# OXIDATION-REDUCTION POTENTIAL AND RADIOLYSIS OF GLUTATHIONE

By

AYOUB ESFANDI

Bachelor of Science

Panhandle State College

Goodwell, Oklahoma

1970

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
MASTER OF SCIENCE
May, 1972

# OXIDATION-REDUCTION POTENTIAL AND RADIOLYSIS OF GLUTATHIONE

Thesis Approved:

George Gorin
Thesis Adviser

Ernest M. Hodnett

Noel Pundie

D. Durham
Dean of the Graduate College

ACKNOWLEDGEMENTS

I am pleased to express my gratitude to Professor George Gorin for calling the following interesting problems to my attention and for his helpful comments, suggestion and supervision throughout these investigations.

I would like to express my gratitude to my wife, Rashel, for her encouragement and patience.

I acknowledge with thanks financial assistance from the Chemistry Department of the Oklahoma State University.

Thanks are also due to Dr. George Guthrie for his help and guidance during my first year of graduate work.

TABLE OF CONTENTS

## LIST OF TABLES

LIST OF FIGURES

# CHAPTER I

## INTRODUCTION

This thesis consists of two parts. One deals with the equilibrium constant for reaction of glutathione with $NADP^+$;

$$NADP^+ + 2GSH \rightleftharpoons NADPH + GSSG + H^+ \qquad (1)$$

From the equilibrium constant for reaction (1) and the potential of the $NADP^+/NADPH$ system the potential for the glutathione system can be obtained. The second part of the thesis deals with radiolysis of glutathione in aqueous neutral solutions in presence and absence of oxygen.

The principal parts of this thesis are Chapter III and Chapter IV, in which the more important findings have been described and discussed in a form which would be suitable for publication in a journal.

Chapter V reports additional experiment details which were not included in parts III and IV for reasons of brevity.

The literature review is reported in Chapter II.

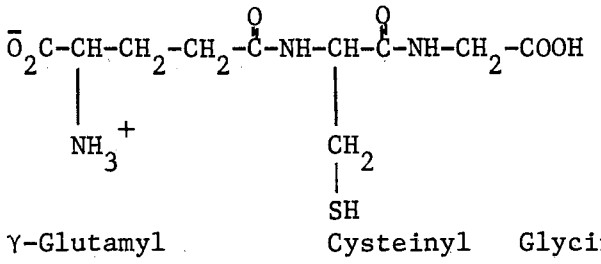
# CHAPTER II

## SELECTED LITERATURE REVIEW

This chapter is divided into several sections: (A) glutathione in general; (B) applicable chemical principles, conventions for oxidation reduction potentials and chemical equilibrium; (C) the oxidation-reduction potentials of the glutathione system; (D) radiation chemistry and chemical dosimetry; (E) radiolysis of glutathione.

### Glutathione

Glutathione is a tripeptide, γ-glutamylcysteinylglycine.




This structure was suggested in 1929 by Pirie and Pinhey.[2] It was confirmed in 1935 by a synthesis carried out by Harington and Mead.[3]

The chemistry of glutathione and its metabolic significance have been intensively investigated. These topics have been covered in several review articles.[5,6,7,8,9] Some special aspects of the subject with selected citations are discussed below, but this survey is not comprehensive. Comprehensive reviews are however presented of the literature on the oxidation-reduction potential of glutathione and on its radioly-

sis.

## Occurrence

Glutathione has been found in many bacteria, yeasts and plants. It has been found in all the animal tissues that have been investigated. Its distribution is exclusively intracellular.[51] In biological materials the reduced form predominates and in animal tissues it constitutes the bulk of the non-protein thiol fraction.[51] In human erythrocytes glutathione is present in concentrations of 160 to 300 µmoles/100 ml of packed cells.[10,51] The concentration of GSSG are much lower: 0 to 15 µmoles/100 ml of packed cells[10], and 4.5 µmoles/100 ml of packed cells.[11]

It is generally believed that glutathione helps to keep sulfhydryl compounds in a reduced state.[12,13] Failure to maintain -SH groups in the membrane of red blood cell results in hemolysis *in vitro* and *in vivo*.[14] In red blood cells the glutathione system is considered to form part of the mechanism serving to prevent oxidative damage. This protective role has been greatly clarified by the discovery of a hereditary defect, which results in an abnormal level of glutathione and glutathione reductase in the erythrocytes. This defect leads to a chronic nonspherocytic hemolytic anemia.[51]

## Glutathione Reductase

The enzyme that catalyzes the reduction of GSSG to GSH by NADPH, glutathione reductase, has been widely investigated. It was identified almost simultaneously by three teams of investigators. In 1951, Mapson and Goddard isolated the enzyme from pea seeds[16], while Conn and

Vennesland discovered the enzyme in wheat germ.[17] In 1952, Rall and Lehninger characterized the enzyme in yeast, pig liver and various tissues of the rat.[18] The stoichiometry of the reaction catalyzed by the enzyme was shown to be

$$H^+ + GSSG + NADPH \rightarrow 2GSH + NADP^+$$

in all three papers. The reverse reaction, oxidation of GSH by $NADP^+$ could not be demonstrated because the equilibrium constant is about $10^{-3}$. Fluorometric analysis made it possible for the first time to demonstrate the formation of NADPH from $NADP^+$ and GSH.[18] NADH can replace NADPH, but with a nucleotide concentration of 100 μmoles the reaction rate is about 100 times slower.[19]

## Review of Applicable Chemical Principles and Conventions

Reactions in which electrons are transferred from one atom, ion or molecule to another are called oxidation-reduction or redox reactions. Oxidation is the loss of one or more electrons, reduction the opposite process.

The convention used in this thesis with respect to the sign of oxidation-reduction potentials is that recommended by the International Union of Pure and Applied Chemistry[20], which is also used by Clark[1] and most commonly accepted among biochemists.

The electrode potential for a half reaction is a measure of the chemical force tending to drive that reaction toward equilibrium. Consequently the electrode potential is zero when a system is at equilib-

rium and becomes larger as the system departs further from this state. Thus the potential of a piece of metallic zinc in pure water is larger relative to a piece of the same metal immersed in a one formal solution of zinc sulfate. In general, then, the concentration of the reactants and products of a half reaction will have a marked effect on electrode potentials and the quantitative aspects of this effect must be considered.

Consider the generalized, reversible reaction

$$aA + bB + \ldots\ldots \rightleftarrows cC + dD + \ldots\ldots$$

It can be shown theoretically as well as experimentally that the potential, E, for this electrode process is governed by the relation

$$E = E^{o} - \frac{RT}{nF} \ln \frac{(C)^{c}(D)^{d}\ldots\ldots}{(A)^{a}(B)^{b}\ldots\ldots} \qquad (1)$$

where $E^{o}$ = potential when the logarithmic term is zero, i.e., the activity quotient is unity. One instance would be when the activities of all of the reactants and products are unity; thus, the standard electrode potential may be defined as the potential of a half-cell reaction versus the hydrogen electrode when the reactants and products are at unit activity.

A standard electrode potential is a relative quantity in the sense that it is really a cell potential in which one of the electrodes is a carefully specified reference electrode that is, the standard hydrogen electrode whose potential is given a value of zero volts at $25^{o}C$. A

standard electrode potential is temperature dependent; so the temperature at which it is determined must be specified.

R = the gas constant = 8.314 volt coulombs/$^{o}$K/mol

T = the absolute temperature

n = number of electrons participating in the reaction as defined by the balanced chemical equation for the half-cell reaction

F = the faraday = 96,493 coulombs

ln = the natural logarithm = 2.303 $\log_{10}$

Substituting numerical values for the various constants into Equation (1) and converting to $\log_{10}$, Equation (1) becomes at 25$^{o}$C

$$E = E^{o} - \frac{0.059}{n} \log \frac{(C)^{c}(D)^{d}\cdots\cdots}{(A)^{a}(B)^{b}\cdots\cdots} \qquad (2)$$

The symbols in parenthesis represent the activities of reacting species. The term following the logarithm can be replaced by the equilibrium constant for the reaction. For very dilute solutions for all practical purposes

$$(A) \cong [A]$$

where square brackets represent concentration

$$K = \frac{(C)^{c}(D)^{d}\cdots\cdots}{(A)^{a}(B)^{b}\cdots\cdots} \cong \frac{[C]^{c}[D]^{d}\cdots\cdots}{[A]^{a}[B]^{b}\cdots\cdots}$$

If the expression for K is substituted in Equation (2)

$$E = E^{o} - \frac{0.059}{n} \log K$$

using this equation oxidation reduction of a system can be calculated if K, equilibrium constant for the reaction is known.

## Oxidation Reduction Potential of Glutathione

Many attempts have been made to determine the value of the redox potential of glutathione. But there has been an unusual amount of disagreement and controversy concerning the results, and there is still some uncertainty about the merits of the respective investigations.

Soon after the discovery of glutathione a number of attempts were made to measure the oxidation reduction potential of glutathione by potentiometric methods. It was observed that the potential with noble metal electrodes was independent of the concentration of the oxidized form of glutathione (GSSG)[21,22,23], i.e., the data fitted an empirical equation of the following form:

$$E = E^{o} - \frac{RT}{F} pH - \frac{RT}{nF} \ln [GSH]$$

instead of the expected form

$$E = E^{o} - \frac{RT}{F} pH - \frac{RT}{nF} \ln \frac{[GSH]^2}{[GSSG]}$$

This indicates that the reaction taking place at the electrodes is not the conversion of GSH into GSSG.

A later attempt was made by Ghosh and Ganguli, who used a galvanic cell with a mercury pool electrode.[24] Immediately prior to making

potential measurements, the mercury electrode was subjected to reduction. The authors believed that this removed a layer of oxide, after which equilibrium could be established. The result was -0.35 V at pH 7.0. Gosh and Ganguli also measured the potential for cysteine-cystine system and found a potential of -0.33 volts. Freedman and Corwin[25] have questioned the validity of the cysteine result.

In 1955 Kolthoff, et al. by means of polarography obtained some results with glutathione and cysteine.[26] The calculated a potential of -0.32 V for glutathione. Some other attempts were made to determine the potential of glutathione by polarography, but most results have been considered invalid.[1]

Fruton and Clark[27] measured the oxidation-reduction potential by equilibration with some oxidation-reduction dyes of known potential. They reported a value of -0.23 V at pH 7.0.

Potentiometric titration was used by Ryklan and Schmidt.[28] They report a value of +0.04 V at pH 7.0, and -0.14 V for cysteine-cystine. However, their results for cysteine could not be reproduced by Freedman and Corwin.[25]

Rall and Lehninger[29] were unable to obtain significant reversal of the reaction

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

with glutathione reductase. They concluded that the potential of glutathione pH 7.0 must be more positive than -0.13 V. The failure of Rall and Lehninger to obtain detectable reaction may have been caused by insufficient amount of enzyme. The substrates inhibit strongly at the concentrations used.

Mapson and Isherwood[30] reported a value of -0.16 V for the redox potential, which corresponds to a conditional equilibrium constant of $10^{-5}$ for reaction of glutathione with $NADP^+$ at pH = 7.0 and 25°C.

$$GSH + NADP^+ \rightleftharpoons GSSG + NADP^+ + H^+ \qquad (1)$$

An independent study by Scott and Duncan[31] led them to estimate a value of $1 \times 10^{-2}$ for the equilibrium constant, and a -0.26 V for the potential of the glutathione system.

The last reported investigation on the subject is by Rost and Rapoport[32] in 1964. They report a value of -0.24 V for the potential of the glutathione system at 40°C under anaerobic conditions. The oxidation reduction potential of the $NADH/NAD^+$ system was taken as -0.33 V at 40°C. They calculated a value of $1 \times 10^{-3}$ for the equilibrium constant for the reaction of glutathione with $NAD^+$.

## Radiation Chemistry

The literature which deals with radiation is very vast so no attempts will be made to give complete coverage to the subject. Only two special aspects of the subject will be considered, chemical dosimetry, as it pertains to experiments performed in this thesis, and a comprehensive review of the radiation chemistry of glutathione.

### Chemical Dosimetry

The ferrous sulfate dosimeter was pioneered by Miller[36] and has been very extensively used. It is one of the most reliable and convenient methods of chemical dosimetry. It is based on the fact that the radiolysis products of water oxidize ferrous ions to ferric. The reac-

tion is usually carried out in aerated solution at pH below 1.5.

Fricke[37] chose 0.8 N sulfuric acid as the solvent. Weiss, Allen and Schwartz[38] have recommended a dosimeter solution containing $10^{-3}$ M ferrous ammonium sulfate, $10^{-3}$ M sodium chloride and 0.8 N sulfuric acid. Chloride is added to the dosimeter solution to inhibit the oxidation of ferrous ions by organic impurities.[39] The water should be very pure (triply distilled). In aerated acid solution $Fe^{++}$ is oxidized by $OH^{\cdot}$, by $HO_2^{\cdot}$, and by $H_2O_2$, so that $Fe^{+++}$ is obtained instead of any of the other products of water radiolysis.

$$OH^{\cdot} + Fe^{++} \rightarrow Fe^{+++} + OH^{-}$$

$$H^{\cdot} + O_2 \rightarrow HO_2^{\cdot}$$

$$HO_2^{\cdot} + Fe^{++} \rightarrow Fe^{+++} + HO_2^{-}$$

$$HO_2^{-} + H^{+} \rightarrow H_2O_2$$

$$H_2O_2 + Fe^{++} \rightarrow Fe^{+++} + HO^{\cdot} + OH^{-}$$

The most common method of measuring the ferric ion formed is by spectrophotometry at 304 nm, the wavelength at which ferric ions show maximum absorption. The mean absorbed dose D in the volume occupied by the dosimeter solution is derived as follows.[34,35] For any chemical system, given that:

G (products) = molecules of product formed/100 eV of energy absorbed.

P = number of molecules of product formed

100 eV = $1.602 \times 10^{-10}$ ergs

The energy E, in ergs, absorbed in the dosimeter solution is:

E = $1.602 \times 10^{-10}$ (ergs/100 eV) P/G (molecules of prod-

uct/100 eV)

1 rad = energy absorption of 100 ergs/g.

The dose D, in rads is given by

$$D = E/W = 1.602 \times 10^{-12} P/GVd$$

where

W = weight of the dosimeter solution, in g

V = volume of dosimeter solution in ml

d = density of dosimeter solution in g/ml

In the ferrous sulfate dosimeter, the concentration of ferric ions formed by irradiation is given by:

$$C_{Fe(III)}(\text{moles/l}) = ((A_i - A_j)/\varepsilon b) \times \frac{1000\ \text{cm}^3}{\text{liter}}$$

where $A_i$ and $A_j$ are the absorbances of the irradiated and unirradiated (blank) dosimeter, respectively, $\varepsilon$ is the molar absorbancy in $cm^2$/mole and b the thickness of sample in cm. P, the ions of $Fe^{3+}$ formed, is given by:

$$P = ((A_i - A_j)/\varepsilon b)(\text{moles/l}) \times 6.02 \times 10^{23}\ (\text{molecules/mole}) \times V(l)$$

and

$$D = 1.602 \times 10^{-12}\ (\text{g rads/100 eV}) \times (6.023 \times 10^{23}) \times ((A_i - A_j)V/\varepsilon b)\ \text{molecules} \times \frac{1}{G(\text{molecules/100 eV})V(\text{ml})d(\text{g/ml})}).$$

For the dosimeter solution; $\varepsilon = 2.193 \times 10^6$ ($cm^2$/mole), b = 1 cm,

G = 15.5, and d = 1.024 (g/ml);

$$D(\text{rads}) = 2.8543 \times 10^4\ (A_i - A_j).$$

## Radiolysis of Glutathione

Hammet[52] studied the effects of β- and γ-rays from radium and qualitatively demonstrated the destruction of glutathione in aqueous solutions.

Woodward[40] studied the effects of ultraviolet light, β- and γ-rays from radium, and x-rays on aqueous solutions of glutathione. She confirmed Hammett's work, but no measurable destruction was observed with x-rays.

Kinsey[41] studied the effects of ionizing radiation on glutathione. He reduced all GSSG to GSH before analyzing. Therefore, only the destruction of the molecule was measured. The destruction of the molecule at pH around 7 occurred with an ionic yield of 0.6.

Barron and Dickman[42,43] showed that enzymes containing essential -SH groups were readily inactivated by ionizing radiations. Furthermore, they showed that the inactivation was reversible, an indication that the -SH group had been oxidized to S-S.

Barron[44] studied the oxidation of glutathione by radiation. He found out that for solutions $2 \times 10^{-4}$ M the extent of oxidation was directly proportional to the radiation dose. He also studied the effect of pH. Between pH 4 and 5, no difference was noted; at these pH values, the degree of dissociation of the -SH group is practically nil. From pH 5 to 8 the ionic yield increased very sharply with pH value. In the absence of oxygen, the oxidation of thiol by ionizing radiations was only 33 per cent of that obtained in the presence of dissolved oxygen. The ionic yield at pH around 7 in presence of oxygen was about 3. There was no reduction of oxidized glutathione on irradiation.

Dale and Davis reported for the first time[45,46] that hydrogen

sulfide is split off, under certain conditions, from cysteine and glutathione when they are irradiated by X-rays. They reported that the production of $H_2S$ is pH dependent, with a maximum and a minimum at pH 4 and 5, respectively, but they give no explanation for the curve.

The most recent study on irradiation of glutathione was conducted by Lal et al.[47]. Under anaerobic conditions they identified hydrogen, hydrogen sulfide, oxidized glutathione, and γ-glutamylalanylglycine (GH), as products; the yields were 1.2, 2.0, 3.0, and 2.3 molecules/100 eV, respectively. The yield for disappearance of glutathione was about 8 molecules/100 eV. The pH was 5.3.

The focal point of chemical change is the -SH group. Since all the yields of γ-radiolysis of glutathione are within the experimental errors the same as those of analogous products from cysteine[48,49] the mechanisms of radiolysis of the two compounds must be similar. The following reactions are involved:

$$GSH + H^{\cdot} \longrightarrow GS^{\cdot} + H_2 \quad (1)$$

$$GSH + H^{\cdot} \longrightarrow G^{\cdot} + H_2S \quad (2)$$

$$GSH + OH^{\cdot} \longrightarrow GS^{\cdot} + H_2O \quad (3)$$

$$GSH + G^{\cdot} \longrightarrow GS^{\cdot} + GH \quad (4)$$

$$GSH + e_{aq} \longrightarrow G^{\cdot} + SH^{-} \quad (5)$$

$$SH^{-} + H^{+} \longrightarrow H_2S \quad (6)$$

$$H^{+} + e_{aq} \longrightarrow H^{\cdot} \quad (7)$$

$$2GS^{\cdot} \longrightarrow GSSG \quad (8)$$

$$2GSH + H_2O_2 \rightarrow GSSG + 2\ H_2O \quad (9)$$

Since GH and GSSG are the only amino-containing products then 2G(GSSG) +

G(GH) must be equal to G(-GSH). This was observed by Lal *et al*. They further say that the yields of GH and $H_2S$ at pH = 5.3 are equal to the value of $G(e_{aq})$. This comes from the fact reactions 5 and 4 are the sources of $H_2S$ and GH in neutral solutions. They also report a decrease in $G(H_2S)$ as pH is lowered, and this trend accompanies an increase in $G(H_2)$. Consequently, they conclude that there is a competition between reactions 5 and 7. The reported value for G(GH) is in all cases about 15% larger than corresponding values of $G(H_2S)$; the authors infer from this observation that a reaction destroys some of the hydrogen sulfide.

## CHAPTER III

(As stated in the Introduction, this chapter is written in a form suitable for publication)

# OXIDATION REDUCTION POTENTIAL OF GLUTATHIONE*


### Summary

The equilibrium constant for the reaction of glutathione with $NADP^+$ has been determined to be $5 \times 10^{-3}$ at pH = 7.0 and $25^{\circ}C$. From this value the oxidation-reduction potential of the glutathione system has been estimated to be -0.25 volts. These values are discussed and compared with literature values.


---

*This paper has been presented in SW & SE Conference of American Chemical Society in December 1970.

# INTRODUCTION

Glutathione (GSH) is a tripeptide formed from glutamic acid, cysteine and glycine. Like other mercaptans, it is easily oxidized to the disulfied (GSSG). Its occurrence in living cells is widespread.[13] It has been found in bacteria, yeasts and plants as well as in all animal tissues in which it has been sought. It is generally believed that reduced glutathione helps to maintain important sulfhydryl compounds, such as enzymes, at the appropriate oxidation-reduction level. In human erythrocytes, its concentration is about 75 mg/100 ml of packed cells.[13] The reducing action of glutathione likely helps to prevent oxidative damage to the cells. Failure to maintain -SH groups in the cell membrane results in hemolysis in vitro and in vivo.[13]

Because of all the important biological functions of glutathione the redox potential of the glutathione system is of great biological interest, and many attempts have been made to measure it. However, as one can see from the values reported in Table I quite discrepant values have been reported. There is controversy even concerning the sign, i.e., whether glutathione is a better reducing agent than hydrogen, or a better oxidizing agent.

The values that have been obtained by determination of the equilibrium constant for the reaction between glutathione and $NADP^+$ and/or $NAD^+$ are in better agreement but there is a difference of 0.13 volts between the largest and the smallest value, and this corresponds to a factor of $10^4$ in the values of the equilibrium constants.

Also pertinent is the comparison between the glutathione system and the cysteine/cystine system.

Three independent studies[9,16,17] indicate the potentials differ

TABLE I

REDOX POTENTIALS OF GLUTATHIONE AT pH 7

| Investigators | Method | Potential (volts) | Ref. |
|---|---|---|---|
| Fruton and Clark (1934) | Equilibrium with Dyes | -0.23 | 1 |
| Ghosh and Ganguli (1935) | Cell Potential | -0.35 | 2 |
| Ryklan and Schmidt (1944) | Potentiometric Titration | +0.04 | 3 |
| Kolthoff and Stricks (1955) | Polarography and Equilibrium | -0.32 | 4 |

POTENTIALS ESTIMATED FROM EQUILIBRIUM CONSTANTS WITH $NAD^+$ AND/OR $NADP^+$

| Investigators | $K_7$ | Potential (volts) | Ref. |
|---|---|---|---|
| Rall and Lehninger (1952) | $<10^{-6}$ | -0.13 | 5 |
| Mapson and Isherwood (1962) | $\sim 10^{-5}$ | -0.16 | 6 |
| Scott and Duncan (1963) | $1.0 \times 10^{-2}$ | -0.26 | 7 |
| Rost and Rapoport (1964) | $1.0 \times 10^{-3}$ | -0.23 | 8 |

by less than 0.02 V. Although the values of the cysteine/cystine potentials has also been the subject of considerable controversy, the preponderance of evidence favors a value of (0.33 - 0.39) V. Especially significance is the calculations based on the heat capacity and the heat of combustion measurements of Huffman and Ellis[10,11] and heat of combustion measurements of Sunner[12]. These calorimetric data, which are certainly sound in principle, lead to a value of -0.39 V for the cysteine-cystine potentials.

Because of the uncertainty which attaches to the reported results, we decided to make additional measurements on the equilibrium:

$$2GSH + NADP^+ \leftrightharpoons GSSG + NADPH + H^+ \qquad (1)$$

The attainment of this equilibrium is facilitated by addition of the enzyme glutathione reductase (E.C. 1·6·4·2). From the equilibrium constant for this reaction, and the potential for the $NADP^+$/NADPH system, the redox potential for the glutathione system can be calculated utilizing the Nernst equation. As it is apparent from its stoichiometry, reaction (1) is pH dependent.

## EXPERIMENTAL

### Materials

Distilled water which was further purified by passage through an ion-exchange resin, distillation from an acid permanganate solution and redistillation in an all glass distillation apparatus. This water was used to prepare all solutions. $NADP^+$ and NADPH, GSH, GSSG and glutathione reductase (suspended in $(NH_4)_2SO_4$) were purchased from Sigma Chemi-

cal Company, disodium ethylenedinitrilotetraacetate (EDTA) from Eastman Chemicals. Phosphate buffer (0.1M) of pH 7.5 was mixed from 1.810 g of $NaH_2PO_4 \cdot H_2O$, 23.30 g of $Na_2HPO_4 \cdot 7H_2O$ and 0.1860 g of EDTA per liter; the phosphate buffer (0.1 M) of pH 7.0 was prepared from 5.520 g of $NaH_2PO_4 \cdot H_2O$, 8.548 g of $Na_2HPO_4$ and 0.1860 g of EDTA per liter. Tris buffer (0.1 M) of different pH was prepared from 24.22 g of tris(hydroxymethyl)aminometane and 0.3720 g of EDTA per liter. Fifty ml of this solution was mixed with appropriate amounts of 0.2 M Hcl per 100 ml.

Method

The absorbances were measured with a Beckman Model DU Spectrophotometer with conventional 1-cm quartz cells. The enzyme stock solution (4 mg/ml) was diluted 10-fold with 0.01 M phosphate buffer of pH 7.0.

The experiment is conducted in the following manner. Exactly 3.50 ml of a reaction mixture containing $NADP^+$ and GSH is measured with a pipet into a dry spectrophotometric cell. Measurements are made at 340 nm against buffer solution as a blank. First, some readings are taken at about 1 minute interval. Then 150 µl of dilute enzyme solution is added, the mixture is stirred vigorously and a reading is taken as soon as possible; additional measurements are then made at appropriate intervals thereafter (Fig. 1) (Curve A). The concentration of NADPH at equilibrium is measured by taking the difference between the initial and equilibrium absorbances. The equilibrium absorbance is estimated by extrapolating to zero time (Curve 1A).

To assay for GSSG at equilibrium an aliquot of the reaction mixture is added to exactly 3.00 ml of NADPH solution (about 0.15 mg/ml) and 150 µl of dilute enzyme solution (Curve 1B).

To shift the reaction back about 200 µl of GSSG solution (about 0.1 mg/ml) is added to the remaining reaction mixture (Curve 1A).

## RESULTS AND DISCUSSION

NADPH has a high extinction coefficients of $6.2 \times 10^6$ $cm^2$/mole at 340 nm.[15] The absorbance of $NADP^+$ is negligible in comparison with NADPH and so are the absorbances of GSH and GSSG at this wavelength. From the initial concentration of $NADP^+$ and GSH and the direct measurements of NADPH and GSSG concentrations at equilibrium, the equilibrium constant for the reaction of glutathione with $NADP^+$ is measured in a buffer medium at constant pH. As will be seen, the equilibrium constant for reaction (1) as written is quite small. At pH 7.0 and approximately $10^{-3}$ M concentration of $NADP^+$ and GSH only small amounts of NADPH are formed. In order to produce easily measurable amounts of all reagents, the GSH concentration was made 5 to 30 times larger than that of $NADP^+$. At high pH the equilibrium is more favored and smaller ratios of $[GSH]/[NADP^+]$ were employed.

Figure 1 represents a typical experiment. From the Curve 1A (open circles) it can be seen that reaction is extremely slow before introduction of the glutathione reductase. Then there is a rapid increase in absorbance and a nearly constant absorbance value is reached in about three minutes; it may be concluded that equilibrium is reached. The slow decrease in absorbance which follows may be ascribed to disappearance of the NADPH formed by autoxidation and hydrolysis.[14]

From this first experiment we obtain the concentration of NADPH at equilibrium. From Equation (1), the concentration of GSSG formed in the reaction is the same. But all samples of GSH contained between 0.5

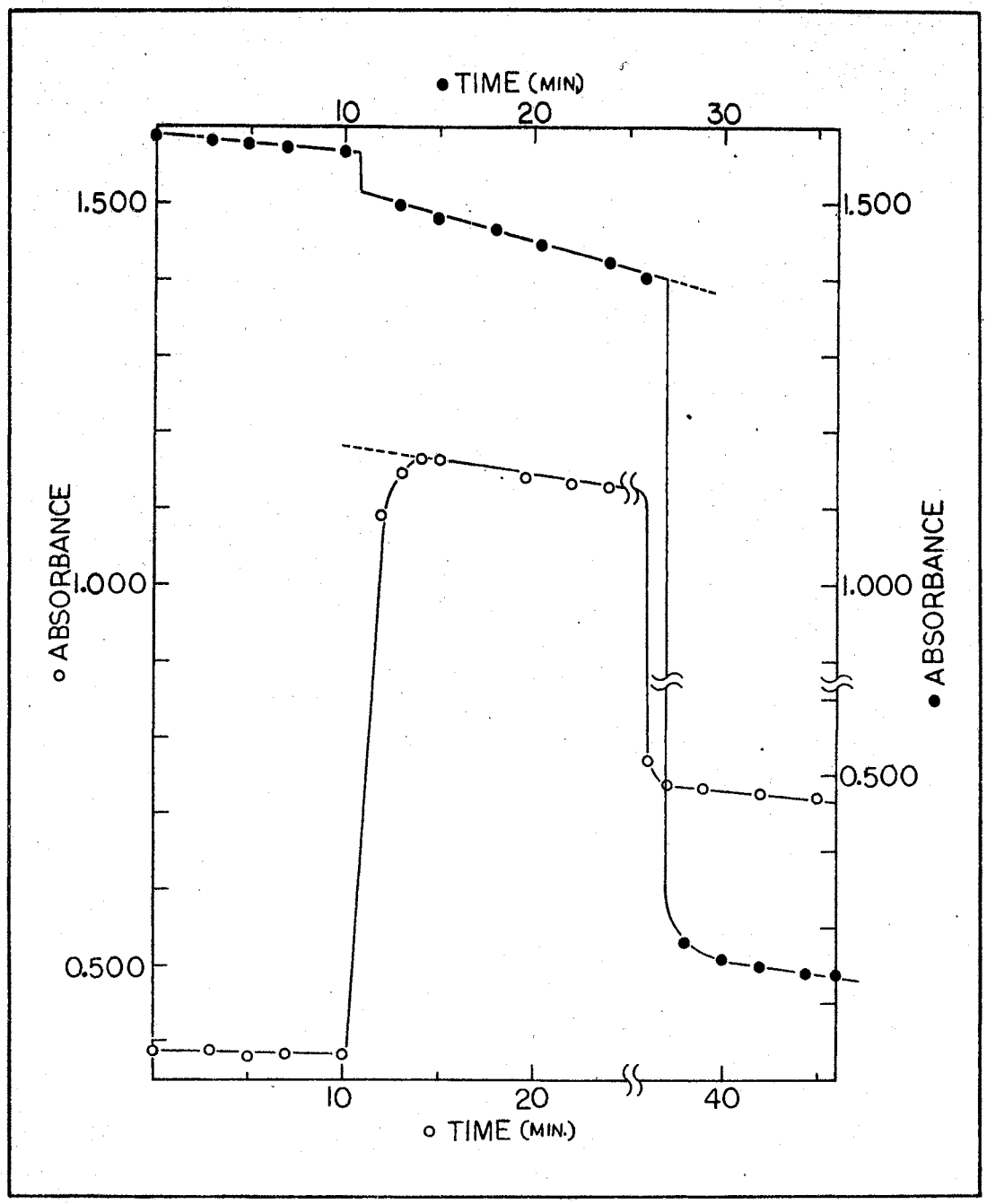



Figure 1. Plots of Absorbance Versus Time for the Reaction of Glutathione With $NADP^+$. The left and the lower axes correspond to the curve A (open circles). Enzyme was added in 10 minutes time. The right and upper axes corresponds to curve B (closed circles) which represents reverse reaction.

to 1.5% of GSSG as impurity. Consequently, the GSSG concentration calculated from stoichiometry is lower than that actually present at equilibrium and, since the amount of GSSG formed is small, neglecting the amounts initially present causes a large error in the value of equilibrium constant. The concentration of GSSG at equilibrium was therefore determined directly by adding an aliquot portion of the solution at equilibrium to an excess of NADPH. As one can see from the plot (Curve 1B) there is a rapid decrease in absorbance, which then becomes constant. At this point because we have excess of NADPH and the equilibrium constant for the reaction is in order of 200, the amount of GSSG left must be less than 2% of its initial value. From this experiment the concentration of GSSG originally present is calculated. From these two measurements, the initial concentration of $NADP^+$ and GSH, and the pH of the solution at equilibrium, the equilibrium constant for reaction 1 is calculated.

If the equilibrium constant calculated in this manner is the true value we should be able to obtain the same value when the equilibrium is approached from the opposite side. To accomplish this, a measured quantity of GSSG is added to the remainder of the reaction mixture that was already at equilibrium. As one can see from the plot (Curve 1A) there is a rapid decrease in absorbance followed by slow disappearance of NADPH indicating equilibrium. From this experiment another value for equilibrium constant is calculated.

Results are tabulated in Table II. Values in column 2 are the pH of the reaction medium; for pH of 7.0 to 7.5 a phosphate buffer was used, and for solutions with pH > 7.5 to 8.5 tris buffer. In column 3 we have tabulated the ratio of $[GSH]/[NADP^+]$ used in the experiments; this has

TABLE II

EQUILIBRIUM CONSTANTS OF GLUTATHIONE WITH $NADP^+$

| 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|
| Experiment | pH | $[GSH]/[NADP^+]$ | $10^3\ K_h^f$ | $10^3\ K_7^f$ | $10^3\ K_7^r$ |
| 1 | 7.00 | 32.0 | 5.4 | 5.4 | 5.0 |
| 2 | 7.00 | 15.4 | 4.9 | 4.9 | 4.3 |
| 3 | 7.00 | 16.0 | 4.9 | 4.9 | 5.2 |
| 4 | 7.00 | 18.0 | 6.4 | 6.4 | 5.9 |
| 5 | 7.82 | 11.0 | 37.3 | 5.7 | 4.5 |
| 6 | 8.00 | 14.3 | 49.0 | 4.9 | (2.6) |
| 7 | 8.47 | 4.6 | 138 | 4.7 | 4.3 |

$AV.K_7^f = (5.3 \pm .5 \times 10^{-3})$ $\qquad$ $E_{h7} = -0.25$ Volts

been varied by a factor of 10. Values of $K_h^f$, the concentration equilibrium constant for reaction 1 divided by antilog of (-pH), are tabulated in column 4. No attempt has been made to estimate the activity. $K_7^f$, values tabulated in column 5 of Table II are the equilibrium constant values at pH 7 divided by antilog (-pH). $K_7^r$ values tabulated in column 6 of Table II are the concentration constant values at pH = 7.0 calculated for experiments in which the equilibrium was approached from the direction opposite to which reaction 1 is written divided by antilog of (-pH).

The average value for equilibrium constant for the forward reaction at pH = 7.0 is $(5.3 \pm 0.5) \times 10^{-3}$ at 25°C. Since in the reverse experiments additional experimental errors will have been introduced, we do not consider these values to be as reliable as those calculated in the "forward" experiment.

From $K_h^f$ a value of -0.25 volts can be calculated for the oxidation reduction potential of the glutathione system taking potential of $NADP^+$/NADPH system to be -0.32 volts.[1] This value compares well with Scott and Duncan value of -0.26 volts, and Rost and Rapoport value of -0.23 volts, and Fruton and Clark value of -0.23 volts.

We feel this is the true value for potential of glutathione system since we have obtained consistent equilibrium constants varying all the possible parameters.

## BIBLIOGRAPHY


(1) Fruton, I. S. and H. T. Clark, J. Biol. Chem. 106, 667 (1934).

(2) Ghosh, J. C. and S. C. Ganguli, Biochem. Z. 279, 296 (1935).

(3) Ryklan, L. R. and G. L. A. Schmidt, Univ. Calif. Pub. Physiol. 8, 257 (1944).

(4) Kolthoff, I. M., W. Stricks, and R. C. Kapoor, J. Amer. Chem. Soc. 77, 4733 (1955).

(5) Rall, T. W. and A. L. Lehninger, J. Biol. Chem. 194, 119 (1952).

(6) Mapson, L. W. and F. A. Isherwood, Biochem. J. 86, 173 (1963).

(7) Scott, E. M., I. W. Duncann, and V. Ekstrand, J. Biol. Chem. 238, 3928 (1963).

(8) Rost, J. and S. Rapoport, Nature 201, 185 (1964).

(9) Eldjarn, L. W. and A. Phil, J. Amer. Chem. Soc. 79, 4589 (1957).

(10) Huffman, H. M. and E. L. Ellis, J. Am. Chem. Soc. 57, 41 (1935).

(11) Huffman, H. M. and E. L. Ellis, J. Am. Chem. Soc. 57, 46 (1935).

(12) Sunner, S., Svensk. Kem. Tidskr. 58, 71 (1946).

(13) Icen, A., Scandinavian J. of Clin and Lab. Invest. Supplementum 96 (1967).

(14) Aravjo, S. M. and G. Cilento, Biochem. J. 8, 2145 (1969).

(15) Stecher, G. P., et al., The Merck Index 8th edition, pp. 710. Merck and Co., Inc., Rahway, N. J., U.S.A. (1968).

(16) Gorin, G. and G. L. Doughty, Arch. Biochem. Biophys. 126, 547 (1968).

(17) Jocelyn, P. C., European J. Biochem. 2, 327 (1967).

# CHAPTER IV

(As was mentioned in the Introduction, this chapter is written in a form suitable for publication.)

## RADIOLYSIS OF GLUTATHIONE IN OXYGEN-CONTAINING SOLUTIONS


### Summary

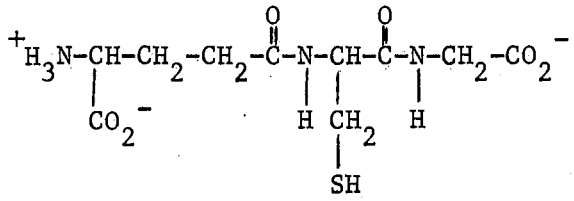
The radiolysis of glutathione in buffer solutions of pH 7 in the presence of oxygen has been investigated. The yields for disappearance of -SH, G(-SH), in 3 x $10^{-3}$ M solutions is about 20 molecules/100 eV. G(-SH) decreases with decreasing GSH concentration, to 2 x $10^{-4}$ M. The major product of radiolysis is oxidized glutathione. A mechanism for the radiolysis is proposed.

# INTRODUCTION

The sulfhydryl and disulfide groups in amino acids, peptides, proteins and enzymes are known to have important biochemical and physiological roles.[1] The effect of ionizing radiations upon these groups is of primary concern in radiation biochemistry. The subject has been reviewed by Barron[2] and by Adams.[20]

The radiolysis of thiols in dilute aqueous solutions containing oxygen has been studied by various workers[7,12,18,19,27,28], who have shown that short chain reactions occur, yielding the corresponding disulfides as the major product.[19] The role of -SH compounds in decreasing radiation injury has been reviewed extensively.[3-5,21] These compounds are presumed to function by various mechanisms; e.g., free radical scavenging, ability to repair by supplying hydrogen[21,23], and formation of mixed disulfides.[21,24,25]

The tripeptide glutathione (GSH)



occurs abundantly in living organisms.[8,21] It has been found in bacteria, yeasts, plants, and in all animal tissues investigated; it is an important constituent of red blood cells.[22] Glutathione has been extensively employed as a radio-protective agent.[21] However, little has been reported on radiolysis of this compound.

In 1932-35, Hammet[9], Woodward[10], and Kinsey[11] conducted the first investigations of the radiolysis of glutathione. They measured

disappearance of -SH but did not identify any products.

Kinsey reduced all the glutathione that had been converted to GSSG before analysis. He reported a yield of 0.6 molecules/100 eV for the disappearance of glutathione.

Barron and his co-workers[7] investigated the radiolytic conversion of glutathione and of thiol-containing proteins to disulfide. Barron reported a G value of 3 molecules/100 eV for glutathione.

Dale and Davis[12] reported the formation of hydrogen sulfide from glutathione and cysteine. $H_2S$ is a minor product, but attracted attention because of its known toxicity.

The most recent study on radiolysis of glutathione has been reported by Lal and his co-workers.[8] In deareated aqueous solutions at pH less than 5.3, they reported a G value of about 8 molecules/100 eV for disappearance of glutathione. They identified the products of radiolysis to be GSSG, $H_2S$, $H_2$, and γ-glutamylalanylglycine (GH); the yields were 3.0, 2.0, 1.2, and 2.3, respectively, in $10^{-3}$ M solutions.

## Materials

Distilled water was further purified by passage through an ion-exchange resin, redistilled with permanganate, and distilled for a third time in an all-Pyrex system. All reagents were of A.C.S.-reagent grade except as otherwise specified. GSH, GSSG, NADPH, and a suspension of glutathione reductase (EC 1.6.4.2) were purchased from Sigma Chemical Co. Phosphate buffer, 0.05 M, was prepared from 2.760 g of $NaH_2PO_4 \cdot H_2O$ and 4.259 g of $Na_2HPO_4$ diluted to a liter; the pH of this solution was 7.0. Phosphate buffer, pH 7.5, was prepared from 0.5984 g of $NaH_2PO_4 \cdot H_2O$ and 6.4870 g of $Na_2HPO_4$. Stock enzyme solution was prepared by addition

of 50 µl of the enzyme suspension to one ml of pH 7.0 phosphate buffer. 5, 5-Dithiobis(2-nitrobenzoic acid)(3-carboxy-4-nitrophenyl disulfide, DTNB) reagent was purchased from Aldrich Chemical Co. The DTNB reagent was prepared by dissolving 90 mg of DTNB in 250 ml of pH 7.5 phosphate buffer.

Procedure

The gamma-ray source was an Atomic Energy of Canada Ltd. Gammacell-200 irradiation unit; it contained a cobalt-60 core, which produced a dose rate of 2700 rads/min. A Beckman model DU spectrophotometer with conventional 1-cm quartz cells was used for absorbance measurements.

A solution of GSH was prepared, one of the samples was kept as a blank, three to six other samples were irradiated from 0.5 to 60 minutes. As soon as possible after irradiation (usually within 10 minutes) the samples were analyzed for total -SH remaining by the DTNB method.[6] Exactly 15 ml portions of DTNB solution was transferred to flasks with a volumetric pipet. To one of these flasks containing the DTNB solution was added 200 µl of pH 7.0 phosphate buffer, to another flask 200 µl of unirradiated GSH solution, and to the remaining flasks 200 µl of irradiated GSH solution. Readings were taken at 412 nm using the first solution as a blank. Measurements were begun as soon as possible and continued until the absorbance remained constant for a few minutes or started decreasing. Absorbance was plotted versus time and an extrapolation was made to the absorbance at zero time. The concentration of -SH remaining was calculated using, $1.36 \times 10^{7}$ $cm^{2}$ $mole^{-1}$ 13600 for the molar absorbancy. The procedure for measurement of GSSG was the same as used in Chapter II. Exactly 3.00 ml of NADPH solution (0.1 mg/ml) was

transferred to a dry spectrophotometer cell, then 250 µl of irradiated solution and 150 µl of dilute enzyme solution were added to the cell. The GSSG concentration was determined from the decrease in absorbance at 340 nm.

## Results and Discussion

The radiolysis of glutathione has been measured in solutions of $2 \times 10^{-4}$ to $3 \times 10^{-3}$ M GSH, at the biologically important pH of 7.0. The G value for total -SH disappearance in presence of oxygen is about 20 molecules/100 eV for $3 \times 10^{-3}$ M solutions. Figure 2 is a typical plot of G(-SH) versus dose. The G value is calculated by extrapolating the first three or two points. As can be seen from the Figure, course of radiolysis undergoes some change after about 25 to 30 Krads, and more will be said about this later. Most probably, a product accumulates then which protects the reduced glutathione. The major product of radiolysis is oxidized glutathione GSSG (see below).

Table III is a table of results. The G(-SH) decreases with decreasing GSH concentration from $3 \times 10^{-3}$ to $2 \times 10^{-4}$ M; the values fall on a smooth curve.

It was thought that the break in $10^{-3}$ M solutions might be due to protection by GSSG. But when we added 5 to 10% GSSG to the radiolysis solutions it did not cause any substantial decrease in G value, therefore the break in Figure 2 might be due to some other product, which acts as a chain stopper, possibly the trisulfide GSSSG. The trisulfide CYSSSCY was produced in significant quantities upon radiolysis of cystine.[26]

TABLE III

G(-SH) FOR RADIOLYSIS OF GLUTATHIONE IN PRESENCE OF OXYGEN

| $[GSH] \times 10^4$ | G |
|---|---|
| 2.1 | 8.6 |
| 4.0 | 9.4 |
| 9.0 | 14.2 |
| 18.0 | 17.0 |
| 35.8 | 21.3 |
| 35.8 | 21.2 |
| 36.0 | 20.0 |
| 36.1 | 20.0 |
| 36.7 | 20.0 |
| 57.6 | 18.5 |
| 36.9a | 24.0 |
| 39.6b | 20.0 |
| 38.0c | 18.0 |

Notes:

a : irradiation solution was oxygenated during the radiolysis.

b : 5 mole % of GSSG was added to radiolysis solution.

c : 10 mole % of GSSG was added to radiolysis solution.

Another explanation for the break on Figure 2 could be depletion of oxygen since the break in all cases occurs after about 10 minutes of irradiation. However, when we increased the ratio $\frac{[O_2]}{[GSH]}$ by decreasing the GSH concentration we found that the yields of -SH decreased (Table III). The decrease in G(-SH) at lower glutathione concentrations may be caused by a further reaction between GSOO· radicals and oxygen, forming products with sulfur in a higher oxidation state. When we bubbled oxygen through the radiolysis solutions in order to keep the solution saturated during the radiolysis we found the yields of G(-SH) to be increased. The break in Figure 2 occurred in the usual place. We then concluded that oxygen depletion could not be the reason for the break on Figure 2.

In the more dilute solutions of glutathione the G values are more scattered but definitely lower. We did not observe a break on the plot of G value versus dose for the more dilute solution, but perhaps in these conditions the break is already past when the first measurement is made. Figure 3 is a plot of [GSH] versus dose for a $4 \times 10^{-4}$ M solution of GSH.

Figure 4 is a plot of percent $\Delta$[-SH] converted to GSSG versus dose. As it is apparent from the plot the total -SH consumed in the radiolysis process up to (about 30% of initial GSH) almost all is converted to GSSG, i.e., it is the major product of radiolysis. The concentration of -SH after radiolysis is somewhat greater than the concentration of GSH because there is a small amount of hydrogen sulfide produced in the radiolysis process. However, the amount of $H_2S$ produced in the presence of oxygen should be quite small. Although no quantitative measurements were made on the production of $H_2S$, from qualitative evidence and re-

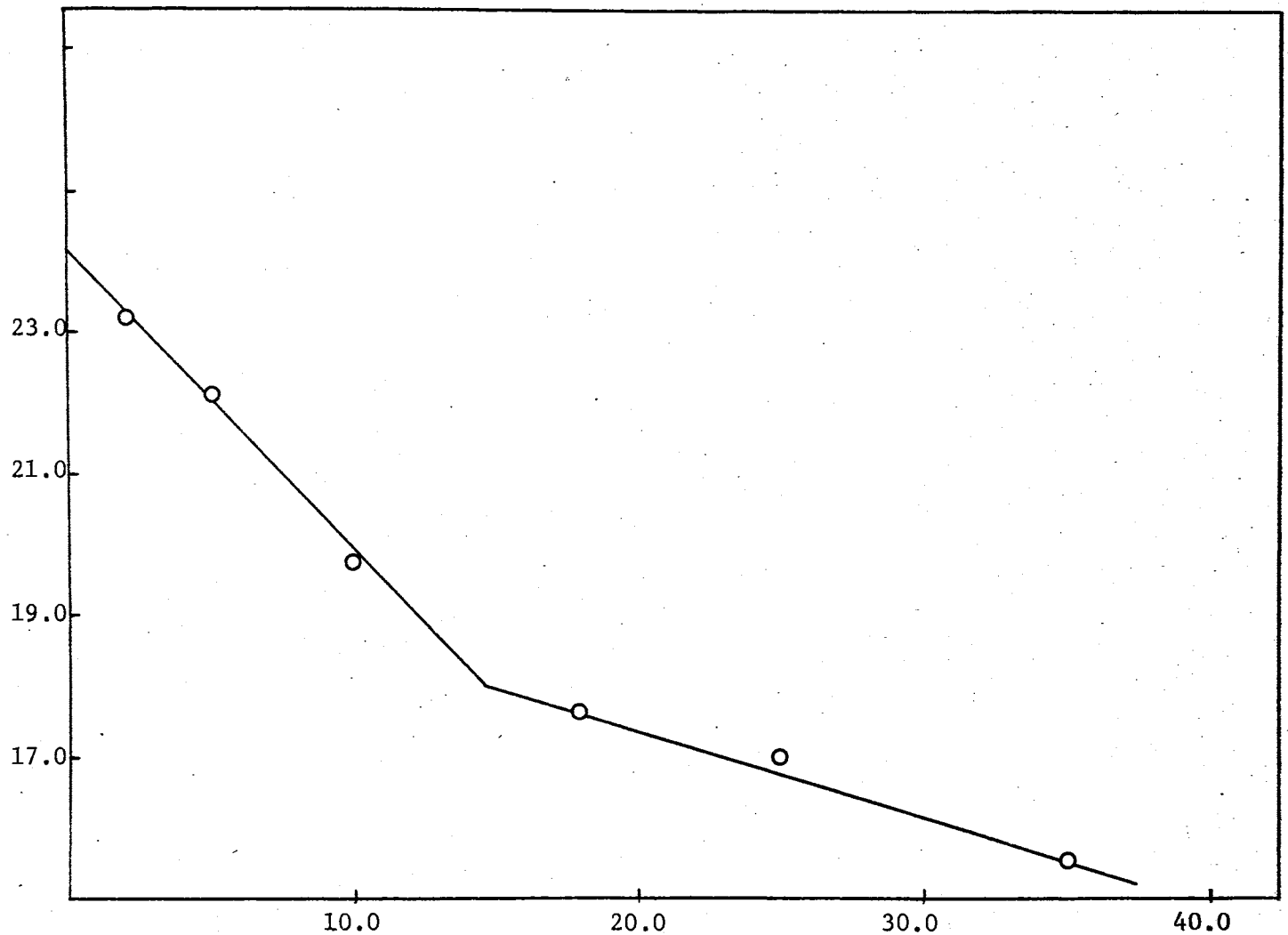



Figure 2. Radiolysis of GSH. A plot of G value versus dose [GSH] = $3 \times 10^{-3}$

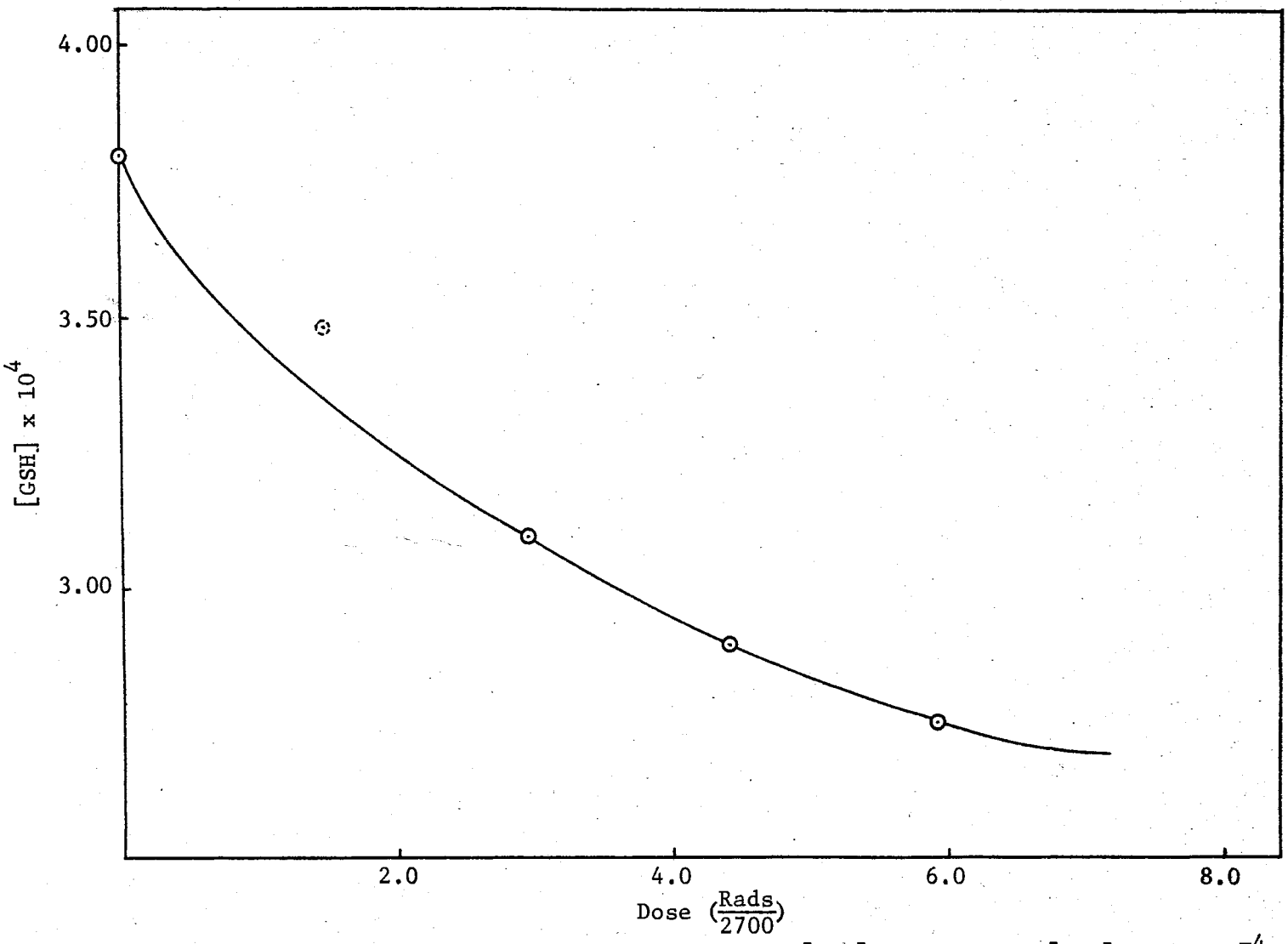



Figure 3. Radiolysis of GSH Solution. A plot of [GSH] versus dose [GSH] = 4 x $10^{-4}$

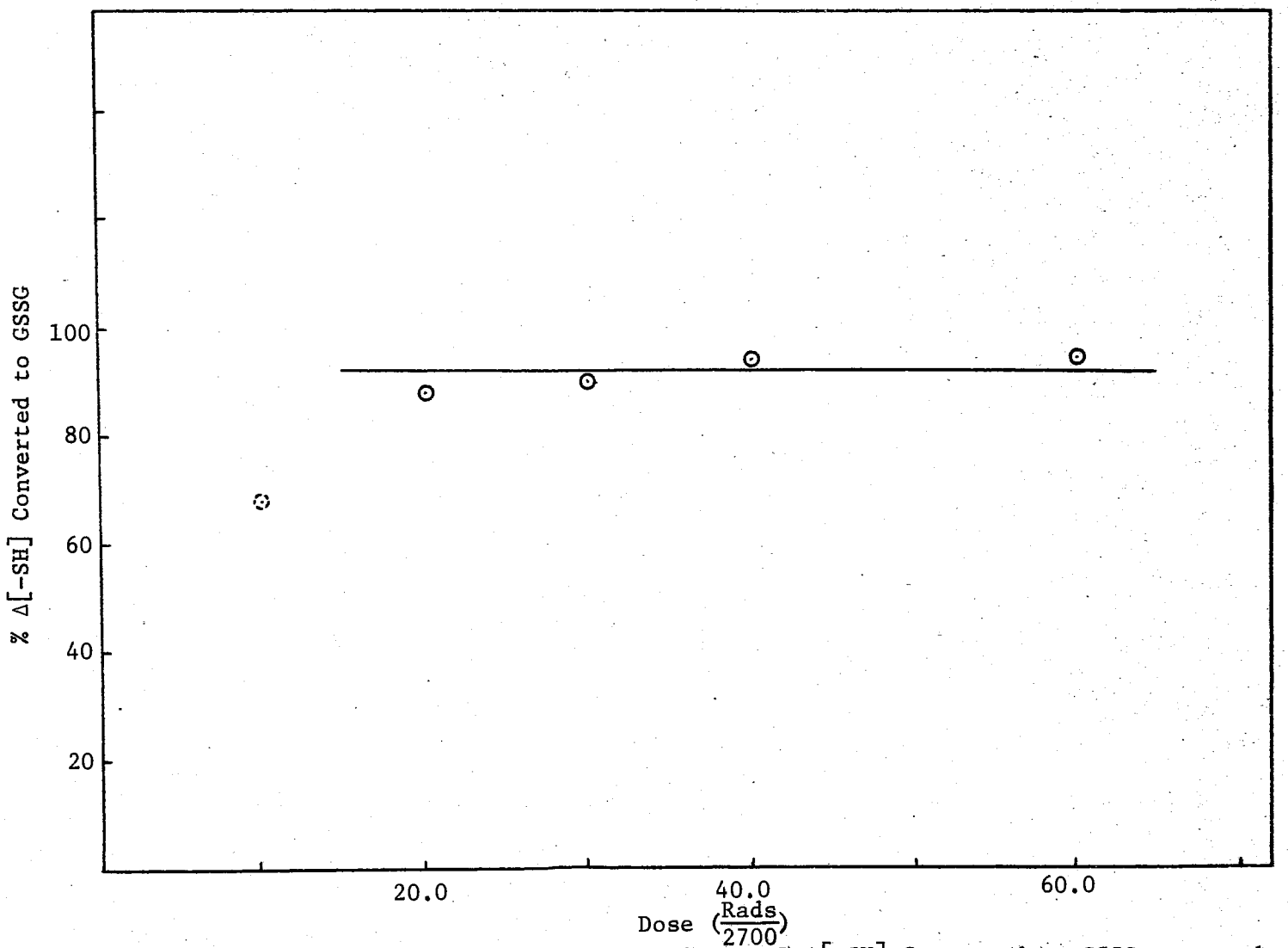



Figure 4. Radiolytic Conversion of GSH to GSSG. A plot of % Δ[-SH] Converted to GSSG versus dose. [GSH] = $3 \times 10^{-3}$

sults obtained for cysteine previously by Packer[19] there are reasons to believe that this is a valid assumption. This point will be cleared as we discuss the mechanism of the radiolysis.

In the absence of oxygen G(-SH) has been reported to be around 8 molecules/100 eV for pH values around 5 or less.[8] The following reactions account for the reported results.

$$GSH + {}^{\cdot}OH \longrightarrow GS^{\cdot} + H_2O \qquad (1)$$

$$GSH + e_{aq} \longrightarrow G^{\cdot} + SH^{-} \qquad (2)$$

$$GSH + H^{\cdot} \longrightarrow G^{\cdot} + H_2S \qquad (3a)$$

$$GSH + H^{\cdot} \longrightarrow GS^{\cdot} + H_2 \qquad (3b)$$

$$G^{\cdot} + GSH \longrightarrow GH + GS^{\cdot} \qquad (4)$$

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O \qquad (5)$$

$$GS^{\cdot} + GS^{\cdot} \longrightarrow GSSG \qquad (6)$$

At this pH the degree of dissociation of the -SH group is practically nil. At pH 7.0 or more, -SH group will dissociate more because the pK value is approached:

$$GSH \rightarrow GS^{-} + H^{+} \qquad (7)$$

The presence of oxygen causes a marked increase in G(-SH) and consequently in G(GSSG). If oxygen is present, there will be competition between oxygen, $GS^{-}$, and thiyl radicals themselves for GS· radicals.

$$GS^{\cdot} + O_2 \rightarrow GSOO^{\cdot} \qquad (8)$$

$$GS^{\cdot} + GS^{-} \leftrightarrows GSSG^{-} \qquad (9)$$

$$GS^{\cdot} + GS^{\cdot} \rightarrow GSSG \qquad (6)$$

$$O_2 + e_{aq} \rightarrow O_2^- \qquad (10)$$

K (the rate constant) for reaction 10 is reported to be $1.88 \times 10^{10}$ 1/mole sec.[13] Braams[14] has shown that at neutral pH's, the rate constant for the reaction between glutathione and solvated electrons is $3.2 \times 10^9$ 1/mole sec. This is an order of magnitude larger than the corresponding rate constants for other small peptides which do not contain the sulfhydryl group. The rate constant for reaction of oxygen with solvated electron (1) is almost six times greater than the rate constant for reaction of glutathione with solvated electron. It is evident that reactions 2 and 10 are competing reactions with reaction 10 the predominating reaction on equimolar bases.

$$GSH + \overline{O_2} \rightarrow GS^{\cdot} + H^{\cdot}O_2 \qquad (11)$$

$$GS^{\cdot} + O_2 \rightarrow GSOO^{\cdot} \qquad (8)$$

$$GSOO^{\cdot} + GSH \rightarrow GSOOH + GS^{\cdot} \qquad (12)$$

$$GSOOH + 3GSH \rightarrow 2GSSG + 2H_2O \qquad (13)$$

A chain reaction like the above should be responsible for the much higher value of G(-GSH) in the presence of oxygen. A possible chain-stopping reaction would be the reaction between GSOO$^{\cdot}$ radicals and oxygen, forming products with sulfur in higher oxidation state. Other possible chain-stopping reactions are reaction 6, and reaction between thiyl radicals and GSSG to produce the trisulfide GSSSG.

Since oxygen is a very efficient scavenger for hydrogen atoms it can combine with hydrogen atoms to produce the perhydroxyl radicals $HO_2^{\cdot}$.

$$H^{\cdot} + O_2 \rightarrow HO_2^{\cdot} \qquad (14)$$

Reactions 3, 14 are competing reactions so we expect, in the presence of oxygen, to get a lower yield for hydrogen sulfide, and a higher yield for oxidized glutathione.

$$GSH + HO_2^{\cdot} \rightarrow GS^{\cdot} + H_2O_2 \qquad (15)$$

In the case of $OH^{\cdot}$ radicals, available information (15-17) implies that the sulfhydryl group is much more reactive than other parts of glutathione. The reaction of $OH^{\cdot}$ radicals should be the same as that in anaerobic conditions so the $OH^{\cdot}$ radicals contribution to the yield of G(-GSH) in the presence of oxygen is the same as anaerobic conditions.

The oxydized glutathione electron adduct $GSSG^{-}$ in anaerobic conditions disappears by reactions -9 and 6. A detailed study of this transient for cysteine has been reported by Adams[15], and it seems that the glutathione system should behave in a similar manner. In the presence of oxygen, the oxydized glutathione adduct $GSSG^{-}$ could react with oxygen to produce oxidized glutathione and $O_2^{-}$ ; the chain could be propagated through reaction 11.

$$GSSG^{-} + O_2 \rightleftarrows GSSG + O_2^{-} \qquad (16)$$

A slow reaction between reduced glutathione and hydrogen peroxide (reaction 5) occurs at this pH, but by determining the yields soon after irradiation, its effects were minimized. Small corrections will slightly increase the value of G(-GSH) from its true value. However, the contribution of $H_2S$ to the total -SH contents will be more than enough to compensate for this correction and will slightly decrease the yield for

disappearance of reduced glutathione. Consequently, the G(-GSH) should be at least 21 molecules per 100 electron volts, and not much greater

## BIBLIOGRAPHY


(1) Barron, E. S. G., Advances in Enzymol. 11, 201 (1951).

(2) Barron, E. S. G., Ann. N.Y., Acad. Sci. 59, 547 (1955).

(3) Pihl, A. and L. Eldjarn, Pharmacol. Revs. 10, 437 (1958).

(4) Patt, H. M., Federation Proc. 19, 549 (1960).

(5) Gordy, W. and I. Miyagwa, Radiation Res. 12, 211 (1960).

(6) Ellman, G. L., Arch. Biochem. Biophys. 82, 70 (1959).

(7) Barron, E. S. G. and V. J. Flood, Gen. Physol. 33, 229 (1950).

(8) Lal, M., D. A. Armstrong, and M. Wieser, Radiation Res. 37, 246 (1969).

(9) Hammett, F. S., Protoplasma 7, 297 (1939).

(10) Woodward, G. E., Biochem. J. 27, 1411 (1933).

(11) Kinsey, V. E., Biol. Chem. 110, 551 (1935).

(12) Dale, W. M. and J. W. Davis, Biochem. J. 48, 129 (1951).

(13) Dorfman, L. F. and M. S. Matheson, Prog. React. Kinet. 3, 65 (1965).

(14) Braams, R., In Pulse Radiolysis (Ebert, M., J. P. Keene, A. J. Swallow, and J. H. Baxendale, eds.), Academic Press, New York (1965), p. 171.

(15) Adams, G. E., J. W. Boag, J. Currant, and B. D. Michael, In Pulse Radiolysis (Ebert, M., J. P. Keene, A. J. Swallow, and J. H. Baxendale, eds.), Academic Press, New York (1965), p. 131.

(16) Scholes, G., P. Shaw, R. L. Willson, and M. Ebert, In Pulse Radiolysis (Ebert, M., J. P. Keene, A. J. Swallow, and J. H. Baxendale, eds.), Academic Press, New York (1965), p. 151.

(17) Scholes, G. and R. L. Willson, Trans. Faraday Soc. 63, 2983 (1967).

(18) Markakis and Tappel, J. Amer. Chem. Soc. 82, 1613 (1960).

(19) Packer, S. E. and R. V. Winchester, Can. J. Chem. 48, 417 (1970).

(20) Adams, G. E., Ann. Rep. Progr. Chem. 66, 207 (1970).

(21) Sanner, T. and A. Phil, In Radiation Protection and Sensitization, (Morosen, H. L. and M. Quantiliani, eds.), Taylor and Francis, Ltd., London (1970), p. 43.

(22) Icen, A., Scandinavian J. of Clin. and Lab. Invest. Supplementum 96 (1967).

(23) Bacq, Z. M. and P. Alexander, Fundamental of Radiolysis, 2nd ed., Pergamon Press, London (1961), p. 457.

(24) Eldjarn, L. and A. Phill, 25th Anniversary publication, Norwegian Radium Hospital (1958), p. 253.

(25) Eldjarn, L. and A. Phil, In Errera and Forssberg's Mechanisms in Radiobiology, vol. II, Academic Press, New York (1960).

(26) Purdie, J. W., J. Am. Chem. Soc. 89, 226 (1967).

(27) Packer, J. E., J. Chem. Soc. 2320 (1963).

(28) Jayson, G. G., D. A. Stirling, and A. J. Swallow, Int. J. Radiat. Biol. 19, 143 (1971).

# CHAPTER V

## ADDITIONAL DETAILS AND CHAPTERS III AND IV

### General Expressions for K and E in Redox Reactions

Consider the following reaction:

$$\text{Oxidant 1} + \text{Reductant 2} \rightarrow \text{Oxidant 2} + \text{Reductant 1}$$

The equilibrium constant for reaction is

$$K = \frac{(\text{Oxidant 2})(\text{Reductant 1})}{(\text{Oxidant 1})(\text{Reductant 2})}$$

where quantities in parentheses represent the activities of the respective reagents. The corresponding expression in terms of reagent concentrations is

$$K' = \frac{[\text{Oxidant 2}][\text{Reductant 1}]}{[\text{Oxidant 1}][\text{Reductant 2}]}$$

K' measures what may be called the concentration equilibrium constant. K and K' are related by the following equation:

$$K' = \left(\frac{f_{01} \times f_{R2}}{f_{02} \times f_{R1}}\right) K$$

where f represents the activity coefficients of the respective reagents.

No attempt has been made to estimate activity coefficient, consequently in our experiments we have determined a concentration equilibrium constant.

The potential for the redox couples are

$$E_1 = E_1^o + \frac{RT}{nF} \ln \frac{[\text{Oxidant 1}]}{[\text{Reductant 1}]}$$

$$E_2 = E_2^o + \frac{RT}{nF} \ln \frac{[\text{Oxidant 2}]}{[\text{Reductant 2}]}$$

At equilibrium $E_1 = E_2$

$$E_1^o = E_2^o - \frac{RT}{nF} \ln K'$$

If n = 2 at 25°C,

$$E_1^o = E_2^o - 0.02958 \log K'$$

Consequently, if $E_2^o$ and K' are known, $E_1^o$ can be calculated.

## CALCULATIONS FOR THE GLUTATHIONE SYSTEM

For the reaction

$$2GSH + NADP^+ \rightarrow NADPH + GSSG + H^+ \qquad (1)$$

the concentration equilibrium expression is

$$K' = \frac{[NADPH][GSSG](H^+)}{[GSH]^2\,[NADP^+]}$$

The reaction takes place in a buffer solution. Consequently, $(H^+)$ is a constant and one may write

$$K_h^f = \frac{K'}{(H^+)} = \frac{[NADPH][GSSG]}{[GSH]^2\,[NADP^+]}$$

The potential for the glutathione system at pH 7., $E_{h7}$ is equal to

$$E_{h7_{GSH}} = E_{h7_{NADP^+/NADPH}} - 0.02958 \log K_7^f$$

SGH samples usually contain GSSG as an impurity. If this is not measured or corrected, some error will be made in estimating the mass of GSH taken initially. In this work the correction is made in the following way. Let f equal the fraction of GSH that has been converted to GSSG. Because two moles of GSH is required to make one mole of GSSG, we can write:

$$[GSH] = (1 - 2f)[GSH]_n$$

Where [GSH] is concentration of GSH at any given time and $[GSH]_n$ is the nominal concentration of GSH calculated from the weight taken, we can write the equilibrium expression in the following form

$$K_h^f = \frac{[NADPH]\ fx\ [GSH]}{\{(1-2f)[GSH]\}^2\,[NADP^+]}$$

or

$$K_h^f = \frac{fx\ [NADPH]}{(1-2f)^2\ [GSH][NADP^+]}$$

We measure [NADPH] and [GSSG] at equilibrium, thus calculates f and $K_h^f$.

The nominal concentrations of GSH and $NADP^+$ are obtained from weight of the samples used in preparation of respective solutions. In calculating the concentration of $NADP^+$ solutions an effective molecular weight specified by the manufacturer has been used which includes the weight of water.

Illustrative Experiment

This experiment consist of three parts (a), (b), (c):

(a) Principal Experiment,

(b) Assay Experiment,

(c) Reverse Experiment.

In the principal experiment the amount of NADPH formed upon addition of glutathione reductase to the reaction mixture is measured. In the assay experiment the equilibrium concentration of GSSG is measured by adding an aliquot portion of the reaction mixture from Part (a) to an excess of NADPH. From these two measurements and initial concentration of the other reagents the equilibrium concentration constant for the reaction of GSH with $NADP^+$ is calculated. In Part (c) the equilibrium is shifted back by adding a measured quantity of GSSG to reaction mixture from Part (a).

Part (a), Principal Experiment

$[GSH]_n$ = Nominal concentration of GSH solution

$= 69.35 \times 10^{-3}$ M

$[NADP^+]_n$ = Nominal concentration of $NADP^+$ solution

$= 4.50 \times 10^{-3}$ M

$[GSH]_d$ = Diluted concentration of GSH after addition of the enzyme

$$= [GSH]_n \left(\frac{V_i}{V_i + V_e}\right)$$

$$= 69.35 \times 10^{-3} \left(\frac{3.50}{3.50 + 0.150}\right)$$

$= 66.50 \times 10^{-3}$ M

where

$V_i$ = Initial volume of reaction mixture

$V_e$ = Volume of enzyme solution added to reaction mixture

$[NADP^+]_d$ = Diluted concentration of $NADP^+$ after addition of enzyme

$$= [NADP^+]_n \times \left(\frac{V_i}{V_i + V_e}\right)$$

$$= 4.50 \times 10^{-3} \left(\frac{3.50}{3.50 + 0.150}\right)$$

$= 4.32 \times 10^{-3}$ M

$A_i$ = Initial absorbance of reaction mixture at 340 nm due to $NADP^+$

$$A_d = 0.302 \times \left(\frac{3.50}{3.50 + 0.150}\right)$$

$$= 0.290 \text{ diluted absorbance}$$

$$A_{eq} = \text{equilibrium absorbance}$$

$$\Delta A = A_{eq} - A_d$$

$$= 0.938 - 0.290$$

$$= 0.648$$

$$\Delta[\text{NADPH}] = \frac{\Delta A}{\varepsilon} = \frac{0.648}{6.2 \times 10^3} = 1.05 \times 10^{-4} \text{ M}$$

$$[\text{NADP}^+]_{eq} = [\text{NADP}^+]_d - \Delta[\text{NADPH}]$$

$$[\text{NADP}^+]_{eq} = 4.32 \times 10^{-3} - 1.05 \times 10^{-4} = 4.215 \times 10^{-3} \text{ M}$$

$$[\text{NADPH}]_{eq} = 1.05 \times 10^{-4} \text{ M}$$

Part (b), Assay Experiment

We denote the symbols by superscript a.

$A_i^a$ = Initial absorbance at 340 nm mostly due to NADPH.

$= 1.112$

$A_d^a$ = Absorbance diluted by addition of enzyme and aliquot from equilibrium reaction mixture

$$= A_i^a \times \left(\frac{V_i^a}{V_i^a + V_e^a + V_{alq}^a}\right)$$

where

$V_i^a$ = Initial volume of NADPH solution

= 3.00 ml

$V_e^a$ = Volume of the enzyme solution added to reaction mixture

= 0.150 ml

$V_{alq}^a$ = Volume of aliquot added to the reaction mixture

$$V_d^a = 1.112 \times \left(\frac{3.00}{3.00 + 0.150 + 0.500}\right)$$

= 0.914

$A_{eq}^a$ = Equilibrium absorbance at 340 nm in the assay experiment

= 0.330

$$A_p^a = A_{eq} \left(\frac{V_{alq}^a}{V_{alq}^a + V_e^a + V_i^a}\right)$$

where $A_p^a$ is absorbance due to the aliquot from Part (a) which is used in the assay experiment.

$A_t^a$ = Total absorbance of the initial mixture in the assay experiment

$= A_d^a + A_p^a$

$$A_p^a = 0.900 \times \left(\frac{0.500}{0.500 + 0.150 + 3.00}\right)$$

= 0.123

$$A_t^a = 0.914 + 0.123 = 1.037$$

$\Delta A^a$ = Change in absorbance in the assay experiment

$$= A_t^a - A_{eq}^a$$

$$= 1.037 + 0.330 = 0.707$$

$$\Delta[NADPH]^a = \frac{\Delta A^a}{\varepsilon}$$

$$= \frac{0.707}{6.2 \times 10^3} = 1.14 \times 10^{-4} \text{ M}$$

$$[GSH]_{eq}^a = [GSH]_d \times \left(\frac{V_{alq}^a}{V_{alq}^a + V_i^a + V_e^a}\right)$$

= Concentration of GSH in the assay experiment

$$= 66.50 \times 10^{-3} \left(\frac{0.500}{3.00 + 0.500 + 0.150}\right)$$

$$= 9.11 \times 10^{-3} \text{ M}$$

Because in the assay experiment the reaction is quantitative and practically all the GSSG is converted to GSH (see the Discussion in Chapter III), the change in concentration of NADPH is equal to the concentration of GSSG to a very good approximation. This is true because from the stoichiometry of the reaction one mole of NADPH is necessary to reduce one mole of GSSG. Consequently,

$$f = \frac{[NADPH]^a}{[GSH]_{eq}^a}$$

$$= \frac{1.14 \times 10^{-4}}{9.11 \times 10^{-3}} = 0.0125$$

$$(1 - 2f)^2 = 0.9506$$

If values obtained for f and all reagent concentrations are substituted in $K_h^f$ expression the value for the constant can be calculated

$$K_h^f = \frac{f \times [NADPH]}{(1 - 2f)^2 [GSH][NADP^+]}$$

$$K_h^f = \frac{0.0125 \times (1.05 \times 10^{-4})}{(0.9506)(66.50 \times 10^{-3})(4.21 \times 10^{-3})}$$

$$K_h^f = 4.9 \times 10^{-3}$$

If this value is substituted in the expression for the redox potential of glutathione we have

$$E_{h7_{GSH}} = E_{h7_{NADP^+/NADPH}} - 0.02958 \log K_h^f$$

$$= -0.32 - 0.02958 \log 4.9 \times 10^{-3}$$

$$= -0.25 \text{ V}$$

Part (c), Reverse Experiment

To calculate $K_h^r$, the equilibrium constant obtained by approaching the equilibrium point from the opposite direction the following experiment is performed. This experiment is denoted by superscript r

$[GSH]_i^r$ = Concentration of GSH initially taken in the reverse experiment

$$= [GSH]_d \times \left(\frac{V_i^r}{V_i^r + V_{GSSG}^r}\right)$$

where

$$V_i^r = V_i + V_e - V_{alq}$$

$$= 3.50 + 0.150 - 0.500$$

$$= 3.15 \text{ ml}$$

$V_{GSSG}^r$ = Volume of GSSG solution added to equilibrium mixture to drive the equilibrium back

$$= 0.200 \text{ ml}$$

$$[GSH]_i^r = 66.50 \times 10^{-3} \times \left(\frac{3.15}{3.15 + 0.200}\right) = 62.53 \times 10^{-3}$$

$[NADP^+]_i^r$ = Concentration of $NADP^+$ in the reverse reaction

$$= [NADP^+]_d \times \left(\frac{V_i^r}{V_i^r + V_{GSSG}^r}\right)$$

$$= 4.21 \times 10^{-3} \times \left(\frac{3.15}{3.15 + 0.200}\right) = 3.97 \times 10^{-3}$$

$A_i^r$ = Initial absorbance at 340 nm in the reverse experiment before addition of GSSG solution

$$= A_{eq} \times \left(\frac{V_i^r}{V_i^r + V_{GSSG}^r}\right)$$

$$= 0.851 \times \left(\frac{3.15}{3.15 + 0.200}\right)$$

$$= 0.800$$

$A_f^r$ = Final absorbance in the reverse experiment

= 0.727

$\Delta A^r = A_i^r - A_f^r$

= 0.800 - 0.727

= 0.073

$\Delta[NADPH]^r$ = Change in concentration of NADPH due to addition of GSSG solution

$= \frac{\Delta A^r}{\varepsilon}$

$= \frac{0.073}{6.2 \times 10^3}$

$= 1.18 \times 10^{-5}$

$[GSSG]_i^r$ = Concentration of GSSG solution added to reaction mixture in the reverse experiment

$= 1.66 \times 10^{-3}$ M

$[GSSG]_d^r$ = Diluted concentration of GSSG after addition to the reaction mixture

$= [GSSG]_i^r \times \left(\frac{V_{GSSG}^r}{V_{GSSG}^r + V_i^r}\right)$

$= 1.66 \times 10^{-3} \times \left(\frac{0.200}{0.200 + 3.15}\right)$

$= 9.91 \times 10^{-5}$ M

$[GSSG]^r$ = GSSG initially present in the reaction mixture

= $f \times [GSH]^r$

= $0.0125 \times 62.53 \times 10^{-3}$

= $7.81 \times 10^{-4}$

$[GSSG]^r_t$ = Total GSSG present in the reverse experiment at equilibrium

= $[GSSG]^r + [GSSG]^r_d - \Delta[NADPH]^r$

= $7.81 \times 10^{-4} + 9.91 \times 10^{-5} - 1.18 \times 10^{-5}$

= $8.7 \times 10^{-4}$ M

$A_d$ = Initial absorbance of reaction mixture in part a diluted

$A^r_d$ = Initial absorbance diluted further by addition of GSSG solution

$$= A_d \left(\frac{V^r_i}{V^r_i + V^r_{GSSG}}\right)$$

$$= 0.290 \left(\frac{3.15}{3.15 + 0.200}\right)$$

= 0.273

$A^r_{eq} = A^r_f - A^r_d$

= 0.727 - 0.273

= 0.454

$[NADPH]^r_{eq}$ = Equilibrium concentration of NADPH in the reverse experiment

$$= \frac{A^r_{eq}}{\varepsilon}$$

$$= \frac{0.454}{6.2 \times 10^3}$$

$$= 7.32 \times 10^{-5} \text{ M}$$

$[NADP^+]^r_{eq}$ = Equilibrium concentration of $NADP^+$ in the reverse experiment

$$= [NADP^+]^r_i + \Delta[NADPH]$$

$$= 3.97 \times 10^{-3} + 1.18 \times 10^{-5} = 3.98 \times 10^{-3} \text{ M}$$

$$[GSH]^r_{eq} = [GSH]_r - 2[GSSG]^r + 2\Delta[NADPH]$$

$$= 62.53 \times 10^{-3} - 2(7.81 \times 10^{-4}) + 2(1.18 \times 10^{-5})$$

$$= 60.99 \times 10^{-3} \text{ M}$$

If all equilibrium concentrations for the reverse reaction are substituted in Equation 2, another value for the equilibrium constant is calculated. We call this constant $K^r_h$ at any given pH, and $K^r_7$ at pH 7.0.

$$K^r_h = \frac{[NADPH][GSSG]}{[GSH]^2\,[NADP^+]}$$

$$K^r_7 = \frac{(7.32 \times 10^{-5})(8.7 \times 10^{-4})}{(60.99 \times 10^{-3})^2\,(3.98 \times 10^{-3})}$$

$$K^r_7 = 4.3$$

PRELIMINARY EXPERIMENTS

When we began investigating the oxidation reduction potential of glutathione, 0.05 M buffer solutions was used. We overlooked the point that glutathione is an acid and that this will change the pH of reaction medium. The hydrogen ion concentration must of course be controlled because the reaction is pH dependent.

$$2GSH + NADP^+ \rightarrow GSSG + NADPH + H^+ \qquad (1)$$

Since pH is a logarithmic function, a small change in pH will make a large difference in value of $K_h^f$.

Since high pH favors the reaction as written, we used a buffer of pH = 8.7. The formation of NADPH at 340 nm was observed and an equilibrium constant was calculated. However, when we measured the pH of reaction mixture at equilibrium the hydrogen ion concentration had been changed by significant amounts.

About 50 experiments were performed this way, and some representative experiments are reported in the following table (Table IV). As can be seen the results are lower than the one reported in Chapter III. This is to be expected since the actual pH would have been lower than nominal value. The median GSH concentration was $16.44 \times 10^{-3}$ M it was determined that this amount of GSH would in this condition lower the pH to 7.7. These experiments were not used in calculating the oxidation reduction potential of the glutathione system, however, if the correction is applied, they add confidence that the value measured in this work is correct. We have used different samples of enzyme, GSH and NADPH we have obtained consistent results.

TABLE IV

PRELIMINARY EXPERIMENTS ON GSH AND $NADP^+$ REACTION

| Exp. No. | Parts $NADP^+$ | Parts GSH | Volume of Enz. Added µl | Concentration mm $NADP^+$ | Concentration mm GSH | Volume of GSSG Added µl | [GSSG] mm | pH | ΔA | $\Delta A^a$ | $\Delta A^r$ | $K_h^f \times 10^{-3}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 24-A | 5 | 4 | 80 | .120 | 9.31 | 250 | 0.760 | | ----- | **0.406** | ----- | ----- |
| 24-P | 5 | 5 | 80 | 0.511 | 10.47 | 250 | 0.760 | | 0.094 | ----- | 0.050 | 30 |
| 26-A | 5 | 1 | 80 | 0.179 | 6.98 | 250 | 0.760 | | ----- | **0.219** | ----- | ----- |
| 26-P | 5 | 3 | 80 | 0.804 | 15.71 | 250 | 0.760 | | 0.184 | ----- | 0.080 | 25 |
| 28-A | 5 | 1 | 150 | 0.176 | 7.13 | 200 | 0.650 | | ----- | **0.251** | ----- | ----- |
| 28-P | 8 | 5 | 150 | 0.932 | 16.44 | 200 | 0.650 | | 0.231 | ----- | 0.120 | 25 |
| 38-A | 5 | 1 | 80 | 0.170 | 7.38 | --- | --- | pH = 8.7 "Nominal" | ----- | **0.225** | ----- | ----- |
| 38-$P_1$ | 8 | 5 | 80 | 1.008 | 17.04 | 50 | 0.168 | | 0.100 | ----- | ----- | 25 |
| 44-$A_1$ | 3 | 0.5 | 150 | 0.113 | 3.81 | --- | --- | | ----- | **0.296** | ----- | ----- |
| 44-P | 1.5 | 1.5 | 150 | 0.905 | 13.24 | 50 | 0.163 | | 0.150 | ----- | ----- | 28 |
| 45-$A_1$ | 3.0 | 0.2 | 150 | 0.1425 | 3.51 | 50 | 0.163 | | ----- | **0.850** | ----- | ----- |
| 45-P | 1.75 | 1.75 | 150 | 0.958 | 28.10 | 50 | 0.163 | | 0.400 | ----- | 0.050 | 20 |
| 47-$A_1$ | 3.0 | 0.2 | 150 | 0.1425 | 3.50 | 50 | 0.163 | | ----- | **0.099** | ----- | ----- |
| 47-P | 1.75 | 1.75 | 150 | 0.985 | 29.07 | 50 | 0.307 | | 0.52 | ----- | 0.110 | 20 |

DETAILS ON TABLE V

A new set of experiments were performed and the data corresponding to these experiments are tabulated in Table V. We have included in Table V the following information. Column 1 is the experiment number. Columns 2 and 3 contain concentrations of GSH and $NADP^+$ respectively. Column 4 is molar percent of GSSG in GSH. Columns 5, 6, and 7 report change in absorbances in assay experiment, principal experiment and the reverse experiment. Column 8 is the pH of the reaction, and columns 9, and 10 report the equilibrium constant for principal and reverse experiment respectively.

GSSG ASSAY

The GSSG concentration was determined by adding 400 µl of GSSG solution to 3.00 ml of NADPH solution followed by addition of 50 µl of a solution of glutathione reductase. The following table contains some of the experiments performed to determine purity of GSSG (Table VI).

SAMPLE CALCULATION

$[GSSG]_s$ = Concentration of stock GSSG solution

$= 7.07 \times 10^{-4}$

$[GSSG]_i$ = Initial concentration of GSSG after dilution

$$= \left(\frac{V_{GSSG}}{V_{GSSG} + V_e + V_{NADPH}}\right) [GSSG]_s$$

$$= \left(\frac{0.400}{0.400 + 0.050 + 3.00}\right)(7.07 \times 10^{-4})$$

TABLE V

DETAILS ON EQUILIBRIUM CONSTANT OF GSH WITH $NADP^+$

| Exp. No. | [GSH] x $10^3$ | [NADP] x $10^3$ | f | $\Delta A^a$ | $\Delta A$ | $\Delta A^r$ | pH | $K_7^f \times 10^3$ | $10^3 \times K_7^r$ |
|---|---|---|---|---|---|---|---|---|---|
| 58 | 126.0 | 3.94 | 1.33% | 0.733 | 1.053 | 0.079 | 7.0 | **5.4** | 5.0 |
| 59 | 69.35 | 4.5 | 1.25% | 0.707 | 0.648 | 0.073 | 7.0 | **4.9** | 4.3 |
| 66 | 75.96 | 4.76 | 1.42% | 0.289 | 0.890 | 0.220 | 7.0 | **4.9** | 5.2 |
| 67 | 82.16 | 4.56 | 1.6 % | 1.700 | 0.803 | 0.209 | 7.0 | **6.4** | 5.9 |
| 62 | 15.36 | 1.40 | 1.6 % | 0.089 | 0.246 | 0.056 | 7.82 | **5.9** | 4.5 |
| 65 | 20.44 | 1.43 | 1.60% | 0.270 | 0.447 | 0.072 | 8.00 | **4.9** | (2.6) |
| 68 | 9.57 | 2.09 | 11.2 % | 0.468 | 0.103 | 0.030 | 8.47 | **4.7** | 4.3 |

TABLE VI

ASSAY OF GSSG SAMPLES

| Experiment | GSSG x $10^5$ | Volume of GSSG µl | Volume of the enzyme µl | Total volume of solution ml | ΔA | % GSSG |
|---|---|---|---|---|---|---|
| 1A | 8.20 | 400 | 50 | 3.45 | 0.432 | 87 |
| 1B | 8.08 | 400 | 100 | 3.50 | 0.425 | 87 |
| 2A | 6.92 | 400 | 50 | 3.45 | 0.372 | 89 |
| 3A | 7.13 | 400 | 50 | 3.45 | 0.363 | 84 |
| 3B | 8.66 | 500 | 50 | 3.55 | 0.459 | 85 |

$$= 8.20 \times 10^{-5}$$

where

$V_{GSSG}$ = Volume of stock GSSG solution

$V_e$ = Volume of the enzyme solution

$V_{NADPH}$ = Volume of NADPH solution

$A_i$ = Initial absorbance of NADPH solution

$A_d$ = Diluted absorbance of reaction mixture

$$= \left(\frac{V_{NADPH}}{V_{NADPH} + V_e + V_{GSSG}}\right)(A_i)$$

$$= \left(\frac{3.00}{3.00 + 0.050 + 0.400}\right)(0.874)$$

$$= 0.759$$

$A_{eq}$ = Equilibrium absorbance of the reaction mixture

$$= 0.327$$

$\Delta A$ = Change in absorbance

$$= A_d - A_{eq}$$

$$= 0.759 - 0.327$$

$$= 0.432$$

$$[GSSG] = \Delta[NADPH] = \frac{\Delta A}{\varepsilon}$$

$$= \frac{0.432}{6.2 \times 10^{6}\ cm^{2}/mole \times cm} (1000\ cm^{3}/l)$$

$$= 6.97 \times 10^{-5}\ ml$$

$[GSSG]_l$ = GSSG left at equilibrium

= 2% of GSSG (at most)

$= 0.02\ (6.97 \times 10^{-5})$

$= 1.4 \times 10^{-6}$

$[GSSG]_t$ = Total GSSG

$= [GSSG]_i + [GSSG]_l$

$= 6.97 \times 10^{-5} + 1.40 \times 10^{-6}$

$= 7.11 \times 10^{-5}$

$$\%GSSG = \frac{[GSSG]_t}{[GSSG]_i} \times 100$$

$$\%GSSG = \left(\frac{7.11 \times 10^{-5}}{8.20 \times 10^{-5}}\right)(100)$$

$= 87\%$

The purity factor for GSSG was not used in the calculation for oxidation reduction potential of the glutathione system because we did not use the reverse equilibrium constant for our calculations, and even if we do apply the correction in the calculations it does not change the

value very much. Because the amount of GSSG added to the equilibrium mixture is only a fraction of GSSG at equilibrium.

DETERMINATION OF -SH TITER

The -SH concentration of GSH was determined by adding 250 µl of GSH stock solution to 15.00 ml of DTNB(5,5-dithiobis-(2-nitrobenzoic acid) reagent solution. Absorbance measurements were taken at 412 nm. The pH of the solution was 7.8. All the determinations were performed after about 30 minutes was past from the time the solutions were prepared. After about 2.5 to 3.0 hours, only about 90% of nominal GSH concentration was left. In the following table we have tabulated measurements corresponding to the -SH determinations.

TABLE VII

-SH CONTENTS OF GSH SAMPLES

| Experiment | [GSH] | %SH |
|---|---|---|
| 1 | $6.19 \times 10^{-3}$ | 94.5 |
| 2 | $9.40 \times 10^{-4}$ | 98.5 |
| 3 | $8.98 \times 10^{-4}$ | 95.4 |
| 4 | $4.62 \times 10^{-3}$ | 94.5 |
| 5 | $3.58 \times 10^{-3}$ | 94.5 |
| 5A* | $3.58 \times 10^{-3}$ | 97.1 |
| | Average = 95.5% | |

*Solution of GSH was deareated.

GSH used in these experiments were from 2 different samples; first 3

experiments from sample 1 and last 2 experiments from sample 2, however, the results are comparable. The average of 95.5% seems reasonable because samples of GSH contained between 1 to 1.5 mole % of GSSG. This is the same as 2 to 3% of GSH. Although we did not make any direct measurements on GSSG content of our GSH samples, but if one calculates indirectly from GSSG concentration in the equilibrium experiment one would come up with this conclusion. Since the -SH concentration of both unirradiated and irradiated GSH solutions were directly measured this would not effect the results of our calculations in any way.

SAMPLE CALCULATION

$[GSH]_n$ = Nominal concentration of GSH solution

$= 8.98 \times 10^{-4}$

$[GSH]_d$ = Diluted concentration of GSH solution

$$[GSH]_d = [GSH]_n \left(\frac{V_{GSH}}{V_{GSH} + V_{DTNB}}\right)$$

$$= 8.98 \times 10^{-4} \left(\frac{1.00}{1.00 + 15.00}\right)$$

$$= 5.61 \times 10^{-5}$$

where

$V_{GSH}$ = Volume of GSH solution used in analysis

$V_{DTNB}$ = Volume of DTNB solution used in the analysis

$A_{eq}$ = Absorbance at equilibrium at 412 nm

$\varepsilon$ = Extinction coefficients

= 13600 M/I

$$[GSH] = \frac{A_{eq}}{\varepsilon}$$

$$= \frac{0.727}{13600}$$

$$= 5.35 \times 10^{-5}$$

$$\%GSH = \frac{[GSH]}{[GSH]_d} \times 100$$

$$= \frac{5.35 \times 10^{-5}}{5.61 \times 10^{-5}} \times 100$$

= 95.4%

G-VALUE DETERMINATION

C(-SH) = Concentration of -SH

$C(-SH)(moles/l) = (A_i - A_j)/\varepsilon$ ($cm^2$/mole)b(cm) x ($\frac{1000\ cm^3}{1}$) (dilution factor) where $A_i$ and $A_j$ are the absorbances of the unirradiated (blank) and irradiated solutions respectively, $\varepsilon$ is the molar absorbancy in $cm^2$/mole and b the thickness of sample in cm.

P, number of molecules of SH disappeared is given by

$$P = C(-SH)(moles/l) \times (6.02 \times 10^{23})(molecules/mole)(V)(1)$$

$$P = (A_i - A_j)/\varepsilon b) \times (6.02 \times 10^{23})(V)(molecules)$$

G(-SH) = Number of -SH molecules disappeared/100 eV

From Equation (1) of Chapter II we have

$$G = 1.602 \times 10^{-12} \times 6.02 \times 10^{23} (A_i - A_j)/\varepsilon b)(V) \left(\frac{1}{\text{Dose} \times V \times d}\right)$$

Dose = Dose rate x time

Dose rate = 2700 rads/minute

The dilution factor must be included in the G value expression because we dilute the GSH solution during the course of analysis. In this particular experiment we added 250 µl of GSH solution to 15.00 ml of DTNB reagent. Consequently, the dilution factor is 61.

$\varepsilon = 1.36 \times 10^7 \text{ cm}^2 \text{ mole}^{-1}$

b = 1 cm

$$G(-SH) = \frac{26.2 (A_i - A_j) \text{ dilution factor}}{\text{time (min)}}$$

$$G(-SH) = \frac{26.2 (A_i - A_j) \times 61}{\text{time (min)}}$$

SAMPLE CALCULATION

$A_i$ = Absorbance of unirradiated GSH solution

= 0.736

$A_f$ = Absorbance of GSH solution irradiated for 5 minutes

$$= 0.686$$

250 µl of GSH solution is added to 15 ml of DTNB reagent.

$$\text{Dilution factor} = \frac{V_{GSH} + V_{DTNB}}{V_{GSH}}$$

$V_{GSH}$ = Volume of GSH solution used in analysis

$$= 0.250 \text{ ml}$$

$V_{DTNB}$ = Volume of DTNB solution used in analysis

$$= 15.0 \text{ ml}$$

$$\text{Dilution factor} = \frac{0.250 + 15.00}{0.250} = 61$$

$$G = \frac{26.2\ (0.736 - 0.686)}{5} \times 61$$

$$= 16.0 \text{ molecules/100 eV}$$

## BIBLIOGRAPHY


(1) Clark, W. M., "Oxidation-Reduction Potential of Organic Systems", The Williams and Wilkins Company, Baltimore, 1960, p. 471.

(2) Pirie, N. W. and K. G. Pinhey, J. Biol. Chem. 84, 321 (1929).

(3) Harington, C. R. and T. H. Mead, Biochem. J. 29, 1602 (1935).

(4) Pirie, N. W., Biochem. J. 25, 1565 (1931).

(5) Bricas, E. and C. Fromagoot, Advan. Protein Chem. 8, 1 (1953).

(6) Colowick, S., A. Lazarow, E. Racker, D. R. Schwarz, E. Stadtman, and H. Waelsch (eds.), Glutathione, A Symposium, Acad. Press Inc., New York, 1954.

(7) Crook, E. M. (ed.), Glutathione, Biochem. Soc. Symposium No. 17, Cambridge University Press, Cambridge 1959.

(8) Knox, W. E. Glutathione, (Boyer, P. D, Lardy, and K. Myrback, eds.), 2nd. ed., Vol. 2, Acad. Press, Inc., New York and London, 1960, pp. 253 - 294.

(9) Waley, S. G. Naturally Occurring Peptides, Adv. Protein Chem. 21, 1 (1966).

(10) Jocelyn, P. C., Biochem. J. 77, 363 (1960).

(11) Guntherberg, H. and J. Rost, Anal. Biochem. 15, 205 (1966).

(12) Allen, D. W. and J. H. Jandl, J. Clin. Invest. 40, 454 (1961).

(13) Jacob, H. S. and J. H. Jandl, J. Clin. Invest. 41, 779 (1962).

(14) Jacob, H. S. and J. H. Jandl, J. Clin. Invest. 41, 1514 (1962).

(15) Jandl, J. H., L. K. Engle, and D. W. Allen, J. Clin. Invest. 39, 1818 (1960).

(16) Mapson, L. W. and D. R. Goddard, Biochem. J. 49, 592-601 (1951).

(17) Conn, E. E. and B. Vennesland, J. Biol. Chem. 192, 17 (1951).

(18) Rall, T. W. and A. L. Lehninger, J. Biol. Chem. 194, 119 (1952).

(19) Mapson, L. W. and P. A. Isherwood, Biochem. J. 86, 173 (1963).

(20) Von Rysselberghe, P., Report of Commission 2, "Proceedings of the 6th Meeting of the International Committee of Electrochemical Thermodynamics and Kinetics" (C.I.T.C.E.) held in Poitiers, 1954, Butterworths Scientific Publications, London, 1955, p. 20-49.

(21) Dixon, M., and J. H. Quastel, J. Chem. Soc. 123, 2943 (1923).

(22) Dixon, M., Proc. Roy. Soc. London, Series B, 101, 57 (1927).

(23) Michaelis, L., and L. B. Flexner, J. Biol. Chem. 79, 689 (1928).

(24) Ghosh, J. C., and S. C. Ganguli, Biochem. Z. 279, 296 (1935).

(25) Freedman, L. D. and A. H. Corwin, J. Biol. Chem. 181, 601 (1949).

(26) Kolthoff, I. M., W. Stricks, and N. Tanaka, J. Am. Chem. Soc. 77, 4749 (1955).

(27) Fruton, J. S. and H. T. Clarke, J. Biol. Chem. 106, 667 (1934).

(28) Ryklan, L. R. and C. I. A. Schmidt, Univ. Calif. Publs. Physiol. 8, 257 (1944).

(29) Rall, T. W., and A. L. Lehninger, J. Biol. Chem. 194, 119 (1952).

(30) Mapson, L. W., and F. A. Isherwood, Biochem. J. 86, 173 (1963).

(31) Scott, E. M., I. W. Duncan, and V. Ekstrand, J. Biol. Chem. 238, 3928 (1963).

(32) Rost, J. and S. Rapoport, Nature 201, 185 (1964).

(33) Weiss, J., Trans. Faraday Soc. 37, 467 (1941).

(34) Allen, A. O., "The Radiation Chemistry of Water and Aqueous Solutions", D. van Nostrand Co., Princeton, New Jersey, 1961.

(35) Spinks, J. W. T., and R. J. Woods, "An Introduction to Radiation Chemistry", John Wiley, New York, 1964.

(36) Miller, N., J. Chem. Phys. 18, 79 (1950).

(37) Fricke, H. and E. J. Hart, J. Chem. Phys. 3, 60 (1935).

(38) Weiss, J., A. O. Allen, and H. A. Schwarz, Proc. Intern. Conf. Peaceful Uses Atomic Energy, United Nations, New York 14, 179 (1956).

(39) Dewhurst, H. A., J. Chem. Phys. 19, 1329 (1951).

(40) Woodward, G. E., Biochem. J. 27, 1411 (1933).

(41) Kinsey, V. E., J. Biol. Chem. 110, 551 (1935).

(42) Barron, E. S. G., S. Dickman, J. A. Muntz, and T. P. Singer, J. Gen. Physiol. 32, 537 (1949).

(43) Barron, E. S. G. and S. Dickman, J. Gen. Physiol. 32, 595 (1949).

(44) Barron, E. S. G. and V. Flood, J. Gen. Physiol. 33, 229 (1950).

(45) Dale, W. M., J. V. Davis, and C. W. Gilbert, Biochem. J. 45, 93 (1949a).

(46) Dale, W. M., J. V. Davis, and C. W. Gilbert, Biochem. J. 45, 543 (1949b).

(47) Lal, M., D. A. Armstrong, and M. Wieser, Radiation Res. 37, 246 (1969).

(48) Wilkening, V. G., Manohar Lal, M. Arends, and D. A. Armstrong, Can. J. Chem. 45, 1209 (1967).

(49) Wilkening, V. G., M. Lal, M. Arends, and D. A. Armstrong, J. Phys. Chem. 72, 185-190 (1968).

(50) Adams, G. E., J. W. Boag, J. Curarant, and B. D. Michael, In: Pulse Radiolysis (M. Ebert, J. P. Keene, A. J. Swallow, and J. H. Bexendale, eds.), Academic Press, New York, 1965, p. 131-143.

(51) Icen, A., Scandinavian J. of Clin. and Lab. Invest. Supplementary 96 (1967).

(52) Hammett, F. S., Protoplasma 7, 297 (1939).

(53) Woodward and Fry, J. Biol. Chem. 97, 465 (1932).

(54) Eldjarn, L. W. and A. Phil, J. Amer. Chem. Soc. 79, 4589 (1957).

(55) Huffman, H. M. and E. L. Ellis, J. Am. Chem. Soc. 57, 41 (1935).

(56) Huffman, H. M. and E. L. Ellis, J. Am. Chem. Soc. 57, 46 (1935).

(57) Sunner, S., Svensk. Kem. Tidskr. 58, 71 (1946).

(58) Aravjo, S. M. and G. Cilento, Biochem. J. 8, 2145 (1969).

(59) Stecher, G. P., et al., The Merck Index 8th edition, pp. 710. Merck and Co., Inc., Rahway, N. J., U.S.A. (1968).

(60) Gorin, G. and G. L. Doughty, Arch. Biochem. Biophys. 126, 547 (1968).

(61) Jocelyn, P. C., European J. Biochem. 2, 327 (1967).

(62) Pihl, A. and L. Eldjarn, Pharmacol. Revs. 10, 437 (1958).

(63) Patt, H. M., Federation Proc. 19, 549 (1960).

(64) Gordy, W. and I. Miyagwa, Radiation Res. 12, 211 (1960).

(65) Ellman, G. L., Arch. Biochem. Biophys. 82, 70 (1959).

(66) Dorfman, L. F. and M. S. Matheson, Prog. React. Kinet. 3, 65 (1965).

(67) Schols, G., P. Shaw, R. L. Willson, and M. Ebert, In Pulse Radiolysis (Ebert, M., J. P. Keene, A. J. Swallow, and J. H. Baxendale, eds.), Academic Press, New York (1965), p. 151.

(68) Schols, G. and R. L. Willson, Trans. Faraday Soc. 63, 2983 (1967).

(69) Markakis and Tappel, J. Amer. Chem. Soc. 82, 1613 (1960).

(70) Packer, S. E. and R. V. Winchester, Can. J. Chem. 48, 417 (1970).

(71) Adams, G. E., Ann. Rep. Progr. Chem. 66, 207 (1970).

(72) Sanner, T. and A. Phil, In Radiation Protection and Sensitization, Morosen, H. L. and M. Quantiliani, eds., Taylor and Francis, Ltd., London (1970), p. 43.

(73) Bacq, Z. M. and P. Alexander, Fundamental of Radiolysis, 2nd ed., Pergamon Press, London (1961), p. 457.

(74) Eldjarn, L. and A. Phil, 25th Anniversary Publication, Norwegian Radium Hospital (1958), p. 253.

(75) Purdie, J. W., J. Am. Chem. Soc. 89, 226 (1967).

(76) Packer, J. E., J. Chem. Soc. 2320 (1963).

(77) Jayson, G. G., D. A. Stirling, and A. J. Swallow, Int. J. Radiat. Biol. 19, 143 (1971).

# VITA

Ayoub Esfandi

Candidate for the Degree of

Master of Science

Thesis: OXIDATION-REDUCTION POTENTIAL AND RADIOLYSIS OF GLUTATHIONE

Major Field: Chemistry

Biographical:

Personal Data: Born in Teheran, Iran, on August 3, 1946, the son of Elias Esfandi and Mrs. Roza Esfandi.

Education: Attended Shahriar Grade School in Tehran, Iran, graduated from Hadaf High School, Tehran, Iran, in June 1964; received the Associate in Science degree from Chicago City Junior College in January 1968 and was granted the Bachelor of Science degree in May 1970 from Panhandle State College, Goodwell, Oklahoma; Majors: Chemistry and Mathematics; Minor: Physics. Completed the requirements for the degree of Master of Science at Oklahoma State University in May 1972.

Professional Experience: Worked as a Chemist for Board of Water Pollution at City of Guymon, Oklahoma, from May 1969 to Sept. 1969. Graduate Research Assistant, Oklahoma State University September 1969 to September 1970. Graduate Teaching Assistant, Oklahoma State University, September 1970 to January 1972. Member of American Chemical Society, Phi Lambda Upsilon Honorary Chemical Society. President of International Student Association.