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STUDIES OF THE MECHANISM OF THE METABOLIC OXIDATION
OF ELEMENTAL MERCURY

© Sherry L. Perkins, B.Sc.

A thesis submitted to the Faculty of
Graduate Studies in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

Department of Chemistry
Carleton University
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April, 1984

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For David,
Heather,
and my parents.

ABSTRACT

This thesis presents an in depth look at the characteristics of the metabolic oxidation of elemental mercury to the mercuric form by Catalase and Catalase Compound I.

A double Magos technique is described for quantitative speciation of Hg(0) and Hg(II) in the cold vapour atomic absorption (CVAA) apparatus. The behaviour of the three forms of inorganic mercury, at trace levels, in the CVAA apparatus under reducing and non-reducing conditions is explored. Quantitative speciation in the presence of Hg_2^{2+} ions was ambiguous due to disproportionation of the mercurous ion in the CVAA apparatus under non-reducing conditions.

Enhancement of the "uptake" of elemental mercury into Catalase solutions in the presence of H_2O_2 was found to be due to oxidation to the mercuric form. H_2O_2 alone exhibited no such oxidation, however, alkyl peroxides enhanced "uptake" of Hg(0) by oxidizing the dissolved elemental mercury to Hg_2^{2+} .

A technique was devised in which Hg^0_{aq} homogeneous solutions could be generated. These solutions were then treated with numerous oxidizing agents. Oxidation was monitored by recording the rate of decrease of the non-reducing CVAA signal. Catalase was found to oxidize elemental mercury to Hg(II) by a one-step 2-electron process. This oxidation process was approximately 1st order in both Catalase and mercury and 2nd order overall.

Oxidation of Hg(0) by Catalase Compound I was found to proceed via a rapid ($k=6 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$), one-step, approximately 2nd order mechanism.

An investigation of the oxidation of Hg_2^{2+} solutions by Catalase

or Catalase Compound I indicated oxidation to the mercuric form occurs at rates comparable to that of the Hg^0 oxidation processes.

No evidence was found to indicate the existence of intermediary Hg(I) species in the oxidation of Hg(0) to Hg(II) by either native Catalase or Catalase Compound I.

Spectroscopic studies indicated no sustained binding of either Hg(0) , Hg(I) , or Hg(II) to Catalase, Catalase Compound I or Catalase Compound II at or near the heme moiety of any of the enzyme species.

Stopped-flow experiments were conducted to investigate the effect of Hg(0) , Hg(I) and Hg(II) on the rate of formation of Catalase Compound I. No effect was detected.

No one particular hypothesis evolved from this work as the sole mechanism for the oxidation of Hg(0) to Hg(II) , however several detailed electron transfer mechanisms are suggested based on the evidence collected in this study.

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INTRODUCTION

Historically, mercury and its compounds have played a fascinating role in the development and advancement of civilization. Perhaps the earliest recorded employment of a mercury compound was the use of cinnabar, mercuric sulphide, as a pigment by prehistoric man (1). Since the 4th century B.C. when mercury compounds became popular among Arab and Greek physicians, elemental mercury and mercury salts have been utilized for a variety of medicinal purposes including: the treatment of syphilis, as dermal preparations for antiseptic action, as a laxative, as a diuretic, and more recently in dental preparations (1,2). Contemporary commercial and industrial uses of elemental mercury and mercury compounds include: electrical apparatus, instruments such as thermometers, barometers, switches, batteries, mercury vapour lamps, neon lamps, in the paint industry for the production of anti-corrosive paints, in the cellulose industry as a preservative of wet pulp from bacterial and fungal biodeterioration, in metal refinement processes, in electrolytic cells for the preparation of Cl_2 and NaOH , as fungicides for seed treatment and as catalysts in the manufacture of plastic (1-4).

The unique physical chemical properties of mercury and the poorly understood biochemistry of this heavy metal present serious problems for toxicological evaluation. Mercury is the only metal which is liquid at room temperature. It boils at 357°C compared to 2212°C for silver, it is ten thousand times more volatile than DDT and has a vapour

pressure of 1.2×10^{-3} compared to 1.5×10^{-7} mm Hg for DDT (1). Hg(0), Hg(I) and Hg(II) represented by elemental mercury, mercurous ion (Hg_2^{2+}), and mercuric ion (Hg^{2+}) respectively, are the three stable oxidation states of mercury. Except for their novelty, in so far as they involve a binuclear cation, the compounds of mercury (I) are typical metallic compounds. The chemical and physical properties of this element are summarized in Table I.

Until the late 1960's, quantitative analysis of mercury at trace levels was conventionally done with colourimetric agents such as diphenylthiocarbazone (5) or by radioactive tracer techniques (6). In 1968 Hatch and Ott (7) revolutionized the quantitative determination of submicrogram quantities of mercury with their cold vapour atomic absorption (CVAA) technique. In this analysis the mercury compounds are chemically reduced, using stannous chloride, to the metallic form which is vapourized from aqueous solution into an inert gas stream. The mercury vapour is carried into a 20-30 cm gas cell in which the quantity of mercury present in the gas stream is measured by UV absorbance measurement of the 254 nm light emitted from a mercury lamp source. Magos and Clarkson (8) subsequently modified this technique to differentiate between total organic and total inorganic mercury in biological samples. The CVAA technique has been studied and modified in a variety of ways over the last 15 years (8-18), however, the basics of the analysis remain virtually unchanged and for a number of reasons including sensitivity, selectivity, availability, reliability and cost, the CVAA technique is usually the method of choice. Table 2 compares and summarizes the currently available analytical techniques.

TABLE I
Chemical and Physical Properties of Mercury

Atomic Number	80
Atomic Volume	14.81 cm ³ g-atom ⁻¹
Atomic Weight	200.59
Atomic Distance (Hg-Hg)	3.005 Å
Density	13.546 g cm ⁻³ at 20°C
Freezing Point	-38.87°C
Boiling Point	356.9°C/760 mm Hg
Vapourization, Heat of	73.2 cal g ⁻¹ at 25°C
Electrode Potentials	
$\text{Hg}_2^{2+} + 2\text{e}^- \longrightarrow 2\text{Hg}^0$	0.789 V
$\text{Hg}^{2+} + 2\text{e}^- \longrightarrow \text{Hg}^0$	0.854 V
$\text{Hg}^{2+} + 2\text{e}^- \longrightarrow \text{Hg}_2^{2+}$	0.920 V
$\text{Hg}_2^{2+} \longrightarrow \text{Hg}^0 + \text{Hg}^{2+}$	-0.115 V
Solubility	6.4x10 ⁻⁵ g L ⁻¹ H ₂ O at 25°C
Ionization Potentials	
1st	10.4 eV
2nd	18.7 eV
3rd	34.3 eV
Electronic Configuration	[Xe] 4f ¹⁴ 5d ¹⁰ 6s ²

TABLE 2

Detection Limits of Various Instrumental Methods
for Mercury Analysis

Analytical Method	Assayed Species	Detection Limit
Cold Vapour Atomic Absorption	Hg^0	0.01 ppb
Flame Atomic Absorption	Hg^{T}	20 ppb
Atomic Fluorescence	Hg^0	3 ppb
Atomic Emission	Hg^{2+}	2 ppb
GC with EC Detector	Hg halides	10^{-11} g
Colourimetry	Hg^{2+}	10 ppb
Polarography	Hg^{2+}	50 ppb
Neutron Activation	^{197}Hg , ^{203}Hg	0.1 ppb
Thin Gold Film Resistance	Hg^0	0.05 ppb
Spark-Source Spectrometry	Hg^{2+}	1 ppb
Ion Selective Electrode	Hg^{2+}	200 ppb
Enzyme Inhibition	Hg^{2+}	2 ppb

Toxicology

There is no known nutritional requirement for mercury thus the widespread and often indiscriminate use of this element and its compounds over the centuries has resulted in several well publicized toxicological disasters. One of the earliest recorded cases of mercurialism was the so-called "mad hatter" syndrome suffered by hat makers during the 19th century and made famous by the "Mad Hatter" character in Alice in Wonderland. The central nervous system disorder suffered by these workers derived from the fact that the hat makers often used "secret formulas" containing mercuric nitrate for the "carroting" of furs to make felt hats (3,4,19).

The most publicized incident in the 20th century of chronic organic mercury poisoning began in 1950 when a mysterious disease broke out among inhabitants, especially fishermen and their families, of Minamata Japan. It took three years before authorities traced the problem to localized contamination of fish and shellfish by mercurial compounds dumped into the bay by a local plastics factory. By 1968, when the final Minamata report was written, 46 out of 121 reported cases of poisoning had died (20).

Symptoms of mercury intoxication and sites of accumulation are varied depending on the route and chemical form of exposure. Compounds of methyl mercury (e.g. CH_3HgCl), formed by environmental methylation of mercuric compounds, are particularly hazardous due to their high volatility and lipid solubility. Inhalation, ingestion or cutaneous exposure to these species may result in adverse toxicological effects. The principal target organ of methyl mercury is the brain.

Poisonings due to inorganic mercury salts usually arise from either accidental or intentional ingestion. Although mercuric chloride taken orally is an unpleasant approach to death, it once ranked sixth in frequency of use as a suicidal agent (21). The oral LD_{50} of mercuric chloride is less than $3-6 \text{ mg Kg}^{-1}$ and the target organs of this "supertoxin" include: Kidney > liver > blood > brain. In contrast to this, elemental mercury, which presents a unique toxicological hazard because of its appreciable vapour pressure at room temperature, has minimal adverse effects when ingested. Short term exposure to $\text{Hg}(0)$ vapour does however, result in acute accumulation in the lungs, while the brain is the critical organ for prolonged chronic exposure (22). This cerebral accumulation is facilitated by the high diffusibility and solubility of $\text{Hg}(0)$ in membrane lipids (23).

Non-specific physiological effects of mercury intoxication include: numbness in the fingers and lips, hearing impairment, tremor in the hands with muscular convulsions, headache, fatigue, weakness, loss of memory, insomnia, loss of hair, depression, loss of appetite and excessive salivation.

At the biochemical level there seems to be little difference between the effects of exposure to $\text{Hg}(0)$ vapour or Hg(II) compounds. In general, Hg^{2+} acts as a potent, non-specific enzyme poison. It inactivates metabolic enzymes, denatures structural proteins, disrupts cell membranes in the cells of target organs, complexes nitrogenous bases of DNA and inhibits protein synthesis. The final mechanism of cell death, according to recent evidence, may be attributed to an uncontrolled influx of calcium into the cell which is toxic to the mitochondria and also

activates lysosome enzymes (21).

The similarity in the toxic effects of elemental mercury and various salt forms prompted researchers in the 1960's to speculate that, once it is dissolved in the blood stream and distributed throughout the body, the elemental mercury becomes oxidized to either Hg_2^{2+} or Hg^{2+} which ultimately produce toxic effects by forming covalent bonds with sulfhydryl groups (6).

The exact mechanism by which this in vivo oxidation of $\text{Hg}(0)$ occurs became a subject of immense interest during the late 1960's and is the subject of this study.

Early experiments in the 1930's showed that the rate of uptake of mercury vapour in isolated suspensions of red blood cells was increased as the oxygen tension was raised. This led to the conclusion that the oxidation step in blood was a chemical process not involving catalysis by enzymes in the blood (23).

Clarkson et al. (6) soon challenged these old theories with the finding that the rate of oxidation in red cells was more rapid than in the plasma, thus implying a more elaborate mechanism of oxidation than a simple chemical process. Nielsen-Kudsk (24,25) performed a series of experiments to investigate the factors influencing the uptake of mercury vapour into the blood and presented the first hypothesis that the Catalase-hydrogen peroxide system was principally responsible for $\text{Hg}(0)$ oxidation in erythrocytes.

In the early 1970's Magos and Clarkson (26,82) performed a series of elegant in vivo experiments to test the effects of agents and conditions, known to effect Catalase activity, on the uptake, accumulation and

distribution of mercury vapour. Further studies by these same researchers (27) with acatalasemic mice led them to propose the following mechanism for Hg(0) oxidation:

Several questions arose from this hypothesis including how could a non-polar, uncharged mercury atom attach itself to Catalase Compound I and then be capable of undergoing oxidation to Hg^{2+} (27), and what, if any, other factors are involved in the oxidation mechanism? A number of studies have been performed since the late 1970's including: an investigation of the role of Glutathione peroxidase in erythrocytic oxidation of Hg(0) (28), the effect of ethanol on the distribution and oxidation of inhaled mercury vapour (29-32), oxidation of Hg(0) by ferric compounds (82), correlation of Catalase activity to the uptake of Hg(0) by rat brain (83), studies of the effect of catalasemia on the in vivo and in vitro uptake of Hg(0) (33-37) as a number of studies of the effects of known Catalase inhibitors such as cyanide, azide and 3-amino-1,2,4-triazole (38). Unfortunately none of these studies have been successful in elucidating a more detailed description of the mechanism of oxidation of Hg(0) by the Catalase-hydrogen peroxide system. Interestingly, even the most recent papers in the literature (39) which suggest an elucidation of the mechanism of Hg(0) oxidation, fail to propose details of the electron transfer process and merely

hypothesize that the reacting sites on the Catalase molecule for mercury and ethyl alcohol are "very close to each other or identical".

Catalase- the enzyme

Catalase is ubiquitously present in mammalian and nonmammalian aerobes which contain a cytochrome system, and with few exceptions only strict anaerobes lack this enzyme (40). Quantitative analysis of Catalase levels in post-mortem tissues of male humans (41) revealed the presence of Catalase in the liver, erythrocytes, renal cortex, renal medulla, thyroid, adrenal gland, testis, cardiac muscle, skeletal muscle, lungs, prostate, duodenal mucosa, spleen, and red bone marrow.

Catalase (EC 1.11.1.6, hydrogen-peroxide: hydrogen-peroxide oxidoreductase) is a large (molecular weight = 240,000-250,000) ferric hemoprotein consisting of four identical 60,000 mol. wt. subunits. The prosthetic group, protohemin, is noncovalently bound to each of the subunits. In most eukaryotic cells, most of the Catalase is localized in the matrix of the peroxisomes. The location of erythrocyte Catalase, which has been studied extensively (42-44), seems to be identical with that of hemoglobin (45).

The physiological function of Catalase appears to be to detoxify the cell of oxygen metabolites such as hydrogen peroxide and superoxide which can inactivate viruses, induce lipid peroxidation, damage membranes and kill cells (45).

In vitro and in vivo studies on the biosynthesis of Catalases from different organisms show that the enzyme is synthesized, at least

predominantly by the following pathway:

Studies of the regulation of Catalase biosynthesis have been unsuccessful in determining the specific control factors involved, however, the presence of hydrogen peroxide, glucose, heme, and oxygen seem to play as yet undetermined roles in the regulation of Catalase levels in the body.

The two apparently contrasting toxicological roles played by Catalase: i) detoxication of hydrogen peroxide and oxygen metabolite radicals and ii) oxidation of the relatively inert elemental mercury form to the highly reactive and toxic mercuric form, present an interesting example of the fallibility of the natural protective systems in the body.

If the mechanism and factors influencing the oxidation of elemental mercury in the body can be determined then one more step will have been taken towards understanding the mechanism and development of in vivo xenobiotic responses.

Statement of the Problem

Although a substantial volume of work has been done over the last 20 years concerning the in vivo oxidation of elemental mercury to the toxic mercuric form, the exact mechanism by which this oxidation occurs

has not yet been elucidated. The objective of this project will be to gain insight into the details of this oxidation process.

The work will be presented in 9 sections. The first will deal with the problems of quantitatively speciating Hg(0), Hg(I) and Hg(II) at submicrogram levels. The second chapter will utilize this speciation technique to repeat and re-evaluate the "uptake-oxidation" experiments performed by Magos and Clarkson (27). Chapters 3 and 4 present the results of a completely new approach to studying the mechanism; kinetic evaluation of Hg(0) and Hg(I) oxidation by Catalase and Catalase Compound I in homogeneous solutions. Chapter 5 is an attempt at quantitatively assessing these kinetic data. Chapter 6 takes a qualitative approach to the problem by studying the effect of Hg(0), Hg(I) or Hg(II) exposure on the visible absorption spectrum of Catalase and Catalase Compound I. Chapter 7 investigates the effect of the three inorganic mercury species on the rate of formation of Catalase Compound I. Quantitative adsorption studies are presented in Chapter 8 and Chapter 9 presents some possible mechanisms of oxidation.

RESULTS AND DISCUSSION

Chapter I: Analytical Chemistry, Speciation and Behaviour of Mercury in the Cold Vapour Atomic Absorption Apparatus

The principal aim of the study was to gain insight into the mechanism of in vivo oxidation of elemental mercury to the toxic divalent form. Since quantitative analysis of products and/or reactants is fundamental to any mechanistic investigation, preparation of homogeneous elemental mercury solutions and $\text{Hg}^0/\text{Hg}^{2+}$ speciation techniques were investigated first.

Preparation of Elemental Mercury Solutions

Elemental mercury, Hg^0 , has a very limited solubility in aqueous solution. Careful perusal of current literature (6, 46, 47) sets the aqueous solubility limit at approximately 20-30 ppb for anaerobic (nitrogen purged) solutions and ~60 ppb for aerobic conditions. Numerous thermodynamic studies have been performed to determine the solubility of the metal in various liquids and gases (48 - 51) and the temperature dependence of aqueous solubility has been determined to be:

$$\log_{10} X_2^0 = -122.811 + 4475.3/T + 40.2205 \log_{10} T$$

where X_2^0 is the mercury mole fraction and T is absolute temperature (52).

Kinetic and speciation studies require the preparation of homogeneous

Hg^0 saturated aqueous solution. Initial attempts at saturating the solvent by placing a drop ($\sim 100 \mu\text{L}$) of elemental mercury directly into the water were unsuccessful due to the oxidation of the Hg^0 to Hg^{2+} within the drop of elemental mercury thus leading to contamination of the solution with the divalent mercuric species. The resulting heterogeneous solution would have rendered any subsequent kinetic studies and calculations ambiguous and complex. Okouchi & Sasaki (51) overcame this oxidation problem by adding phosphinic acid reducing agent to an aqueous solution containing a drop of liquid mercury. These researchers found this technique to satisfactorily maintain all the dissolved mercury in the Hg^0 form, however this approach was rejected for the purposes of this study due to the probable adverse effects of residual reducing agent on the delicate enzyme system to be added to the mercury solution. Homogeneity was eventually achieved by exploiting the large vapour pressure property of elemental mercury in a nitrogen purged apparatus. Figure 1 illustrates the technique wherein a double-necked 250 mL round-bottom flask was fitted with a side-arm and a septum. 100 μL of metallic mercury was placed in the side-arm, the solvent (water or buffer) was purged with nitrogen and the flask was shaken overnight to permit homogeneous saturation of the solution with Hg^0 . The actual concentration of Hg^0 in these solutions was found to vary from about 25 to 40 ppb depending on the length of the incubation period, the volume of solution, and the amount of liquid mercury placed in the side-arm.

Speciation of Trace Quantities of Mercury using a Double Magos Determination

In reviewing the literature relevant to quantitative mercury

D I F F U S I O N A P P A R A T U S

Figure 1

speciation it became evident that compared to the extensive literature (20,46,53,54) on over a dozen different analytical methods for total mercury quantitation, much less attention appears to have been paid to the question of mercury speciation, especially at the trace level. One way in which the $\text{Hg}^0/\text{Hg}^{2+}$ speciation analysis may be accomplished is by separate determinations of Hg^{2+} and of total mercury; the concentration of Hg^0 then being taken, in the absence of organic mercury or mercurous ion, as the difference between these two determinations, i.e. $\text{Hg}^0 = \text{Hg}^T - \text{Hg}^{2+}$. In this approach, total mercury is determined by the Magos procedure involving stannous chloride reduction of all mercury to Hg^0 , followed by cold vapour atomic absorption analysis of this species (55). Divalent mercury may then be determined either by use of an ion selective electrode (46) or by dithizone extraction followed by colourimetric analysis of the Hg-dithizone complex (5).

The problem with this approach is that while the atomic absorption method has a limit of detection of approximately 1 ppb; the other parts of the analysis do not. The literature was somewhat divergent on the more sensitive of the two methods mentioned for Hg^{2+} analysis, this being colourimetric analysis of the Hg^{2+} -dithizone complex. Elly (5) states that the method was linear between 2 and 40 ppb, while other workers (46) summarize the detection limit as 50 or 200 ppb.

Under these circumstances, it was surprising that the potential of the cold vapour atomic absorption procedure (55) as a single technique to provide the required speciation analysis, did not appear to have been fully exploited. The method had, in the past, been used with stannous chloride as the reductant to quantify total inorganic

mercury and with a stannous chloride-cadmium chloride mixture to provide a total inorganic and organic mercury analysis (55).

The method did not appear to have been used without a reducing agent, in which case, Hg^0 would be the only species analyzed. The approach to the $\text{Hg}^0/\text{Hg}^{2+}$ speciation analysis thus becomes simply two analyses using the usual Magos cold vapour atomic absorption apparatus, one utilizing a reducing agent (SnCl_2) to give total inorganic mercury, and the other in the absence of reducing agent to give Hg^0 , the difference between the two being taken as Hg^{2+} . A triple speciation in the presence of organic mercury would, in principle, also be possible by adding in the variable of CdCl_2 to obtain, by three analyses, Hg^0 , total inorganic, and total inorganic and organic mercury:

$$\text{Hg}^{2+} = \text{Hg}_{\text{inorg.}}^{\text{T}} - \text{Hg}^0$$

$$\text{Hg}_{\text{org.}} = \text{Hg}^{\text{T}} - \text{Hg}_{\text{inorg.}}^{\text{T}}$$

In order to explore this approach, measurements of mercury-containing solutions were made using the Magos procedure, both in the presence and absence of stannous chloride. Initial results were surprising; solutions containing Hg^{2+} but not Hg^0 gave high mercury analyses, even in the absence of the reducing agent. This potentially serious and curious problem was eventually attributed to trace quantities of stannous chloride adhering to the surface of the reaction vessel, sufficient to reduce the trace quantities of Hg^{2+} in solution, even after thorough rinsing of the vessel. Various ways of preventing this problem were considered, and a remedy was found in the procedure of including a hydrogen peroxide rinse of the reaction vessel. This procedure, although it essentially solved the problem, still gave rise to a very small reading for Hg^0 , even when none was present. In practice, this "spillover" of Hg^{2+} appearing as Hg^0 (1.2%) needed to be taken into consideration (added to Hg^{2+} and subtracted from Hg^0), as shown in Tables 3 & 4. In contrast to this, there was no problem with Hg^{2+} contaminating Hg^0 solutions. Hg^0 solutions analyzed with and without the reducing agent yielded identical results.

In order to test this approach as an analytical method, solutions containing various amounts of Hg^0 and Hg^{2+} were analyzed both by this method and by the Magos-dithizone combination approach mentioned above. Table 3 and Figures 2 and 3 show comparison data for Hg^{2+} analysis in solutions containing Hg^{2+} and Hg^0 . Plots of Magos Hg^{2+} analysis versus both the gravimetrically determined concentration and the dithizone determination showed excellent correlations.

The Hg^0 analysis results were much less straight forward. Figure 4

TABLE 3

Comparison of Hg^{2+} concentrations by gravimetric, Magos and Dithizone Techniques

Solution	Gravimetric Hg^{2+} (ppb)	Magos Hg^{Total} (a) (ppb)	Magos Hg^{O} (b) (ppb)	Magos $\text{Hg}^{\text{O}*}$ (c)=(b-1.2%a) (ppb)	Magos Hg^{2+} a-c (ppb)	Dithizone Hg^{2+} (ppb)
A	65.1±0.7	70.4±0.7	5.0±1.0	4.2±1.0	66.2±1.7	68.5±2.5
B	49.3±0.9	53.6±1.3	4.9±0.4	4.3±0.4	49.3±1.7	50.5±2.4
C	33.3±0.4	38.0±0.2	4.4±0.3	3.9±0.3	34.1±0.5	31.0±2.3
D	14.7±0.3	19.6±1.5	3.6±0.2	3.4±0.2	16.2±1.7	17.5±2.3
E	64.9±0.9	74.3±0.7	6.6±0.8	5.7±0.7	68.6±1.4	68.5±2.5
F	49.0±0.9	55.2±0.6	6.8±0.8	6.1±0.7	49.1±1.3	50.5±2.5
G	32.9±1.2	40.4±1.6	6.4±0.2	5.9±0.2	34.5±1.8	30.5±2.5
H	13.3±0.1	19.9±0.3	5.6±0.3	5.4±0.3	13.7±0.9	18.0±2.5
I	64.9±0.7	76.7±0.8	12.3±1.2	11.4±1.1	65.3±1.9	68.0±2.4
J	48.5±0.3	59.3±0.8	11.8±1.3	11.1±1.2	48.2±2.0	48.0±2.4
K	33.2±1.0	43.0±0.8	10.8±0.1	10.3±0.1	32.7±0.8	37.0±2.4
L	14.3±0.8	22.6±0.5	10.4±1.3	10.1±1.2	12.5±1.7	13.0±2.3

* values corrected for 1.2% Hg^{2+} "spillover"

All values represent mean ± SD of three estimations

Figure 2: Correlation of the double Magos (●) and Dithizone (□) Methods of Hg(II) analysis of mixed Hg(II)-Hg(0) solutions to the gravimetric Hg(II) value

Figure 3:

Correlation of the double Magos Hg(II) analysis
of mixed Hg(II)-Hg(0) solutions to the Dithizone method

Figure 4: Correlation of the Magos Hg(0) analysis of mixed Hg(II)-Hg(0) solutions to the Dithizone method

TABLE 4

Hg^0 concentrations by Magos and Dithizone Techniques

Solution	Magos Hg^{0*} (ppb)	Magos Hg^{Total} (a) (ppb)	Dithizone Hg^{2+} (b) (ppb)	Dithizone Hg^0 (a-b) (ppb)
A	4.2±1.0	70.4±0.7	68.5±2.5	1.9±3.2
B	4.3±0.4	53.6±1.3	50.5±2.4	3.1±3.7
C	3.9±0.3	38.0±0.2	31.0±2.3	7.0±2.5
D	3.4±0.2	19.6±1.5	17.5±2.3	2.1±3.8
E	5.7±0.7	74.3±0.7	68.5±2.5	5.8±3.2
F	6.1±0.7	55.2±0.6	50.5±2.5	4.7±3.1
G	5.9±0.2	40.4±1.6	30.5±2.5	9.9±4.1
H	5.4±0.3	19.1±0.3	18.0±2.5	1.1±2.8
I	11.4±1.1	76.7±0.8	68.0±2.4	8.7±3.2
J	11.1±1.2	59.3±0.8	48.0±2.4	11.3±3.2
K	10.3±0.1	43.0±0.8	37.0±2.4	6.0±3.2
L	10.1±1.2	22.6±0.5	13.0±2.3	9.6±2.8

*values corrected for Hg^{2+} "spillover"

All values represent mean ± SD of three estimations

shows the correlation between the two analyses with considerable scatter. The data in Table 4 reveals the reason for this; due to solubility considerations, the concentration of Hg^0 in aqueous solution was always very small. Whereas the present double Magos method gave this value as a direct determination, the Magos-dithizone analysis yields only a small difference between two large numbers; giving rise to extremely large errors in the method, as shown in Table 4. For this reason, it appeared justified to interpret the scatter in Figure 4 as an inadequacy of the Magos-dithizone procedure and that the presently proposed double Magos determination represents a substantial improvement in methodology. The double Magos determination appears to be an excellent and simple analytical method for mixed $\text{Hg}^0/\text{Hg}^{2+}$ determinations at the ppb level.

Speciation and Behaviour of Mercurous Ion in Cold Vapour Atomic Absorption Analysis

While it is generally agreed (6,23,28,34) and will be shown in this study that the ultimate product of elemental mercury oxidation in vivo is the divalent mercuric form, consideration was given to the possible existence of a mercury (I) intermediate in a multistep oxidation mechanism.

Mercury exists in three distinct oxidation states, commonly referred to as Hg^0 , Hg_2^{2+} and Hg^{2+} . While elemental mercury and the mercuric ion exist as simple monomeric species, there are many lines of evidence (56) demonstrating that the mercurous ion is a dimer.

Because it was not clear what analytical data would result from double Magos cold vapour atomic absorption analysis of solutions containing mercurous ion, a study of the behaviour of mercurous solutions

under a range of possible conditions in the CVAA apparatus was conducted.

1. Mercury solutions analyzed under non-reducing conditions in the CVAA apparatus:

Table 5 shows some typical results of CVAA analysis of trace mercury solutions (Hg(0), Hg(I) and Hg(II)) under reducing (addition of SnCl_2) and non-reducing conditions. As discussed above, analysis of aqueous elemental mercury solutions gives all the mercury signal either with or without a reducing agent. In contrast, mercuric chloride yields less than 1.2% of the total mercury in the absence of reducing agent. The results of CVAA analysis of two mercurous solutions- nitrate and chloride- were similar and unexpected. Although the total mercury determinations (reducing agent present) gave the expected analysis, very close to that predicted from the original weighing and subsequent dilutions of the sample, the results in the absence of reducing agent were consistently 35-40% of the total mercury content. Apparently 35-40% of the mercurous mercury was being converted to elemental mercury in the CVAA apparatus. Aside from direct oxidation or reduction, the three inorganic mercury species are related by a disproportionation reaction:

According to this equation one might expect a 0% signal for mercurous solutions analyzed under non-reducing conditions if disproportionation did not occur, or 50% of the total mercury signal if disproportionation occurred completely. A volatile non-reducing signal of 35-40% of the total mercury-corresponding to 70-80% disproportionation was difficult

TABLE 5

CVAA Analysis of Different Mercury Compounds in Aqueous Solution

Mercury species	Volatile Hg (ppb) (non-reducing signal)	Total Hg (ppb) (reducing signal)	Percentage of total appearing as "Hg ⁰ "
● Hg ⁰	48.7 ± 0.8	46.6 ± 1.1	104
HgCl ₂	0.40 ± 0.08	50.5 ± 1.5	0.01
HgNO ₃ · H ₂ O	21.5 ± 0.9	52.2 ± 1.0	41.2
Hg ₂ Cl ₂	19.2 ± 1.0	51.6 ± 1.0	37.2

* All values represent mean ± SD of three estimations

to rationalize unless the rate of disproportionation was comparable to the CVAA analysis. To test whether reaction rates were a factor in the analysis, the analyzed solution was re-analyzed a few minutes later to determine if additional volatile mercury (0) was produced. Under various time periods it was not found possible to obtain any further signal.

The only report in the literature of a mercurous solution being analyzed in a CVAA apparatus claims a 50% total mercury non-reducing signal (57) implying complete disproportionation. Despite many attempts this claim could not be reproduced under these conditions.

No significant difference between mercurous chloride and mercurous nitrate behaviour was detected; signals varied between 35 and 42% of the total mercury signal, and, within the limited concentration range possible, did not appear to be concentration dependent.

2. Effect of chloride ion:

From an analytical viewpoint, the above result was highly unsatisfactory. Firstly, the fact that mercurous ion solutions give a signal at all makes the analysis of Hg^0 ambiguous. Secondly, that this signal bears a curious and not obvious relationship to mercurous ion concentration, and that it is not particularly reproducible, creates a totally unworkable situation for analysis. In an effort to improve matters, consideration was given to the addition of chloride ion, in the hope that formation of the insoluble mercurous chloride complex would prevent disproportionation. If this occurred, a non-reducing signal would not be produced from any mercurous ion in solution, thus removing the interference from the Hg^0 analysis. The possibility of

getting a Hg_2^{2+} analysis from a double analysis in the presence and absence of chloride ion could then be considered.

The failure of this idea is evident in Figure 5. While the signal from mercurous nitrate in the absence of chloride ion (open circles) shows a linear relationship with concentration, and the signal from mercuric nitrate (triangles) remains essentially zero, the signal from mercurous nitrate in the presence of a large excess of chloride ions (closed circles) shows ambivalent behaviour. At low concentrations (0-120 ppb) the signal rises with concentration in a manner indistinguishable from that in the absence of chloride ion. The signal then flattens off and beyond 160 ppb remains essentially constant. Although the approach is clearly valid for higher concentrations, it is foiled at trace levels by the small but significant solubility of mercurous chloride ($K_{sp} \approx 10^{-18}$ (58)) with its behaviour up to 120 ppb apparently being that of a normal soluble salt.

3. Mercuric ion complexation:

In order to confirm that the CVAA signal from mercurous ion solutions was indeed due to mercury vapour, arising from mercurous ion disproportionation, the parallel formation of Hg^{2+} according to equation [1] was tested. A series of analyses of mercurous nitrate solutions were conducted in which the Hg^0 was determined by CVAA and Hg^{2+} was determined by colourimetric analysis following dithizone (diphenylthiocarbazone) complexation (59). The first four entries of Table 6 show that despite the extent of disproportionation being erratic, and certainly less than 100%, there is indeed a close parallel between the analyses for these

Figure 5: Proportion of mercury volatilized under non-reducing conditions from solutions of $\text{Hg}(\text{NO}_3)_2 \cdot \text{H}_2\text{O}$ in 0.01 M HNO_3 (○), $\text{Hg}(\text{NO}_3)_2 \cdot \text{H}_2\text{O}$ in 0.1 M chloride ion (●), and HgCl_2 (△)

two oxidation states of mercury. The fifth entry is typical of determinations taken at the low limit of determination for the CVAA technique, in that measurements at around 5 ppb become significantly less reliable despite an apparent reproducibility as indicated in the error limits.

A curious observation was made when these double analyses were conducted in the presence of 0.1 M chloride ion. Consistent with the previous observation that chloride ion made no impact on the extent of mercurous ion disproportionation at these (2-40 ppb) concentrations, the extent of Hg^0 formation remained at its usual value of approximately 35% of the total mercury present. However, in the dithizone determination for Hg^{2+} , the analysis jumped to well over the theoretical maximum of 50% corresponding to complete disproportionation. The final five entries in Table 6 indicate typical data in this regard; once again data obtained on samples at ≤ 5 ppb appear anomalous. Whether these observations are due to chloride ion interference of the dithizone determination or genuine mercury oxidation remains unanswered, however some previous studies (80) have indicated a severe chloride interference effect on the dithizone extraction procedure.

4. Effect of other reagents:

In order to probe the origin of the curious CVAA signal arising from mercurous solutions, corresponding to 70-80% disproportionation, further mercurous solutions were analyzed in the presence of various reagents relating to normal use of CVAA for mercury analysis. These results are summarized in Table 7. Clearly none of the reagents tried gave

TABLE 6

Mercurous nitrate solutions analyzed by CVAA for
 Hg^0 and by the dithizone technique for Hg^{2+}

Other ions present	Hg^{Total} (CVAA) (ppb)	Volatile Hg (CVAA non- reducing signal)(ppb)	Percentage appearing as Hg^0	Hg^{2+} (dithizone) (ppb)	Percentage appearing as Hg^{2+}
None	128.8±6.4	54.8±1.7	42.5	58	45.0
None	57.7±0.9	24.4±0.7	42.3	25.5	44.2
None	44.2±0.1	18.3±0.3	41.4	18	40.7
None	28.1±0.9	11.7±0.4	41.6	10.5	37.4
None	15.4±0.6	5.35±0.2	34.7	3	19.5
0.1M NaCl	89.3±0.8	37.4±0.5	41.9	67	75.0
0.1M NaCl	62.9±0.2	21.2±1.4	33.7	50	79.5
0.1M NaCl	42.9±3.3	14.3±0.0	33.3	33	76.9
0.1M NaCl	20.6±0.4	6.53±0.00	31.7	11.5	55.8
0.1M NaCl	8.16±0.23	2.08±0.80	25.5	3	36.8

* All values represent mean ± SD of three estimations.

TABLE 7

Effect of CVAA Conditions on the Non-Reducing
Signal from Mercurous Ion

Reagent added	Percentage of total mercury appearing as Hg^0	
	$\text{HgNO}_3 \cdot \text{H}_2\text{O}$ in 0.1 M NaCl	$\text{HgNO}_3 \cdot \text{H}_2\text{O}$ in 0.01 M HNO_3
5.0 mL H_2O	26.3	33.0
5.0 mL H_2SO_4 - cysteine reagent	43.1	47.3
5.0 mL 35% NaOH	71.7	76.2
2.5 mL H_2SO_4 -cysteine reagent, 2.5 mL 35% NaOH	31.5	43.6
2.5 mL H_2SO_4 -cysteine- SnCl_2 reagent, 2.5 mL 35% NaOH	100	100

rise to a clear signal corresponding to 100% disproportionation. The normal reducing conditions (entry 5), as expected, reduced the mercurous ion completely to Hg^0 , giving an accurate and quantitative mercury assay. In this regard, the presence of mercurous ion does not interfere with total mercury analysis. The most striking result is the analysis conducted in the presence of sodium hydroxide (entry 3), in which more than 50% of total mercury appears as the mercury vapour signal. Clearly this cannot be explained by disproportionation. This observation has been previously reported by Hildebrand (58), who suggested the following oxidation-reduction chemistry to account for the large signal:

and

Two other reagents were examined- EDTA and excess cysteine- to explore whether complexation of Hg^{2+} would induce a greater degree of mercurous ion complexation. However neither reagent caused any noticeable effect on the size of the non-reducing CVAA signal.

5. Effect of sparging:

In order to probe the availability of residual mercury that apparently would not disproportionate in the CVAA apparatus, sparging experiments to remove Hg^0 were conducted. Opinions on the rate of disproportionation vary from essentially instantaneous (60) to very slow ($t_{1/2} = 47.9$ days) (61). Figure 6 shows the CVAA signal from a

Figure 6:

Effect of sparging on mercurous nitrate. Disappearance of the reducing signal (○) and non-reducing signal (●) in CVAA analysis

mercurous solution under both reducing and non-reducing conditions as a function of the time that nitrogen gas was allowed to bubble through the solution to drive off volatile Hg^0 . Consistent with previous results, the initial reducing signal corresponded to total mercury in solution, and the initial non-reducing signal was approximately one third of this signal corresponding to about 70% disproportionation. As time elapsed, the non-reducing signal (closed circles) decayed to zero (approximately 20 minutes) indicating no further mercurous ion present in solution that would disproportionate. Interestingly, at this point the total mercury determination (open circles) also became constant with 32.5% of the original mercury having been sparged from solution. This 32.5% corresponds very well to the 60-80% disproportionation consistently observed in the usual analysis. Clearly there remains in solution a residue of mercury that is not volatilizable either under non-reducing CVAA analysis or other sparging conditions. In order to test whether the disproportionation equilibrium might take several days to re-establish itself the sparged solutions were left for periods of up to 2 weeks. None of these solutions regained any ability to generate a CVAA signal under non-reducing conditions. No further mercury could be sparged from solution.

At this point it was of interest to enquire what is the nature of this curious non-volatilizable (the difference between 60-70% and 100% disproportionation) residue of mercury. Clearly it is not "normal" mercurous ion as this would eventually disproportionate completely and give 50% of the total mercury signal under non-reducing conditions. It is not Hg^0 since this would volatilize directly. It is not Hg^{2+}

since this external oxidation (as opposed to disproportionation) would cause a discrepancy between CVAA analysis of Hg^0 and dithizone analysis of Hg^{2+} . And, finally, this curious residue cannot be released either by sparging or by the various reagents in CVAA analysis mentioned above.

6. Effect of pH:

Conditions for achieving 100% disproportionation and elimination of the "non-volatilizable residue" mentioned above were finally found by a study involving variation of the pH of the solution in which the mercurous ion was analyzed under non-reducing conditions. Data shown in Figure 7 indicate a striking dependence of the CVAA non-reducing signal size as a function of the acidity of the solution. Within the range pH 1-2 the signal corresponds to 30-40% of the total mercury appearing as Hg^0 , i.e. the ubiquitous 60-80% disproportionation. At lower hydrogen ion concentrations this signal size increases to the theoretically satisfying 50% of total mercury, corresponding to complete disproportionation. As a matter of interest, the signal size at higher hydrogen ion concentration was also investigated. As Figure 7 shows, the signal continues to decrease as the hydrogen ion concentration is increased, eventually reaching a signal size corresponding to less than 10% of total mercury present.

From these data, the CVAA technique appears to be far slower than mercurous ion disproportionation and, in view of the behaviour mentioned above, does not appear promising as a mercury speciating analysis when mercurous ion may be present. Careful attention must be paid to the

Figure 7: Effect of pH on the CVAA non-reducing signal from mercurous nitrate solutions

fact that in the presence of mercurous ion, the volatile non-reducing signal observed in the CVAA analysis may be due to the sum of the dissolved Hg^0 present in solution and the fraction of Hg_2^{2+} which disproportionates in the reaction vessel:

$$\text{"Hg}^0\text{" volatile} = \text{Hg}^0_{(\text{aq})} + X(\text{Hg}_2^{2+})$$

where X depends on pH, and/or chelating ligands present in solution and can range from 0.1 to 0.5.

This study clearly indicates that, at trace levels, the double Magos CVAA analysis provides a rapid and accurate speciation of Hg^0 and Hg^{2+} , however in the presence of equimolar concentrations of Hg_2^{2+} , the analysis becomes ambiguous and extreme caution must be exercised in interpreting the non-reducing signal.

Chapter II: "Uptake" and Oxidation of Elemental Mercury

Uptake and Oxidation of Hg^0

As discussed in the Introduction and in Chapter I, elemental mercury has a very limited solubility in aqueous solution hence the uptake and accumulation of mercury in the body after exposure to elemental mercury vapour is thought to be augmented by the rapid oxidation of the dissolved Hg^0 to the more soluble Hg^{2+} form. Magos & Clarkson (23,27) pioneered the investigation of this rapid oxidation by monitoring the "uptake" of mercury from Hg^0 saturated atmospheres into solutions containing various oxidizing agents. Of the numerous biological oxidizing agents tested, the Catalase-hydrogen peroxide combination was found to be the most efficient at causing mercury "uptake" leading these authors to the hypothesis that the Catalase Compound I intermediate was the major species in vivo, responsible for the oxidation of Hg^0 to Hg^{2+} . Early studies (40) described the reaction of Catalase and hydrogen peroxide as a two step oxidation/reduction process leading to the formation of molecular oxygen and water. The first step was thought to involve oxidation of a molecule of hydrogen peroxide to form a complex which was designated Catalase Compound I. This enzymatic intermediate then reacts to reduce the second molecule of hydrogen peroxide ultimately regenerating native Catalase and producing oxygen and water:

Magos & Clarkson reasoned that the oxidation of mercury vapour could be described by a reaction with Compound I involving a two-electron transfer as indicated in the reaction below:

These researchers further assumed that any "uptake" of total mercury (by the test solutions) above solubility levels of Hg^0 would be indicative of oxidation to the more soluble Hg^{2+} form.

It seemed desirable in view of the availability of the CVAA inorganic mercury speciating technique to confirm that mercury "uptake" actually did represent oxidation. In order to test this point, the Magos & Clarkson experiments were repeated in normal Thunberg flasks. Solutions containing reagents to be tested were placed in the central compartment of the flask, metallic mercury was placed in the side-arm and the flasks were incubated for various time periods. At the end of the incubation time samples were analyzed for "uptake" of mercury using a double Magos speciation. The results of these experiments are summarized in Table 8 which represents typical values from an extensive body of data. These experiments essentially parallel those of Magos & Clarkson (27) and confirm both that "uptake" and oxidation proceed simultaneously and that the reagents causing mercury "uptake" are in fact causing conversion of mercury to the toxic divalent state.

TABLE 8

Uptake and Oxidation of Mercury by Phosphate Buffer Solution with Different
Supplements

Catalyst	Time of Incubation	H ₂ O ₂	Volatile Hg (CVAA non- reducing signal) (ppb)	Hg ^{Total} (CVAA Reducing signal) (ppb)
None	160 minutes	-	26.1±3.8	24.7±0.9
		+	29.2±1.5	31.4±2.3
Catalase (8X10 ⁻¹⁰ mol)		-	23.5±1.6	22.6±1.0
		+	32.6±4.3	84.5±4.2
None	70 minutes	-	24.2±1.1	24.0±1.6
		+	23.6±1.0	22.0±2.0
Catalase (8X10 ⁻¹⁰ mol)		-	36.4±4.7	32.2±1.9
		+	29.0±1.2	39.3±3.4

* All values represent mean ± SD of three estimations

Uptake and Oxidation of Elemental Mercury by Catalase Compound I

At this point in the study it seemed prudent to address the question of whether Compound I was in fact the principal agent responsible for enhancing "uptake" of elemental mercury.

As discussed previously, Catalase is somewhat of a unique enzyme in that its normal substrate, hydrogen peroxide, is both oxidized and reduced in the detoxication process. For this reason it is difficult to study the formation of Compound I with hydrogen peroxide since the rapid second step impedes complete conversion to Compound I.

Substituted peroxides have been used extensively to examine the formation and properties of Compound I (43,63,63,64).

Catalase reacts with pseudosubstrates such as peracetic acid ($\text{CH}_3\text{C(=O)OOH}$), ethyl hydroperoxide ($\text{CH}_3\text{CH}_2\text{OOH}$), and methyl hydroperoxide (CH_3OOH) to form identical Compound I intermediates which are stable for several minutes under non-excess conditions.

Palcic & Dunford (43) have reported 100% conversion of native Catalase to Compound I on treatment of $1.5 \mu\text{M}$ Catalase with $80 \mu\text{M}$ peracetic acid. These experiments were reproduced and the spectral changes after reaction of Catalase with peracetic acid are shown in Figures 8 & 9. The Soret peak of native Catalase at 405 nm decreases to less than half the original intensity after Compound I formation and is shifted to 399 nm. Other changes include the appearance of a distinct band at 662 nm and the loss of resolution and masking of α & β $\pi \rightarrow \pi^*$ transitions. Recent visible, CD and MCD spectroscopic studies (65, 66, 67, 68, 69) of Compound I and native Catalase have led researchers to discard the early postulation of Deisseroth

Figure 8: Visible absorption spectra of Catalase and Catalase Compound I

& Dounce (40) that Compound I is the species depicted in equation [2].

The formation of Compound I is now thought to be bimolecular reaction between Catalase and hydroperoxide, following second order kinetics over a wide range of hydroperoxide concentrations. It is a 2-electron oxidation of the native enzyme (Fe^{3+} high spin) however the exact location of the oxidizing equivalents has been a controversial subject. A number of studies (66) have established that Compound I is a formal Fe (V) oxy-cation species however this designation does not imply that both oxidizing equivalents (above the native enzyme Fe (III)) reside upon the iron itself. Considerable evidence has been accumulated (62, 63, 65, 68, 69) to suggest that the Catalase Compound I intermediate is actually an oxy-ferryl-porphyrin cation radical ($\text{O}=\text{Fe}(\text{IV})\text{P}^{\cdot+}$) in a $^2\text{A}_{1\text{u}}$ ground state. The reaction is very complex and essentially irreversible. The Compound I spectrum is independent of the oxidant used in its preparation (43) but studies of the formation of Compound I with organic hydroperoxides reveal significant steric constraints upon access of the substrate to the active site (69). Only aliphatic hydroperoxides with small end groups are acceptable and there is a parallel between rate attenuation and increasing Van der Waal's volume of the hydroperoxide. In the case of the readily ionizable peracetic acid the neutral peracid is an acceptable substrate but the anion is not, implying that electrostatic factors may play an important role in Compound I formation. One hypothesis which has been presented suggests that negatively charged protein residues near the entrance to the enzyme active site may act as an "electrostatic gate", permitting neutral molecules to diffuse freely in and out of the active site but

reflecting anions (both present in the external environment and formed within the active site). Alternatively crystal structure data suggests that discrimination against charged ligands may arise from a hydrophobic environment around the ferrihaem (69). The formation of Compound I can best be represented as:

With this evidence and information in mind the Magos-Clarkson uptake experiments were repeated using peracetic acid and ethyl hydroperoxide Compound I species as well as a Catalase-hydrogen peroxide mixture as potential oxidizing agents. Results of these experiments are shown in Tables 9 & 10. The CVAA signal under reducing (SnCl_2) conditions corresponds to total mercury present; the signal under non-reducing conditions can, as discussed previously, arise from either or both of elemental mercury or mercurous ion, the latter giving a signal due to Hg^0 from disproportionation.

Perusal of the results in Tables 9 & 10 reveal various points. Firstly,

TABLE 9

Uptake and Oxidation of Mercury by Catalase in the Presence of Different Peroxides

		Oxidant		H_2O_2 ($\times 10^{-7}$ mol mL ⁻¹)	CVAA Signal		Oxidation %
		Catalase ($\times 10^{-9}$ mol mL ⁻¹)	CH_3CO_3H ($\times 10^{-7}$ mol mL ⁻¹)		Reducing Conditions (Hg ^{Total}) (ppb)	Non-Reducing Conditions (Hg ⁰ , Hg ₂ ²⁺) (ppb)	
I	(a)	-	-	0.294	48.5±2.1	48.3±0.2	0.4
	(b)	-	-	1.47	53.6±1.0	46.5±1.4	13.2
	(c)	-	-	2.94	60.3±0.1	44.1±1.8	26.9
	(d)	-	-	11.8	60.1±1.7	44.2±1.8	26.5
II	(a)	1.92	-	0.294	139±2	19.2±0.1	86.2
	(b)	1.92	-	1.47	147±4	18.9±0.8	87.1
	(c)	1.92	-	2.94	170±4	15.8±0.4	90.7
	(d)	1.92	-	11.8	164±4	16.8±0.4	89.8
III	(a)	-	-	-	31.5±0.6	44.2±1.2	0*
	(b)	-	0.175	-	2965±21	323±6	89.1
	(c)	-	1.05	-	2214±26	214±10	90.3
	(d)	-	2.10	-	1510±6	162±2	89.3
	(e)	-	10.5	-	1863±91	271±7	85.5
IV	(a)	1.92	-	-	100±1	17.1±1.2	82.9
	(b)	1.92	0.175	-	305±21	13.7±0.9	95.5
	(c)	1.92	1.05	-	561±8	13.7±0.4	97.6
	(d)	1.92	2.10	-	652±50	16.5±0.9	97.5
	(e)	1.92	10.5	-	1543±31	25.7±1.0	98.3

* See note in Materials and Methods

All values represent mean ± SD of three estimations

TABLE 10

Uptake and Oxidation of Mercury by Catalase in the
Presence of Various Peroxides

		Oxidant				CVAA Signal	
		Catalase ($\times 10^9$ mol mL ⁻¹)	CH ₃ CO ₃ H ($\times 10^7$ mol mL ⁻¹)	CH ₃ CH ₂ OOH ($\times 10^7$ mol mL ⁻¹)	H ₂ O ₂ ($\times 10^7$ mol mL ⁻¹)	Non- Reducing Conditions (ppb)	Reducing Conditions (ppb)
I	(a)	-	-	-	-	27.2 $\pm$ 0.9	24.5 $\pm$ 1.4
	(b)	-	0.175	-	-	282 $\pm$ 9	1305 $\pm$ 43
	(c)	-	1.05	-	-	132 $\pm$ 6	968 $\pm$ 2
	(d)	-	2.10	-	-	7.3 $\pm$ 2.0	1826 $\pm$ 76
II	(a)	-	-	0.172	-	24.4 $\pm$ 0.1	19.0 $\pm$ 0.7
	(b)	-	-	1.29	-	21.1 $\pm$ 0.2	18.5 $\pm$ 0.0
	(c)	-	-	2.15	-	19.7 $\pm$ 0.4	17.3 $\pm$ 1.3
III	(a)	-	-	-	0.294	27.1 $\pm$ 0.1	26.7 $\pm$ 0.6
	(b)	-	-	-	1.18	27.0 $\pm$ 2.3	30.1 $\pm$ 0.2
	(c)	-	-	-	2.35	27.5 $\pm$ 0.3	28.2 $\pm$ 0.8
IV	(a)	0.771	-	-	-	21.6 $\pm$ 1.2	79.8 $\pm$ 2.7
	(b)	0.771	0.175	-	-	21.8 $\pm$ 3.1	81.8 $\pm$ 2.7
	(c)	0.771	1.05	-	-	18.1 $\pm$ 1.6	115 $\pm$ 0
	(d)	0.771	2.10	-	-	20.0 $\pm$ 1.2	115 $\pm$ 4
V	(a)	0.771	-	0.172	-	21.2 $\pm$ 1.9	70.0 $\pm$ 0.9
	(b)	0.771	-	1.29	-	21.1 $\pm$ 1.0	81.3 $\pm$ 0.2
	(c)	0.771	-	2.15	-	17.8 $\pm$ 3.0	86.7 $\pm$ 1.4
VI	(a)	0.771	-	-	0.294	25.6 $\pm$ 1.8	64.5 $\pm$ 2.2
	(b)	0.771	-	-	1.18	23.9 $\pm$ 1.2	56.8 $\pm$ 1.6
	(c)	0.771	-	-	2.35	21.4 $\pm$ 1.5	61.3 $\pm$ 0.1

* All values represent mean $\pm$ SD of three estimations

oxidation of elemental mercury by hydrogen peroxide is modest at best, whereas oxidation with Catalase/ H_2O_2 , Catalase/ CH_3COOOH , or Catalase/ $\text{CH}_3\text{CH}_2\text{OOH}$ gives substantially complete oxidation (> 80%) to the divalent mercuric ion. These results are not conclusive, but they do provide further evidence for the hypothesis that Catalase Compound I is the principal active intermediate of the Catalase/ H_2O_2 pathway responsible for the in vivo oxidation of $\text{Hg}(0)$ to $\text{Hg}(\text{II})$. Secondly, oxidations involving Catalase, in conjunction with either hydrogen peroxide, ethyl hydroperoxide, or peracetic acid, give a non-reducing CVAA signal which never exceeds 40-50 ppb, consistent with the limited solubility (~ 60 ppb) of elemental mercury in aerobic aqueous solution. Thirdly and most importantly, the set of data using buffered peracetic acid, while still showing predominant oxidation to Hg^{2+} shows non-reducing signals far in excess of the 60 ppb solubility limit of elemental mercury. Since this signal cannot therefore be due to Hg^0 , the results clearly indicate that in the oxidation of elemental mercury by peracetic acid, significant quantities of mercurous ion are formed.

Two important points are indicated from these results. The first is that Catalase in the presence of concentrations of alkyl peroxide known to cause Compound I formation, will enhance the uptake and oxidation of $\text{Hg}(0)$ to $\text{Hg}(\text{II})$ with no apparent accumulation of Hg_2^{2+} . Control samples of Catalase alone will also take up and oxidize $\text{Hg}(0)$ to $\text{Hg}(\text{II})$ however the magnitude of the uptake and oxidation is substantially less than that observed in the presence of alkyl peroxides. Secondly, in the non-enzymatic alkyl peroxide control solutions two effects were observed: i) hydrogen peroxide and ethyl hydroperoxide did not oxidize any of the dissolved

Hg(0) (reducing and non-reducing signals were essentially identical) but ii) oxidation of elemental mercury by peracetic acid produced substantial quantities of both mercurous and mercuric ion. This behaviour illustrates that while the CVAA speciation analysis of Hg^0 , Hg_2^{2+} , and Hg^{2+} is ambiguous at trace levels, the situation becomes progressively less serious as total mercury concentration exceeds 63 ppb. This solubility limit of Hg^0 in aqueous solution may be utilized to draw conclusions about oxidation state analysis. This point is parallel to the analysis of mercurous ion in the presence of chloride ion (discussed in Chapter I) which prevents mercurous ion disproportionation but only at levels above 120 ppb--the solubility limit of mercurous chloride. The question of why under non-enzymatic conditions peracetic acid oxidized Hg(0) to both Hg(I) and Hg(II) but ethyl hydroperoxide and hydrogen peroxide showed no such oxidation was addressed in the next experiment.

Uptake & Oxidation of Elemental Mercury by Organic Peracids & Alkyl Peroxides

Results from the previous section indicate that in the absence of Catalase, the peracid group seems to be a far more effective oxidant than the peroxide group. Furthermore, the peracid group appears to oxidize Hg(0) to both Hg(I) and Hg(II). A study was undertaken to compare and evaluate the oxidizing ability of various commercially available peracids and peroxides including hydrogen peroxide, ethyl hydroperoxide, tert-butyl hydroperoxide, peracetic acid and m-chloroperbenzoic acid. The results of this investigation are summarized in Tables 1.1 & 1.2, perusal of these data reveal a number of interesting points. Firstly, the Hg(0) reactions with H_2O_2 , $\text{C}_2\text{H}_5\text{OOH}$ and t- $\text{C}_4\text{H}_9\text{OOH}$ show little oxidation

TABLE 11
Uptake and Oxidation of Elemental Mercury by
Alkyl Peroxides and Organic Peracids

Oxidizing Reagent ($\times 10^{-8}$ mol mL $^{-1}$)		CVAA Signal	
		Non-Reducing Conditions (ppb)	Reducing Conditions (ppb)
CH ₃ COOOH	1.40	40.3 $\pm$ 1.0	66.5 $\pm$ 2.6
	10.5	64.6 $\pm$ 0.0	218 $\pm$ 7
	17.5	105 $\pm$ 2	397 $\pm$ 8
	26.3	153 $\pm$ 3	528 $\pm$ 15
	35.1	237 $\pm$ 3	671 $\pm$ 6
2.90	36.8 $\pm$ 1.5	334 $\pm$ 25
	11.6	109 $\pm$ 7	(1.08 $\pm$ 0.01) $\times 10^3$
	19.3	189 $\pm$ 14	(2.10 $\pm$ 0.01) $\times 10^3$
	38.6	120 $\pm$ 5	(1.70 $\pm$ 0.01) $\times 10^3$
	193	28.7 $\pm$ 2.7	(1.93 $\pm$ 0.01) $\times 10^3$
CH ₃ COOH	23.3	42.6 $\pm$ 1.0	30.6 $\pm$ 0.6*
27.3	39.7 $\pm$ 1.9	32.8 $\pm$ 1.1
H ₂ O		41.0 $\pm$ 0.1	42.3 $\pm$ 1.0

* See note in Materials and Methods

All values represent mean $\pm$ SD of three estimations

TABLE 12
Uptake and Oxidation of Elemental Mercury by
Alkyl Peroxides and Organic Peracids

Oxidizing Reagent ($\times 10^{-8}$ mol mL ⁻¹)	CVAA Signal		
	Non-Reducing Conditions (ppb)	Reducing Conditions (ppb)	
CH ₃ COOOH	1.75	31.3 $\pm$ 1.1	117 $\pm$ 11
	10.5	64.2 $\pm$ 0.2	245 $\pm$ 5
	21.0	132 $\pm$ 1	574 $\pm$ 2
Cl COOOH	2.89	55.5 $\pm$ 1.1	351 $\pm$ 11
	11.6	476 $\pm$ 21	(1.70 $\pm$ 0.03) $\times 10^3$
	21.2	42.2 $\pm$ 1.6	(1.86 $\pm$ 0.04) $\times 10^3$
H ₂ O ₂	2.94	36.0 $\pm$ 0.6	33.6 $\pm$ 2.2
	11.8	30.8 $\pm$ 1.6	35.9 $\pm$ 4.9
	23.5	35.5 $\pm$ 1.7	31.7 $\pm$ 0.8
(CH ₃) ₃ COOH	2.59	36.1 $\pm$ 2.5	27.3 $\pm$ 0.4
	20.7	36.5 $\pm$ 1.3	93.5 $\pm$ 2.1
CH ₃ CH ₂ OOH	2.68	35.1 $\pm$ 1.6	25.3 $\pm$ 3.0
	10.7	33.9 $\pm$ 2.3	35.8 $\pm$ 2.0
	21.5	36.4 $\pm$ 1.4	27.1 $\pm$ 2.1

* All values represent mean $\pm$ SD of three estimations

and the results of these experiments reflect the diffusion controlled formation of a Hg^0 aqueous solution. In some cases (eg. [t-butyl hydroperoxide] $20.7 \times 10^{-8} \text{ mol mL}^{-1}$) the total mercury solution is greater than 63 ppb, but this is mostly in the mercuric form and there is no suggestion of the non-reducing CVAA signal exceeding, or approaching this value. These observations are consistent with the previous chemical and enzymatic uptake/oxidation experiments (Tables 9 & 10). The oxidations by the two peracids, in contrast, give substantial uptake and oxidation of mercury, in some cases approaching 2000 ppb. Thirdly, although most of this mercury signal appears only in the CVAA analysis under reducing conditions, and is therefore mercury in the Hg^{2+} oxidation state, oxidations both by peracetic acid and by m-chloroperbenzoic acid at several concentrations show CVAA signals under non-reducing conditions substantially in excess of 63 ppb. This observation shows the presence of the mercurous ion in these oxidations. Finally, within the concentration range studied, there appears to be an interesting contrast between oxidations by peracetic acid and m-chloroperbenzoic acid. Oxidations by peracetic acid show the formation of mercurous ion at $[\text{CH}_3\text{COOOH}] = 10.5 \text{ mol mL}^{-1}$ and at higher concentrations. This mercurous signal continually keeps increasing. In contrast, the oxidation with m-chloroperbenzoic acid also show the mercurous ion becoming evident at approximately $[\text{RCO}_3\text{H}] = 10 \text{ mol mL}^{-1}$, but then rising to a maximum value at approximately $[\text{RCO}_3\text{H}] = 20 \text{ mol mL}^{-1}$ and dropping at higher $[\text{RCO}_3\text{H}]$. At very high $[\text{RCO}_3\text{H}]$ the signal is once again submerged below the solubility limit of Hg^0 .

A number of conclusions appear to follow from these observations.

Firstly, the peracids are evidently far more effective oxidants than the peroxides and, since these "acids" are not particularly acidic ($pK_a = 7.1-8.2$ (70)), it appears likely that the carbonyl group may well be playing a role in the peracid oxidations. Although a cyclic mechanism involving mercury and the carbonyl group, such as shown in Figure 10a is an attractive notion, and obviously a mechanism restricted to the peracids and not the peroxides, the mechanism could be as simple as the displacement shown in Figure 10b. In this case the ease of displacing an acetate or benzoate group as opposed to hydroxide or alkoxide (71) could account for the greater oxidative capacity of the peracids.

The oxidation to the mercurous ion presents some intriguing questions. The consistent increase in the non-reducing CVAA signal with increasing $[CH_3CO_3H]$, in contrast to the rise and fall behaviour in the *m*-chloroperbenzoic acid oxidations is an interesting difference. However, the present data do not allow the determination of whether Hg^{2+} is arising directly from Hg^0 via a 2-electron oxidation (Figures 10 a & b) or whether it is primarily being formed via the mercurous ion. Simple differences in the values of the oxidation rate constants in the two oxidations would certainly account for the observations and the data do not require any fundamental difference in chemistry or mechanism.

The formation of mercurous ion in the peracid oxidations presents a mechanistic challenge apparently requiring two mercury atoms and one-electron shifts. A suggestion is made in Figure 10c in which one peracid molecule oxidizes two mercury atoms to the mercurous dimer. This is clearly speculative and doubtless other variations on this

mechanism, which bears some resemblance to Norrish photochemical mechanisms, could be considered, along with other substantially different mechanisms.

Figure 10 a

Figure 10 b

Figure 10 c

Chapter III: Oxidation of Homogeneous Elemental Mercury Solutions

Results to this point indicate several points: i) Mercury will diffuse and dissolve into solution up to a limit of approximately 60 ppb (aerobic conditions) ii) Various "oxidizing agents" will enhance the "uptake" of mercury above this solubility limit iii) Speciation of the mercury in solutions containing Catalase, Catalase/H₂O₂ or Catalase/alkyl peroxides indicates this enhanced "uptake" is due to the oxidation of the Hg⁰ to the Hg₂²⁺ or Hg²⁺ state, and iv) Presynthesized Catalase Compound I appears to be the most efficient at promoting uptake-oxidation of the elemental mercury to the divalent state.

One obvious and serious question seemed to arise from these results: was the "uptake" in fact an accurate reflection of the oxidation process? More specifically, to what extent do physical chemical factors such as surface properties of the solutions effect the degree and rate of dissolution of the mercury vapour into the test solutions? To circumvent the influence of these extraneous factors, consideration was given to the possibility of following the oxidation of mercury in homogeneous solution and perhaps developing this approach to the point of being able to obtain true kinetic data on the reaction. This would represent a completely new development in the investigation of the metabolic oxidation of elemental mercury. The idea of evaluating and elucidating reaction mechanisms via reaction kinetics is by no means a revolutionary approach, however the unique physical-chemical properties of elemental mercury make the study of even the fundamental kinetics of the oxidation of this element a challenge. Two major difficulties which had to be overcome were the problems of creating a homogeneous Hg(0) solution to

which various potential oxidizing agents could be added and the devising of a rapid analytical method which could monitor and identify any changes in the oxidation state of the mercury.

The Thunberg flasks used by Magos & Clarkson for their uptake experiments provided a novel diffusion technique for the preparation of homogeneous elemental mercury solutions. In this modified approach, a homogeneous $\text{Hg}(0)$ aqueous solution was prepared by simply allowing mercury vapour to diffuse into nitrogen purged water solvent from the side-arm of the flask. Changes in the oxidation state of the mercury in this pre-prepared $\text{Hg}(0)$ solution after treatment with various oxidizing agents could then be monitored.

The dynamics of kinetic studies are such that a continuous monitoring system is required to follow the conversion of reactant(s) to product(s). Perhaps the most useful tool for such studies is time-drive spectroscopy at the wavelength maxima of either product(s) or reactant(s). Unfortunately neither of the products, Hg_2^{2+} or Hg^{2+} , nor the substrate, Hg^0 , of interest to this study, have the necessary absorptivity in the U.V. or visible regions for the required sensitivity even if 10 cm absorption cells are used (72). The extinction coefficient of Hg^0 is so small ($7.35 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}$) that a minimum pathlength of 30 cm (17,18,49,73) must be used to monitor trace levels of gaseous Hg^0 at 253.7 nm in the CVAA apparatus. After careful consideration of the current methodology (16,18,20,74,75,76,77) available for the proposed kinetic study it became apparent that time-aliquot CVAA (non-reducing signal) sampling would give the most sensitive analysis of the $\text{Hg}^0 \longrightarrow \text{Hg}^{2+}$ oxidation process.

Preliminary studies were performed to assess the apparent requirement

for the presence of both hydrogen peroxide and Catalase in the oxidation of elemental mercury to the mercuric species. Figure 10 d illustrates the rate of decrease of the non-reducing volatile mercury signal of a homogeneous elemental mercury solution after treatment with Catalase, hydrogen peroxide, Catalase + hydrogen peroxide or simple phosphate buffer (as a control). Both the hydrogen peroxide and phosphate buffer controls indicate some loss of Hg^0 from solution, a phenomenon which will be discussed in detail in a later chapter. More interestingly however, is the apparent ambivalent behaviour of the substrate, Hg^0 , to the enzyme alone and to the enzyme in the presence of hydrogen peroxide. In parallel with the "uptake" experiments, Catalase + hydrogen peroxide is a much more efficient oxidizing combination than the enzyme alone. In contrast however, to the "uptake" experiments, Catalase alone seems to have a small but significant oxidizing effect on homogeneous Hg^0 solutions.

To explore the characteristics of the Catalase (no hydrogen peroxide) oxidation of elemental mercury an extensive series of experiments were performed varying either initial substrate concentration (Hg^0) or enzyme concentration. The results of these studies are shown in Figures 11-17 and summarized in Tables 13-15.

The major difficulty in analyzing these results arises from the fact that it is impossible to determine what fraction, if any, of the observed "non-reducing volatile mercury" signal arises from the formation of Hg_2^{2+} (which subsequently disproportionates in the CVAA apparatus) and what fraction accurately represents unreacted Hg^0 . If there is intermediary Hg_2^{2+} being formed then the overall rate of oxidation of Hg^0 to Hg^{2+} as determined from the decrease in the non-reducing CVAA signal will be

FIGURE 10 d: Oxidation of Hg^0 by $1.71 \times 10^{-11} \text{ mol mL}^{-1}$ Catalase in the absence (□—□) and presence (▼—▼) of $8.8 \times 10^{-9} \text{ mol mL}^{-1} \text{ H}_2\text{O}_2$. (●--●) H_2O_2 control (○--○) Phosphate buffer control.

FIGURE 11: Oxidation of Hg^0 by $1.7 \times 10^{-10} \text{ mol mL}^{-1}$ Catalase in the absence of H_2O_2

FIGURE 12:

Oxidation of Hg^0 by $1.37 \times 10^{-12} \text{ mol mL}^{-1}$
Catalase in the absence of H_2O_2

Figure 13:

Oxidation of Hg^0 by 1.37×10^{-12} mol mL⁻¹
Catalase in the absence of H_2O_2

Figure 14:

Oxidation of Hg^0 by $6.83 \times 10^{-12} \text{ mol mL}^{-1}$
Catalase in the absence of H_2O_2

Figure 15: Oxidation of Hg^0 by 6.83×10^{-12} mol mL $^{-1}$ Catalase in the absence of H_2O_2

Figure 16: Oxidation of Hg^0 by $1.37 \times 10^{-12} \text{ mol mL}^{-1}$ (—) and $1.71 \times 10^{-10} \text{ mol mL}^{-1}$ (—) Catalase in the absence of H_2O_2

Figure 17:

Oxidation of Hg^0 by 6.83×10^{-12} mol ml^{-1} Catalase in the absence of H_2O_2

artificially low (possibly 50% low due to $\text{Hg}_2^{2+} \longrightarrow \text{Hg}^0 + \text{Hg}^{2+}$).

A second but probably insignificant problem is the question of whether the observed decreases in the non-reducing signal actually arise from the "oxidation" of the substrate Hg^0 to Hg^{2+} (or Hg_2^{2+}), or are simply a binding or physical-chemical effect of the reagent Catalase on the Hg^0 , reducing the volatility of the Hg^0 but not actually altering its oxidation state. This last point was rejected since analysis of the mercury total concentration of the solution throughout the kinetic runs revealed minimal loss of total mercury implying the observed decreases in the non-reducing signal were truly a reflection of a change in the oxidation state of the mercury. If the possible existence of a Hg_2^{2+} intermediate is temporarily ignored then several points are indicated by the observed results: i) Oxidation of Hg^0 by Catalase will occur in the absence of H_2O_2 . ii) This oxidation occurs in a two-step manner; a rapid initial primary oxidation followed by a gradual secondary oxidation over several minutes and iii) The rate and extent of the oxidation is dependent on the relative concentrations of both the enzyme Catalase and the substrate Hg^0 . This latter point is illustrated in Figure 17 where the % oxidation of initial elemental mercury by $6.83 \times 10^{-12} \text{ mol mL}^{-1}$ Catalase is plotted as a function of the initial $[\text{Hg}^0]$. The relationship is by no means linear however a definite inverse relationship does appear to exist.

The reproducibility of this oxidation may be assessed by inspecting the data of Tables 13 & 14. The trend of increased oxidation with decreased initial $[\text{Hg}^0]$ is consistent, however the actual degree of oxidation (by a constant [Catalase]) at any specific initial substrate concentration

TABLE 13

Summary of Oxidation of Hg^0 by Native Catalase

[Catalase] (mol mL ⁻¹)	Initial [Hg^0] (mol mL ⁻¹)	% Oxidation of Initial Hg^0 after 3.5 minutes
1.37 x 10 ⁻¹²	11.1 x 10 ⁻¹¹	30.9
	7.83x 10 ⁻¹¹	36.3
	4.99x 10 ⁻¹¹	49.0
	2.24x 10 ⁻¹¹	62.2
6.83 x 10 ⁻¹²	11.7 x 10 ⁻¹¹	41.4
	8.95x 10 ⁻¹¹	45.3
	5.6 x 10 ⁻¹¹	48.2
	3.4 x 10 ⁻¹¹	60.3
6.83 x 10 ⁻¹²	11.7 x 10 ⁻¹¹	31.4
	9.27x 10 ⁻¹¹	37.6
	5.13x 10 ⁻¹¹	47.5
	3.39x 10 ⁻¹¹	73.5
6.83 x 10 ⁻¹²	9.87x 10 ⁻¹¹	40.4
	7.93x 10 ⁻¹¹	40.6
	4.64x 10 ⁻¹¹	49.2
	3.04x 10 ⁻¹¹	51.5
Control	8.89x 10 ⁻¹¹	7.3

TABLE 14

Summary of Oxidation of Hg^0 by Native Catalase

[Catalase] (mol mL ⁻¹)	Initial [Hg^0] (mol mL ⁻¹)	% Oxidation of Initial Hg^0 after 3.5 minutes
6.83×10^{-12}	10.7×10^{-11}	43.5
	7.75×10^{-11}	52.3
	5.5×10^{-11}	56.4
	1.9×10^{-11}	71.1
6.83×10^{-12}	11.0×10^{-11}	30.3
	7.88×10^{-11}	57.9
	5.08×10^{-11}	64.2
	2.54×10^{-11}	72.5
6.83×10^{-12}	10.8×10^{-11}	35.2
	7.33×10^{-11}	67.7
	5.08×10^{-11}	69.6
	2.44×10^{-11}	81.6

may vary by 10% (20% in the extreme of very low initial $[\text{Hg}^0]$).

Figures 11-12 and 16 illustrate the effect of performing the kinetic study under aerobic (Figures 11 & 12) and anerobic (Figure 16) conditions. Careful examination of these graphs reveals no observable difference in the kinetics of the oxidation under nitrogen purged or atmospheric conditions.

Finally, Figure 16 and Table 15 illustrate the significant effect of an approximately hundred fold increase in the concentration of Catalase on the extent of oxidation.

The most striking conclusion from this data is the apparent existence of two processes for the oxidation of elemental mercury by Catalase. The first slower mechanism occurs in the absence of hydrogen peroxide. This oxidation requires a low ratio of $[\text{Hg}^0]$ to $[\text{Catalase}]$ indicating the process is probably "non-catalytic". Moreover, the mechanism appears to proceed in a stepwise manner including an initial rapid oxidation, reflected by the steep shape of the first minute of the kinetic curve, followed by a slower subsequent oxidation which proceeds for several minutes. This oxidation in the absence of hydrogen peroxide is probably a redox type mechanism whereby the Catalase acts as an "electron sink". This mechanism is not "catalytic" in that each Catalase molecule after participating in the oxidation reaction is not returned to its native state thus oxidation will only approach 100% when excess ($\geq 100x$) Catalase is present. The apparent two mechanisms of oxidation, in the absence of H_2O_2 , indicated by the two steps of the kinetic curves may simply represent different rates of access to two separate electrophilic sites on the enzyme. Alternatively, the oxidation may be proceeding

TABLE 15

Summary of Oxidation of Hg^0 by Native Catalase

[Catalase] (mol mL ⁻¹)	Initial [Hg^0] (mol mL ⁻¹)	% Oxidation of Initial Hg^0 After:			
		0.25 min	1.0 min	3.0 min	6.0 min
1.37×10^{-12}	10.6×10^{-11}	11.3	20.2	36.2	57.7
	7.53×10^{-11}	4.6	20.5	31.7	41.7
	5.04×10^{-11}	11.9	22.8	38.6	60.4
	2.69×10^{-11}	20.4	33.3	44.4	66.7
1.71×10^{-10}	10.7×10^{-11}	49.8	51.2	57.7	79.8
	7.8×10^{-11}	39.7	41.0	50.6	76.9
	5.25×10^{-11}	42.9	48.6	67.6	-
	2.4×10^{-11}	52.1	72.9	96.7	100

by a two step mechanism involving the formation of a Hg (I) intermediate. This latter possibility is discussed further in Chapter V.

The second process by which homogeneous elemental mercury solutions may be oxidized is a catalytic mechanism involving Catalase and hydrogen peroxide. This oxidation is extremely rapid and approaches 100% conversion to Hg(II) even when $[Catalase] \ll [Hg^0]$.

An attempt at quantitative kinetic assessment of these two oxidation processes is made in Chapter V.

Oxidation of Homogeneous Hg(0) solutions by Presynthesized Catalase Compound I and Catalase Compound II

The considerable enhancement of the rate and extent of Hg^0 oxidation by the addition of hydrogen peroxide (Figure 10d) is a clear indication that in homogeneous solution, hydrogen peroxide has definite role to play in the oxidation of elemental mercury to mercury (II).

The logical extension of these "homogeneous" studies was to investigate the effect of treating Hg^0 (aq) solutions with presynthesized Catalase Compound I. Paralleling the "uptake" experiments performed at the onset of this study, this approach would circumvent the problem of elucidating which step of the Catalase/hydrogen peroxide oxidation/reduction process was involved in the oxidation of the elemental mercury. Figure 18 illustrates the results of treating ~20 ppb elemental mercury solutions with presynthesized peracetic acid-Catalase Compound I. Two points are indicated by these results: i) Native Catalase at concentrations 100x less than the elemental mercury substrate shows little detectable oxidation above control levels ii) Presynthesized Compound I rapidly oxidizes

Figure 18: Oxidation of Hg^0 by Pre-synthesized Catalase/Peracetic Acid Compound I

$(\bullet-\bullet)$ $1.23 \times 10^{-11} \text{ mol mL}^{-1}$, $(\square-\square)$ $0.613 \times 10^{-11} \text{ mol mL}^{-1}$, $(\blacktriangle-\blacktriangle)$ $0.123 \times 10^{-11} \text{ mol mL}^{-1}$,

$(\circ-\circ)$ Phosphate buffer/peracetic acid control.

elemental mercury, the rate and extent of oxidation depending on the relative concentrations of Compound I and Hg^0 .

Treatment of Catalase Compound I with excess alkyl peroxide has been shown to result in a one-electron reduction of Compound I to a non-radical low spin iron (IV) species known as Compound II (62, 68, 69):

$2.39 \mu\text{M}$ native Catalase was treated with $4.78 \mu\text{M}$ peracetic acid to form Compound I. Figures 19 & 20 illustrate the overlay of the visible spectra of the initial native Catalase and the resulting Compound I. Subsequent treatment of the same native Catalase with $19 \mu\text{M}$ peracetic acid yielded the species shown in Figures 21 & 22. The dominating characteristic of this spectrum is the shift of the Soret band from 405 nm (for native Catalase) and 399 nm (for Compound I) to 427 nm . This shift coupled with the apparent lack of charge transfer bands in the visible region lead to the positive identification of this species as the aforementioned Compound II.

The one-electron structural difference between the Compound I and

Figure 19: Visible absorption spectra of Native Catalase and Catalase Compound I

W	Abs	Source	WAVE
700.0	4.6484	Visible	Scan

Figure 20: Visible absorption spectra of Native Catalase and Catalase

Compound I

λ	Abs	SOURCE	MODE
700.0	0.0547	Visible	Scan

Figure 21: Visible absorption spectra of Native Catalase and Catalase

WAVELENGTH	WAVELENGTH	WAVELENGTH
400.0	415.0	430.0
445.0	460.0	475.0
490.0	500.0	515.0

Figure 22: Visible absorption spectra of Native Catalase and Catalase Compound II

Compound II species presented a useful tool for investigating and possibly identifying the portion of the enzyme complex responsible for the oxidation of the mercury. Specifically, if presynthesized Compound I did oxidize but presynthesized Compound II did not oxidize $\text{Hg}(0)$ to $\text{Hg}(\text{II})$, then the porphyrin π -radical of Compound I would be implicated as being principally involved in the oxidation mechanism. The results of the treatment of 20 ppb homogeneous $\text{Hg}(0)$ solutions with presynthesized Compound II are shown in Figure 23. Clearly Compound II is equally capable of rapid Hg^0 oxidation and in fact superimposition of Figures 18 & 23 reveal little if any difference in Hg^0 oxidation by Catalase Compound I or Catalase Compound II. This result lends further evidence to the hypothesis that it is the

portion of Compound I which is principally responsible for the oxidation of Hg^0 .

End product inhibition studies were also carried out by adding 30-50 fold excess of Hg^{2+} to the Hg^0 solution before treatment with Catalase or Catalase/ H_2O_2 . No effect on the rate of Hg^0 oxidation was observed.

One serious question which arises from the results presented in this chapter is the possible intermediacy of the $\text{Hg}(\text{I})$ species in the oxidation of $\text{Hg}(0)$ to $\text{Hg}(\text{II})$. More specifically, is Catalase or Catalase Compound I capable of oxidizing Hg_2^{2+} and if so how do the kinetics of this oxidation qualitatively compare to those of the Hg^0 oxidation process? This question is addressed in Chapter IV.

Figure 23: Oxidation of Hg^0 by Pre-synthesized Catalase/peracetic acid Compound II
 (Δ) $1.23 \times 10^{-11} \text{ mol mL}^{-1}$, ($\bullet$) $0.613 \times 10^{-11} \text{ mol mL}^{-1}$, ($\square$) $0.306 \times 10^{-11} \text{ mol mL}^{-1}$,
 ($\circ$) $0.123 \times 10^{-11} \text{ mol mL}^{-1}$, and by (Δ) $0.123 \times 10^{-11} \text{ mol mL}^{-1}$ native Catalase.
 ($\circ-\circ$) Phosphate buffer/peracetic acid control

2

Chapter IV: Oxidation of Homogeneous Hg_2^{2+} Solutions by Catalase and and Catalase Compound I

Oxidation of Hg_2^{2+} by Catalase and Catalase/ H_2O_2

The question of the possible intermediacy of the Hg_2^{2+} species in the oxidation of Hg^0 to Hg^{2+} by Catalase or Catalase Compound I formed the basis for the kinetic studies presented in this section. It was hoped that an investigation of the oxidation of Hg_2^{2+} by these enzyme species and comparison to the previously discussed Hg^0 oxidation would shed some light on the details of the reaction mechanism. This approach was facilitated by the volatile behaviour of mercurous solutions in the CVAA apparatus under non-reducing conditions and by the high solubility of the Hg(I) species compared to that of Hg(0) .

An extensive series of experiments were performed to investigate the reaction, if any, between Hg_2^{2+} and Catalase or Catalase/ H_2O_2 .

The results of these studies are shown in Figures 24-32. The major difficulty in analyzing these results is evaluating the significance of a decrease in the non-reducing signal from a mercurous solution.

Chapter I discussed in detail the behaviour of mercurous solutions in the CVAA apparatus including the effects of the presence of chelating ligands such as proteins. Clearly the possibility that the observed decreases in the non-reducing signal arose simply from binding to the enzyme cannot be ignored, however an argument for oxidation is also merited. Careful examination of these figures reveals a parallel to the results observed for Hg^0 solutions. Specifically, catalase reduces the non-reducing signal (above control levels) when the enzyme is present in

quantities where $[\text{Hg}_2^{2+}] : [\text{Catalase}]$ is less than 20. The Catalase/ H_2O_2 combination however, produces a much more dramatic decrease at levels 100x less than the substrate Hg_2^{2+} . A quantitative assessment of this kinetic data is attempted in Chapter V.

In all of these studies analysis of Hg^{Total} before and after the kinetic run revealed minimal loss of mercury from the solution.

Three mechanisms exist which may account for the observed results. The first is a binding of the Hg_2^{2+} to the enzyme, probably the apoprotein, resulting in decreased disproportionation of the Hg_2^{2+} in the CVAA apparatus and thus less volatile mercury under non-reducing conditions. Alternatively, the enzyme (native Catalase or Compound I) may be undergoing a one electron redox reaction with the mercurous species to yield non-volatile mercuric ion. Finally, the enzyme may be driving the disproportionation of the mercurous dimer to form Hg^0 and Hg^{2+} and then subsequently oxidizing the Hg^0 to Hg^{2+} as was discussed in Chapter III. Of these three mechanisms the first is the least likely in view of the substantial decreases in the non-reducing signal even when $[\text{Hg}_2^{2+}] \gg [\text{Compound I}]$. Of the other two mechanisms, it is quite possible that the observed behaviour is due to a combination of both of these processes although the speed of the reaction indicates enzymatic oxidation of the Hg_2^{2+} species is probably the dominating process. This enzymatic oxidation mechanism is further supported by the typical "enzyme" behaviour of the system: i) substantial decreases in the volatile signal occur even when $[\text{Hg}_2^{2+}] \gg [\text{Catalase}]$; and ii) the rate and extent of the drop in the non-reducing signal depends on the relative concentrations of Catalase, hydrogen peroxide and mercurous ion.

Figure 24: Oxidation of Hg_2^{2+} by Catalase in the absence of H_2O_2

Figure 25: Oxidation of Hg_2^{2+} by Catalase in the presence of $7.20 \times 10^{-10} \text{ mol mL}^{-1} \text{ H}_2\text{O}_2$.

Figure 26:

Oxidation of Hg_2^{2+} by Catalase in the presence of
 $1.80 \times 10^{-7} \text{ mol mL}^{-1} \text{ H}_2\text{O}_2$

Figure 27: Oxidation of Hg_2^{2+} by Catalase in the absence of H_2O_2

Figure 28: Oxidation of Hg_2^{2+} by Catalase in the presence of 7.20×10^{-10} mol mL $^{-1}$ H_2O_2

Figure 29:

Oxidation of Hg_2^{2+} by Catalase in the presence of 1.80×10^{-7} mol mL⁻¹ H_2O_2

Figure 30: Oxidation of Hg_2^{2+} by Catalase in the absence of H_2O_2

Figure 31:

Oxidation of Hg_2^{2+} by Catalase in the presence of
 $7.20 \times 10^{-10} \text{ mol mL}^{-1} \text{ H}_2\text{O}_2$

Figure 32 :

Oxidation of Hg_2^{2+} by Catalase in the presence of
 1.80×10^{-7} mol mL⁻¹ H_2O_2

Effect of 3-Amino-1,2,4-triazole on the Oxidation of Hg_2^{2+} by Catalase
and Catalase Compound I

Tephly et al. (78) have shown that the herbicide 3-amino-1,2,4-triazole (AT) reacts with the Catalase-hydrogen peroxide Compound I intermediate to become irreversibly bound to this enzyme complex resulting in enzyme inactivation. These workers found the degree of inhibition to be dependent on the availability of hydrogen peroxide and thus liver Catalase is much more sensitive to AT than erythrocyte Catalase due to the inherent ability of liver cells to generate hydrogen peroxide. The possible involvement of Catalase Compound I in the oxidation and uptake of mercury in erythrocytes prompted Nielsen-Kudsk (25) to investigate the effect of AT on $\text{Hg}(0)$ oxidation. This author's results indicated the high rate of mercury uptake and oxidation, in vitro, in human erythrocytes in the presence of a hydrogen peroxide generating system is unaffected by the presence of AT. This initially lead Nielsen-Kudsk to conclude that the Catalase Compound I species was not involved in the oxidation of elemental mercury. Later, in vitro studies by this same author (79) with liver Catalase revealed a distinct inhibition effect of AT on elemental mercury uptake and oxidation by liver Catalase in the presence of hydrogen peroxide. Studies of this curious ambivalent behaviour of the two Catalase isozymes in vitro were extended by Magos et al. (26) to in vivo investigations of the effect of AT on the inhalation, oxidation and accumulation of mercury in rats. These workers confirmed Nielsen-Kudsk's observations that AT does not effect the in vitro mercury uptake of red blood cells exposed to mercury vapour. The in vivo experiments produced a surprising finding in that the mercury content of livers of AT treated rats actually increased. This was in

contrast to their in vitro observation, which confirmed Nielsen-Kudsk's (79) results that AT inhibited mercury uptake and oxidation in liver homogenates. These peculiar results have since been further complicated by results from the study of the effect of AT on the in vitro uptake and oxidation of $\text{Hg}(0)$ by mushroom Catalase extracts which indicated 94% inhibition by AT (38).

In this study the effect of AT on the oxidation of mercurous solutions by Catalase and Catalase/ H_2O_2 was investigated. Typical results of these experiments are shown in Figures 33-36. Despite substantial variations of $[\text{H}_2\text{O}_2]$, [Catalase], and $[\text{Hg}_2^{2+}]$ no conditions were found where AT consistently produced a detectable effect on the oxidation process. These results may reflect a non-involvement of the "peroxide active" portion of Compound I in the oxidation of Hg_2^{2+} to Hg^{2+} , but it is more likely that the non-effect of AT is due to insufficient incubation time of the AT with the Catalase/ H_2O_2 mixture to produce enough inactive enzyme species (26). This would indicate that on simultaneous mixing, Hg_2^{2+} reacts with Catalase Compound I at a rate equal to, or quicker than that of AT.

Figure 33:

Oxidation of Hg_2^{2+} by Catalase in the absence of H_2O_2 and AT

Figure 34:

Oxidation of Hg_2^{2+} by Catalase in the presence of
 $2.43 \times 10^{-6} \text{ mol mL}^{-1}$ AT.

Figure 35: Oxidation of Hg_2^{2+} by Catalase in the presence of $2.89 \times 10^{-7} \text{ mol mL}^{-1} \text{ H}_2\text{O}_2$

Figure 36:

Oxidation of Hg_2^{2+} by Catalase in the presence of
 $2.89 \times 10^{-7} \text{ mol mL}^{-1} \text{ H}_2\text{O}_2$ and $2.43 \times 10^{-6} \text{ mol mL}^{-1} \text{ AT}$

Chapter V: Attempts at Quantitative Kinetic Evaluation of the
Oxidation of Hg^0 or Hg_2^{2+} by Catalase and/or Catalase Compound I

The temptation to derive some quantitative kinetic description (i.e. K , V_{max} , etc.) of the oxidation of elemental mercury by Catalase, Compound I or Compound II from the enormous volume of data collected in this study must be tempered with a consideration of the conditions and assumptions utilized during the derivation of the mathematical models available. In view of the ambiguity of the non-reducing CVAA signal and the complexity of the kinetics of the normal Catalase-peroxide reaction, quantitative analysis of the kinetics of Hg^0 and Hg_2^{2+} oxidation by Catalase and Catalase-peroxide will be approached with a great deal of caution.

For an enzymic reaction, initial velocity is defined as the reaction rate at the early phase of catalysis during which the formation of product is linear with respect to time. This linear phase is achieved when the enzyme and substrates reach a steady state or quasi equilibrium. Three other assumptions basic to the derivation of initial rate equations for enzymatic reactions are: i) the enzyme and substrate form a complex, ii) the substrate concentration is much greater than the enzyme concentration, so that free substrate concentration is equivalent to the total concentration and the amount of product formed is small so that the reverse reaction or product inhibition is negligible and iii) during the reaction pH, temperature and ionic strength remain constant.

The kinetic experiments discussed in Chapters III and IV fulfilled

few if any of these steady-state assumptions. Perhaps the most serious problem encountered in these studies was the limited solubility of Hg^0 in aqueous solution which resulted in a very narrow working substrate concentration range. Limited oxidation was observed when Catalase concentrations were brought down to the 10^{-9} - 10^{-10} M region, however, these conditions represented a $[\text{Hg}^0] : [\text{Catalase}]$ ratio of less than 20, approximately five-fold less than the accepted ratio for enzyme kinetic studies. For these reasons the possibility of fitting the accumulated kinetic data to a steady-state enzymatic model was not pursued.

Given the numerous restraints inherently placed on the quantitative interpretation of this kinetic data the most that may be realistically determined from these experiments is an approximate order of magnitude for the rate constants for the oxidation of Hg^0 and Hg_2^{2+} by Catalase or Catalase Compound I. The inappropriateness of an enzymatic steady-state approach forced a purely physical chemical kinetic evaluation of the four reactions: $\text{Hg}^0 + \text{Catalase}$, $\text{Hg}_2^{2+} + \text{Catalase}$, $\text{Hg}^0 + \text{Compound I}$ and $\text{Hg}_2^{2+} + \text{Compound I}$.

Preliminary attempts were made to determine if the observed kinetic curves represented zero, first or second order reactions.

Plots of $\ln \frac{[\text{Hg}^0_{t=0}]}{[\text{Hg}^0_{t=t_1}]} = kt_1$ (1st order) and

$$\frac{1}{[\text{Hg}^0_{t=0}] - [\text{Catalase}_{t=0}]} \ln \frac{[\text{Catalase}_{t=0}] [\text{Hg}^0_{t=t_1}]}{[\text{Hg}^0_{t=0}] [\text{Hg}^0_{t=t_1}]} = k_2 t_1 \quad (2\text{nd order})$$

produced scatter plots and indicated the reactions could probably not be trusted to progress to any appreciable extent without becoming involved in labyrinthine complications. This latter point is further evidenced by the steep-shallow dual characteristics of the individual kinetic curves.

For complicated reactions it is often desirable to utilize the initial reaction rate method for surveying the kinetics. This approach focuses solely on the initial reaction rate and the effect of reactant concentrations on the magnitude of that rate, so that:

$$\frac{d x}{d t} = k a^{n_1} b^{n_2} c^{n_3} \dots$$

where $\frac{d x}{d t}$ is the initial rate, k is the rate constant and a, b, c are

the initial reactant concentrations.

For reactions investigated in this study the mathematical relationships are:

$$v = \frac{-d [\text{Hg}^0]}{d t} = k [\text{Hg}^0]^{n_1} [\text{Catalase}]^{n_2}$$

$$v = \frac{-d [\text{Hg}^0]}{d t} = k [\text{Hg}^0]^{n_1} [\text{Compound I}]^{n_2}$$

$$v = \frac{-d [\text{Hg}_2^{2+}]}{d t} = k [\text{Hg}_2^{2+}]^{n_1} [\text{Catalase}]^{n_2}$$

$$v = \frac{-d [\text{Hg}_2^{2+}]}{d t} = k [\text{Hg}_2^{2+}]^{n_1} [\text{Compound I}]^{n_2}$$

Here if n_2 is found to be 0 then the reaction is 1st order with respect to initial mercury. Conversely, if n_1 is found to be 0 then the reaction is 1st order with respect to enzyme. If $n_1 = n_2 = 1$ then the reaction is second order overall and 1st order in both initial [mercury] and [enzyme].

Several assumptions were made in fitting the experimental data to this initial-rate model: i) $\frac{d[\text{Hg}]}{dt}$ was taken as the change

in the non-reducing signal after the first minute of the reaction,

ii) for Hg_2^{2+} , a constant degree of disproportionation in the CVAA apparatus under non-reducing conditions was assumed so that the observed volatile signal was taken as being representative of non-oxidized Hg_2^{2+} ,

iii) correlation of the data to the mathematical model was assessed

by least-squares linear regression analysis, iv) presynthesized

Catalase Compound I (from peracetic acid) was utilized for the Hg^0

kinetic studies, however, Hg_2^{2+} Compound I oxidations were performed

by treating native Catalase with 10^5 fold excess H_2O_2 at the initiation

of the Hg_2^{2+} oxidation reaction with the assumption that maximum

formation of Compound I would occur, v) the non-reducing volatile

mercury signal observed during the oxidation of Hg^0 by Catalase or

Catalase Compound I was taken to quantitatively represent unreacted

Hg^0 and was assumed not to arise from the disproportionation of any

Hg_2^{2+} possibly formed during the reaction.

The results of the initial-rate interpretation of the kinetic data are listed in Tables 16-36 and summarized in Tables 37-38.

Several conclusions may be drawn from these data: i) oxidation of

Hg^0 and Hg_2^{2+} by native Catalase appears to be approximately 1st order, with respect to initial mercury concentration and Hg^0 oxidation is also 1st order with respect to Catalase concentration. The poor correlation ($r=0.29$) of Hg_2^{2+} oxidation to a 1st order reaction with native Catalase is particularly interesting in view of the much better fit of the data to a 2nd order ($r=0.86$) treatment. Whether this behaviour is indicative of a mixed-order reaction or simply reflects the small but often significant error in single CVA analysis at very low mercury concentrations can only be speculated. Comparison of the 1st and 2nd order rate constants for native Catalase oxidation of Hg^0 and Hg_2^{2+} reveals an interesting result in that Hg_2^{2+} is consistently oxidized at a faster rate than Hg^0 . This result essentially eliminates the possibility that the two step characteristic of the Hg^0 kinetic curves is due to a primary oxidation to Hg_2^{2+} and a subsequent slower oxidation to Hg^{2+} . Furthermore, this result points to a non-catalytic redox type reaction of these two mercury species with the native enzyme. In such a mechanism the direct one electron oxidation of Hg(I) to Hg(II) would proceed at a faster rate than the two electron oxidation of Hg(0) to Hg(II) . ii) Examination of the rate constants for the oxidation of Hg^0 and Hg_2^{2+} by Catalase Compound I indicates a less ambiguous situation. The reaction appears to be first order in mercury substrate, first order in Catalase Compound I and second order overall. Comparison of the 1st and 2nd order rate constants for native Catalase and Catalase Compound I oxidation of either Hg^0 or Hg_2^{2+} reveals that: a) Compound I oxidation of Hg^0 proceeds at a rate approximately 40x that of native Catalase oxidation. b) Hg_2^{2+}

oxidation by Compound I is also more efficient than native Catalase oxidation, however, this enhancement of the reaction rate is not as pronounced as that observed for Hg^0 . This may be a reflection of not using presynthesized Compound I. c) The magnitude of the 2nd order rate constant for Compound I oxidation of Hg^0 or Hg_2^{2+} ($\approx 10^6 \text{ M}^{-1} \text{ sec}^{-1}$) is about 10 fold lower than the 2nd order rate constant ($\approx 10^7 \text{ M}^{-1} \text{ sec}^{-1}$) for the reaction of Catalase with H_2O_2 (66). Thus it is doubtful that the in vivo oxidation of dissolved Hg^0 by Compound I interferes to any great extent with the normal Catalase detoxication reaction of H_2O_2 in the body.

The similarity in the values obtained for the 2nd order rate constants for Compound I oxidation of Hg^0 ($k = 6.0 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$) and Hg_2^{2+} ($k = 6.96 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$) again indicates that the two step characteristic of the Hg^0 oxidation curves is probably not due to oxidation to Hg(I) and then subsequent oxidation to Hg(II) . The observed rate constants imply that such a mechanism would probably proceed at a constant rate thus exhibiting an almost linear oxidation curve. For Hg_2^{2+} , enzymatic oxidation to Hg_2^{2+} by Compound I probably occurs via a two step mechanism; disproportionation to Hg^{2+} and Hg^0 and then subsequent oxidation of this resulting Hg^0 to Hg^{2+} by the same mechanism through which dissolved Hg^0 is oxidized. In this mechanism the rate determining step would be the enzymatic oxidation of Hg^0 to Hg^{2+} which would also be the driving force for the disproportionation reaction:

TABLE 16

1st Order Treatment: Constant $[\text{Catalase}] = 6.83 \times 10^{-9} \text{ M}$

$$\frac{d[\text{Hg}^0]}{dt} = k[\text{Hg}^0]$$

Initial $[\text{Hg}^0]_{t=0}$ (M)	$[\text{Hg}^0]_{t=1\text{min}}$ (M)	$\frac{d[\text{Hg}^0]}{dt}$ (M min^{-1})
10.7×10^{-8}	7.7×10^{-8}	3.25×10^{-8}
7.75×10^{-8}	5.1×10^{-8}	2.65×10^{-8}
5.5×10^{-8}	3.4×10^{-8}	2.1×10^{-8}
1.9×10^{-8}	1.1×10^{-8}	0.8×10^{-8}

Linear regression analysis: $k = \text{slope} = 2.78 \times 10^{-1} \text{ min}^{-1}$
correlation coefficient = 0.99

TABLE 17

1st Order Treatment:

Constant [Catalase] = 1.37×10^{-9} M

$$\frac{d[\text{Hg}^0]}{dt} = k[\text{Hg}^0]$$

Initial $[\text{Hg}^0]_{t=0}$ (M)	$[\text{Hg}^0]_{t=1\text{min}}$ (M)	$\frac{d[\text{Hg}^0]}{dt}$ (M min ⁻¹)
10.7×10^{-8}	8.4×10^{-8}	2.3×10^{-8}
7.6×10^{-8}	5.5×10^{-8}	2.1×10^{-8}
5.1×10^{-8}	3.9×10^{-8}	1.2×10^{-8}
2.7×10^{-8}	1.8×10^{-8}	0.9×10^{-8}

Linear regression analysis: $k = \text{slope} = 2.9 \times 10^{-1} \text{ min}^{-1}$
correlation coefficient = 0.96

TABLE 18

1st Order Treatment:

Constant [Catalase] = $1.71 \times 10^{-7} \text{ M}$

$$\frac{d [\text{Hg}^0]}{d t} = k [\text{Hg}^0]$$

Initial $[\text{Hg}^0]_{t=0}$ (M)	$[\text{Hg}^0]_{t=1 \text{ min.}}$ (M)	$\frac{d [\text{Hg}^0]}{d t}$ (M min ⁻¹)
10.7×10^{-8}	5.4×10^{-8}	5.3×10^{-8}
7.8×10^{-8}	4.8×10^{-8}	3.0×10^{-8}
5.3×10^{-8}	2.8×10^{-8}	2.5×10^{-8}
2.4×10^{-8}	0.65×10^{-8}	1.75×10^{-8}

Linear regression analysis: $k = \text{slope} = 4.1 \times 10^{-1} \text{ min}^{-1}$

correlation coefficient = 0.95

TABLE 19

1st Order Treatment: Constant $[\text{Hg}^0]_{t=0} = 10.7 \times 10^{-8} \text{ M (21.4 ppb)}$

$$\frac{d[\text{Hg}^0]}{dt} = k [\text{Catalase}]$$

$[\text{Catalase}]$ (M)	$\frac{d[\text{Hg}^0]}{dt}$ (M min ⁻¹)
----------------------------	--

1.37×10^{-9}

2.3×10^{-8}

6.83×10^{-9}

3.25×10^{-8}

1.71×10^{-7}

5.3×10^{-8}

Linear regression analysis: $k = \text{slope} = 1.5 \times 10^{-1} \text{ min}^{-1}$
correlation coefficient = 0.96

TABLE 20

1st Order Treatment:

$$\text{Constant } [\text{Hg}^0]_{t=0} = 7.6 \times 10^{-8} \text{ M (15.1 ppb)}$$

$$\frac{d [\text{Hg}^0]}{d t} = k [\text{Catalase}]$$

[Catalase]
(M)

$\frac{d [\text{Hg}^0]}{d t}$ (M min⁻¹)

$$1.37 \times 10^{-9}$$

$$2.1 \times 10^{-8}$$

$$6.83 \times 10^{-9}$$

$$2.65 \times 10^{-8}$$

$$1.71 \times 10^{-7}$$

$$3.0 \times 10^{-8}$$

Linear regression analysis:

$$k = \text{slope} = 3.82 \times 10^{-2} \text{ min}^{-1}$$

$$\text{correlation coefficient} = 0.81$$

TABLE 21

1st Order Treatment:

Constant $[\text{Hg}^0]_{t=0} = 5.2 \times 10^{-8} \text{ M (10 ppb)}$

$$\frac{d[\text{Hg}^0]}{dt} = k[\text{Catalase}]$$

[Catalase]
(M)

$\frac{d[\text{Hg}^0]}{dt}$ (M min⁻¹)

1.37×10^{-9}

1.2×10^{-8}

6.83×10^{-9}

2.1×10^{-8}

1.71×10^{-7}

2.5×10^{-8}

Linear regression analysis:

$k = \text{slope} = 5.22 \times 10^{-2} \text{ min}^{-1}$

correlation coefficient = 0.76

TABLE 22

1st Order Treatment: Constant $[\text{Hg}^0]_{t=0} = 2.5 \times 10^{-8} \text{ M}$ (5 ppb)

$$\frac{d[\text{Hg}^0]}{dt} = k [\text{Catalase}]$$

[Catalase]
(M)

$\frac{d[\text{Hg}^0]}{dt}$ (M min⁻¹)

1.37×10^{-9}

0.9×10^{-8}

6.83×10^{-9}

1.3×10^{-8}

1.71×10^{-9}

1.75×10^{-8}

Linear regression analysis:

$k = \text{slope} = 3.95 \times 10^{-2} \text{ min}^{-1}$

correlation coefficient = 0.90

TABLE 23

2nd Order Treatment:

$$\frac{d [\text{Hg}^0]}{d t} = k [\text{Hg}^0] [\text{Catalase}]$$

[Catalase] (M)	Initial $[\text{Hg}^0]_{t=0}$ (M)	[Catalase] $[\text{Hg}^0]_{t=0}$ (M ²)	$\frac{d [\text{Hg}^0]}{d t}$ (M min ⁻¹)
6.83 x 10 ⁻⁹	10.7 x 10 ⁻⁸	7.31 x 10 ⁻¹⁶	3.25 x 10 ⁻⁸
6.83 x 10 ⁻⁹	7.75 x 10 ⁻⁸	5.29 x 10 ⁻¹⁶	2.65 x 10 ⁻⁸
6.83 x 10 ⁻⁹	5.5 x 10 ⁻⁸	3.76 x 10 ⁻¹⁶	2.1 x 10 ⁻⁸
6.83 x 10 ⁻⁹	1.9 x 10 ⁻⁸	1.30 x 10 ⁻¹⁶	0.8 x 10 ⁻⁸
1.37 x 10 ⁻⁹	10.7 x 10 ⁻⁸	1.47 x 10 ⁻¹⁶	2.3 x 10 ⁻⁸
1.37 x 10 ⁻⁹	7.6 x 10 ⁻⁸	1.03 x 10 ⁻¹⁶	2.1 x 10 ⁻⁸
1.37 x 10 ⁻⁹	5.1 x 10 ⁻⁸	0.69 x 10 ⁻¹⁶	1.2 x 10 ⁻⁸
1.37 x 10 ⁻⁹	2.7 x 10 ⁻⁸	0.37 x 10 ⁻¹⁶	0.9 x 10 ⁻⁸
1.71 x 10 ⁻⁷	10.7 x 10 ⁻⁸	1.83 x 10 ⁻¹⁴	5.3 x 10 ⁻⁸
1.71 x 10 ⁻⁷	7.8 x 10 ⁻⁸	1.33 x 10 ⁻¹⁴	3.0 x 10 ⁻⁸
1.71 x 10 ⁻⁷	5.3 x 10 ⁻⁸	8.98 x 10 ⁻¹⁵	2.5 x 10 ⁻⁸
1.71 x 10 ⁻⁷	2.4 x 10 ⁻⁸	4.1 x 10 ⁻¹⁵	1.75 x 10 ⁻⁸

Linear regression analysis:

$$k = \text{slope} = 1.49 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$$

correlation coefficient = 0.76

TABLE 24

2nd Order Treatment:

$$\frac{d [\text{Hg}^0]}{d t} = k [\text{Hg}^0] [\text{Compound I}]$$

[Compound I] (M)	Initial [Hg ⁰] _{t=0} (M)	[Compound I] [Hg ⁰] (M ²)	$\frac{d [\text{Hg}^0]}{d t}$ (M min ⁻¹)
1.23 x 10 ⁻⁸	9.3 x 10 ⁻⁸	11.4 x 10 ⁻¹⁶	9.25 x 10 ⁻⁸
0.61 x 10 ⁻⁸	9.0 x 10 ⁻⁸	3.82 x 10 ⁻¹⁶	6.27 x 10 ⁻⁸
0.31 x 10 ⁻⁸	8.3 x 10 ⁻⁸	1.07 x 10 ⁻¹⁶	3.5 x 10 ⁻⁸
0.123 x 10 ⁻⁸	8.5 x 10 ⁻⁸	0.25 x 10 ⁻¹⁶	2.0 x 10 ⁻⁸

Linear regression analysis:

$$k = \text{slope} = 6.04 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$$

correlation coefficient = 0.96

TABLE 25

1st Order Treatment: Constant [Catalase] = $9.95 \times 10^{-9} \text{ M}$

$$\frac{d [\text{Hg}_2^{2+}]}{d t} = k [\text{Hg}_2^{2+}]$$

Initial $[\text{Hg}_2^{2+}]_{t=0}$ (M)	$[\text{Hg}_2^{2+}]_{t=1 \text{ min}}$ (M)	$\frac{d [\text{Hg}_2^{2+}]}{d t} (\text{M min}^{-1})$
11.9×10^{-8}	8.2×10^{-8}	3.7×10^{-8}
30.5×10^{-8}	22.3×10^{-8}	8.2×10^{-8}
213×10^{-8}	129×10^{-8}	84×10^{-8}

Linear regression analysis:

$$k = \text{slope} = 4.06 \times 10^{-1} \text{ min}^{-1}$$

$$\text{correlation coefficient} = 0.99$$

TABLE 26

1st Order Treatment:

$$\text{Constant}[\text{Catalase}] = 5.05 \times 10^{-9} \text{ M}$$

$$\frac{d[\text{Hg}_2^{2+}]}{dt} = k[\text{Hg}_2^{2+}]$$

Initial $[\text{Hg}_2^{2+}]_{t=0}$ (M)	$[\text{Hg}_2^{2+}]_{t=1 \text{ min}}$ (M)	$\frac{d[\text{Hg}_2^{2+}]}{dt}$ (M min ⁻¹)
11.9×10^{-8}	8.6×10^{-8}	3.3×10^{-8}
31.0×10^{-8}	24.9×10^{-8}	6.1×10^{-8}
202×10^{-8}	138×10^{-8}	64×10^{-8}

Linear regression analysis:

$$k = \text{slope} = 3.27 \times 10^{-1} \text{ min}^{-1}$$

$$\text{correlation coefficient} = 0.99$$

TABLE 27

1st Order Treatment:

Constant [Catalase] = 1.02×10^{-9} M

$$\frac{d[\text{Hg}_2^{2+}]}{dt} = k [\text{Hg}_2^{2+}]$$

Initial $[\text{Hg}_2^{2+}]_{t=0}$
(M)

$[\text{Hg}_2^{2+}]_{t=1\text{min}}$
(M)

$\frac{d[\text{Hg}_2^{2+}]}{dt}$ (M min⁻¹)

 12.6×10^{-8} 9.0×10^{-8} 3.6×10^{-8} 30.3×10^{-8} 22.7×10^{-8} 7.6×10^{-8} 198×10^{-8} 144×10^{-8} 54×10^{-8}

Linear regression analysis:

 $k = \text{slope} = 2.74 \times 10^{-1}$

correlation coefficient = 0.99

TABLE 28

1st Order Treatment:

$$\text{Constant } [\text{Hg}_2^{2+}]_{t=0} = 11.9 \times 10^{-8} \text{ M}$$

$$\frac{d [\text{Hg}_2^{2+}]}{d t} = k [\text{Catalase}]$$

[Catalase]
(M)

$\frac{d [\text{Hg}_2^{2+}]}{d t}$ (M min⁻¹)

9.95×10^{-9}

3.7×10^{-8}

5.05×10^{-9}

3.3×10^{-8}

1.02×10^{-9}

3.6×10^{-8}

Linear regression analysis:

$$k = \text{slope} = 1.37 \times 10^{-1} \text{ min}^{-1}$$

$$\text{correlation coefficient} = 0.29$$

TABLE 29

1st Order Treatment:

$$\text{Constant } [\text{Hg}_2^{2+}]_{t=0} = 30.3 \times 10^{-8} \text{ M}$$

$$\frac{d[\text{Hg}_2^{2+}]}{dt} = k [\text{Catalase}]$$

[Catalase]

(M)

$\frac{d[\text{Hg}_2^{2+}]}{dt}$ (M min⁻¹)

dt

$$9.95 \times 10^{-9}$$

$$8.2 \times 10^{-8}$$

$$5.05 \times 10^{-9}$$

$$6.1 \times 10^{-8}$$

$$1.02 \times 10^{-9}$$

$$7.6 \times 10^{-8}$$

Linear regression analysis:

$$k = \text{slope} = 8.00 \times 10^{-1} \text{ min}^{-1}$$

$$\text{correlation coefficient} = 0.33$$

TABLE 30

1st Order Treatment:Constant $[\text{Hg}_2^{2+}]_{t=0} = 200 \times 10^{-8} \text{ M}$

$$\frac{d [\text{Hg}_2^{2+}]}{d t} = k [\text{Hg}_2^{2+}]$$

[Catalase]
(M)

$\frac{d [\text{Hg}_2^{2+}]}{d t}$ (M min⁻¹)

 9.95×10^{-9} 84×10^{-8} 5.05×10^{-9} 64×10^{-8} 1.02×10^{-9} 54×10^{-8}

Linear regression analysis:

 $k = \text{slope} = 3.39 \times 10^1 \text{ min}^{-1}$

-correlation coefficient = 0.99

TABLE 31

2nd Order Treatment:

$$\frac{d[\text{Hg}_2^{2+}]}{dt} = k[\text{Hg}_2^{2+}][\text{Catalase}]$$

[Catalase] (M)	Initial [Hg ₂ ²⁺] (M)	[Catalase] [Hg ₂ ²⁺] (M ²)	$\frac{d[\text{Hg}_2^{2+}]}{dt}$ (M min ⁻¹)
9.95 × 10 ⁻⁹	11.9 × 10 ⁻⁸	1.18 × 10 ⁻¹⁵	3.7 × 10 ⁻⁸
9.95 × 10 ⁻⁹	30.5 × 10 ⁻⁸	3.03 × 10 ⁻¹⁵	8.2 × 10 ⁻⁸
9.95 × 10 ⁻⁹	213 × 10 ⁻⁸	2.12 × 10 ⁻¹⁴	84 × 10 ⁻⁸
5.05 × 10 ⁻⁹	11.9 × 10 ⁻⁸	6.01 × 10 ⁻¹⁶	3.3 × 10 ⁻⁸
5.05 × 10 ⁻⁹	31.0 × 10 ⁻⁸	1.57 × 10 ⁻¹⁵	6.1 × 10 ⁻⁸
5.05 × 10 ⁻⁹	202 × 10 ⁻⁸	1.02 × 10 ⁻¹⁴	64 × 10 ⁻⁸
1.02 × 10 ⁻⁹	12.6 × 10 ⁻⁸	1.29 × 10 ⁻¹⁶	3.6 × 10 ⁻⁸
1.02 × 10 ⁻⁹	30.3 × 10 ⁻⁸	3.09 × 10 ⁻¹⁶	7.6 × 10 ⁻⁸
1.02 × 10 ⁻⁹	198 × 10 ⁻⁸	2.02 × 10 ⁻¹⁵	54 × 10 ⁻⁸

Linear regression analysis:

$$k = \text{slope} = 3.93 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$$

$$\text{correlation coefficient} = 0.86$$

TABLE 32

1st Order Treatment:

$$\text{Constant [Compound I]} = 1.02 \times 10^{-9} \text{ M}$$

$$\frac{d[\text{Hg}_2^{2+}]}{dt} = k[\text{Hg}_2^{2+}]$$

Initial $[\text{Hg}_2^{2+}]_{t=0}$ (M)	$[\text{Hg}_2^{2+}]_{t=1 \text{ min}}$ (M)	$\frac{d[\text{Hg}_2^{2+}]}{dt}$ (M min ⁻¹)
11.1×10^{-8}	7.3×10^{-8}	3.8×10^{-8}
30.2×10^{-8}	21.5×10^{-8}	8.7×10^{-8}
213×10^{-8}	143×10^{-8}	70×10^{-8}

Linear regression analysis:

$$k = \text{slope} = 3.3 \times 10^{-1} \text{ min}^{-1}$$

$$\text{correlation coefficient} = 0.99$$

TABLE 33

1st Order Treatment:Constant [Compound I] = 5.05×10^{-9} M

$$\frac{d[\text{Hg}_2^{2+}]}{dt} = k[\text{Hg}_2^{2+}]$$

Initial $[\text{Hg}_2^{2+}]_{t=0}$ (M)	$[\text{Hg}_2^{2+}]_{t=1 \text{ min}}$ (M)	$\frac{d[\text{Hg}_2^{2+}]}{dt}$ (M min ⁻¹)
11.0×10^{-8}	2.3×10^{-8}	8.7×10^{-8}
30.7×10^{-8}	8.5×10^{-8}	22.2×10^{-8}
209×10^{-8}	87.5×10^{-8}	121.5×10^{-8}

Linear regression analysis:

 $k = \text{slope} = 5.6 \times 10^{-1} \text{ min}^{-1}$

correlation coefficient = 0.99

TABLE 34

1st Order Treatment:Constant [Compound I] = $9.95 \times 10^{-9} \text{ M}$

$$\frac{d[\text{Hg}_2^{2+}]}{dt} = k[\text{Hg}_2^{2+}]$$

Initial $[\text{Hg}_2^{2+}]_{t=0}$ (M)	$[\text{Hg}_2^{2+}]_{t=1 \text{ min}}$ (M)	$\frac{d[\text{Hg}_2^{2+}]}{dt}$ (M min ⁻¹)
10.2×10^{-8}	1.3×10^{-8}	8.9×10^{-8}
29.4×10^{-8}	7.8×10^{-8}	21.6×10^{-8}
207×10^{-8}	68×10^{-8}	139×10^{-8}

Linear regression analysis:

$$k = \text{slope} = 6.61 \times 10^{-1} \text{ min}^{-1}$$

correlation coefficient = 1.00

TABLE 35

1st Order Treatment: Constant $[\text{Hg}_2^{2+}]_{t=0} = 11 \times 10^{-8} \text{ M}$

$$\frac{d[\text{Hg}_2^{2+}]}{dt} = k [\text{Compound I}]$$

$[\text{Compound I}]$ (M)	$\frac{d[\text{Hg}_2^{2+}]}{dt}$ (M min ⁻¹)
------------------------------	---

9.95×10^{-9}	8.9×10^{-8}
5.05×10^{-9}	8.7×10^{-8}
1.02×10^{-9}	3.8×10^{-8}

Linear regression analysis:

$k = \text{slope} = 5.5 \text{ min}^{-1}$

correlation coefficient = 0.86

TABLE 36

2nd Order Treatment:

$$\frac{d [\text{Hg}_2^{2+}]}{d t} = k [\text{Hg}_2^{2+}] [\text{Compound I}]$$

[Compound I] (M)	$[\text{Hg}_2^{2+}]_{t=0}$ (M)	[Compound I] $[\text{Hg}_2^{2+}]_{t=0}$ (M ²)	$\frac{d [\text{Hg}_2^{2+}]}{d t}$ (M min ⁻¹)
1.02×10^{-9}	11.1×10^{-8}	1.13×10^{-16}	3.8×10^{-8}
1.02×10^{-9}	30.2×10^{-8}	3.08×10^{-16}	8.7×10^{-8}
1.02×10^{-9}	213×10^{-8}	2.17×10^{-16}	70×10^{-8}
5.05×10^{-9}	11.0×10^{-8}	5.56×10^{-16}	8.7×10^{-8}
5.05×10^{-9}	30.7×10^{-8}	1.56×10^{-15}	22.2×10^{-8}
5.05×10^{-9}	209×10^{-8}	1.06×10^{-14}	121.5×10^{-8}
9.95×10^{-9}	10.2×10^{-8}	1.01×10^{-15}	8.9×10^{-8}
9.95×10^{-9}	29.4×10^{-8}	2.93×10^{-15}	21.6×10^{-8}
9.95×10^{-9}	207×10^{-8}	2.06×10^{-14}	139×10^{-8}

Linear regression analysis:

$$k = \text{slope} = 6.96 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$$

correlation coefficient = 0.91

TABLE 37

Summary

Rate Equation	Mercury Substrate	Constant Parameter	Order	k	r
$\frac{d[\text{Hg}]}{dt} = k[\text{Hg}]$	Hg^0	$[\text{Catalase}] = 1.37 \times 10^{-9} \text{ M}$	1st	$1.9 \times 10^{-1} \text{ min}^{-1}$	0.96
	Hg_2^{2+}	$[\text{Catalase}] = 1.02 \times 10^{-9} \text{ M}$	1st	$2.74 \times 10^{-1} \text{ min}^{-1}$	0.99
	Hg^0	$[\text{Catalase}] = 6.83 \times 10^{-9} \text{ M}$	1st	$2.78 \times 10^{-1} \text{ min}^{-1}$	0.99
	Hg_2^{2+}	$[\text{Catalase}] = 5.05 \times 10^{-9} \text{ M}$	1st	$3.27 \times 10^{-1} \text{ min}^{-1}$	0.99
$\frac{d[\text{Hg}]}{dt} = k[\text{Catalase}]$	Hg^0	$[\text{Hg}^0] = 10.7 \times 10^{-8} \text{ M}$	1st	$1.5 \times 10^{-1} \text{ min}^{-1}$	0.96
	Hg_2^{2+}	$[\text{Hg}_2^{2+}] = 11.9 \times 10^{-8} \text{ M}$	1st	$1.37 \times 10^{-1} \text{ min}^{-1}$	0.29
$\frac{d[\text{Hg}]}{dt} = k[\text{Catalase}][\text{Hg}]$	Hg^0	-	2nd	$0.49 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$	0.76
	Hg_2^{2+}	-	2nd	$3.93 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$	0.86

TABLE 38

Summary

Rate Equation	Mercury Substrate	Constant Parameter	Order	k	r
$\frac{d[\text{Hg}]}{dt} = k[\text{Hg}]$	Hg_2^{2+}	[Cmpd. I] = 1.02×10^{-9} M	1st	$3.3 \times 10^{-1} \text{ min}^{-1}$	0.99
	Hg_2^{2+}	[Cmpd. I] = 5.05×10^{-9} M	1st	$5.6 \times 10^{-1} \text{ min}^{-1}$	0.99
$\frac{d[\text{Hg}]}{dt} = k[\text{Cmpd. I}]$	Hg^0	$[\text{Hg}^0] = 9 \times 10^{-8}$ M	1st	6.5 min^{-1}	0.99
	Hg_2^{2+}	$[\text{Hg}_2^{2+}] = 11 \times 10^{-8}$ M	1st	5.5 min^{-1}	0.86
$\frac{d[\text{Hg}]}{dt} = k[\text{Hg}][\text{Cmpd. I}]$	Hg^0	-	2nd	$6.0 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$	0.96
	Hg_2^{2+}	-	2nd	$6.96 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$	0.91

Chapter VI: Spectroscopic Binding Studies

The recent development of highly sensitive spectroscopic techniques has generated improved methods for detailed examination of electronically fine infrastructures. The 18π electron arrangement of the porphyrin ring and the ligand field effects of the apoprotein on the central iron atom of the Catalase molecule combine to produce an electronically complex configuration. The optical absorption spectrum of this enzyme provides an elaborate "map" for careful examination of the iron protoporphyrin moiety as well as the ligands occupying the 5th and 6th coordination positions of the heme. Binding of substrates, inhibitors or other chemicals with the enzyme may be characterized by examining the spectroscopic changes that take place as a result of changes in the electronic configuration of the enzyme. The sensitivity of the absorption spectrum technique to changes in the heme environment, including changes in the spin state of the iron, has been well documented (40,44,85,86), including the following observations from ligand exchange studies (87): i) Addition of NaF to native Catalase is characterized by a shift of the charge transfer band at 622 nm to 596 nm. In the Soret region the absorption spectrum shows only a slight reduction in intensity indicating the iron remains in the Fe(III) high spin state, ii) Treatment of native Catalase with NaN_3 produces an absorption spectrum characteristic of an intermediate spin heme complex. This high-low mixed spin configuration is characterized by a shift of the Soret band to 411 nm, iii) The low spin nature of the CN^- complex is reflected by the

reduction in the Soret band intensity and the shift to 423 nm.

These results illustrate the spectral changes that characteristically accompany ligation exchange at the 6th coordination position of the heme ring. Other studies (69) have shown that the apoprotein of the Catalase molecule regulates the magnitude of the ligand field effect felt by the iron and thus interaction of a protein binding species with the amino acid side chain of the heme moiety may also induce spectral changes.

In this work a series of binding studies were performed to investigate the effect, if any, on the visible absorption spectra of native Catalase, Catalase Compound I and Catalase Compound II after treatment with elemental mercury, mercury (I), and mercury (II). It is noteworthy that a unique and significant problem arises when attempting to do such a study in that ideally the enzyme should be treated with at least an equivalent amount and preferably an excess of the potential binding chemical (87). The solubility limit of elemental mercury in aqueous solution is approximately 1.0×10^{-7} M, an equivalent amount of native Catalase at 405 nm would have an absorption of approximately 0.04 ($\epsilon = 3.97 \times 10^5$ (42)). Clearly bringing the Catalase concentration down below these levels would result in the loss of considerable sensitivity, however, this problem may be somewhat circumvented by prolonged exposure of a Catalase solution to a Hg^0 saturated atmosphere. Both the direct mixing of Hg^0 and Catalase solutions and Catalase exposure to a Hg^0 saturated atmosphere approaches were utilized in this study.

Direct Mixing of Catalase with Hg^0 or Hg^{2+} Solutions

Figures 37-39 are the visible spectra of 0.4×10^{-7} M Catalase before and after treatment with 25×10^{-7} M Hg^{2+} or 0.8×10^{-7} M Hg^0 solutions. Careful examination of the overlay of these three spectra (Figure 40) reveals that (within the errors of pipetting) there is no detectable difference in the visible spectra of these species. These spectra illustrate the serious signal to noise problems encountered when the enzyme concentration is decreased to levels where $A = 0.020$; the charge transfer bands are still visible at these levels but resolution of slight peak shifts becomes virtually impossible. These results, while not conclusive, indicate that at a 1:1 direct mixing level it is unlikely that either Hg^0 or Hg^{2+} bind with either the iron, the porphyrin ring or the axial ligands of the 5th or 6th coordination positions of native Catalase.

Prolonged Exposure of Catalase to a Hg^0 Saturated Atmosphere

Figures 41-44 depict the visible spectra of 2.58 μM Catalase incubated overnight in two Thunberg flasks, one flask was equipped with an empty side-arm, the other flask's side-arm contained 200 μL of metallic mercury. Specific absorbances at structurally relevant wavelengths for these two spectra are listed in Table 39. Comparison of Figures 41-42 to 43-44 and careful examination of the data in Table 39 reveals that prolonged exposure of native Catalase to a Hg^0 saturated atmosphere appears to have no detectable effect on the electronic

Scan # 01.04

0.020

Display Mode
[Single]Function
[Abs]

TABLE			
W	Abs	SOURCE	MODE
700.0	0.0018	Visible	Scan
	485.5		Abs
	0.0154		

Figure 37: 0.4 x 10⁻⁷ M Catalase absorption spectrum

Figure 38: 0.4×10^{-7} M Catalase + 25×10^{-7} M Hg^{2+} absorption spectrum

Scan # 01.05

0.020

Display Mode
[Single]Function
[Abs]

0.000

375

600 nm/min

TPE

WAVE	ABS	WAVE	ABS
700.0	0.0017	Visible	Scan
		435.5	0.001

Figure 39: 0.4×10^{-7} M Catalase + 0.8×10^{-7} M Hg^0 absorption spectrum

Scan # 01.02 01.04 01.05

0.025

Display Mode
(Overlay)Function
[Abs]

W	ABS	SOURCE	MODE
700.0	3.6992	Visible	Scan

Figure 40:

Overlay of absorption spectra of Native Catalase,

Catalase + Hg^{2+} , Catalase + Hg^0

Figure 41: 2.58×10^{-6} M Catalase incubated overnight in the absence of Hg^0

Scan # 01.05

0.000

Display Mode
[Single]Function
[Abs]

0.000

450

600 nm/min

700

TRACE

λ	ABS	SOURCE	MODE
700.0	0.0532	Visible	Scan

λ	ABS
622.0	0.1064

Figure 43: 2.5×10^{-6} M Catalase incubated overnight in the presence of a saturated Hg^0 atmosphere.

Figure 44: 2.58×10^{-6} M Catalase incubated overnight in the presence of a saturated Hg^0 atmosphere

TABLE 39

Effect of Overnight Exposure of Native Catalase
to a Hg^0 Saturated Atmosphere

3.0 mL Catalase

λ nm	A
405	1.0281
485.5	0.1621
531	0.1330
586	0.1001
622	0.1064

 $\text{Hg}^0 = 0$ ppb $\text{Hg}^T = 0$ ppb3.0 mL Catalase + Hg^0 Side-arm

λ nm	A
405	1.0255
485.5	0.1621
531	0.1325
586	0.0987
622	0.1057

 $\text{Hg}^0 = 18.7 \pm 0.5$ ppb $\text{Hg}^T = 80.1 \pm 2.9$ ppb

structure of the Catalase. This apparent non-effect is particularly interesting in view of the fact that the mercury speciation analysis of these solutions indicated that the Catalase had reacted with and oxidized the Hg^0 to the Hg^{2+} form.

Two hypotheses exist which may account for these results: i) The electronics of the active site of the Catalase molecule responsible for oxidizing Hg^0 to Hg^{2+} are not directly reflected in the visible absorption spectrum of Catalase or, ii) Catalase is reduced in the process of oxidizing Hg^0 however residual oxidizing agents present in solution rapidly react with the reduced Catalase to return it to its native state.

Binding of Hg^0 with Compound I

To parallel the long term exposure of native Catalase to a Hg^0 saturated atmosphere, Catalase Compound I and Compound II species were prepared, and incubated overnight in Thunberg flasks with and without metallic mercury in the side-arms. An important point which has been neglected in the previous discussion, but which will now be considered, is the stability of these two species. Chapters III, IV and V discussed in detail the characteristics of Catalase Compound I and Compound II spectra including the importance of the changes in the magnitude and position of the Soret band when identifying and differentiating between native Catalase, Compound I and Compound II.

Figures 45-56 illustrate the results of repetitive scanning, at 2 minute intervals, of 1.92×10^{-9} mol mL⁻¹ Catalase after treatment with either a) 1.75×10^{-8} mol mL⁻¹, b) 10.5×10^{-8} mol mL⁻¹, c) 21.0×10^{-8} mol mL⁻¹ or d) 105×10^{-8} mol mL⁻¹ peracetic acid.

At the two lower concentrations of peracetic acid (Figures 47-50), Compound I is seen to form initially ($t=0$), as shown by the decrease in the Soret band intensity relative to native Catalase (Figures 47 & 49), and by the appearance of the 662 nm band (Figures 48 & 50). After 2 minutes however, the Compound I species has begun to degenerate to native Catalase as indicated by the increase in the magnitude of the Soret band and the disappearance of the 662 nm band. After 4 minutes, regeneration to the native enzyme is complete. This short term stability of the Catalase Compound I species and regeneration of free Catalase at low (< 50) concentration ratios of peracetic acid:Catalase has been well documented (64, 43). This two electron reductive regeneration process is sensitive to the presence of oxidizable materials in the solutions (adventitious donors (64)). The rapid native Catalase regeneration rate obtained in this work may be the result of the reductive powers of the residual bile pigments present in the Bovine Liver Catalase utilized.

At the higher concentration ratios of peracetic acid:Catalase (>100) (Figures 51-56), formation of Compound II occurs as evidenced by the broadening of the Soret band towards longer wavelengths and by the appearance of the 555 nm band. As discussed previously, Compound II is an inactive species formed from a one electron peroxidatic reduction reaction of Compound I with excess peracetic acid:

Figure 45: Absorption spectrum of 1.92×10^{-9} mol mL^{-1} native Catalase before treatment with Peracetic acid

Figure 46: Absorption spectrum of 1.92×10^{-9} mol mL⁻¹ native Catalase before treatment with Peracetic acid

W	Abs	SOURCE	MODE
700.0	0.0065	Visible	Repscan

Figure 47: 2 minute repetitive scans of $1.92 \times 10^{-9} \text{ mol mL}^{-1}$ Catalase after treatment with $1.75 \times 10^{-8} \text{ mol mL}^{-1}$ Peracetic acid

Scan # 01.01 01.02 01.03

0.200

Display Mode
[Overlay]Function
[Abs]

t = 0 min.

t = 2,4 min.

0.000

450

700

λ	Abs	SOURCE	MODE
700.0	0.0367	Visible	Repscan

Figure 48:

2 minute repetitive scans of 1.92×10^{-9} mol mL^{-1} Catalase after
treatment with 1.75×10^{-8} mol mL^{-1} Peracetic acid

Scan # 01.04 01.05 01.06

λ	Abs	Source	Mode
700.0	0.0462	Visible	Repscan

Figure 49: 2 minute repetitive scans of $1.92 \times 10^{-9} \text{ mol mL}^{-1}$ Catalase after treatment with $10.5 \times 10^{-8} \text{ mol mL}^{-1}$ Peracetic acid

Scan # 01.04 01.05 01.06

0.200

• Display Mode
[Overlay]

Function
[Abs]

t = 0 min.
t = 2 min.
t = 4 min.

0.000

450

700

W	Abs	SOURCE	MODE
700.0	0.0418	Visible	RepScan

Figure 50: 2 minute repetitive scans of 1.92×10^{-9} mol mL⁻¹ Catalase after treatment with 10.5×10^{-8} mol mL⁻¹ Peracetic acid

Scan # 01.13 01.14 01.15

0.000

Display Mode
[Overlay]Function
[Abs]

W	Abs	SOURCE	MODE
700.0	0.0001	Visible	Repscan

Figure 51: 2 minute repetitive scans of 1.92×10^{-9} mol mL^{-1} Catalase after treatment with 21.0×10^{-8} mol mL^{-1} Peracetic acid

Scan # 01:13 01:14 01:15

λ	Abs	SOURCE	MODE
700.0	0.0560	Visible	Repscan

Figure 52: 2 minute repetitive scans of 1.92×10^{-9} mol mL^{-1} Catalase after treatment with 21.0×10^{-8} mol mL^{-1} Peracetic acid

Figure 53: Absorption Spectrum of 1.92×10^{-9} mol mL^{-1} Catalase 20 minutes

Scan # 01.18

λ	ABS	SOURCE	MODE	λ	ABS
700.0	-0.0160	Visible	RepScan	588.0	0.1222

Figure 54: Absorption Spectrum of 1.92×10^{-9} mol mL^{-1} Catalase 20 minutes after treatment with 21.0×10^{-8} mol mL^{-1} Peracetic acid.

Scan # 01.19 01.20 01.21 ☐

0.600

Display Mode
[Overlay]Function
[Abs]

λ	Abs	SOURCE	MODE
700.0	0.0574	Visible	RepScan

Figure 55: 2 minute repetitive scans of 1.92×10^{-9} mol mL⁻¹ Catalase after treatment with 105×10^{-8} mol mL⁻¹ Peracetic acid

W	Abs	SOURCE	MODE
700.0	0.0564	Visible	Repscan

Figure 56: 2 minute repetitive scans of 1.92×10^{-9} mol mL⁻¹ Catalase after treatment with 105×10^{-8} mol mL⁻¹ Peracetic acid. \

Examination of Figures 51 & 52, 55 & 56 reveals that the Compound II species was still being formed at the 0 and 2 minute time intervals. After 4 minutes the solutions appear to be a mixture of native Catalase and Compound II indicating a one electron reduction of the Compound II species by excess peracetic acid had taken place (62):

This apparent instability of both Compound I and Compound II over long periods of time necessitated a re-evaluation of the interpretation of the uptake experiments discussed in Chapter II. Early work by Magos & Clarkson (27) and subsequent results from this work indicated increased uptake and oxidation of elemental mercury by Catalase in the presence of hydroperoxides may be due to the oxidation of the mercury by Compound I. To explore this discrepancy between the apparent instability of the Compound I species and the enhancement of Hg^0 uptake/oxidation by the addition of hydroperoxides to Catalase solutions, the overnight uptake-oxidation experiments were repeated using the same peracetic acid-Catalase mixtures as were used in the above repetitive scanning experiments. At the end of the incubation period solutions were analyzed for mercury by the double Magos procedure and absorption spectra were recorded. The resultant spectra are shown

in Figures 57-63. Mercury concentrations are listed in Table 40. Careful examination of the Soret region and the charge transfer bands of the spectra of the incubated enzyme solutions (Figures 57-63) reveals complete regeneration of native Catalase in all cases except in Figure 63 where some denaturation appears to have occurred. The data in Table 40 however, indicates that despite this decomposition to native Catalase, the presence of peracetic acid appears to stimulate the oxidation of Hg^0 to Hg^{2+} by Catalase. Catalase in the absence of peracetic acid (entry II a) does demonstrate some limited oxidation to the mercuric form. Peracetic acid alone (entries I b, c, d, e), as was discussed in Chapter II, appears to oxidize Hg^0 to the mercurous state. The instability of the peracetic acid-Catalase Compound I complex is not conclusive enough evidence to vitiate the Hg^0 -Compound I oxidation hypothesis, however, some re-evaluation of the possible mechanisms involved is necessary: i) Pre-synthesized Catalase Compound I in the presence of ~50 fold excess of peracetic acid is stable for less than 10 minutes, ii) Overnight incubation of these degenerating enzymic solutions in a Thunberg flask equipped with a side-arm containing 200 μL of metallic mercury results in substantial uptake and oxidation of the elemental mercury to the mercuric form compared to control solutions of Catalase alone or peracetic acid alone which stimulates uptake and oxidation to the mercurous form, iii) The absorption spectra of the incubated solutions are typical of native Catalase indicating no detectable binding of mercury to the heme site.

The difference in the oxidation state of the mercury taken up in peracetic acid solutions in the presence and absence of Catalase implies

Figure 57: Solution II a- Absorption Spectrum of $1.92 \times 10^{-9} \text{ mol mL}^{-1}$ native Catalase incubated overnight in Hg^0 saturated atmosphere

Figure 58: Solution II a- Absorption Spectrum of 1.92×10^{-9} mol mL^{-1} native Catalase incubated overnight in Hg^0 saturated atmosphere

Figure 59:

Solution II b- Absorption Spectrum of $1.92 \times 10^{-9} \text{ mol mL}^{-1}$ Catalase +

$1.75 \times 10^{-8} \text{ mol mL}^{-1}$ Peracetic acid incubated overnight in Hg^0 saturated atmosphere

Figure 60:

Solution II b- Absorption Spectrum of 1.92×10^{-9} mol mL⁻¹ Catalase +

1.75×10^{-8} mol mL⁻¹ Peracetic acid incubated overnight in Hg⁰-saturated atmosphere

Figure 61: Solution 11 c- Absorption Spectrum of 1.92×10^{-9} mol mL^{-1} Catalase +

10.5×10^{-8} mol mL^{-1} Peracetic acid incubated overnight in Hg^0 saturated atmosphere

Solution II c-- Absorption Spectrum of 1.92×10^{-9} mol mL^{-1} Catalase +

10.5×10^{-8} mol mL^{-1} Peracetic acid incubated overnight in Hg^0 saturated

Figure 62:

Figure 63: Solution II d- Absorption Spectrum of 1.92×10^{-9} mol mL^{-1} Catalase +

21.0×10^{-8} mol mL^{-1} Peracetic acid incubated overnight in Hg^0 saturated atmosphere

TABLE 40

Uptake and Oxidation of Hg^0 by Catalase in the
Presence and Absence of Peracetic Acid

Solution	Oxidant		Signal	
	[Catalase] ($\times 10^{-9}$ mol mL^{-1})	[CH_3COOOH] ($\times 10^{-8}$ mol mL^{-1})	Non-reducing ($\text{Hg}^0, \text{Hg}_2^{2+}$) (ppb)	Reducing (Hg^{TOTAL}) (ppb)
I	a)	-	44.2 ± 1.2	31.5 ± 0.6
	b)	1.75	323 ± 6	2965 ± 21
	c)	10.5	214 ± 10	2214 ± 26
	d)	21.0	162 ± 2	1510 ± 6
	e)	105	271 ± 7	1863 ± 91
II	a)	1.92	17.1 ± 1.2	100 ± 1
	b)	1.92	13.7 ± 0.9	305 ± 21
	c)	1.92	13.7 ± 0.4	561 ± 8
	d)	1.92	16.5 ± 0.9	652 ± 50
	e)	1.92	25.7 ± 1.0	1543 ± 31

* All values represent mean $\pm$ SD of three estimations

an enzymatic mechanism. It is possible that either a) a small but significant concentration of Compound I is present at any given time, which is capable of oxidizing any dissolved Hg^0 to Hg^{2+} and which is itself in the process reduced to native Catalase or b) the peracetic acid in solution may stimulate uptake and oxidation of the Hg^0 to the Hg_2^{2+} state as was described in Chapter II. Small residual quantities of Compound I may then oxidize the Hg_2^{2+} to 2Hg^{2+} , thus reducing the Compound I species to native Catalase:

Binding of Hg(0), Hg(I) and Hg(II) with Compound I and Compound II

Figures 64-89 illustrate the results of an extensive study of the effects of treating Catalase Compound I and Catalase Compound II with the three forms of inorganic mercury. Native Catalase was treated with three different concentrations of peracetic acid for various periods of time to yield control samples of either Compound I ($t=0$ min.) or Compound II ($t=6$ min.). The experiments were then repeated substituting aqueous solutions of $\text{Hg}(0)$, $\text{Hg}(I)$, or $\text{Hg}(II)$ for the water solvent used in the control spectra. Careful inspection of Figures 64-89 reveals little if any change in the electronic structure of either Compound I or Compound II after treatment with any of the three mercury forms. This

Figure 64: Absorption Spectrum of 1.33×10^{-9} mol mL⁻¹ native Catalase

Figure 65: Absorption Spectrum of 1.33×10^{-9} mol mL^{-1} native Catalase

Figure 66: Absorption Spectrum of 1.33×10^{-9} mol mL^{-1} Catalase after treatment with 1.2×10^{-7} mol mL^{-1} Peracetic acid

λ	Abs	SOURCE	MODE
700.0	-0.0287	Visible	Scan

Figure 67: Absorption Spectrum of 1.33×10^{-9} mol mL^{-1} Catalase after treatment with 1.2×10^{-7} mol mL^{-1} Peracetic acid

Figure 68: Absorption Spectrum of 1.33×10^{-9} mol mL⁻¹ Catalase after

treatment with 1.2×10^{-7} mol mL⁻¹ Peracetic acid and 9.09×10^{-11} mol mL⁻¹ H₂O

W	Abs	SOURCE	MODE
700.0	-0.0264	Visible	Scan

Figure 89: Absorption Spectrum of 1.33×10^{-9} mol mL⁻¹ Catalase after treatment with 1.2×10^{-7} mol mL⁻¹ Peracetic acid and 9.09×10^{-11} mol mL⁻¹ Hg⁰

λ	Abs	SOURCE	MODE
700.0	-0.0242	Visible	Scan

Figure 70: Absorption Spectrum of $1.33 \times 10^{-9} \text{ mol mL}^{-1}$ Catalase after treatment with $1.2 \times 10^{-7} \text{ mol mL}^{-1}$ Peracetic acid and $1.7 \times 10^{-8} \text{ mol mL}^{-1} \text{ Hg(I)}$

Figure 71: Absorption Spectrum of 1.33×10^{-9} mol mL^{-1} Catalase after treatment with 1.2×10^{-7} mol mL^{-1} Peracetic acid and 1.7×10^{-8} mol mL^{-1} Hg(I)

λ	ABS	SOURCE	MODE
700.0	-0.0251	Visible	Scan

Figure 72: Absorption Spectrum of 1.33×10^{-9} mol mL⁻¹ Catalase after treatment

λ	WGS	SOURCE	MODE
700.0	-0.0247	Visible	Scan

Figure 73: Absorption Spectrum of 1.33×10^{-9} mol mL⁻¹ Catalase after treatment

Scan # ~~01.01~~ 01.02 ☐

0.400

Display Mode
[Overlay]

Function
[Abs]

W	AES	SOURCE	MODE
700.0	-0.0012	Visible	Scan

Figure 74: Absorption Spectrum of 1.33×10^{-9} mol mL⁻¹ Catalase after treatment with 4.78×10^{-7} mol mL⁻¹ Peracetic acid

λ	Abs	Source	Mode
700.0	-0.0312	Visible	Scan

Figure 75: Absorption Spectrum of $1.33 \times 10^{-9} \text{ mol mL}^{-1}$ Catalase after treatment with $4.78 \times 10^{-7} \text{ mol mL}^{-1}$ Peracetic acid

Figure 76: Absorption Spectrum of 1.33×10^{-9} mol mL⁻¹ Catalase after treatment with 4.78×10^{-7} mol mL⁻¹ Peracetic acid and 9.09×10^{-11} mol mL⁻¹ Hg⁰

Wavelength (nm)	Abs	Source	Mode
700.0	-0.0007	Visible	Scan

Figure 77: Absorption Spectrum of 1.33×10^{-9} mol mL^{-1} Catalase after treatment

3 3

OF / DE

Scan # 01.05 01.05

0.400

Display Mode
[Over]Function
[Abs]

A	Abs	SOURCE	MODE
700.0	-0.0004	Visible	Scan

Figure 78: Absorption Spectrum of $1.33 \times 10^{-9} \text{ mol mL}^{-1}$ Catalase after treatment with $4.78 \times 10^{-7} \text{ mol mL}^{-1}$ Peracetic acid and $1.7 \times 10^{-8} \text{ mol mL}^{-1} \text{ Hg(II)}$

λ	Abs	SOURCE	MODE
700.0	-0.0001	Visible	Scan

Figure 79: Absorption Spectrum of 1.33×10^{-9} mol mL^{-1} Catalase after treatment with 4.78×10^{-7} mol mL^{-1} Peracetic acid and 1.7×10^{-8} mol mL^{-1} Hg(I)

Figure 80: Absorption Spectrum of $1.33 \times 10^{-9} \text{ mol mL}^{-1}$ Catalase after treatment with $4.78 \times 10^{-7} \text{ mol mL}^{-1}$ Peracetic acid and $1.1 \times 10^{-8} \text{ mol mL}^{-1} \text{ Hg(II)}$

λ	Abs	SOURCE	MODE
700.0	-0.0297	Visible	Scan

Figure 81: Absorption Spectrum of 1.33×10^{-9} mol mL^{-1} Catalase after treatment with 4.78×10^{-7} mol mL^{-1} Peracetic acid and 1.1×10^{-8} mol mL^{-1} Hg(II)

λ	ABS	SOURCE	MODE
700.0	-0.0000	Visible	Scan

Figure 82: Absorption Spectrum of 1.33×10^{-9} mol mL^{-1} Catalase after treatment with 23.9×10^{-7} mol mL^{-1} Peracetic acid

Scan # 01.01 01.02

0.150

Display Mode
[Overlay]Function
[Abs]

t = 6 min

t = 0 min

0.000

450

700

λ	Abs	Source	Mode
700.0	-0.0010	Visible	Scan

Figure 83:

Absorption Spectrum of 1.33×10^{-9} mol mL^{-1} Catalase after treatment
with 23.9×10^{-7} mol mL^{-1} Peracetic acid.

Scan # ~~01.03~~ 01.04 ☐

0.400

Display Mode
[Overlay]Function
[Abs]

λ	Abs	Source	Mode
700.0	-0.0002	Visible	Scan

Figure 84: Absorption Spectrum of 1.33×10^{-9} mol mL^{-1} Catalase after treatment with 23.9×10^{-7} mol mL^{-1} Peracetic acid and 9.09×10^{-11} mol mL^{-1} Hg^0

Figure 85:

Absorption Spectrum of 1.33×10^{-9} mol mL^{-1} Catalase after treatment

with 23.9×10^{-7} mol mL^{-1} Peracetic acid and 9.09×10^{-11} mol mL^{-1} Hg^0

Scan # ~~01.05~~ 01.06

0.400

Display Mode
[Overlay]Function
[Abs]

λ	ABS	SOURCE	MODE
700.0	-0.0004	Visible	Scan

Figure 86: Absorption Spectrum of $1.33 \times 10^{-9} \text{ mol mL}^{-1}$ Catalase after treatment with $23.9 \times 10^{-7} \text{ mol mL}^{-1}$ Peracetic acid and $1.7 \times 10^{-8} \text{ mol mL}^{-1} \text{ Hg(I)}$

λ	Abs.	SOURCE	MODE
700.0	-0.0002	Visible	Scan

Figure 87: Absorption Spectrum of 1.33×10^{-9} mol mL⁻¹ Catalase after treatment

with 23.9×10^{-7} mol mL⁻¹ Peracetic acid and 1.7×10^{-8} mol mL⁻¹ Hg(I)

λ	ABS	SOURCE	MODE
700.0	-0.0000	Visible	Scan

Figure 88: Absorption Spectrum of 1.33×10^{-9} mol mL⁻¹ Catalase after treatment with 23.9×10^{-7} mol mL⁻¹ Peracetic acid and 1.1×10^{-8} mol mL⁻¹ Hg(II)

Figure 89: Absorption Spectrum of 1.33×10^{-9} mol mL⁻¹ Catalase after treatment with 23.9×10^{-7} mol mL⁻¹ Peracetic acid and 1.1×10^{-8} mol mL⁻¹ Hg(II)

TABLE 41
Absorption Values at Structurally Important Wavelengths
(from Figures 64-89)

Wavelength (nm)	1.33 x 10 ⁻⁹ mol mL ⁻¹ Catalase + 1.20 x 10 ⁻⁷ mol mL ⁻¹ Peracetic Acid							
			Hg(0)		Hg(I)		Hg(II)	
	0 min.	6 min.	0 min.	6 min.	0 min.	6 min.	0 min.	6 min.
399	0.2910		0.3191		0.3248		0.3051	
405		0.2778		0.3100		0.3171		0.2954
427		0.2385		0.2805		0.2887		0.2648
485.5	0.0694	0.0694	0.0795	0.0792	0.0827	0.0830	0.0770	0.0770
499	0.0599		0.0697		0.0721		0.0664	
534	0.0484	0.0542	0.0571	0.0645	0.0591	0.0675	0.0555	0.0622
555.5	0.0464	0.0522	0.0548	0.0625	0.0578	0.0658	0.0540	0.0602
585	0.0475	0.0474	0.0545	0.0550	0.0565	0.0571	0.0535	0.0530
622	0.0448	0.0400	0.0511	0.0455	0.0538	0.0480	0.0505	0.0447
662	0.0492	0.0434	0.0560	0.0482	0.0587	0.0505	0.0550	0.0475
405/622		6.95		6.81		6.61		6.61
399/662	5.91		5.7		5.53		5.55	

TABLE 42
Absorption Values at Structurally Important Wavelengths
(from Figures 64-89)

1.33 x 10 ⁻⁹ mol mL ⁻¹ Catalase + 4.78 x 10 ⁻⁷ mol mL ⁻¹ Peracetic Acid								
Wavelength (nm)	Hg(0)		Hg(I)		Hg(II)			
	0 min.	6 min.	0 min.	6 min.	0 min.	6 min.	0 min.	6 min.
399	0.3041		0.3111		0.3114		0.3141	
405		0.3011		0.3095		0.3084		0.3131
427		0.2724		0.2828		0.2804		0.2880
485.5	0.0740	0.0738	0.0765	0.0762	0.0767	0.0764	0.0775	0.0771
499	0.0657		0.0674		0.0680		0.0680	
534	0.0555	0.0588	0.0542	0.0610	0.0545	0.0610	0.0545	0.0621
555.5	0.0521	0.0560	0.0514	0.0582	0.0517	0.0581	0.0517	0.0591
585	0.0507	0.0491	0.0511	0.0511	0.0512	0.0511	0.0510	0.0522
622	0.0468	0.0405	0.0472	0.0417	0.0480	0.0421	0.0475	0.0433
662	0.0534	0.0447	0.0541	0.0454	0.0542	0.0458	0.0542	0.0470
405/622		7.43		7.42		7.33		7.23
399/662	5.69		5.75		5.75		5.80	

TABLE 43

Absorption Values at Structurally Important Wavelengths

(from Figures 64-89)

1.33 x 10 ⁻⁹ mol mL ⁻¹ Catalase + 23.9 x 10 ⁻⁷ mol mL ⁻¹ Peracetic Acid								
Wavelength (nm)	Hg(0)		Hg(I)		Hg(II)			
	0 min.	6 min.	0 min.	6 min.	0 min.	6 min.	0 min.	6 min.
399	0.3400		0.3415		0.3314		0.3428	
405		0.3345		0.3350		0.3237		0.3351
427		0.3250		0.3225		0.3117		0.3220
485.5	0.0860	0.0827	0.0868	0.0861	0.0840	0.0814	0.0864	0.0847
499	0.0762		0.0761		0.0732		0.0750	
534	0.0651	0.0700	0.0638	0.0727	0.0605	0.0680	0.0624	0.0707
555.5	0.0610	0.0672	0.0621	0.0700	0.0577	0.0655	0.0591	0.0682
585	0.0585	0.0564	0.0611	0.0595	0.0558	0.0555	0.0571	0.0578
622	0.0532	0.0444	0.0562	0.0480	0.0508	0.0438	0.0524	0.0462
662	0.0608	0.0471	0.0631	0.0510	0.0577	0.0467	0.0601	0.0495
405/622		7.53		6.98		7.39		7.25
399/662	5.64		5.41		5.74		5.70	

point is clearly illustrated in Tables 41-43 where specific absorptions of the various spectra have been recorded. Within the limits of errors in pipetting, the spectra are essentially identical in the absence or presence of Hg(0), Hg(I), or Hg(II).

These results coupled with those of the previous sections are strongly suggestive that there is little if any binding of Hg(0), Hg(I), or Hg(II) with the heme moiety of Compound I, Compound II or native Catalase.

Chapter VII: Stopped-flow Experiments. Effect of Hg(0), Hg(I) and
Hg(II) on the Rate of Formation of Catalase Compound I

Effect of Mercury on the Kinetics of H₂O₂ Decomposition

The formation of Catalase Compound I is conventionally written as if it were an elementary bimolecular reaction:

In the early literature the process was written as an addition and a reverse dissociation reaction was included (66, 88):

It has now been well established (69, 89) that Compound I is not an enzyme-substrate complex in the conventional sense. The reaction is an oxidation-reduction process of considerable complexity and is essentially irreversible.

The observed second order rate constant for the overall catalytic reaction of the enzyme is in the order of $10^7 \text{ M}^{-1} \text{ s}^{-1}$ (66). The overall reaction may be monitored by spectroscopic measurement of the decrease in the absorptivity of the H₂O₂ substrate solution ($\lambda_{\text{max}} = 240 \text{ nm}$) after treatment with buffered enzyme. An unsatisfactory aspect of this approach is that hydrogen peroxide acts as both an oxidizing and a reducing substrate for the enzyme so that the observed rate of decomposition of the substrate reflects the kinetics of both the formation and

decomposition of Compound I:

In view of the apparent, but as yet unspecified, relationship between Catalase, Catalase Compound I and elemental mercury oxidation it seemed pertinent to investigate the effect of mercury on the kinetics of the normal Catalase- H_2O_2 reaction. Preliminary experiments were performed to evaluate the effect of Hg (0) and Hg (II) on the overall rate of decomposition of hydrogen peroxide by Catalase. Table 44 summarizes the effect of the presence of excess mercuric ion on the decomposition of hydrogen peroxide by Catalase. These results indicate minimal inhibitory effects until $[\text{Hg}^{2+}] \geq 240 \times [\text{Catalase}]$. These results are not unexpected; early work by Abe *et al.* (58) has shown that alkylation of six of the sulfhydryl groups of Catalase with iodoacetamide had no significant influence on the enzymatic reaction. Mercuric ions have a similar affinity for sulfhydryl groups, thus the non-effect of Hg^{2+} on the catalytic reactions presents further evidence that sulfhydryl groups do not actively participate in the configuration of the active site of the enzyme. The observed inhibition at 240x excess Hg(II) probably reflects changes in the 3^o structure of the enzyme.

The effect of prolonged exposure to a Hg(0) saturated atmosphere on the H_2O_2 decomposition activity of Catalase was investigated by overnight incubation of native Catalase in a Thunberg flask containing 200 μL of

TABLE 44

Effect of Hg(II) on the Decomposition of H_2O_2 by Catalase

[Catalase] (mol mL ⁻¹)	[H ₂ O ₂] (mol mL ⁻¹)	[Hg ²⁺] (mol mL ⁻¹)	V (mM H ₂ O ₂ min ⁻¹)
1.38 × 10 ⁻¹²	1.9 × 10 ⁻⁵	-	11.9 ± 0.6
		1.67 × 10 ⁻¹¹	11.9 ± 0.1
		3.33 × 10 ⁻¹¹	11.9 ± 0.3
		8.33 × 10 ⁻¹¹	11.0 ± 0.0
		10.0 × 10 ⁻¹¹	10.5 ± 0.0
		16.7 × 10 ⁻¹¹	10.1 ± 0.4
		33.3 × 10 ⁻¹¹	7.6 ± 0.8

metallic mercury in the side-arm. At the end of this incubation period the enzyme's ability to catalyze hydrogen peroxide degradation was compared to that of a control enzyme sample. No difference in catalytic activity was detected.

Stopped-flow Kinetics of Compound I Formation

Kinetic studies play a crucial role in elucidating mechanisms and recent improvements in stopped-flow rapid kinetic instrumentation has enabled researchers to accurately determine rate constants for individual steps in complex mechanisms such as the Catalase- H_2O_2 system. Abe *et al.* (58) using stopped-flow techniques have studied the pH dependency of the kinetic parameters of the Catalase- H_2O_2 system. These researchers found the decomposition of H_2O_2 by Catalase to involve a ternary complex (ESS):

At pH 7.00 they found the ternary complex decomposition rate to be about 100 times faster than ESS formation.

Perusal of the current literature (58, 64, 66) reveals a plethora of information on these kinetics, including pH, temperature and stereochemical dependence of the Catalase-peroxide reaction. These studies were not without difficulties. Assay of the catalytic activity of the enzyme is complicated by: 1) the very high specific activity of the enzyme,

ii) inactivation of the enzyme in the absence of substrate at convenient [Catalase] (a phenomenon related to the dissociation of the tetramer followed by denaturation) iii) a time dependent inactivation of the enzyme by H_2O_2 . Factors i) and ii) restrict the range of [Catalase] for accurate measurement with conventional techniques to 0.1-1 nM and factor iii) requires that either fairly rapid techniques or extrapolation procedures be employed.

Since hydrogen peroxide reacts rapidly both as an oxidizing and reducing substrate for the enzyme, maximal formation of Compound I with H_2O_2 is only 24-40%. Stopped-flow studies with organic hydroperoxides such as peracetic acid permit the investigation of the kinetics of 100% Compound I formation. Subsequent addition of a peroxidatic donor (such as ethanol) reduces Compound I to native Catalase:

These studies emulate the kinetics of the Catalase-hydrogen peroxide reaction; however, calculation of k_1 and k_4 from the data is not straight forward in that the steady state approximation cannot realistically be applied to the maximal formation of Compound I under conditions where experiments are feasible.

Determination of the effects, if any, of $Hg(0)$, $Hg(I)$ and $Hg(II)$ on the complex kinetics of Compound I formation require the use of both a stopped-flow apparatus and an artificial organic hydroperoxide

substrate.

The optical spectra of Bovine Liver Catalase and its Compound I and Compound II derivatives have been discussed in Chapters III and IV. The most dramatic change in the spectrum of native Catalase associated with the formation of Compound I is the halving of the absorptivity of the Soret maxima. Thus for fixed wavelength stopped-flow kinetic studies, changes in the absorptivity of the Soret peak provides the best resolution for monitoring the rate of Compound I formation.

Figure 90 illustrates the change in absorption of the Soret band (405 nm) during stopped-flow mixing of 1.46 μM Bovine Liver Catalase and 5.26 mM peracetic acid in a Model II A Tri-tech Dynamic Stopped-Flow Apparatus. Time for formation of Compound I was about 60 ms. Figures 91-92 are the overlays of the optical spectra of native Catalase and the endproduct Compound I. The sigmoidal curve in Figure 90 represents the 13.9% transmittance change (0.1044 absorbance units) in the Soret peak seen in Figure 91. The sensitivity of the Tri-tech stopped-flow apparatus is listed as 2% transmittance change; however, comparison of Figures 90 & 91 indicates a requirement of at least 10% transmittance change for useful resolution and determination of reaction rates. Thus while the appearance of the 662 nm peak would provide a much more specific tool for monitoring Compound I formation, this change of ~4.2% transmittance was barely detectable with the apparatus on hand, consequently restricting all studies to monitoring Soret band changes.

To investigate the effect of the three inorganic mercury species on the rate of formation of Compound I, native Catalase ($1.46 \times 10^{-9} \text{ mol mL}^{-1}$) was pretreated with excess Hg(0) ($4.57 \times 10^{-7} \text{ mol mL}^{-1}$), Hg(I) ($9.02 \times 10^{-6} \text{ mol mL}^{-1}$) or Hg(II) ($1.11 \times 10^{-5} \text{ mol mL}^{-1}$). Each of these Catalase-mercury

Figure 90:

Change in absorption of Soret band (405 nm) during stopped-flow mixing of 1.46 μM Bovine Liver Catalase and 5.26 mM Peracetic Acid

Figure 91: Absorption spectra of native Catalase (1.46 μ M) and Compound I (from 5.26 mM peracetic acid)

λ	Abs	SOURCE	MODE
700.0	0.0005	Visible	Scan

Figure 92: Absorption spectra of native Catalase (1.46 μ M) and Compound I (from 5.26 μ M peracetic acid)

Figure 93: Change in absorption of Soret band (405 nm) during stopped-flow mixing of 1.46 μM Bovine Liver Catalase and 5.26 mM Peracetic Acid in the presence of Hg(0) ($\times$ -), Hg(I) (- -), or Hg(II) ($\times$ -) and in the absence of mercury (—).

Wavelength [nm]	Abs	Source	Unit
300.0	0.0051	Visible	Scan

Figure 94: Absorption spectra of 1.46 μM native Catalase and 1.46 μM Catalase +

$4.57 \times 10^{-7} \text{ mol mL}^{-1} \text{ Hg(0)}$

λ	ABS	SOURCE	MODE
700.0	-0.0554	Visible	Scan

Figure 95: Absorption spectra of 1.46 μM native Catalase and 1.46 μM Catalase +

$4.57 \times 10^{-7} \text{ mol mL}^{-1} \text{ Hg(0)}$

ABS	SOURCE	MODE
700.0	0.0281	Visible Scan

Figure 96: Absorption spectra of 1.46 μM native Catalase and 1.46 μM Catalase +
 1.11×10^{-5} mol mL^{-1} Hg(II)

A	ABS	SOURCE	MODE
700.0	0.0287	Visible	Scan

Figure 98: Absorption spectra of 1.46 μM native Catalase and 1.46 μM Catalase +

9.02×10^{-6} mol mL^{-1} Hg(I).

λ	ABS	SOURCE	MODE
450.0	0.0527	Visible	Scan

Figure 99:

Absorption spectra of 1.46 μM native Catalase and 1.46 μM Catalase +

9.02 × 10⁻⁶ mol mL⁻¹ Hg(I)

mixtures were then subjected to stopped-flow mixing with $5.26 \times 10^{-6} \text{ mol mL}^{-1}$ peracetic acid. The results of these tests are shown in Figure 93.

Figures 94-99 are the optical spectra of the Catalase-mercury solutions prior to stopped-flow mixing with the peracetic acid. Table 45 summarizes the rate constant, intercept and correlation coefficients of the curves after fitting the data to the computer "Kinetic Ver 6" program (Cantech Scientific Ltd.: $A = \log (1 + \log (1 + T_i - z) - C_0))$ where T_i is the instantaneous value of intensity, z is the offset of I ($t=0$), and C_0 is $\log I_0$). (A second set of data is also presented in Table 45

representing parallel experiments done with more concentrated peracetic acid, $2.63 \times 10^{-5} \text{ mol mL}^{-1}$). Several points are clear from these results:

i) A minimum of 10% transmittance change is required for resolution at the stopped-flow time level using the Tri-tech Dynamics apparatus.

Such a change is restricted to the Soret band for monitoring the

formation of Catalase Compound I, ii) Approximately 1000 fold excess of peracetic acid is required to induce complete formation of Compound I, iii) Pre-

treatment of native Catalase with excess of any of the three inorganic forms of mercury had no apparent effect on the rate of formation of

Compound I. Treatment of Catalase with $\text{Hg}(0)$, $\text{Hg}(I)$, or $\text{Hg}(II)$ had no

effect on the optical spectrum of the enzyme. Whether this apparent non-effect on the kinetics reflects an actual non-interference of mercury

with the formation of the Compound I complex or whether it is a result of almost instantaneous re-reaction of Catalase with the excess peracetic acid after oxidation of elemental mercury as suggested in Figure 100 cannot be determined from these studies.

Figure 100: Possible reaction sequence in stopped-flow
Experiments

TABLE 45

Summary of the Effect of Hg(0), Hg(I), and Hg(II) on the
Kinetics of Catalase Compound I Formation

[Catalase] (mol mL ⁻¹)	[Peracetic Acid] (mol mL ⁻¹)	Mercury Species	[Mercury] (mol mL ⁻¹)	Rate Constant	Correlation Coefficient
1.85 x 10 ⁻⁹	2.63 x 10 ⁻⁵	-	-	29.34	0.97
		0	6.63x10 ⁻⁷	29.82	0.97
		1	4.73x10 ⁻⁶	29.54	0.96
		2	6.53x10 ⁻⁶	28.24	0.94
1.46 x 10 ⁻⁹	5.26 x 10 ⁻⁶	-	-	14.14	0.98
		0	4.57x10 ⁻⁷	14.49	0.96
		1	9.02x10 ⁻⁶	14.66	0.99
		2	11.1 x10 ⁻⁶	14.55	0.97

Chapter VIII: Protein Binding and Adsorption of Hg^0 , Hg_2^{2+} , Hg^{2+}
onto Glassware

The problems associated with maintaining constant levels of submicrogram quantities of mercury ($\text{Hg} (0)$, $\text{Hg} (I)$ or $\text{Hg} (II)$) in solution have been well documented (90, 91, 92). These reports attribute decreases in concentration with storage to: i) vaporization after reduction to metallic mercury (in the case of $\text{Hg}(I)$ and $\text{Hg}(II)$) by residual reducing agents within the system, ii) adsorption onto container walls, iii) disproportionation of any Hg_2^{2+} present to Hg^0 and Hg^{2+} and subsequent vaporization of the metallic mercury thus produced, and iv) association with particulate matter not homogeneous to the solution.

The majority of these problems have been overcome by: i) lowering the pH to 0.5 with nitric acid, ii) avoiding reagents possibly containing contaminant reducing agents thus preferentially maintaining all of the mercury in the non-volatile divalent state and iii) avoiding polyvinyl chloride containers.

While these recommendations are suitable for most quantitative environmental studies where the oxidation state of the analyte is unimportant, the approach is not applicable to a study such as this where a saturated $\text{Hg}(0)$ solution is required.

Careful examination of the control curves (Figures 10 d, 13, 14) of the kinetic studies of the oxidation of homogeneous $\text{Hg}(0)_{\text{aq}}$ solutions clearly reveals that these experiments were not without adsorption and volatilization storage problems. Loss of elemental mercury from 20 mL

of an approximately 20 ppb aqueous solution in a stoppered 25 mL round bottom Pyrex* flask averaged about 4.5 ppb over 10 minutes. Similarly, Hg (I) and Hg(II) solutions were also found to show variable loss with short-term storage.

This adsorption/volatilization phenomenon presented a sensitive tool for detecting binding of very small quantities of any of the three forms of inorganic mercury to Catalase. In this experiment 20 mL of $\sim 1 \times 10^{-10}$ mol mL⁻¹ Hg(0), Hg(I), or Hg(II) was placed in an acid cleaned 25 mL round bottom flask. 1.0 mL of 1.64×10^{-9} mol mL⁻¹ of test binding reagent was added (phosphate buffer for control, or Catalase, or Catalase Compound I or Catalase-CN complex). Total mercury content was monitored over a period of 7.5 minutes. The results of this study are shown in Table 46.

These results demonstrate a substantial reduction in the amount of mercury (Hg (0), Hg(I), or Hg(II)) lost from solution when Catalase, Catalase Compound I or Catalase-CN complex are added to the solution. As would be expected, the Hg⁰ and Hg₂²⁺ control samples (entries I a,b,c and II a,b,c) showed the largest loss of total mercury over the test period. The mercury (II) samples were more stable but also showed slight losses (entries III a,b,c). Of the test reagents added, Catalase Compound I was the most efficient at preventing Hg⁰ or Hg₂²⁺ loss from solution. This observation is not unexpected in view of the rapid oxidation of both of these species by the Compound I complex. The ability to oxidize Hg⁰ or Hg₂²⁺ seems to parallel the ability to maintain the total mercury concentration of the solution. Virtually no loss of mercury was experienced when the mercuric solution

was treated with either Catalase, Catalase Compound I or Catalase-CN complex. These three enzyme species differ in the ligand occupying the 6th axial position of their heme moieties, thus presenting evidence that it is unlikely that mercuric binding occurs at this position. This is consistent with the previously discussed visible region spectroscopic binding studies (Chapter VI) which indicated no apparent binding of mercury to the heme portion of the enzyme nor to the immediately adjacent ligands.

By the process of elimination only one possible site of binding remains; the apoprotein of the enzyme. There is relatively little detailed information about the location of the amino acids of the peptide backbone around the four heme groups of the enzyme (62). Recently isomorphous and molecular replacement techniques have been used to propose a three dimensional structure for beef liver Catalase which consists of four domains: an extended non-globular amino terminal arm which stabilizes the quaternary structure, an anti-parallel eight stranded β -band providing the residues on the distal side of the heme, a rather random "wrapping domain" around the subunit exterior including the proximal heme ligand, and a final α -helical structure (93). One subunit of Catalase has 506 amino acid residues with an additional 10 to 15 residues at the C-terminal of a small fraction of the subunits. The typical high cysteine content of such a large protein provides a readily available binding target for soft acids such as Hg^{2+} .

Changes in the secondary structure of the protein may be detected by examining shifts in the ultraviolet region (42). $1.7 \times 10^{-9} \text{ mol mL}^{-1}$ Bovine Liver Catalase was treated with $2.49 \times 10^{-9} \text{ mol mL}^{-1} \text{ Hg}^{2+}$ and

$3.56 \times 10^{-9} \text{ mol mL}^{-1} \text{ Hg}_2^{2+}$ and spectroscopically examined in the U.V. region. Figure 101 illustrates the apparent non-effect of mercury (I) or mercury (II) on the 2^0 structure of the enzyme. These results are not totally unexpected since treatment was almost at the 1:1 level and thus only a very small portion of the apoprotein would be affected by the binding of one molecule of mercury. Nonetheless these results do indicate that binding probably occurs at amino acid residues which are not integral parts of the 3^0 structure of the enzyme. This last point eliminates the hypothesis that in oxidizing Hg^0 to Hg^{2+} , Catalase is acting as a "suicide" enzyme, since the end product seems to have little effect at the mole to mole level. One must not however neglect the fact that over a long period of time Hg^{2+} may accumulate to such excess levels that the enzyme does experience some detrimental effects.

Scan # 01.01 01.02 01.03

2.100

Display Mode
[Overlay]

Function
[Abs]

0.000

250

325

λ	ABS	SOURCE	MODE
325.0	-0.1077	Vis-UV	Scan

Figure 101:

Overlay of absorption spectra of 1.7×10^{-9} mol mL^{-1} native Catalase,

1.7×10^{-9} mol mL^{-1} Catalase + 2.49×10^{-9} mol mL^{-1} Hg^{2+} , and 1.7×10^{-9} mol mL^{-1}

Catalase + 3.56×10^{-9} mol mL^{-1} Hg_2^{2+}

TABLE 46

Binding of Hg(0), Hg(I) and Hg(II) to Catalase,
Catalase Compound I and Catalase-CN

Mercury Solution			Binding Reagent	Decrease in Hg ^{Total} after 7.5 minutes (ppb)
I	a)	Hg(0)	Phosphate buffer	4.1
	b)		Phosphate buffer-Peracetic Acid	4.2
	c)		Cyanide	4.7
	d)		Catalase	2.6
	e)		Catalase Compound I	0.1
	f)		Catalase-CN	0.7
II	a)	Hg(I)	Phosphate buffer	5.9
	b)		Phosphate buffer-Peracetic acid	2.7
	c)		Cyanide	2.8
	d)		Catalase	0.0
	e)		Catalase Compound I	0.0
	f)		Catalase-CN	0.3
III	a)	Hg(II)	Phosphate buffer	1.3
	b)		Phosphate buffer-Peracetic Acid	1.6
	c)		Cyanide	0.0
	d)		Catalase	0.0
	e)		Catalase Compound I	0.2
	f)		Catalase-CN	0.0

Chapter IX: Summary, Conclusions and Possible Mechanisms for
the Oxidation of Elemental Mercury

The data and information collected during the course of this study have provided some fascinating insights into both the chemistry of mercury and the mechanism of the in vivo oxidation of elemental mercury. No one particular hypothesis evolved from this work as the sole mechanism for the oxidation process, however, in summary, several questions may be addressed and a number of conclusions drawn about the oxidation of $\text{Hg}(0)$ to $\text{Hg}(\text{II})$:

1. Is the oxidation of $\text{Hg}(0)$ to $\text{Hg}(\text{II})$ a one-step 2-electron or a two-step 1-electron process?
2. What, if any, is the role of mercurous ion in the oxidation?
3. Is mercurous ion oxidized under the same conditions, and, if so, how does the rate compare with that of Hg^0 oxidation?
4. What is the species of enzyme that causes oxidation?
5. What is the kinetic order with respect to each component and what is the overall kinetic rate equation?
6. What is the active site on the enzyme responsible for the reaction?
7. Is the process truly enzymic- i.e. catalyzed,- or is the enzyme consumed in the process?

8. What is the overall mechanism of oxidation?

The first three questions presented above may be addressed together. Kinetic studies discussed in Chapters III IV and V revealed that Catalase alone oxidizes Hg(0) to Hg(II) at a rate very similar to the rate at which Catalase oxidizes Hg(I) to Hg(II). Similarly, studies with pre-synthesized Catalase Compound I indicated that this enzyme species also oxidizes Hg(0) and Hg(I) at comparable rates. These results indicate that the oxidation of Hg(0) to Hg(II) by native Catalase and/or Catalase Compound I probably proceeds via a one-step 2-electron process not involving Hg(I) intermediates..

Comparison of the rates of oxidation of Hg(0) by native Catalase and Catalase Compound I reveals that Compound I oxidation proceeds at a much greater rate than native Catalase oxidation. Thus in answer to question 4 above, both native Catalase and Catalase Compound I are the species of enzyme that cause Hg(0) oxidation, however, the Compound I process is probably the dominating mechanism.

Data presented in Chapter V indicate that the oxidation reactions of Hg(0) and Catalase and Catalase Compound I are somewhat mixed order but approximately 1st order in both enzyme and mercury and second order overall. These results are consistent with data collected in this laboratory (94) on the oxidation of Hg(0) and Hg(I) by Peroxidase Compound I which indicate that the oxidation process is 1st order in enzyme and mercury and second order overall. The oxidation rates measured in the Peroxidase study showed the oxidation of Hg(0) and Hg(I) to also proceed at approximately the same rate but considerably slower ($k = 8 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$) than that observed in this study for Catalase Compound I oxidation

($k = 6 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$).

The last three questions presented above may be addressed together. Careful consideration must be given to the fact that the in vitro aqueous experiments conducted in this study do not properly emulate the complex chemical environments encountered in vivo. Nonetheless, some comments and conclusions may be made based on the behaviour of the in vitro system. The most important observation made in this study was the fact that the rate of oxidation of Hg(0) by Catalase Compound I is approximately 10x slower than the rate of formation of Compound I. This indicates that the reaction of Catalase with H_2O_2 (to form Compound I) and the oxidation of Hg(0) to Hg(II) by Compound I is not a concerted mechanism.

It appears most likely that in a non-aqueous biological environment oxidation of Hg(0) to Hg(II) by Catalase occurs via a number of mechanisms. The lipophilic nature of the Hg(0) species presents interesting possibilities for access and attack of the Hg(0) species on the enzyme (either native Catalase or Catalase Compound I); it is quite possible that the Hg(0) may approach the active site(s) of the enzyme through lipid pockets in the membrane environment in which the enzyme is situated. More important is the fact that in constrained configurations and environments such as membranes and proteins it is often the electrons and protons which migrate through the biological matrices, and not actually the substrates.

The exact nature of the Compound I species has been, and still appears to be, a controversial subject. Many researchers (62, 63, 66) depict the Compound I complex as a resonance radical species:

where $\text{P}^{\bullet+}$ is the porphyrin ring radical. It is unlikely that the 7 coordinate form is an accurate representation of this intermediate species. It is more likely that the formation of Compound I is a two-step process involving peroxide binding with the native enzyme and then cleavage of the peroxide ligand by the filling of the O-O antibonding orbitals with electrons from the iron and the porphyrin ring to give the six coordinate ferryl-oxene structure:

If this iron(IV)-oxene structure is assumed to be the more accurate representation of the Catalase Compound I structure then numerous possibilities exist for attack by elemental mercury. Perhaps the simplest and most chemically appealing mechanism for the aqueous oxidations performed in this study is simple attack at the oxene oxygen:

This mechanism is quite similar to that originally proposed by Magos and Clarkson (27) in that it involves $\text{Hg}(0)$ reaction with the ligand in the 6th axial position of Compound I:

The new mechanism differs from that originally proposed by Magos and Clarkson only in the nature of the location of the oxidizing equivalents on the Compound I species. The problem of how a neutral mercury atom would attach itself to this intermediate enzyme species is still not resolved with this new concept, however, delocalization of electrons from the oxygen to the iron through the π orbitals would render the oxene functionality slightly electrophilic and thus more disposed to reaction with an electron dense species such as $\text{Hg}(0)$ than the old Fe^{3+}OOH structure.

Regeneration of the native Catalase would result from the flow of electrons either directly to the iron and the porphyrin ring (1-electron reduction of each) or to the iron initially (2-electron reduction to form Fe^{II}) and then a subsequent 1-electron reduction of the porphyrin ring. The inherent availability of water molecules would facilitate exchange of the Hg-O- ligand for a water molecule at the 6th axial position thus completing the regeneration of the native enzyme. As mentioned previously, evidence collected during the course of this study indicates that the oxidation reaction is a one-step 2-electron process with 1st order kinetics in each component. The mechanism discussed above is the simplest way of performing such a reaction.

The short bond length of the high spin Fe^{IV} -oxene Compound I complex as well as the peptide backbone of the apoprotein enveloping the heme moiety provides a sterically constrained environment into which the $\text{Hg}(0)$.

atom would have to move if oxidation occurred via attack at the Fe^{IV} -oxene site. The probability of the large, electron dense, $\text{Hg}(0)$ atom attacking the oxene functionality in vivo is highly unlikely. It is possible that the mechanism may involve hydration of the neutral $\text{Hg}(0)$ substrate in the protein environment and then attack at the oxene. Alternatively, direct attack of the $\text{Hg}(0)$ on the electron deficient porphyrin ring may occur from the side:

In this mechanism the flow of electrons from the mercury would regenerate the 18π porphyrin ring and Fe^{III} components of native Catalase. The replacement of the d electron into the iron orbitals would force cleavage of the oxene-iron double bond. The thus produced $\text{Fe}^{\text{III}}-\text{O}^-$ would rapidly become protonated in the biological environment to reproduce the native Catalase $\text{Fe}^{\text{III}}-\text{H}_2\text{O}$ form, while the mercuric ion would either take up hydroxide ions or complex with any soft bases (eg. sulphur) present in the biological environment.

MATERIALS AND METHODS

General

All reagents were analytical grade except where stated and were selected for their low mercury content. All glassware was soaked in 30% v/v nitric acid for 48 hours, rinsed with distilled water and oven dried prior to use. All reagents were prepared with distilled deionized water (Millipore Corporation, Bedford Mas.).

Mercury Solutions

- i) Mercuric chloride solutions; Fresh stock solution was prepared each month by dissolving 0.6767 g of mercuric chloride (Anachemia Ltd., Toronto Canada, AC-5672) in 1.00 L water. Serial dilutions were prepared daily as required.
- ii) Mercurous nitrate solutions; Fresh stock solutions were prepared weekly by dissolving 1.0000 g mercurous nitrate (Fisher Scientific, Toronto Canada, M-168) in 1.00 L of 0.1% v/v nitric acid (CanLab McGraw Ltd.). Working solutions were prepared daily as required by serial dilution of this stock solution.
- iii) Elemental mercury solutions; Aqueous solutions of elemental mercury were prepared in a 3-neck round bottom flask (250 mL) equipped with two side-arms and a septum. Distilled deionized water was placed in the flask, boiled for 10 minutes and then purged

with nitrogen gas through a medium porosity glass frit for 30 minutes. Metallic mercury (0.2 mL) was placed in the side-arms and the flask was shaken overnight in a metabolic shaker. Solutions were prepared fresh daily as required.

Catalase Solutions (E.C. No. 1.11.1.6)

Catalase solutions were prepared fresh daily as required by dissolving the desired amount of Bovine Liver Catalase (Sigma Chemical Co., St. Louis Mo., C-100) in phosphate-phosphate buffer (pH=7.00 0.05M).

Hydrogen Peroxide Solutions

All working solutions were prepared by serial dilutions of a 30% stock solution (Anachemia Ltd., Montreal Canada)

Peracetic Acid Solutions,

40% solution was obtained from FMC Corporation, Buffalo, N.Y., Working solutions were prepared as required by serial dilutions of this stock.

1. Double Magos Cold Vapour Atomic Absorption Analysis

Reagents

- i) Stannous chloride reducing agent; was prepared by dissolving 0.40 g L-cysteine (Sigma Chemical Co.) and 4.00 g stannous chloride (Analar BDH Chemicals, Toronto Canada) in 200 mL sulfuric acid-sodium chloride solution (256 mL sulfuric acid, Aristar BDH Chemicals Poole England + 20.1 g sodium chloride, Fisher Scientific, made up

to 2.00 L with distilled deionized water).

- ii) Sodium Hydroxide; 35% w/v sodium hydroxide solution was prepared from sodium hydroxide pellets (Natriumhydroxid, Riedel-De Haen a.g., Seelze-Hannover).
- iii) Non-reducing reagent; distilled deionized water was used as the non-reducing reagent.

Apparatus

A schematic diagram of the cold vapour atomic absorption apparatus is shown in Figure 102. A mercury U.V. Monitor Model 1235 (Laboratory Data Control) was used. The inlet of the gas cell was connected successively to Tygon tubing, a midget impinger of 25 mL volume (containing 10 mL deionized water and 20 drops antifoam solution) and then to a 30 mL reaction vessel fitted with a long stem funnel. The outlet of the gas cell was connected by Tygon tubing to a Neptune Dyna Pump (CanLab Cat. # P8395-3) through a flowmeter (Dwyer 5 L.P.M. Air Furnace Eng. Co., Montreal, Cat. # VFA-23-FSV).

The output was measured by a Digitec Voltmeter H.T. Series 2120-Multimeter (United States Corporation), and a Minigrator (Spectra Physics) all connected as shown in Figure 102. Reducing and non-reducing reagents were dispensed into the reaction vessel using Brinkmann Dispensettes (0-5.0 mL).

The reaction vessel is diagrammatically illustrated in Figure 103.

Figure 102: Schematic diagram of Cold Vapour Atomic Absorption Apparatus

Figure 103: Reaction Vessel

Procedure

For total inorganic mercury analysis the following procedure was utilized. The reaction vessel was rinsed with tap water, the drainage valve was closed, the integrator was switched on, the sample was injected into the reaction vessel immediately followed by 2.5 mL stannous chloride reducing agent and 2.5 mL 35% sodium hydroxide solution. After the peak area was recorded by the integrator the drainage valve was opened, the reaction vessel was rinsed with water and the next sample was prepared for injection.

For "non-reducing" volatile mercury analysis essentially the same procedure was followed except that the reaction vessel was rinsed for approximately 20 minutes with 30% hydrogen peroxide prior to analysis to remove residual reducing agent. Following sample injection 5.0 mL distilled deionized water was dispensed into the reaction vessel instead of the $\text{SnCl}_2/\text{NaOH}$ reagents. Integrated peak area was recorded and the reaction vessel was rinsed and prepared for the next sample.

Sample aliquots varied from 10 μL to 1.0 mL depending on the relative mercury concentration of the sample solution.

Standard curves for inorganic Hg^{Total} were prepared monthly to ensure accurate calibration and sensitivity of the apparatus.

2. Dithizone Hg^{2+} Determination

The procedure used was essentially that used by Elly (5). A 100 mL sample containing 10-100 ppb Hg^{2+} was placed in a 250 mL flask.

Concentrated sulfuric acid (3.0 mL Aristar BDH Chemical Co.) was added,

the solution mixed, cooled to room temperature, and transferred to a 250 mL separatory funnel. After adding 8.0 mL 6N acetic acid (J.T. Baker, Phillipsburg N.J.) and 5.0 mL chloroform (Anachemia Ltd.), the solution was shaken vigorously for 1 minute. When the phases were separated completely, the chloroform layer was withdrawn and discarded. Dithizone solution (10.0 mL of 0.001% Dithizone, J.T. Baker Chemical Co. in chloroform, Anachemia Ltd.) was added and the solution shaken again for 1 minute. The layers were allowed to separate and the dithizone-mercury solution was withdrawn. The absorbance at 495 nm was measured on a Beckman Model 25 Spectrophotometer in quartz cells previously rinsed with chloroform. A standard curve was constructed by use of a series of standard Hg^{2+} solutions.

3. Speciation and Behaviour of Mercurous Ion in Cold Vapour Atomic Absorption Analysis

Chloride ion effect

Mercurous nitrate solutions ($0-300 \text{ ug L}^{-1}$) were treated with sodium chloride (Fisher Scientific A.C.S.) to yield a 0.1 M chloride solution. No mercurous chloride precipitate was evident in any of these solutions despite the 10^5 excess of chloride over mercury. Chloride treated solutions were analyzed by the double Magos CVAA and dithizone techniques described above.

Variation of reagents

Cold vapour atomic absorption analysis was performed on mercurous ion solutions (in either 0.1 M NaCl or 0.01 M HNO_3) with a variety

of reducing and non-reducing agents. All reagents were prepared and dispensed as described above.

Mercurous nitrate solution were treated with EDTA (10^4 excess, disodium EDTA, dihydrate, J.T. Baker Chemical Co.) and L-cysteine (10^3 excess, Sigma Chemical Co.). Solutions were then analyzed by the double Magos CVAA technique.

Variation of pH

Mercurous nitrate solutions ($0-300 \text{ ug L}^{-1}$) were treated with 35% w/v sodium hydroxide or 70% v/v nitric acid to adjust pH. pH was determined using a Corning pH/ion meter 135. Solutions were then analyzed by the double Magos CVAA technique.

Sparging experiments

Mercurous nitrate solutions were placed in 250 mL round bottom flasks and sparged with nitrogen gas through a medium porosity glass frit. Samples were removed and analyzed by the double Magos CVAA technique.

4. Uptake Experiments

Reagents

- i) t-Butyl Hydrogen Peroxide; t-butyl hydrogen peroxide solutions were prepared by serial dilution of t-Butyl Hydroperoxide-70 (Wallace and Tiernan Inc., Buffalo N.Y.).
- ii) Ethyl Hydrogen Peroxide; solutions were prepared by serial dilutions of 10% aqueous Ethyl Hydrogen Peroxide (Polysciences Inc., Warrington P.A.).

iii) m-Chloroperoxybenzoic Acid; 0.5 g m-chloroperoxybenzoic acid (Aldrich Chemical Co., Milwaukee Wis.) was dissolved in 10.0 mL absolute ethanol (Consolidated Alcohols Ltd., Toronto Canada). Reagent solutions were prepared by serial dilutions of this stock solution with distilled water.

Catalase, hydrogen peroxide and peracetic acid were prepared as previously described.

Incubation and Analysis

Enzyme solution (3.0 mL) or buffer solution (phosphate-phosphate pH=7.00, 0.05 M, 3.0 mL) or water (3.0 mL) was placed in the main body of each Thunberg flask. The test reagent (20 uL) was added and the flasks were stoppered with a side-arm containing metallic mercury (0.1 mL). The flasks were incubated overnight in a metabolic shaker. At the end of the incubation period the side-arms were removed, flasks were sealed with parafilm and solutions were analyzed by the double Magos CVAA technique.

5. Kinetic Experiments with Homogeneous Solutions

20.0 mL of homogeneous elemental mercury or mercurous nitrate solution was placed in a 25 mL round bottom flask. The flask was fitted with a rubber septum and a sample aliquot was removed and analyzed for "non-reducing" volatile mercury by the CVAA technique. At t=0 minutes the test oxidizing agent(s) was added, sample aliquots were removed at regular time intervals and analyzed for volatile

"non-reducing" mercury in the CVAA apparatus. Control runs were carried out to monitor mercury total concentration (i.e. stability of reducing signal) over the reaction time interval.

Inhibition studies with 3-amino-1,2,4-triazole were performed by adding the desired amount of 3-Amino-1,2,4-triazole (Sigma Chemical Company).

6. Spectroscopic Studies

Spectroscopic studies of Catalase, Catalase Compound I and Catalase Compound II were performed with a Beckman DU 7 U.V./Vis. Spectrophotometer. All scans were done using 1.5 mL quartz cells at a rate of 600 nm min^{-1} .

7. Stopped Flow Experiments

Catalase, peracetic acid and mercury solutions were prepared as previously described. The concentration of Catalase was determined spectrophotometrically at 405 nm using a molar extinction coefficient of $3.97 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (43). All stopped-flow kinetic experiments were carried out in a Tri-tech Dynamic Instruments Model IIA Stopped-flow Apparatus (Cantech Scientific Ltd., Winnipeg Canada). For kinetic measurements one reservoir of the stopped-flow apparatus contained 1.85 nM Catalase pre-spiked with Hg(0), Hg(I), or Hg(II). For control experiments water was added instead of mercury solution. The other reservoir contained at least 25 uM peracetic acid. The formation of Compound I was followed by monitoring the decrease in absorbance as a function of time at 405 nm. The observed rate constants were

determined from a non-linear least squares analysis- Kinetic Ver 6- (Cantech Scientific Ltd.). Parallel mixing experiments were performed in the Beckman DU 7 spectrophotometer and the visible spectra of the resulting compounds were recorded for identification purposes.

* N.B. In some experiments the signal under non-reducing conditions in the CVAA apparatus appeared to exceed that under reducing conditions. This was due to unavoidable time lags between determinations, resulting in the loss of Hg^0 from solution.

REFERENCES

1. J.G. Saha, K.S. McKinlay, Toxicology and Environmental Chemistry Reviews 1, 271 (1973).
2. L.J. Casarett, J. Doull, in "Toxicology The Basic Science of Poisons", p. 484, MacMillan Publishing Co., New York, 1975.
3. W.E. Donaldson, in "Trace Element Toxicity", p. 330,
4. A.K. Shrivastava, S.G. Tandon, Toxicology and Environmental Chemistry 5, 311 (1982).
5. C.T. Elly, Journal of the Water Pollution Control Federation 45, 940 (1973).
6. T.W. Clarkson, J. Gatz, C. Dalton, in "Studies on the Equilibration of Mercury Vapor with Blood", University of Rochester A.E.P. Report V.R. 582 (1961).
7. W.R. Hatch, W.L. Ott, Analytical Chemistry 40, 2085 (1963).
8. P. Coyle, T. Hartley, Analytical Chemistry 53, 254 (1981).
9. R. Yamamoto, H. Satoh, T. Suzuki, A. Nagamura, N. Imura, Analytical Biochemistry 101, 254 (1980).
10. K. Minagawa, Y. Takizawa, Analytica Chimica Acta 115, 103 (1980).
11. K. Tanabe, J. Takahashi, H. Haraguchi, K. Fuwa, Analytical Chemistry 52, 453 (1980).
12. D.C. Stuart, Analytica Chimica Acta 106, 411 (1979).

13. J.F. Chapman, L.S. Dale, *Analytica Chimica Acta* 101, 203 (1978).
14. L. Ebdon, J.R. Wilkinson, K.W. Jackson, *Analytica Chimica Acta* 128, 45 (1981).
15. I. Sanemasa, E. Takagi, T. Deguchi, H. Nagai, *Analytica Chimica Acta* 130, 149 (1981).
16. A.M. Ure, *Analytica Chimica Acta* 76, 1 (1975).
17. C.E. Oda, J.D. Ingle, *Analytical Chemistry* 53, 2305 (1981).
18. C.E. Oda, J.D. Ingle, *Analytical Chemistry* 53, 2030 (1981).
19. C.A. McAuliffe, "The Chemistry of Mercury", 1st ed., The MacMillan Press Ltd., New York, 1977.
20. J.F. Uthe, F.A.J. Armstrong, *Toxicological and Environmental Chemistry Reviews* 2, 45 (1974).
21. C.L. Winek, F.W. Fochtman, J.D. Bricker, C.H. Wecht, *Clinical Toxicology* 18, 261 (1981).
22. L. Magos, *Environmental Research* 1, 323 (1967).
23. T.W. Clarkson, S. Halbach, L. Magos, Y. Sugata, in "Molecular Basis of Environmental Toxicity", Chapter 18, Ann Arbor Science, 1980.
24. Nielsen-Kudsk, *Acta Pharmacologica et toxicologica* 27, 161 (1969).
25. F. Nielsen-Kudsk, *Acta Pharmacologica et toxicologica* 27, 149 (1969).
26. L. Magos, T. Sugata, T.W. Clarkson, *Toxicology and Applied Pharmacology* 28, 367 (1974).

27. L. Magos, S. Halbach, T.W. Clarkson, *Biochemical Pharmacology* 27, 1373 (1978).
28. S. Halbach, T.W. Clarkson, *Biochimica et Biophysica Acta* 523, 522 (1978).
29. J.D. Dunn, T.W. Clarkson, L. Magos, *British Journal of Industrial Medicine* 35, 241 (1978).
30. J.B. Hursh, M.R. Greenwood, T.W. Clarkson, J. Allen, S. Demuth, *The Journal of Pharmacology and Experimental Therapeutics* 214, 520 (1980).
31. T.W. Clarkson, L. Magos, *The Journal of Pharmacology and Experimental Therapeutics* 216, 19 (1981).
32. J.D. Dunn, T.W. Clarkson, L. Magos, *Science* 213, 1123 (1981).
33. M. Palcic, H.B. Dunford, *Canadian Journal of Biochemistry* 57, 321 (1979).
34. Y. Sugata, S. Halbach, J. Allen, T.W. Clarkson, *Journal of Environmental Science and Health* 613, 97 (1979).
35. I., Eide, T.L.M. Syverson, *Acta Pharmacologica et toxicologica* 51, 371 (1982).
36. Y. Sugata, T.W. Clarkson, *Biochemical Pharmacology* 28, 3474 (1979).
37. M. Ogata, M. Ikeda, Y. Sugata, *Archives of Environmental Health* 34, 218 (1979).
38. M. Ogata, K. Kenmotsu, N. Hirota, M. Naito, *Bulletin of Environmental Contamination and Toxicology* 27, 816 (1981).
39. M. Ogata, H. Aikoh, *Physiological Chemistry and Physics and Medicinal N.M.R.* 15, 89 (1983).
40. A. Deisseroth, A.L. Dounce, *Physiological Reviews* 50, 319 (1970).

41. J.W. Hartz, S. Funakoshi, H.F. Deutsch, Clinica Chimica Acta 46, 125 (1973).
42. M. Palcic, H.B. Dunford, Canadian Journal of Biochemistry 57, 321 (1979).
43. M. Palcic, H.B. Dunford, Journal of Biological Chemistry 255, 6128 (1980).
44. M. Palcic, H.B. Dunford, Archives of Biochemistry and Biophysics 211, 245 (1981).
45. H. Ruis, Canadian Journal of Biochemistry 57, 1122 (1979).
46. N.R.C. Associate Committee on Scientific Criteria for Environmental Quality, "effects of Mercury in the Canadian Environment", NRCC No. 16789.
47. H.C. Moser, A.F. Voigt, Journal of the American Chemical Society 79, 1837 (1957).
48. J.N. Spencer, A.F. Voigt, Journal of Physical Chemistry 72, 464 (1968).
49. R.R. Kuntz, G.J. Mains, The Journal of Physical Chemistry 68, 408 (1964).
50. S. Okouchi, S. Sasaki, Bulletin of the Chemical Society of Japan 54, 2513 (1981).
51. S. Okouchi, S. Sasaki, Bulletin of the Chemical Society of Japan 54, 2821 (1981).
52. D.N. Glew, D.A. Hames, Canadian Journal of Chemistry 49, 3114 (1971).
53. S. Chilov, Talanta 22, 205 (1975).

54. The Mercury Analysis Working Party of the Bureau International
Technique du Chlore, *Analytica Chimica Acta* 109, 209 (1979).
55. M.R. Greenwood, P. Dhahir, T.W. Clarkson, J.-P. Farent, A. Chartrand,
A. Khayat, *Journal of Analytical Toxicology* 1, 265 (1977).
56. N.V. Sidgwick, in "The Chemical Elements and their Compounds", Volume 1,
p289, Oxford at the Clarendon Press, Oxford, 1950.
57. R.J. Baltisberger, D.A. Hildebrand, D. Griehle, T.A. Ballintine,
Analytica Chimica Acta 111, 111 (1979).
58. D.A. Hildebrand, Ph.D. Thesis, University of North Dakota, 1977.
59. D.C. Wifgield, S.L. Perkins, *Journal of Analytical Toxicology* 6,
279 (1982).
60. R. Davies, B. Kipling, A.G. Sykes, *Journal of the American Chemical
Society* 95, 7250 (1973).
61. R.J. Baltisberger, C.L. Knudson, *Analytica Chimica Acta* 73, 265 (1974).
62. W.R. Browett, M.J. Stillman, *Biochimica et Biophysica Acta* 623, 21 (1980).
63. W.R. Browett, M.J. Stillman, *Biochimica et Biophysica Acta* 660, 1 (1981).
64. P. Jones, D.N. Middlemiss, *Biochemical Journal* 130, 411 (1972).
65. D. Dolphin, *Accounts of Chemical Research* 7, 26 (1973).

66. P. Jones, H.B. Dunford, *Journal of Theoretical Biology* 69, 457 (1977).
67. M. Kajiyoshi, F.K. Anan, *Journal of Biochemistry* 81, 1319 (1977).
68. D. Dolphin, in "The Biological Chemistry of Iron", p. 283, D. Riedel Publishing Co., Dordrecht, Holland, 1982.
69. P. Jones, in "The Biological Chemistry of Iron", p. 427, D. Riedel Publishing Co., Dordrecht, Holland, 1982.
70. C.R. Møller, in "Chemistry of Organic Compounds", p. 201, W.B. Saunders Company, 3rd ed., Philadelphia, 1966.
71. P.R. Wells, *Chemical Reviews* 63, 171 (1963).
72. E. Onat, *Journal of Inorganic and Nuclear Chemistry* 36, 2029 (1974).
73. D. Littlejohn, G.S. Fell, J.M. Ottaway, *Clinical Chemistry* 22, 1719 (1976).
74. R. Andruzzi, A. Trazza, *Talanta* 28, 839 (1981).
75. Z. Wittmann, *Talanta* 28, 271 (1981).
76. G. Somor, *Analytical Chemistry* 53, 2143 (1981).
77. W.H. Shroeder, *Environmental Science and Technology* 16, 394 A (1982).
78. T.R. Tephly, G.J. Mannering, R.E. Parks, *Journal of Pharmacology and Experimental Therapeutics* 134, 77 (1961).

79. F. Nielsen-Kudsk in "Mercury, Mercurials & Mercaptans", M.W. Miller
T.W. Clarkson, Eds., Charles C. Thomas Publisher, Springfield, Ill., 1973.
80. H.M.N.H. Irving, in "Dithizone", p61 The Chemical Society, Burlington
House, London, 1977.
81. L. Magos, Journal of the Association of Official Analytical Chemists
55, 966 (1972)
82. L. Magos, T.W. Clarkson, M.R. Greenwood, Toxicology and Applied
Pharmacology 26, 180 (1973).
83. I. Eide, T.L.M Syversen, Acta Pharmacologica et toxicologica 52,
217 (1983)
84. M. Ogata, K. Kenomotsu, N. Hirota, H. Aikoh, Archives of Toxicology 50,
93 (1982).
85. A. S. Brill, R.J.P. Williams, Biochemical Journal 78, 246 (1961).
86. B. Chance, Journal of Biological Chemistry 179, 1299 (1949).
87. W.R. Browett, M.J. Stillman, Biochimica et Biophysica Acta 577,
291 (1979).
88. B. Chance, Nature 161, 914 (1948).
89. P. Jones, A. Suggett, Biochemical Journal 110, 621 (1968).

90. T.Y. Toribara, C.P. Shields, L. Koval, Talanta 17, 1025 (1970).
91. R.M. Rosain, C.M. Wai, Analytica Chimica Acta 65, 279 (1973).
92. R.A. Carr, P.E. Wilkniss, Environmental Science and Technology 7, 62 (1973).
93. M.R.N. Murthy, T.J. Reid, A. Scicignano, N. Tanaka, M.G. Rossmann, in "The Biological Chemistry of Iron", p. 439, D. Riedel Publishing Co., Dordrecht, Holland, 1982.
94. S. Tse, M.Sc. Thesis in progress.

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