	37.9	
	45 min.	32.5	4.8	37.3	
	75 min.	28.8	8.0	36.8	
	72 hrs.	30.6	5.5	36.1	

in the light of the present findings and will be discussed later. Incidentally it might be mentioned here that this investigation was started before the publication of the above mentioned paper. None of the animals (rabbits 1, 13, 14, and 20) which survived the dehydroascorbic acid injections (Table 11) showed consistent hyperglycemia; only rabbit No. 1 showed a slight rise in the blood sugar for a few days, and when the dehydroascorbic acid injection was repeated another transient rise in the blood sugar was observed (Fig. 8), but, the truly diabetic type of maintained hyperglycemia was not observed. In this animal the blood GSH was lower than could be accounted for by expected errors of the analysis 24 and 72 hours after the injection of DHA - i.e. when there was hyperglycemia. This may be significant, but unfortunately the observation has not yet been repeated since none of the other rabbits has shown comparable hyperglycemia. A somewhat similar fall in the blood GSH appeared in the control animals No. 6, 23 in which case, however, it was within the border limit of experimental errors. It is interesting to note that rabbit No. 20 which received 2 g. of DHA for three consecutive days shows a tremendous rise in both the reduced and total glutathione values of blood 15 minutes after the completion of the third injection. It is rather difficult to conclude from this experiment whether the above observation is due to a true rise in the glutathione content of blood or is due to the interference from ascorbic acid. Since the rabbit was injected with massive

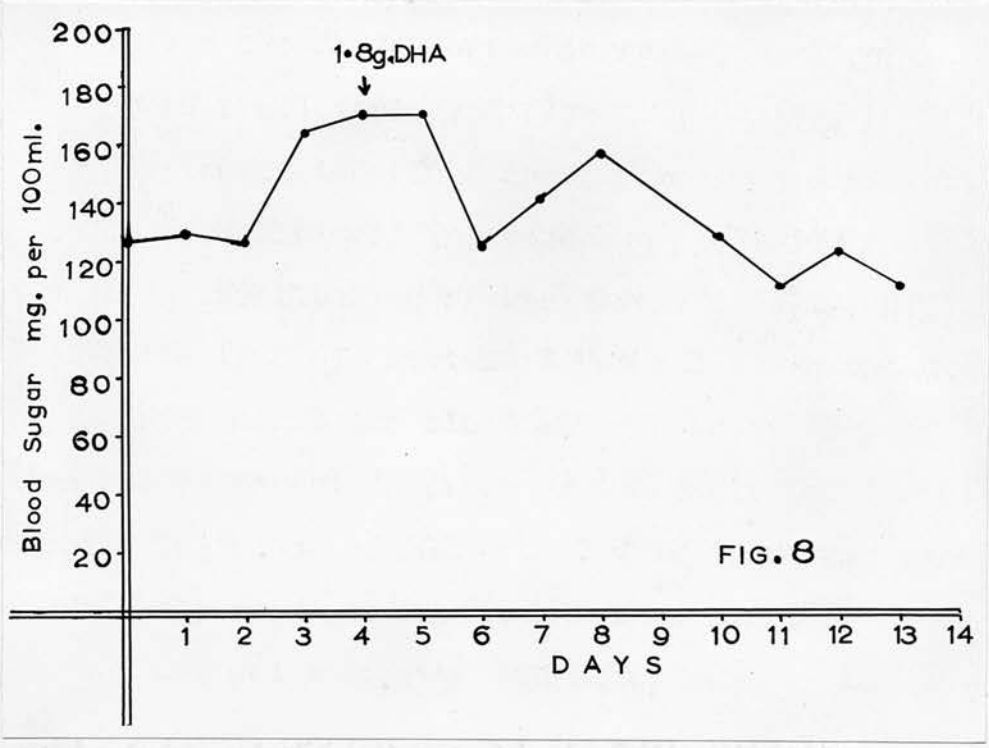


FIG. 8. Effect of dehydroascorbic acid on the blood sugar of rabbit No. I. The figure only shows the second injection; the first injection (1.5 g. DHA) was given 4 days prior to the second injection.

doses of DHA it is quite possible that the ascorbic acid level in the blood of this animal was very high. However, as will be evident from the results of the experiments reported below, where glutathione estimations were done by the glyoxalase method and the interference due to ascorbic acid was not present, rabbits when injected repeatedly with DHA showed a similar rise in the blood glutathione values. Table 11 shows that the GSSG values in the blood of the dehydroascorbic acid injected animals tend to rise. The rise in the GSSG values of blood is not characteristic of the DHA-injected animals; the hydroquinone-injected animal and saline-injected animal also show a similar trend.

Since the method for the estimation of glutathione lacked specificity in the foregoing experiments, it was decided to repeat those experiments estimating the reduced and total glutathione in the blood by the specific manometric method. The results of such experiments are presented in Table 12. It is clear from this table that there is no significant fall in the blood glutathione (rabbits No. 25, 26, 27, 28, 29, 38, 44) immediately following the injections of massive doses of DHA. On the contrary, rabbit No. 29 which received repeated injections of DHA shows, like Rabbit No. 20 of Table 11, a tremendous rise in the glutathione and total glutathione values of blood. In this animal the blood glutathione analyses were done on the first day before any injection and after the final dose at different intervals of time; again on the third day

TABLE 12

Concentration of Glutathione (Manometric Estimation) in Rabbit Blood before and after Dehydroascorbic Acid Injection

GSH - Reduced glutathione GSSG-Oxidized glutathione DHA - Dehydroascorbic acid
D.D. - Desensitizing dose F.D. - Final dose

Rabbit No. and Weight in g.	Time	GSH	GSSG	Total GSH	Hematocrit	Treatment
		mg.%	mg.%	mg.%	%	
No. 25, 1800	Contr.	23.9	5.2	29.1	-	D.D. 200 mg. DHA
	15 min.	25.0	3.6	28.6	-	F.D. 1500 mg. DHA
	45 min.	25.0	5.9	30.9	-	Dead
No. 26, 1850	Contr.	22.4	7.3	29.7	-	D.D. 300 mg. DHA
	15 min.	23.5	6.4	29.9	-	F.D. 1500 mg. DHA
	45 min.	22.7	7.8	30.5	-	Dead
No. 27, 1750	Contr.	33.7	8.0	41.7	-	D.D. 400 mg. DHA
	5 min.	39.0	0	39.0	-	F.D. 1500 mg. DHA
	60 min.	41.6	-	40.5	-	Dead
No. 28, 1800	Contr.	26.2	12.2	38.4	46	D.D. 400 mg. DHA
	"0" min.	24.7	13.1	37.8	45	F.D. 1300 mg. DHA
	30 min.	27.0	9.7	36.7	43	Dead
No. 38, 1500	Contr.	38.7	7.5	46.2	41	D.D. 400 mg. DHA
	"0" min.	41.2	2.5	43.7	43	F.D. 800 mg. DHA
	15 min.	38.7	5.0	43.7	42	
No. 44, 2800	Contr.	41.7	17.6	59.3	44	D.D. 500 mg. DHA
	15 min.	47.5	18.7	66.2	46	F.D. 500-800 mg. DHA

TABLE 12 (Contd.)

Rabbit No. and Weight in g.	Time	GSH	GSSG	Total GSH	Hematocrit	Treatment
		mg.%	mg.%	mg.%	%	
No. 29, 2100	Contr.	17.2	10.4	27.6	40	D.D. 400 mg. DHA
	"0" min.	19.1	7.3	26.4	42	F.D. 1200 mg. DHA
	20 min.	17.2	10.1	27.3	40	
	Received 1800 mg. of DHA for 2 days					
	Before 3rd inj.	29.8	14.2	44.0	40	
	"0" min. after 3rd inj.	28.8	15.2	44.0	40	Dead
	30 min. after 3rd inj.	35.8	6.3	42.1	38	
No. 33, 2000	Contr.	22.5	6.8	29.3	38	D.D. 200 mg. DHA
	Received 500 mg. of DHA for 6 days					
	24 hrs. after last inj.	28.7	6.9	35.6	36	
No. 34, 2800	Contr.	26.0	10.5	36.5	45	D.D. 200 mg. DHA
	Received 500 mg. of DHA for 6 days					
	24 hrs. after last inj.	26.8	11.2	38.0	40	

TABLE 12 (Contd).

Rabbit No. and Weight in g.	Time	GSH	GSSG	Total GSH	Hematocrit	Treatment
		mg.%	mg.%	mg.%	%	
No. 50, 1600	Contr.	35.0	13.2	48.2	40	Received 0.6 g. of DHA/Kg. in 4 equal doses in our hour after 12 hrs. fasting
	9 days after first inj.	34.3	11.7	46.0	38	
No. 51, 1800	Contr.	43.1	10.9	54.0	43	Same as above
	9 days after first inj.	34.3	13.2	47.5	38	

before and after the third injection as indicated in the table. The " 0 " time in Table 12 signifies that the blood sample was drawn while the last portion of the DHA was injected; this technique is similar to that of Leech and Bailey (93) when they studied the effects of alloxan on blood glutathione. No significant change in blood sugar was observed in the surviving animals (rabbits No. 38, 44).

Patterson (79) originally reported that rats could be made diabetic with a single injection of 1.1 g. of DHA per Kg. body weight. Patterson and Lazarow (75) made rats diabetic by injecting 0.7 g. of DHA per Kg. of body weight for 3 consecutive days. Tables 11 and 12 show that the mortality rate in rabbits is very high when dehydroascorbic acid is injected around dose level of 1.1 g./Kg. It has been found in the course of this work that, it is almost impossible to inject 0.7 g. of DHA per Kg. into rabbits for 3 consecutive days; out of the 10 rabbits tried only two survived - No. 20 (Table 11) and No. 29 (Table 12). Unfortunately, the latter rabbit died within an hour after the 3rd injection on the third day, therefore, its blood sugar changes could not be followed, and as has been stated elsewhere in the former rabbit no significant change in the blood sugar was observed. It was decided, therefore, to inject DHA repeatedly into rabbits in small doses for longer periods of time. Rabbits No. 33, and 34 (Table 12) were injected for six consecutive days with 500 mg. of DHA. Twenty-four hours after the completion of the

injections blood samples were taken for glutathione and sugar analyses. It can be seen from Table 12 that the hematocrit value in the blood of rabbit No. 33 has decreased, but the reduced and total glutathione values have increased; in rabbit No. 34 the hematocrit value has decreased by 5% but the reduced and total glutathione values remain unchanged. These findings indicate that the glutathione and total glutathione contents have increased in the red cells. None of these rabbits was hyperglycemic for five days after the injections. Leech and Bailey (93) reported the same type of increase in the blood glutathione after repeated alloxan injections in small doses. This increased glutathione production in the body is quite conceivably an attempt of the latter to increase its resistance against such toxic substances as alloxan and dehydroascorbic acid.

Princiotto (81) reported that rabbits when starved for 12-24 hours could be made diabetic by injecting 0.6 g./Kg. of DHA in four equal doses at 15 minute intervals. In this work the same procedure was tried on rabbits No. 50 and 51 (Table 12). These animals were starved for 12 hours and then injected with the aforesaid amount of DHA at the specified time intervals. Blood sugar analyses were done for four days after the injections and varied between 84-110 mg.%. On the fifth day, after 24 hours fasting, the same rabbits were injected again with the same dose of DHA in the same way. Blood sugar analyses were done for the next four days and showed no change. Blood glutathione analyses were done after 12 hours fasting just before the first

injection of DHA and again on the 9th day after 12 hours fasting. Reference to Table 12 shows that the hematocrit values as well as the glutathione and total glutathione contents of the blood have decreased in these animals. In these cases, however, the increase in the glutathione and total glutathione values of blood as observed in rabbits No. 29, 33 and 34 is not present. The most plausible explanation is that the injections of DHA were given too far apart.

Reduction of Dehydroascorbic Acid by Rabbit Blood:

Several investigators have suggested that glutathione is responsible for the reduction of DHA (7,21,69,74). Since the preceding experiments did not show any significant change in the blood glutathione after dehydroascorbic acid injection, it was, therefore, decided to investigate whether DHA is appreciably reduced to ascorbic acid in the rabbit blood.

The following experimental procedure was adopted: In Vivo: Rabbits weighing between 2100-2900 g. were injected with 1000-1500 mg. of DHA in divided doses within 30 minutes. Blood samples were collected before and 15 minutes after the completion of the injections and analyzed for ascorbic acid and total ascorbic acid. Immediately upon collection the blood samples were saturated with carbon monoxide (to prevent oxidation of ascorbic acid by hemoglobin during deproteinization) and then centrifuged. Ascorbic acid and total ascorbic acid estimations were done in the plasma and red cells by the indophenol (83)

and phenylhydrazine (87) methods. The latter method does not differentiate between dehydroascorbic acid (DHA) and diketo-gulonic acid (DKA), hence H_2S reduction was applied to the metaphosphoric acid filtrate, as described by Levenson et al. (91), to differentiate between the two substances. In Part I of this thesis it has been stated that because of the lack of specificity of H_2S reduction it cannot be applied to differentiate between DHA and DKA when these substances are present in small amounts (0.07-0.3 mg.%) in biological materials. In this experiment, however, the concentrations of DHA in the red cells and plasma, after DHA injection, were very high (vide infra). At such high levels of DHA the figures obtained after H_2S reduction of the latter, by the indophenol method are reasonably accurate; the interfering substances constitute only a fraction of the amount estimated. In Vitro: Ascorbic acid or dehydroascorbic acid solutions in 0.9% NaCl were added to whole blood (saturated with CO) and incubated for 1 hour at 37°. At the end of incubation period the blood was centrifuged and ascorbic acid determinations were done in the red cells and plasma by the indophenol method (83).

Table 13 shows that when DHA is injected into rabbits the ascorbic concentration in the red cells greatly increases. The results of the in vitro experiment in Table 14 show that the levels of the ascorbic acid in the red cells of rabbits rise to a significant extent when the red cells are incubated with DHA. Both the results of the in vivo and in vitro

TABLE 13

Concentrations of Ascorbic Acid and Total Ascorbic Acid in Plasma and Red Cells of Rabbits before and after Dehydroascorbic Acid Injection

AsA --- Ascorbic acid DHA --- Dehydroascorbic acid

Rabbit No. and Weight in g.	Material analyzed	Indophenol method AsA	Phenyl-hydrazine method Total AsA	After H_2S reduction Total AsA	Treatment
		mg.%	mg.%	mg.%	
No. 39, 2100	Control plasma	0.5	0.413	-	1000 mg. DHA
	Plasma after inj.	19.0	112.5	-	
No. 42, 2700	Control plasma	0.7	0.875	-	1500 mg. DHA
	Plasma after inj.	31.0	181.2	82.5	
	Contr. red cells	0.5	0.55	-	
	Red cells after inj.	24.5	61.0	42.5	
No. 43, 2900	Control plasma	1.75	1.7	-	1500 mg. DHA
	Plasma after inj.	26.0	140.0	32.5	
	Contr. red cells	0	1.37	-	
	Red cells after inj.	15.7	37.5	27.5	

TABLE 14

Concentrations of Ascorbic Acid in Plasma and Red Blood Cells before and after Addition of Dehydroascorbic Acid or Ascorbic Acid to Whole Blood

AsA -- Ascorbic acid DHA - Dehydroascorbic acid

Species	Concentration and form of substance added	Material analyzed	Ascorbic acid found
	mg. per cent		mg. per cent
Rabbit	25 DHA	Control plasma	0.9
		Plasma after incubation	2.4
		Control red cells	0.3
		Red cells after incubation	1.5
Rabbit	24.3 AsA	Control plasma	0.48
		Plasma after incubation	23.8
		Control red cells	0.35
		Red cells after incubation	0.38
Rabbit	24.3 AsA	Control plasma	0.48
		Plasma after incubation	23.1
		Control red cells	0.35
		Red cells after incubation	0.38

experiments strongly suggest that DHA is reduced to AsA in the red cells. The results of the in vitro experiments in Table 14 show that ascorbic acid does not enter into the red cells of rabbits. Other observers (21,167) have also reported that both in vivo and in vitro the erythrocytes of human, cat, dog, rat, sheep, pig, and bovine are impermeable to AsA. Hence the increase in the ascorbic acid content of the red cells as observed in the in vivo and in vitro experiments above is not due to the entry of ascorbic acid into the red cells.

It is clear from the results of the above experiments and those in the last section that, in vivo DHA is reduced to a considerable degree in the red cells of rabbits and concurrent with the reduction of the DHA no significant change in the blood glutathione takes place. There is a possibility that the GSH is regenerated from the GSSG very rapidly by the glutathione reductase in blood (68); i.e. the second of the following reactions is very effective:

(1) $\quad DHA + 2GSH \rightleftharpoons AsA + GSSG$

(2) $\quad GSSG + TPNH_2 \longrightarrow 2GSH + TPN$

However, Rall and Lehninger (68) have also shown that the specific activity of the glutathione reductase in the rat liver is higher than in the blood. Since in the course of this work a fall in the liver glutathione of rats after DHA injections under similar experimental conditions has been

observed (vide infra), the above possibility may be ruled out. It can be concluded, therefore, that glutathione is not responsible for the reduction of dehydroascorbic acid in the rabbit blood.

The data in Table 13 also show that the AsA concentration in the plasma rises after DHA injection. The results in Table 14 show that the ascorbic acid concentration in the plasma increases after incubation of whole blood with DHA. The rise in the plasma ascorbic acid may be due to the reduction of DHA in the plasma or to the passage of AsA from the red cells and other cells in the body. Experiments have not been done to verify whether rabbit plasma reduces DHA to AsA; because the main object of this experiment was to observe whether glutathione is responsible for the reduction of DHA and plasma does not contain any glutathione. If DHA is reduced in the plasma the reducing agent is certainly not glutathione.

It is easily discernible from Table 13 that after the DHA injections the total ascorbic acid values in the plasma and red cells are higher than the corresponding ascorbic acid values. This indicates that the whole of the injected DHA has not been reduced to AsA but is present either as DHA or DKA. Since the total ascorbic acid value, after the DHA injections, in the plasma and red cells as obtained by the phenylhydrazine method are higher than those obtained by the indophenol method after H_2S reduction, it indicates that a considerable amount of the

injected DHA has been converted to DKA. The degree of conversion of the injected DHA to DKA is far greater in plasma than in red cells.

Effect of Dehydroascorbic Acid Injection on Blood and Tissue Glutathione Contents of Rats:

Since diabetes could not be produced in rabbits with dehydroascorbic acid, it was of interest to know whether rats could be made diabetic with the latter substance as has been claimed by Patterson (79,80).

The following experimental procedure was adopted: Rats (♂ or ♀) weighing between 145-160 g. were divided into two groups in such a way that the distribution of the body weight and sex remained approximately the same in both groups. One group was kept as control and the rats in the other group were injected with DHA (experimental). The experimental animals were injected with 0.7-0.8 ml. of dehydroascorbic acid solution (100 mg./ml.) per 100 g. body weight for three consecutive days. Before the first injection the rats were desensitized with 0.3 ml. of the above dehydroascorbic acid solution per 100 g. body weight. The control animals were injected with 0.7-0.8 ml. of ether-extracted hydroquinone solution per 100 g. body weight. Both the control and the experimental animals were starved for 12 hours before the first injection. Because of experimental limitations, the control and experimental animals were not

injected at the same time; the experimental animals were injected first. From 4-10 days after the last injection, the experimental animals were sacrificed a few at a time. The animals were anesthetized with nembutal and laparotomized; blood was collected from the abdominal aorta for glutathione and sugar analyses. Glutathione analyses were also performed in the liver and pancreas. The same experimental procedure was followed for the control animals after hydroquinone injection. The blood, liver, and pancreas were analyzed for glutathione. The tissues (pancreas, liver, kidney, thyroid, and suprarenal) were taken from control and experimental animals for histological examination.

Table 15 shows that 100% of the rats became hyperglycemic after the injections of dehydroascorbic acid. Nevertheless, the glutathione contents of the blood, liver, and pancreas of the control animals are not significantly different from those of the diabetic animals. The total and oxidized glutathione concentrations in the blood of experimental animals are almost similar to those of the control animals. It is interesting to note that the total glutathione values are not consistently higher than the glutathione values in the liver and pancreas of both diabetic and normal rats. This indicates that the oxidized glutathione is not present in the tissues of control animals and it does not appear in the tissues of diabetic rats. It is concluded, therefore, that in established dehydroascorbic acid-diabetic rats the glutathione concentrations in the blood, liver, and pancreas

TABLE 15

Glutathione Contents of Blood and Tissues of Control and DHA-Diabetic Rats

GSH - Reduced glutathione GSSG - Oxidized glutathione DHA - Dehydroascorbic acid

Control rats

Blood						Liver						Pancreas					
Rat No.	Control sugar	After inj. sugar	GSH	GSSG	Total GSH	Rat No.	Control blood sugar	After inj. blood sugar	GSH	GSSG	Total GSH	Rat No.	Control blood sugar	After inj. blood sugar	GSH	GSSG	Total GSH
	mg.%	mg.%	mg.%	mg.%	mg.%		mg.%	mg.%	mg.%	mg.%	mg.%		mg.%	mg.%	mg.%	mg.%	mg.%
11	102	102	18.2	25.6	43.8	1	108	89	135.5	-	-	3	105	-	48.3	-	47.1
12	98	-	17.5	24.4	41.9	2	-	147	193.1	0	193.1	4	105	-	55.8	-	51.4
13	-	-	20.7	23.1	43.8	3	105	-	231.7	-	-	7	99	-	57.5	-	47.2
14	-	-	22.5	22.5	45.0	4	105	-	241.5	-	-	8	107	140	62.5	-	-
15	-	-	24.4	23.8	48.2	5	99	96	147.6	-	-						
16	114	-	20.0	18.2	38.2	6	107	124	229.5	-	228.3						
17	98	-	18.0	19.5	37.5	7	99	-	225.2	-	-						
18	114	-	15.0	21.3	36.3	8	107	140	200.7	-	-						
19	105	-	18.8	25.6	44.4	9	108	138	174.8	7.0	181.8						
20	105	-	17.5	20.5	38.0	10	107	124	199.4	-	-						
Mean GSH 19.2 ± 2.72						Mean GSH 197.9 ± 36.1						Mean GSH 56.0 ± 5.9					

TABLE 15 (Contd.)

DHA-Diabetic rats

Blood						Liver						Pancreas					
Rat No.	Control sugar	After inj. sugar	GSH	GSSG	Total GSH	Rat No.	Control blood sugar	After inj. blood sugar	GSH	GSSG	Total GSH	Rat No.	Control blood sugar	After inj. blood sugar	GSH	GSSG	Total GSH
	mg.%	mg.%	mg.%	mg.%	mg.%		mg.%	mg.%	mg.%	mg.%	mg.%		mg.%	mg.%	mg.%	mg.%	mg.%
17	-	333	21.3	24.4	45.7	3	126	378	179.6	0	179.6	4	112	416	73.5	-	58.8
18	-	336	18.8	16.2	35.0	4	112	416	214.7	0	214.7	8	101	423	85.2	-	60.2
19	-	461	26.2	15.8	42.0	6	126	392	164.6	1.2	165.8	9	124	392	52.7	0	52.7
23	-	436	23.8	15.2	39.0	7	118	423	218.0	-	208.3	12	99	349	48.5	0	48.5
27	-	500	23.2	16.8	40.0	8	101	423	237.3	34.4	271.7						
28	-	235	18.2	20.0	38.2	9	124	392	188.3	1.9	190.2						
						13	94	428	204.5	-	-						
						16	120	427	241.5	-	-						
Mean GSH		21.9 ± 3.08				Mean GSH		206.0 ± 27.2				Mean GSH		64.9 ± 17.4			

remain practically unaltered.

At this stage, it was decided to estimate the glutathione contents of the blood, liver, and pancreas of rats immediately after DHA injections. In vivo/and in vitro (pH 7.3-7.4) the half life of DHA is only a few minutes (23,168,169) and therefore, it was expected that any change in the glutathione contents of the blood and tissues would be manifested within a short time after the DHA injection.

The experimental and technical procedure used was as follows: Rats/(♀) weighing between 130-160 g. were injected with 0.7 ml. of dehydroascorbic acid solution (100 mg./ml.) per 100 g. body weight for two consecutive days. The desensitizing dose was the same as in the previous experiment. On the third day, the rats were anesthetized with nembutal and injected with the aforesaid amount of DHA. The animals were laparotomized and at 10, 15 and 17 minutes after the DHA injection blood, liver and pancreas were collected respectively for glutathione analyses. Blood samples were taken before the third injection for sugar analyses.

The same experimental procedure was repeated on a control group of rats of similar body weight which were injected with ether-extracted hydroquinone solution instead of DHA.

It is clear from Table 16 that the glutathione and total glutathione concentrations in the blood of the control animals are not significantly different from those of the experimental animals. The glutathione concentrations in the liver of the

TABLE 16

Glutathione Contents of Blood and Tissues of Control and DHA-Injected Rats

GSH - Reduced glutathione GSSG - Oxidized glutathione DHA - Dehydroascorbic acid

Control rats

Blood					Liver					Pancreas				
Rat No.	Sugar before 3rd inj.	GSH	GSSG	Total GSH	Rat No.	Blood sugar before 3rd inj.	GSH	GSSG	Total GSH	Rat No.	Blood sugar before 3rd inj.	GSH	GSSG	Total GSH
	mg.%	mg.%	mg.%	mg.%		mg.%	mg.%	mg.%	mg.%		mg.%	mg.%	mg.%	mg.%
1	124	16.3	23.7	40.0	1	124	221.0	0	221.0	1	124	53.8	-	-
3	108	16.3	19.9	36.2	2	114	219.8	-	-	2	114	63.4	-	-
4	101	16.3	21.2	37.5	3	108	204.8	-	-	3	108	60.1	-	-
5	117	15.0	21.2	36.2	4	101	241.0	0	241.0	4	101	62.1	-	-
6	113	16.5	19.1	35.6	5	117	201.9	-	-	5	117	59.7	-	-
7	-	18.8	19.9	38.7	6	113	208.8	-	-	6	113	54.3	-	-
Mean GSH 16.5 ± 1.24					Mean GSH 216.0 ± 14.4					Mean GSH 58.9 ± 3.99				

TABLE 16 (Contd.)

DHA-Injected rats

Blood					Liver					Pancreas				
Rat No.	Sugar before 3rd inj.	GSH	GSSG	Total GSH	Rat No.	Blood sugar before 3rd inj.	GSH	GSSG	Total GSH	Rat No.	Blood sugar before 3rd inj.	GSH	GSSG	Total GSH
	mg.%	mg.%	mg.%	mg.%		mg.%	mg.%	mg.%	mg.%		mg.%	mg.%	mg.%	mg.%
1	402	18.5	23.5	42.0	1	402	231.0	-	215.1	1	402	96.6	-	-
7	363	16.9	21.2	38.1	2	-	147.9	4.4	152.3	2	-	52.6	-	-
8	214	18.8	17.4	36.2	7	363	132.2	-	120.4	7	363	102.5	-	-
9	328	14.0	17.2	31.2	8	214	137.6	0.4	138.0	8	214	69.5	-	-
10	230	16.3	19.3	35.6	9	328	118.7	-	-	9	328	68.1	-	-
11	122	18.5	19.0	37.5	10	230	167.5	-	-	10	230	61.2	-	-
										11	122	72.8	-	-
Mean GSH 17.1 ± 1.74					Mean GSH 155.8 ± 40.29					Mean GSH 74.7 ± 18.25				

DHA-injected animals are lower than those of the control animals. This difference is significant ($P < 0.01$). It is interesting to note that the glutathione contents of the pancreas of the experimental animals are higher than those of the control animals. This difference is most probably due to the variation in the water content, because, the whole pancreas of a DHA-injected animal weighs less than that of a control animal. The significance of these results will be discussed later.

Effect of Dehydroascorbic Acid Injection on Liver Glutathione Contents of Rats:

The above experiment has shown that the glutathione content of the liver falls after DHA injection. This observation has been further verified by experiments of different nature .

The technique was as follows: A rat was laparotomized under nembutal anesthesia and a suitable amount of liver tissue was taken out for glutathione analysis. The bleeding was controlled by mosquito hemostats and pads of cotton wool. Then the rat was given a priming dose of 20-25 mg. of DHA per 100 g. body weight; 15 minutes later another dose of 60-140 mg. of DHA per 100 g. body weight was injected. Another sample of liver tissue was taken out for glutathione analysis 15 minutes after the last injection. The same experiment was repeated on rats injected with normal saline.

Similar experiments were done in vitro. The experimental and technical procedure used was as follows: A. The whole liver of a rat was extracted with phosphate buffer at pH 7.4. The tissue debris was not separated. To the extract DHA was added to make a concentration of 20 mg. of DHA per 100 ml. in the extract. The sample was incubated for 30 minutes at 37°. Samples of the extract were taken out for glutathione analyses before and after incubation with dehydroascorbic acid.
B. The whole liver of a rat was extracted with phosphate buffer at pH 7.4; the extract was centrifuged and filtered. A sample of the filtrate was taken out for glutathione analysis. The rest of the filtrate was divided into two equal parts; one half was used as control and to the other half DHA was added to give a concentration of 100 mg. of DHA per 100 ml. in the filtrate. Immediately upon addition of DHA a portion of the filtrate was taken out for glutathione analysis. Both the control and the sample to which DHA had been added were incubated for an hour at 37°. Aliquots of the samples were taken out after 30 minutes and at the end of the incubation period for glutathione analyses.

The results of these in vivo and in vitro experiments are shown graphically in Fig. 9. The top half of the figure shows the results of the in vivo experiments and the lower half those of the in vitro experiments. To facilitate presentation, the concentrations of glutathione in the liver extracts are expressed in mg. of GSH per 100 g. of liver.

It is easily discernible from Fig. 9 that the glutathione concentration in the liver of both the DHA-injected animals (DHA_1 and DHA_2) has fallen to a significant extent. On the other hand, the saline-injected animals ($Saline_1$ and $Saline_2$), under the same experimental conditions, do not show any change in the liver glutathione concentration. It should be pointed out in this context that the choice of saline instead of ether-extracted hydroquinone solution for injection into the control animals was purely arbitrary. It has been observed in the course of this investigation that hydroquinone does not cause any change in the glutathione contents of liver or blood.

Curve A in the above figure shows the result of 30 minutes incubation of liver brei with DHA (20 mg. of DHA per 100 ml. extract). A definite fall in the glutathione concentration of the liver extract is clearly shown.

Curve B_2 in Fig. 9 shows the effect of incubation, of liver filtrate (without cell debris) with DHA (100 mg. of DHA per 100 ml. filtrate), on the glutathione concentration of the liver filtrate. The parallel control of the same filtrate (without DHA) is represented by curve B_1. It can be observed (curve B_2) that within 3 minutes of the addition of DHA the concentration of GSH in the filtrate has started falling and after 30 minutes of incubation the GSH content is reduced to zero value. On the other hand, some loss of GSH occurs in the control filtrate (curve B_1) within 30 minutes but after that

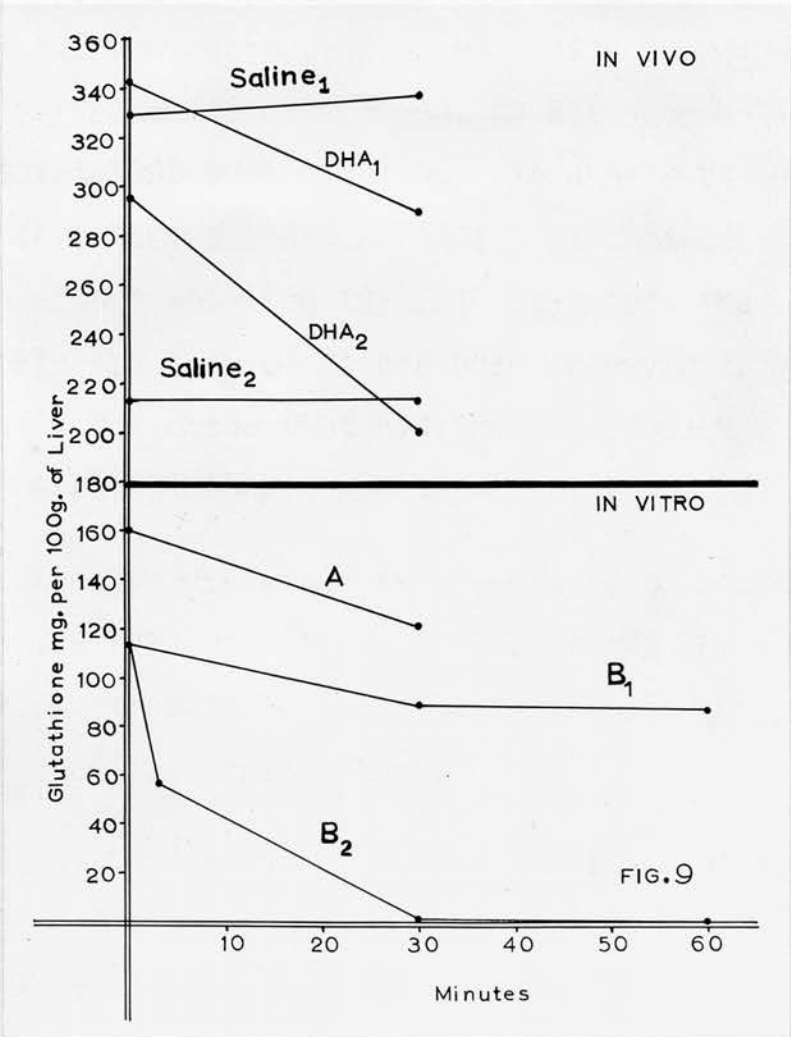


FIG. 9. IN VIVO: Glutathione concentration in rat liver before and 30 min. after DHA or saline injection. DHA_I, 80 mg. of DHA injected per 100 g. body wt.; DHA_2, 164 mg. of DHA injected per 100 g. body wt; $Saline_I$ and $Saline_2$, 4 ml. of 0.9% NaCl injected.

IN VITRO: Glutathione concentration in liver extracts (liver extracted with phosphate buffer at pH 7.4) of rats before and after incubation at 37° with or without DHA. A, DHA was added to the extract to give a concentration of 20 mg.%; B_I and B_2 are the two halves of the same extract; to B_I no DHA was added and to B_2 DHA was added to give a concentration of 100 mg.%.

the loss of GSH is very small. The fall in the GSH content of the control filtrate may be expected, because glutathione is very unstable at pH 7.4. However, the difference between the control sample and the sample to which DHA was added is quite marked. The changes in the ascorbic acid values of the sample to which DHA had been added (curve B_2) are shown below.

Concentration of ascorbic acid before the addition of DHA ---- 17.6 mg./100 g. of liver.
Concentration of ascorbic acid after 30 min. incubation with DHA ---- 130.6 mg. per 100 g. of liver.
Concentration of ascorbic acid after 60 minutes incubation with DHA ---- 152.0 mg. per 100 g. of liver.

The results of this experiment and those of the previous section strongly suggest that glutathione is responsible for the reduction of dehydroascorbic acid in the liver.

Effect of Alloxan Injection on Blood Glutathione of Rabbits:

Leech and Bailey (93) originally reported that immediately after alloxan injection a sharp drop in the glutathione concentration of blood occurs. They, however, did not use a specific method for the estimation of glutathione. It was, therefore, of interest to repeat the work of the above authors and to observe whether similar changes can be demonstrated by the specific glyoxalase method.

The following experimental procedure was adopted: Rabbits (♂ or ♀) weighing between 1500-3000 g. were injected intravenously with 200 mg./Kg. body weight of alloxan. Blood samples were collected at various intervals of time for glutathione analyses by the glyoxalase method as well as by the method of Benedict and Gottschall (114) as modified by Potter and Franke (115). The latter method was used by Leech and Bailey and it measures only GSH.

It is easily discernible from Table 17 that a precipitous fall in the blood glutathione immediately after alloxan injection can be demonstrated by both the methods. The glutathione values obtained by the method of Benedict and Gottschall are higher than those obtained by the manometric method. This is quite conceivable, because, the former method is not specific for glutathione. The total glutathione values obtained by the glyoxalase method immediately after alloxan injection are lower than before alloxan injection. This indicates that the reduced glutathione has not been simply oxidized by the alloxan, but the latter substance has formed, probably, an addition compound with glutathione as has been suggested by Lazarow and his coworkers (170,171). Dr. Robson, working in this laboratory, has confirmed the above finding.

TABLE 17

Concentration of Glutathione in Rabbit Blood before and after Alloxan Injection

GSH -- Reduced glutathione GSSG -- Oxidized glutathione

Rabbit No. and Weight in g.	Time	Hemato-crit	Glyoxalase method			Benedict and Gottschall's method
			GSH	GSSG	Total GSH	GSH
		per cent	mg.%	mg.%	mg.%	mg.%
No. 35, 1500	Contr.	44	52.5	20.0	72.5	69.6
	"0" min.	42	17.5	28.2	45.7	32.0
	15 min.	-	-	-	-	27.5
No. 36, 1600	Contr.	42	55.0	12.5	67.5	70.0
	"0" min.	42	23.1	3.1	26.2	32.1
No. 37, 2100	Contr.	40	20	10.6	30.6	-
	15 min.	39	9.4	15.6	25.0	-
No. 40, 3000	Contr.	42	24.0	16.0	40.0	-
	"0" min.	43	4.0	27.2	31.2	-
No. 41, 2500	Contr.	48	25.0	21.3	46.3	-
	"0" min.	51	6.0	22.1	28.1	-
	60 min.	43	21.0	17.1	38.1	-
No. 49, 1600	Contr.	40	30.0	13.0	43.0	-
	"0" min.	45	18.0	6.4	24.4	-

DISCUSSION

Soon after the discovery by Patterson (79,80) that dehydroascorbic acid is diabetogenic in rats, Princiotto (81) claimed that rabbits could be made diabetic with the same substance. In a recent communication, however, Banerjee et al. (165) reported that they could not produce diabetes in rabbits with dehydroascorbic acid. It may be pointed out in this context that, the experimental technique of Banerjee et al. is a little different from that of Princiotto. Banerjee et al. injected dehydroascorbic acid intravenously into non-fasting rabbits at a dose level of 1 g./Kg. or 1.5 g./Kg. in two injections at 10 minute intervals; a dose which is diabetogenic in rats (79). On the other hand, Princiotto starved the rabbits for 12-24 hours and then injected intravenously 0.6 g./Kg. of dehydroascorbic acid in four equal doses at intervals of 15 minutes. In the present work, dehydroascorbic acid was injected intravenously into rabbits at varying dose levels using different techniques including that used by Princiotto. In no instance, however, rabbits could be made diabetic with dehydroascorbic acid. Neither Patterson nor Lazarow has injected dehydroascorbic acid into rabbits (personal communication). The results of the present work and those of Banerjee et al. make it unlikely that dehydroascorbic acid is diabetogenic in rabbits.

In the course of the present investigation, attempts were made initially to measure the blood glutathione of rabbits, after

dehydroascorbic acid injections, using the iodometric method of Woodward and Fry (101). No significant change in the blood glutathione was observed. Later, however, the above method of glutathione estimation was discarded in view of the fact that the ascorbic acid level in the blood increases greatly after dehydroascorbic acid injections and ascorbic acid at high concentration interferes with the iodometric method giving falsely high glutathione value. In view of this, it is surprising that Banerjee et al. (165) using the same method of glutathione estimation (101) could observe a fall in the blood glutathione of rabbits after dehydroascorbic acid injections. Furthermore, the half life of dehydroascorbic acid in vivo/or in vitro (pH 7.3-7.4) is only a few minutes (23,168,169); if glutathione is responsible for the reduction of dehydroascorbic acid, then any change in the blood glutathione should be apparent a few minutes after the injection of dehydroascorbic acid. The observation of Banerjee et al. is inconsistent with this idea since they reported the fall in the blood glutathione 1-24 hours after the injection of dehydroascorbic acid. The present author, however, using the specific glyoxalase method for the estimation of glutathione, did not observe any significant change in the blood glutathione of rabbits during the first hour after dehydroascorbic acid injections. On the other hand, when dehydroascorbic acid was injected repeatedly into rabbits, a rise in the blood glutathione was observed. A similar rise in the blood glutathione of rabbits after repeated injections of alloxan in small doses has been observed by Leech and Bailey

(93). The significance of this change has been discussed elsewhere.

It is well established that, in vivo and in vitro dehydroascorbic acid is reduced to ascorbic acid in the blood and tissues (18-24,73,74,173-176). In the present work an increase in the ascorbic acid concentration of rabbit erythrocytes has been observed both after the injection of dehydroascorbic acid and after incubation of whole blood with dehydroascorbic acid. The possibility that the increase in the ascorbic acid concentration of the erythrocytes is due to the entry of ascorbic acid into the red blood cells has been excluded by in vitro experiments. Since no significant change in the blood glutathione has been observed after dehydroascorbic acid injections into rabbits, it has been concluded that glutathione is not responsible for the reduction of dehydroascorbic acid in the rabbit blood.

The results also indicate that when large doses of dehydroascorbic acid are injected into rabbits, a considerable portion of this substance undergoes an irreversible transformation into diketogulonic acid.

In contrast to rabbits, rats were easily and regularly made diabetic with dehydroascorbic acid. This confirms the work of Patterson (79,80) and Patterson and Lazarow (75). Histological studies (see appendix) indicated that dehydroascorbic acid-induced diabetes is pancreatic in origin. The glutathione values in the blood, liver, and pancreas of the permanently diabetic animals

were not significantly different from those of the control animals. This is quite consistent with the fact that the turnover of glutathione is very rapid both in vitro and in vivo (52-55), so that the changes in the glutathione concentration of blood or tissue which might occur immediately after dehydroascorbic acid injections would not be manifested in the permanently diabetic condition.

However, no significant change in the blood glutathione could be observed in rats immediately after dehydroascorbic acid injections; on the other hand, in common with other workers it has been observed that alloxan causes a precipitous fall in the blood glutathione of rabbits. This raises the question whether the fall in the blood glutathione is of any significance in the production of experimental diabetes. A survey of the literature shows that ninhydrin, a non-diabetogenic substance, also causes a fall in the blood glutathione which is similar to that induced by alloxan (135). Furthermore, a fall in the blood glutathione of guinea pigs immediately after alloxan injection has been demonstrated (136,137), although these animals are resistant to the diabetogenic action of alloxan (136,137,177). Judging from these facts it can be concluded that a fall in the blood glutathione is not specifically related to the production of experimental diabetes.

In the present work a consistent fall in the liver glutathione of rats immediately after dehydroascorbic acid injections has been observed; a fall in the glutathione content of liver extracts

has also been shown to occur on incubation with dehydroascorbic acid. This is probably due to the reduction of dehydroascorbic acid in the liver by glutathione. It is of interest to note that Bruckmann and Wertheimer (135) did not observe any change in the liver glutathione of rats immediately after alloxan injection; Brada (178), on the other hand, observed a fall in the liver glutathione of rats after alloxan injection. Nevertheless, the significance of the fall in the liver glutathione following alloxan or dehydroascorbic acid injection in relation to the production of experimental diabetes is obscure.

Immediately following the injection of dehydroascorbic acid the glutathione contents of the pancreas of rats were not significantly different from those of the control animals. The data in the literature on the glutathione content of the pancreas following the injection of alloxan are subject to considerable controversy. Bruckmann and Wertheimer (135) could not observe any significant change in the glutathione content of the pancreas of rats following the injection of diabetogenic dose of alloxan. Brada, (178), however, found a significant fall in the glutathione concentration of the pancreas of rats after alloxan injection. The present author believes, in common with Lazarow (82), that very little information regarding the concentration of glutathione in the beta-cells of the islets of Langerhans of the pancreas can be gained from the results of glutathione analyses of the whole pancreas, because the beta-cells constitute only a fraction (0.5%) of the total weight of the organ. Unfortunately, however, at the

present time there is no method available for the quantitative separation of the beta-cells from the acinar tissues of the pancreas, so that in the present work only indirect methods could be applied.

GENERAL CONSLUSIONS AND SUMMARY

GENERAL CONCLUSIONS AND SUMMARY

1. The ascorbic acid and total ascorbic acid contents of the plasma of rabbits have been estimated under different experimental conditions. The limitations of the various methods used for the accurate estimation of dehydroascorbic acid (DHA) have been discussed. The results suggest that appreciable quantities of DHA do not exist in the plasma of normal or of alloxan-diabetic rabbits.

2. The reduced glutathione (GSH) content of blood can be estimated equally well by the iodometric and glyoxalase methods. Only the latter method is reliable for the estimation of GSH in tissues. By comparative study it has been shown that the iodometric method is unreliable for the accurate estimation of total glutathione in blood. Certain modifications have been suggested for the accurate estimation of total glutathione (reduced plus oxidized) in blood by the combined use of electrolytic reduction and the glyoxalase method. It has been demonstrated by the modified technique that considerable amounts of oxidized glutathione (GSSG) are present in the blood of humans, rabbits, and rats. Unlike blood, tissues do not contain any measurable quantity of GSSG.

3. DHA is reduced to a significant extent in the red blood cells of rabbits both in vitro and in vivo. Since no significant change in blood GSH takes place concurrent with the reduction of DHA it is concluded that GSH is not responsible for the reduction of DHA in rabbit blood. When DHA is injected intravenously into rabbits to give very high blood concentrations, a considerable portion of

the DHA in plasma and in red blood cells appears to undergo an irreversible change to diketogulonic acid.

4. It has not been possible to produce diabetes in rabbits by the intravenous injection of DHA, whereas rats have been found to be easily and regularly susceptible to the diabetogenic action of DHA. The GSH contents of blood, liver, and pancreas of rats made diabetic with DHA are not significantly different from those of the control animals. GSSG does not appear in the tissues of diabetic rats nor does the amount of this substance in the blood of diabetic rats increase.

5. The GSH concentration in blood of rabbits and rats does not alter immediately after intravenous injection of DHA. In contrast to this a precipitous, but temporary, fall in blood GSH of rabbits after alloxan injection has been observed. The significance of this fall in blood GSH has been discussed and it has been concluded that the fall in blood GSH is not specifically related to the production of experimental diabetes.

6. The GSH content of rat liver falls immediately after DHA injection but that of the pancreas remains unaltered. It has been observed also that GSH content of rat liver extracts falls after incubation of the latter with DHA and it has been concluded that GSH is responsible for the reduction of DHA in the liver. The significance of the changes in GSH concentrations of tissues after injections of diabetogenic substances has been discussed.

APPENDIX

Histological Examination of the Tissues of Dehydroascorbic Acid-Diabetic Rats:

The liver, kidney, suprarenal, and thyroid of the diabetic rats showed no significant histological changes. The islets of Langerhans in the pancreas of the diabetic rats, however, showed signs of degenerative changes. From Fig. 11 the following degenerative changes can be easily seen;

a) disruption of the normal structure of the islet,

b) vacuolation or loss of cytoplasm of the islet cells,

c) pyknosis and variation in size and shape of the nuclei.

In the actual stained section, however, some further changes were observed; viz. the degenerative cells are almost all beta-cells, the alpha-cells being relatively unaffected; there is loss of normal peripheral arrangement of the alpha-cells; lymphocytic infiltration is present in the islets.

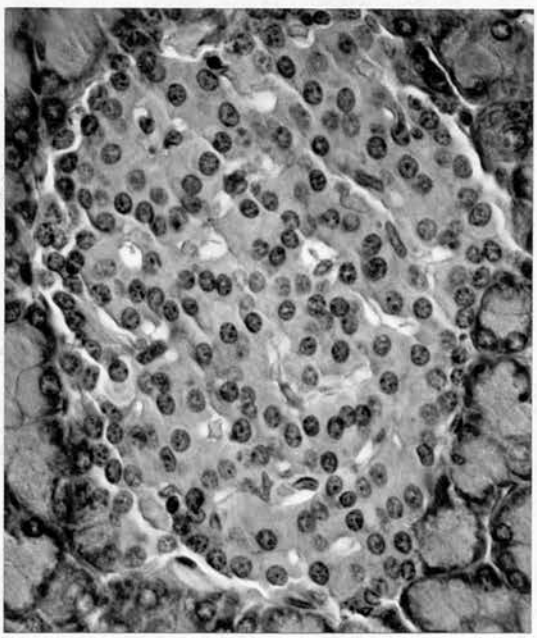


FIG. 10. An islet of Langerhans in the pancreas of a hydroquinone injected rat (control) × 550

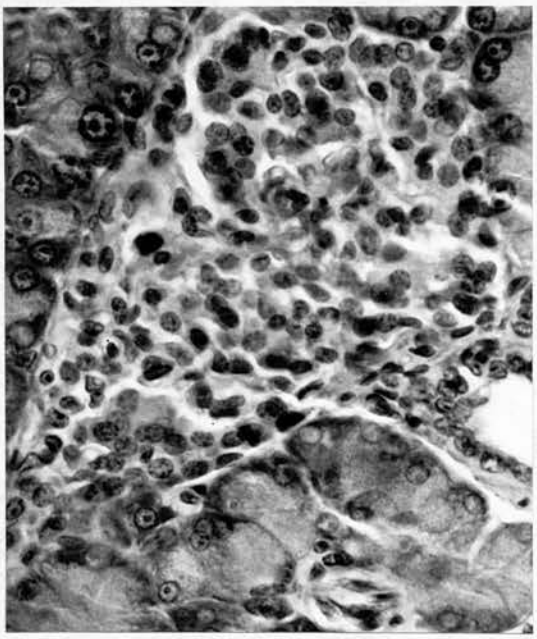


FIG. 11. An islet of Langerhans in the pancreas of a rat made diabetic with dehydroascorbic acid ×550

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