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STUDIES ON THE PHARMACODYNAMICS AND TOXICITY
OF CHLORINE-DIOXIDE IN DRINKING WATER IN RAT
AND CHICKEN.

THE OHIO STATE UNIVERSITY, PH.D., 1979
STUDIES ON THE PHARMACODYNAMICS AND TOXICITY OF
CHLORINE DIOXIDE IN DRINKING WATER IN RAT AND CHICKEN

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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I would like to express my most sincere thanks to my faculty adviser, Professor Daniel Couri, whose constant stimulation, guidance and criticisms have provided much help to me not only in my graduate studies but also in my life in my new country. Dr. Couri's enthusiasm for science is an inspiration to his students. Also, my appreciation is extended to Dr. Couri's family.

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I would like to especially recognize Lynn Hetland, John Young and Karl Ebner for their encouragement and advice.

To the group, Al, Bob, Joe, Pam, Steve, Mike, Paul, Jon and all persons in Toxicology, I want to thank them for their assistance and friendship during my graduate studies.
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INTRODUCTION

A. WATER POLLUTION

"To live is to pollute. This condition of existence man shares with all creatures. Even, however, as evolution granted man greater gifts to control environment, so did it enable him to dirty it most. Once used, urban water is a waste carrier", Kogan (1). The people of the United States use water lavishly. Swimming pools and lawn sprinklers help make this nation the greatest per capita water consumers. The average citizen uses no less than five gallons a day to shave, wash and brush his teeth. Flushing the toilet once requires five gallons. A minute under a running shower spends five gallons. The character of the waste reflects the times. Civilization and industries create a new era and every new era has brought new wastes. Detergents long ago replaced the strong lye soaps of another generation. Tissues have almost wholly relaced handkerchiefs. Leftovers from dinner, carefully preserved by the thrifty housewife of yesteryear, are now fed to the pig. All this and more finds its way into sewage pipes.

Human wastes are not the only problem. Farm animals produce ten times as much organic waste as the human population. In some areas waste is just collected by sewer pipes that lead it unpurified to the nearest river. At
least this removes the problem from immediate sight. To all these pollu-
tions one must add over 500,000 different chemicals that find their way
into U. S. streams.

In 1965 Congress passed the Water Quality Act and in 1966 it passed
the Water Pollution Control Act (1). They are largely concerned with the
protection of surface water, rivers, lakes and streams. Disposal of
wastes in deep wells also needs controls. Many of these wastes are highly
toxic, containing a variety of substances from cyanides to radioactive
wastes. Brine waste injected deep into Canaian earth has ended up in
Michigan, Crossland and Brodine (2). Also, by injecting the wastes into
the earth in earthquake-prone areas, a gradual dislocation may be induced.
All of this indicates that the dimensions of this nation's water pollution
are indeed enormous.

In a water treatment plant, water purification was accomplished
primarily by filtration and chlorination. Safe drinking water is one of the
triumphs of modern public health. Even before chlorine was introduced into
the United States as a water disinfectant in 1908, filtration alone could
provide considerable protection against waterborne disease (1). Among
the most common of such diseases are typhoid fever and cholera. These
microbial infections are transmitted by human feces and urine. Chlorina-
tion brought bacteriological safety and it does not cost much more to
purify highly polluted water than relatively safe water.
B. THE CHLORINATED ORGANIC PROBLEM

Chlorine is, and has been, the most widely used disinfectant in the treatment of water supplies. Because it is the least expensive means of preventing the spread of waterborne disease, it has been employed by virtually every municipal water supply in the United States since early in the 20th century. However, recent studies have demonstrated that the interaction of chlorine with various organic substances in the water results in the formation of trihalomethanes, Rook (3). Industrial wastes, domestic sewage and agricultural runoff all contribute to the problem of water pollution by organic chemicals. The awareness of the widespread occurrence of this type of pollution is due, in large part, to recent development in techniques for the identification of trace organic contaminants. In particular the availability of reliable gas chromatograph-mass spectrometer equipment has led to a proliferation of information in this area. Rook (4), Bellar et al. (5), Stevens et al. (6) reported that chlorinated organics are formed during the chlorination step in water treatment. Rook (4) found that the chlorination of Rhine River water brought about the formation of chloroform (CHCl$_3$), bromodi-chloromethane (CHBrCl$_2$), dibromochloromethane (CHBr$_2$Cl) and bromoform (CHBr$_3$). Bellar et al. (5) found that the concentration of CHCl$_3$, CHBrCl$_2$ and CHBr$_2$Cl increased each time chlorine was added within the treatment scheme.

The four trihalomethanes were not a result of impurities in the chlorine being used but were a result of the reaction of chlorine with precursor. Morris (7) made the point that the number of possible
trihalomethane precursors is extensive and includes ethanol, acetaldehyde and the methyl ketones. Stevens et al. (6) showed that acetaldehyde and acetone did not contribute to observed formation of trihalomethanes in waterworks upon chlorination at pH 7. Rook (4) agreed with Morris (7) that the bromide ion present in a natural water is rapidly oxidized by aqueous chlorine to hypobromous acid (HOBr), and that this acid reacts with precursor to account for the formation of the brominated species. This principle was demonstrated when bromide salts were added to Missouri River water prior to chlorination yielded higher concentrations of brominated trihalomethanes than when the water was chlorinated without the added bromide. Stevens et al. (6) used samples from a pilot plant to examine the chlorine-precursor-trihalomethane relationship. The water treated in this study was Ohio River water. It was collected on a regular basis at the raw water pump station at the Cincinnati water works and trucked to the E.P.A. research facility where it was transported to the pilot plant storage tank. The pilot plant was designed specifically for the study of organics in drinking water. The plant was constructed almost entirely of stainless steel, Teflon and glass, in order to minimize any organic contamination. Among their findings near pH 7 and 25°C were the following:

1. When chlorinated, raw (Ohio River) water yielded higher levels of trihalomethanes than did coagulated and settled water. When chlorinated, coagulated and settled water yielded higher levels of trihalomethanes than did water which had been coagulated, settled and filtered through granular activated carbon (GAC).
2. CHCl₃ production for chlorinated settled water increased with increasing pH or increasing temperature.

Because of the widespread concern for organics in drinking water, the United States Environmental Protection Agency (EPA) undertook an examination of the water supplies of eighty communities in the nation to determine the extent of trihalomethane presence. From this survey Symons et al, (8) reported:

1. As a result of the chlorination step in water treatment, CHCl₃, CHBrCl₂, CHBr₂Cl and CHBr₃ were widespread in U. S. drinking water supplies.

2. Using non-volatile total organic carbon as an indicator of the precursor, trihalomethane concentrations were generally related to the organic content of the raw water, provided a free chlorine residual was present in the finished water.

3. Generally, higher concentrations of trihalomethane were found in the finished water where raw water was from a surface source.

C. HEALTH EFFECT OF TRIHALOMETHANES

Studies of the health effects of trihalomethanes in drinking water were begun after the widespread trihalomethane presence was detected. The predominant product, chloroform, has been declared a carcinogen by the National Cancer Institute in that it caused liver cancer in laboratory animals (9). EPA reported in 1957, that although carcinogenesis data
for chloroform was limited, laboratory animal studies had shown hepatomas produced by chloroform (10). According to Von Oettingen (11), the outstanding effect of CHCl₃ is depression of the central nervous system, but it is also toxic to the liver and, to a lesser degree, the kidneys. Studies undertaken to look for statistically significant correlation between cancer mortality and CHCl₃ in drinking water have produced contradictory results, EPA (12). Druckrey (13) reported that, highly chlorinated drinking water, containing 100 mg of free chlorine per liter, was well tolerated when given on a daily basis to seven successive generations of 236 BD II rats during their entire lifetime. The research did not supply any evidence of toxic effects upon the propagation, growth, blood picture and histology of liver, spleen, kidneys or other organs. The incidence of malignant tumor was identical in test animals and control groups.

Epidemiological studies have demonstrated statistical associations between increased cancer mortality and the practice of chlorination of drinking water, Cantor (14), Page et al. (15), Kuzma et al. (16), Salg (17), Kruse (18) and Alavenja et al. (19). These findings have tended to support laboratory studies which demonstrated the occurrence of liver cancer in selected rat and mouse strains exposed to chloroform, Roe (20). In contrast to the above reported association between trihalomethane exposure and cancer mortality, not all epidemiological studies have supported the proposed cancer association, Tuthill and Moore (21). Also Stokinger (22) has criticized the extrapolation of Roe (20) studies with CHCl₃ since their equivalent level of human exposure in drinking water would require the consumption of from 57,000 to 114,000 liters of water.
per day from sources showing the highest chloroform levels (0.311 ppm). However, the National Academy of Sciences (NAS) report that a lifetime exposure (70 years) to 40 µg CHCl₃/day or (20 µg/liter) would result in one excess case of cancer per 33,333 persons (23).

D. TREATMENT FOR THE REMOVAL OR PREVENTION OF TRIHALOMETHANES

Because of the possibility of injurious health effects of chloroform and the questionable toxicity of the other trihalomethanes, EPA (24) undertook to examine treatment methods that could be recommended to the waterworks community to lower or eliminate the presence of trihalomethanes in drinking water. According to EPA several criteria must be met if any alternative method of disinfection is to replace the widespread use of free chlorine. The criteria include:

a) easy generation
b) history of successful use in communities
c) good biocidal action
d) easily measurable residual
e) cost effective
f) non-toxic to humans

The disinfectants available as potential alternatives to chlorination include bromide, chloride, chloramine, chlorine dioxide, heat, iodine, ionizing radiation, ozone, permanganate, pH, silver, ultra-violet radiation. However, chlorine dioxide and ozonation are the methods receiving the most widespread attention. At this time chlorine dioxide (ClO₂) seems to best satisfy the above listed criteria. This is based on its good
disinfection properties, and its easily measurable residual as compared to ozonation, Augenstein (25).

E. BACKGROUND INFORMATION ON CHLORINE DIOXIDE

Chlorine dioxide (ClO$_2$) gas has an unpleasant odor, is unstable in light, but stable in the dark. The ClO$_2$ molecule has 19 electrons in its valence shell. The O-Cl-O angle is only slightly smaller than the bond angle of sulphur dioxide, the short bond length and high valence force constant similarly testify to the strength of chlorine-oxygen bond, Marino and Hirota (26). ClO$_2$ presents an interesting example of an odd-electron molecule which is stable towards dimerization.

ClO$_2$ has approximately five times more oxidation capacity than chlorine. A major use of ClO$_2$ in the United States is for pulp bleaching, White (27), Rapson (28), but it is also used for other purposes, including the treatment of swimming pool water, odor destruction in scrubbing towers for the control of air pollution, Malpas (29), microbiological control in the cooling waters at the aluminum refining industry, ammonia synthesis, vegetable oil refinery and in steel industries, Ward (30), and in the manufacture of chlorite salts.

a. ClO$_2$ Reactions

Gordon et al. (31) reported that ClO$_2$ is light sensitive and slowly decomposed to chlorate (ClO$_3^-$), chlorite (ClO$_2^-$) and chloride (Cl$^-$).

\[
2\text{ClO}_2 + \text{H}_2\text{O} \rightarrow 2\text{H}^+ + \text{ClO}_2^- + \text{ClO}_3^-
\]

\[
3\text{ClO}_2^- \rightarrow \text{Cl}^- + 2\text{ClO}_3^-
\]
White (27) reported that ClO_2 does not disproportionate in water. It appears that this disproportionation is rate dependent on pH. Tabue and Dodgen (32) reported disproportionation in neutral solution to chlorite (ClO_2^-) and ClO^- is slow, but Gordon et al. (31) stated that at strongly alkaline pH, ClO_2^- disproportionated rapidly to give equal amounts of ClO_2^- and ClO^-.

However, Gordon et al. (31) in an extensive review of the chemistry of ClO_2 concluded that this disproportionation had not been sufficiently investigated.

ClO_2 is 52.6% chlorine by weight. In the process of reduction to Cl^-, ClO_2 undergoes five reduction steps:

\[
\text{ClO}_2 + 5 \text{e}^- \rightarrow \text{Cl}^- + 2 \text{O}^-
\]

In its process of reduction to Cl^-, Cl_2 undergoes one reduction step:

\[
\frac{1}{2} \text{Cl}_2 + \text{e}^- \rightarrow \text{Cl}^-
\]

In terms of oxidation capacity, then 1.0 mg/l of ClO_2 equals 2.63 (0.526 x 5) times the oxidizing power of chlorine. Similarly, 1.0 mg/l of ClO_2^- equals 2.1 mg/l of chlorine.

An important characteristic of the gas is its explosiveness. White (27) reported that a rise in temperature, exposure to light or a sudden shaking of its container may cause an explosion. ClO_2 is, then never transported, but is manufactured at its point of use. Ward (30) and White (27) stated that the gas can be handled safely if it is approximately 10% of an air mixture. ClO_2 is quite soluble in water depending on temperature and pressure. In aqueous solution, ClO_2 is quite safe.
It forms a greenish-yellow solution which is visible at approximately 4 mg/l and has a noticeable odor at approximately 10 mg/l. The aqueous chemistry of chlorine dioxide is very complex. In water treatment involving ClO₂, the chloro-species, might include ClO₂, free Cl₂, combined (Cl₂; ClO₂⁻ and its acid HClO₂; ClO₃⁻ and its acid HClO₃ and Cl⁻).

b. Preparation and Manufacture of ClO₂

ClO₂ can be prepared either by reduction of chlorates or by oxidation of chlorites. The large scale commercial operation of bleaching pulp with chlorine dioxide uses reduction of chlorate:

1) \[ 5\text{NaClO}_3 + 6\text{HCl} \rightarrow 6\text{ClO}_2 + 5\text{NaCl} + 3\text{H}_2\text{O} \]

Dobryshin (33) noted that the reduction of ClO₃⁻ by hydrochloric acid in temperature above 35°C leads to an appreciable increase in (ClO₂ + Cl₂) gas mixture.

2) \[ 6\text{NaClO}_3 + \text{Cr}_2(\text{SO}_4)_3 + 2\text{H}_2\text{O} \rightarrow 6\text{ClO}_2 + 2\text{H}_2\text{CrO}_4 + 3\text{Na}_2\text{SO}_4 \]

3) \[ 2\text{HClO}_3 + \text{SO}_2 \rightarrow 2\text{ClO}_2 + \text{H}_2\text{SO}_4 \]

2 faradays

4) \[ 2\text{NaCl} + 2\text{NaClO}_2 + 2\text{H}_2\text{O} \rightarrow 2\text{ClO}_2 + 2\text{NaCl} + 2\text{NaOH} + \text{H}_2 \]

White (27) reported that the electrochemical method was expensive and difficult to control.
There are several methods by which ClO₂ may be efficiently produced prior to its application to the drinking water. The two principal ways by which ClO₂ is produced are:

a) \[ \text{Cl}_2 + 2\text{NaClO}_2 \rightarrow 2\text{ClO}_2 + 2\text{NaCl} \]

b) \[ 2\text{KClO}_3 + (\text{COOH})_2 + 2\text{H}_2\text{SO}_4 \rightarrow 2\text{ClO}_2 + 2\text{CO}_2 + 2\text{H}_2\text{O} + 2\text{KHSO}_4 \]

The formation of CO₂ tends to reduce the explosion hazard.

c. **Determination of Chlorine Dioxide and Other Active Chlorine Compounds**

The literature indicates that several analytical procedures for the determination of ClO₂ in drinking water are available: Iodometric (34, 35, 36), orthotolidine (37), tyrosine (38), DPD (39, 40) and acid chrome violet K (41). The United States Public Health Service (42) conducted a comparative study of the methods and concluded that the DPD titrimetric method showed the best overall. Also, Adams et al. (43) and Ward (30) reported that DPD was most reliable and the procedure of choice.

The Palin method for the determination of Cl₂, ClO₂, ClO⁻ uses the reagent diethyl-p-phenylene diamine (DPD). A red color results from the reaction of this reagent with either chlorine or chlorine dioxide at pH 7. Cl₂ is reduced to Cl⁻ and ClO₂ to ClO₂⁻ at this pH. When acidified, ClO₂⁻ liberates iodine from iodide. In the presence of DPD, iodine produces a red color upon return to neutral pH. The red color may be titrated to colorless with ferrous ammonium sulfate (FAS). Malonic acid can prevent Cl₂ from interfering with the determination, Adams et al. (43).
The optimum pH for the red color is 6.4 and buffer is used for this purpose (27).

d. Historical Uses of Chlorine Dioxide as a Disinfectant

The first reported use of ClO$_2$ in the treatment of drinking water occurred in 1944 at the Niagara Falls water treatment plant, Kroke (44). The use of ClO$_2$ in this instance resulted because of the availability of a large inventory of sodium chlorite for military use during World War II, Musil et al. (45). In addition to both taste and odor control, ClO$_2$ has been used to enhance the precipitation of iron and manganese and for final disinfection. Both iron and manganese can be oxidized by ClO$_2$. ClO$_2$ changes the soluble ferrous ion to the ferric ion for precipitation as ferric hydroxide (27).

\[ \text{ClO}_2 + \text{Fe}^{2+} + 3\text{OH}^- \rightarrow \text{Fe(OH)}_3 \downarrow + \text{ClO}^- \]

ClO$_2$ has been used for this purpose when ammonia prevented the use of Cl$_2$ as the oxidant, since ClO$_2$ does not react with ammonia (46, 47). ClO$_2$ also oxidized manganese in a basic solution, Griffin (48).

\[ 2\text{ClO}_2 + \text{Mn}^{2+} + 4\text{OH}^- \rightarrow \text{MnO}_2 \downarrow + 2\text{ClO}^- + 2\text{H}_2\text{O} \]

The extent of ClO$_2$ use and control in water treatment practice is not well known based on responses by 56 users. Granstrom and Lee (35) reported that 27% used ClO$_2$ specifically for disinfection. 93% reported that it was beneficial for taste and odor problems, mainly phenolic. All users used excess chlorine besides ClO$_2$, and the majority added chlorine ahead of ClO$_2$. Only 40% reported measuring ClO$_2$. 
concentration and personnel commented that analytical techniques were troublesome. The inventory of municipal water supplies noted that only 8 of 11,590 water treatment plants used ClO₂ (49). Among the states in which ClO₂ was most extensively used were Georgia, Ohio, Pennsylvania and Michigan. ClO₂ has been used not only in the United States, but the EPA (50) has reported that Canada has 10 facilities and Europe several thousand, principally in West Germany, Switzerland and France.

F. HEALTH EFFECT OF ClO₂ AND END PRODUCTS

a. Chlorine Dioxide

ClO₂ was found not to form trihalomethanes in drinking water, and reacted to reduce the concentration of precursors such that if Cl₂ was also used, the resulting trihalomethanes production would be diminished, Miltner (51). There are, however, some problems associated with the use of ClO₂.

1. When ClO₂ reacts with natural waters, the end products include chlorite and chlorate. 48% of the applied chlorine dioxide is reduced to chlorite, and 22% converted to chlorate, Miltner (52). The potential of significant quantities of chlorite and chlorate to be present in drinking water exists particularly if ClO₂ is used as a substitute for, and in similar concentration as chlorine, as a primary disinfectant.
2. Although ClO₂ does not form chloroform, it is not known what other harmful compounds may be produced by its reaction with natural waters.

The literature on ClO₂ toxicity is sparse, concerned primarily with exposure in air. Casarett (53) reported that there is a definite odor at concentration of 2 ppm ClO₂ in air, and eyes become irritated. The throat becomes irritated at 6 ppm. Thirty ppm causes intense coughing spasm, while exposure to 50-1000 ppm in air for one hour may cause lethal effects. Acute inhalation causes bronchitis and pulmonary edema, coughing, wheezing, respiratory distress, nasal discharge and throat irritation.

Elkins (54) and Sax (55) estimated 1 ppm in the air is the maximum allowable concentration for working conditions. Two cases of illness, one fatal, were reported in the paper industry. A concentration of 10 ppm ClO₂ inside a bleach tank caused the death of one worker. It is stated that, 0.1 ppm in the air is well tolerated with a considerable margin of safety, Paulet and Desbrousses (56).

The action of a discontinuous exposure to ClO₂ was observed in rats exposed to 5, 10 and 15 ppm ClO₂ for 15 min/day for 1 month. Neither blood nor lung tissue was altered by 5 ppm ClO₂, but 10 or 15 ppm caused bronchitis, bronchiolitis and alveolar infiltration. These lesions were healed within 15 days after the end of the treatment (57). The 2.5 ppm concentration appeared to mark the threshold of bronchopulmonary pathology in rats and rabbits under exposure conditions of 7 hour inhalation/day for 1 month (56).
In chronic animal toxicity studies, no impact was noted on the health of rats treated with 10 mg/l ClO₂ in drinking water for a period of 2 years. However, rats drinking water containing 100 mg/l ClO₂, exhibited an increased mortality rate at the end of the two year period, Holler and Northgraves (58).

Subsequent Soviet studies by Fridlyand and Kagan (59) revealed that a 6 month exposure of 10 mg of ClO₂/l to rats did not result in any adverse health effects with respect to total body weight, relative organs weights, activity of oxidase enzyme (e.g. Catalase) and Vitamin C levels of internal organs. At 100 mg/l of ClO₂, the only difference between the treatment and control groups was a slower weight gain in the treatment group.

There is very little published on the effect of ClO₂ in drinking water on humans. The available reports reveal limited physiologic effects from the concentration of ClO₂ as high as 0.7 mg/l, Enger (60). Fridlyand and Kagan (59) reported the occurrence of significant increases of leukocytes in oral washing of human subjects. The range of concentration was from 0.5 mg/l to 10 mg/l. Significant increases above the control levels were noted at 1.0 mg/l. No significant changes occurred at 0.5 mg/l. Also, they investigated what happens to the chlorine dioxide as it enters the oral cavity and proceeds to the stomach area. It was found that approximately 30% of ClO₂ was fixed in the mouth and 70% may actually enter the stomach. However, they did not report the species of chemicals to which ClO₂ may have been converted.
Lackett et al. (61) reported that the longevity of honey bees increased when bees were treated with 10 and 100 mg/1 ClO₂ in sucrose solution, but all bees fed 1000 mg/1 ClO₂ died within one week. Before death, bees regurgitated a greenish liquid and exhibited excessive defecation. These bees became very weak and had difficulty climbing and maintaining balance.

b. Chlorates and Chlorites

It is important to consider the health effects of ingesting chlorite and chlorate, primary products resulting from ClO₂ disinfection of surface water. The oral administration of chlorates has been shown to produce methemoglobinemia (Methb) with blood destruction, Richardson (62). Jung and Kuon (63) reported that the rate of Methb formation by chlorate was nearly proportional to the square root of the chlorate concentration.

Chlorite is thought to oxidize hemoglobin more rapidly than chlorate (64). Hopf (65) reported that the toxicity of chlorite might be similar to chlorate and indicated that subjects sensed a weak furry feeling after drinking sodium chlorite concentration of more than 0.3 mg/1. Musil et al. (45) demonstrated chlorite to be a powerful producer of Methb in rats and recommended that water for consumption contain no ClO₂⁻, since it might prove toxic to neonates. Heffernan et al. (66) reported that ClO₂⁻ induced decreases in glutathione in vivo, which was accompanied by an increase in hydrogen peroxide. Also hemolytic anemia occurred as noted by an increased turnover of red cells in cats exposed to ClO₂⁻ orally. There were few other signs of chlorite toxicity observed.
At 500 mg ClO<sub>2</sub>/l a significant increase in kidney/body weight ratio was observed, which may indicate some renal toxicity (66).

G. BIOLOGICAL ROLE OF GLUTATHIONE

Glutathione (GSH) is a tripeptide of cysteine, glutamic acid and glycine. Glutathione is recognized as being a primary component in protecting hemoglobin, enzymes and membrane of the red blood cell from oxidative damage, Schneider (67) and Wintrobe et al. (68). This protection system is dependent not only on glutathione but also on glutathione-related enzymes; glutathione reductase (G-R), glutathione peroxidase (G-Px), glucose-6-phosphate dehydrogenase (G-6-PD) and NADPH. The reactions are shown in Figure 1. It is essential to maintain a normal level of GSH if oxidation of hemoglobin and enzymes is to be kept under control, and this is a function of glutathione reductase. The control of peroxide is the function of glutathione peroxidase, however, Nicholls (69) reported that glutathione-peroxidase and catalase exert a synergistic action and they compete to remove H<sub>2</sub>O<sub>2</sub> in the red cell. If red cells are exposed to an oxidant chemical agent, GSH is restored by the action of glutathione-peroxidase, reductase and catalase. A deficiency of glutathione-peroxidase leads to a drug-related hemolytic anemia, Necheles (70) on the basis of increased susceptibility to oxidative injury by increased hydrogen peroxide concentration. The same is true of glutathione reductase deficiency, Blume (71). The integrity of the glutathione system is also dependent on the activity of G-6-PD. A deficiency of G-6-PD makes the affected individual exceptionally susceptible to the development of drug-induced hemolytic anemia (72).
THE GLUTATHIONE DEPENDENT SYSTEM

\[ \gamma-L-glutamylcysteine + \text{Glycine} \rightarrow \text{G-Synthetase} \]

\[ \begin{align*}
\text{Hb(OX)} & \rightarrow \text{H}_2\text{O} + \text{O}_2 \\
\text{H}_2\text{O}_2 & \rightarrow \text{G-Px} \\
\text{G-S-S-G} & \rightarrow \text{G-R} \\
\text{NADPH} & \rightarrow \text{G-6-PD} \\
\text{Glucose} & \rightarrow \text{Glucose-6-P}
\end{align*} \]

FIGURE 1
Traditionally, the hemolytic anemias secondary to enzymatic defects are called "nonspherocytic", type I, to distinguish them from the classic hereditary spherocytosis, type II, Selwyn and Dacie (73). However, the classification of these anemias into types I and II is of limited usefulness since there is much overlap between the two types of reaction. The occurrence of hemolytic anemia in black soldiers receiving the anti-malarial drug, Primaquine, led to the discovery that this type of hemolytic anemia is caused by a deficiency of G-6-PD in erythrocytes. When deficient red blood cells are challenged by any one of several drugs, there is depletion of glutathione, formation of Heinz bodies and inhibition of glucose utilization. All three factors favor membrane damage and hemolysis. The hemolysis is characterized by three rather distinctive features in the affected red cells:

1. The appearance of brownish or greenish derivatives of hemoglobin including hemoglobin.

2. The appearance of irreversible hemochromes such as sulfhemoglobin.

3. The formation within red cells of water-in soluble, stainable granules, generally termed Heinz bodies.

There are many reasons to suspect the involvement of sulfhydryl (thiol) groups in the processes described above. Such groups are readily oxidized under physiological conditions and are involved in maintaining the integrity of the red cell, Fegler (74) and the physiological activity
of hemoglobin, Riggs (75). Also, Beutler et al. (76) have established an association between intracellular reduced glutathione levels and the susceptibility \textit{in vitro} and \textit{in vivo} of red cells to injury by oxidant drugs.

H. EFFECTS OF THIOL OXIDATION ON GROWTH AND ON PROTEIN SYNTHESIS

Another means of exploring roles of glutathione in biological systems is the possible involvement in mitotic events and all growth, Mano (77) and Jocelyn (78). The addition of diamide (thiol oxidizing agent) to a suspension of E. Coli inhibited growth for various time intervals, depending on the amount of diamide used, Wax et al. (79). Also, the addition of diamide to fertilized sea urchin eggs led to a delay in the first division of 10-50 minutes depending on diamide concentration. Higher concentration of diamide ($2 \times 10^{-3}$M) prevented any division (80). The addition of G-S-S-G to lysate of reticulocytes, in the absence of any other oxidant, leads to inhibition of protein synthesis after an initial lag of 6-9 min (81). The reduction of G-S-S-G to GSH by adding glucose and NADP to the lysate at the beginning of the lag period prevents the inhibition of protein synthesis. Once initiation is halted, G-S-S-G reduction is not associated with recovery of the ability to synthesize protein. This information indicates that the GSH/G-S-S-G level within the cell may well be of central importance to a variety of cell functions.
STATEMENT OF THE PROBLEM

Although the shift from Cl₂ to ClO₂ in water treatment plants began in European countries and North America three decades ago, very little work has been done to evaluate the biochemical fate of chlorine dioxide. Specific information is lacking on the absorption, distribution and excretion of ClO₂ in man and laboratory animals. Studies in rat will be conducted to provide information on the disposition and metabolism of ³⁶ClO₂. These studies will include: ³⁶ClO₂ absorption, distribution in different organs and elimination time course from plasma following oral administration. Excretion patterns of radiolabelled Cl-compounds in expired air, urine and feces will be examined throughout 72 hrs from the time of administration. Also, liver subcellular localization of ³⁶Cl compounds will be determined.

ClO₂ and its breakdown products, chlorite and chlorate are strong oxidizing agents. Chlorite and chlorate have been reported to cause methemoglobin formation when administered in bolus doses either orally or intraperitoneally. The oral administration of ClO₂ also causes a decrease in blood glutathione level. This work will investigate the influence of Cl-compounds (ClO₂, ClO⁻ and ClO₃⁻) on rat and chicken blood parameters following chronic administration of Cl-compounds in drinking water. These studies will include: hematologic analysis, glutathione level, osmotic fragility, methemoglobin, 2-3 diphosphoglycerate and the morphology of erythrocytes.
It has been demonstrated that glutathione reductase, glutathione peroxidase and catalase are responsible for maintaining the level of glutathione when red cells are exposed to an oxidizing chemical agent. Therefore, the activity of these enzymes in rat blood, liver and kidney from animals drinking various concentrations of Cl-compounds for 6 months will be determined.

Additional action of glutathione may be involved in mitosis and cell growth. An increase in G-S-S-G/GSH ratio has been shown to inhibit protein synthesis. It may be possible that the oxidant stress of Cl-compounds oxidizes sulfhydryl groups, causing change in the G-S-S-G/GSH ratio. Therefore, \(^3\)H-thymidine incorporation in treated rat kidney, liver, testes and small intestine mucosa will be studied. Also, the effect of drinking Cl-compounds on the growth of rat, chicken and mice will be determined in this work.

An attempt will be made to correlate the results from these experiments to determine the relative safety and tolerability of Cl\(\text{O}_2\) and its end products.
METHODS AND MATERIALS

A. ANIMALS AND MATERIALS

Sprague-Dawley male rats (150-170 gm), male white leghorn chickens (250-300 gm) and male Swiss-Webster mice (15-17 gm) were used in this study. The animals were maintained in an environment of 73-74°F, 55 percent humidity and 12 hr light/day cycles beginning at 6:00 A.M. Animals were given food ad libitum for all experiments. All the animals were given different concentrations of ClO₂, ClO₂⁻ and ClO₃⁻ in double distilled water 20 hrs/day, 7 days/week for 12 months in drinking water. ClO₂ was generated daily and its concentration determined by diethyl-p-phenylene diamine (DPD) (Palin, 1967).

H³⁶Cl, thymidine (methyl, 1',2'-³H), aquasol-2 and protosol were purchased from New England Nuclear. Potassium chlorate, sodium chlorite and chlorate, ferrous ammonium sulfate and oxalic acid were purchased from Pfaltz, Bauer, Inc. All other chemicals and solvents were of analytical reagent grade quality.
The $\text{K}^{36}\text{ClO}_3$ was synthesized according to the method of Pitman (82) from approximately 600 $\mu$Ci $\text{H}^{36}\text{Cl}$ in the following manner:

1) A carbon, stainless steel electrode electrolysis cell was made using a 10 ml multidose glass vial and a cylinder of stainless steel surrounding a carbon electrode, all inside the vial.

2) The $\text{H}^{36}\text{Cl}$ was added to the cell and neutralized with 2.06 ml 1.93 N KOH. To this solution was added 0.86 ml KCl solution (120.2 milligrams KCl/ml). Total final KCl in solution before electrolysis was 400 milligrams considering both the neutralization of HCl and the addition of KCl solution.

3) Electrolysis was begun after adjusting the volume with distilled water to approximately 8 ml. Electrolysis was allowed to proceed at 100 milliamps for 24 hrs.

4) After electrolysis was completed, the final product was crystallized from solution after evaporating to about 2 ml volume using an ice bath.

5) Actual weight of product 486.7 mg.

---

The synthesis of $\text{K}^{36}\text{ClO}_3$ was first produced by John D. Jones at Phoenix Memorial Laboratory in the University of Michigan, Ann Arbor.
The generation of $^{36}\text{ClO}_2$ from $^{36}\text{ClO}_3$ was accomplished by the following reaction:

$$2^{36}\text{ClO}_3 + (\text{COOH})_2 + 2\text{H}_2\text{SO}_4 \rightarrow 2^{36}\text{ClO}_2 + 2\text{KHSO}_4 + 2\text{CO}_2 + 2\text{H}_2\text{O}$$

The $^{36}\text{ClO}_2$ gas was trapped in ice cold water.

Oxygen Measured by Fast Neutron Activation in $\text{KCIO}_3$, $\text{KCl}$ and $\text{Synthesized KCIO}_3$

The method used to confirm the purity of $\text{KCIO}_3$ produced by carbon-stainless steel electrode electrolysis is (in essence) to determine the percent oxygen present in a cold sample prepared identically to the radioactive sample. The rational of this test is that the starting compound $\text{KCl}$ has zero oxygen content whereas $\text{KCIO}_3$ the final product contains 39.15% oxygen.

Method of Analysis - Fast Neutron activation analysis was chosen as the method of analysis because it is fast and unaffected by interferences. It consists of irradiating each sample and oxalic acid standards in a special boron nitride shield in the 2MW Ford Nuclear Reactor research reactor for 16 seconds followed by a 16 second wait before counting on a 3" x 3" NaI scintillation detector interfaced to an automatic computer analyzer system. A series of known oxygen standard were irradiated and counted and weighed quantities of reagent grade $\text{KCIO}_3$ and $\text{KCl}$ were

---

b The NAA was conducted at the University of Michigan, Ann Arbor by John D. Jones.
processed in exactly the same manner. Finally, a weighed sample of the dry cold synthesized KCIO$_3$ was irradiated and counted.

The Recovery of KCIO$_3$ Synthesis

The analysis of synthesized KCIO$_3$ by fast neutron activation analysis is shown in Table 1. The determined percentage $O_2$ was 39.2, 0, 37.7 in reagent KCIO$_3$, reagent KCl and synthesized KClO$_3$ respectively, in close agreement with percentage $O_2$ of 39.7, 0 and 39.7.

C. DETERMINATION OF $^{36}$Cl$^-$, $^{36}$ClO$_2^-$ and $^{36}$ClO$_3^-$ IN BIOLOGICAL FLUIDS

The Synthesis of $^{36}$Cl Derivatives

a) $^{36}$ClO$_3^-$ was synthesized as potassium salt as described. $^{36}$ClO$_2^-$ was formed by the reaction:

$$2^{36}\text{ClO}_2^- + 2I^- \rightarrow 2^{36}\text{ClO}_2^- + I_2$$

b) H$^{36}$Cl was neutralized with 1 N NaOH to form $^{36}$Cl$^-$.

Recovery of $^{36}$Cl derivatives was studied by the addition of $4.3 \times 10^4$, $6.5 \times 10^4$, $1.1 \times 10^5$ and $4.8 \times 10^4$ DPM of $^{36}$Cl$^-$, $^{36}$ClO$_3^-$, ($^{36}$Cl$^- + ^{36}$ClO$_3^-$) and ($^{36}$Cl$^- + ^{36}$ClO$_2^-$) respectively to plasma, and $6.8 \times 10^3$ DPM of $^{36}$ClO$_2^-$ also added to H$_2$O.

Experimental Assay

1. 200 µl of plasma containing the $^{36}$Cl-compounds was pipetted in duplicate into 13 x 100 culture tubes.
### TABLE 1

**Oxygen Measurement by Fast Neutron Activation in KCIO₃**

<table>
<thead>
<tr>
<th>Material</th>
<th>Weight (gms)</th>
<th>Determined % O₂</th>
<th>Theoretical % O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent KCIO₃</td>
<td>0.2459</td>
<td>39.2</td>
<td>39.7</td>
</tr>
<tr>
<td>Reagent KCl</td>
<td>0.2480</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cold Synthesized KCIO₃</td>
<td>0.2469</td>
<td>37.7</td>
<td>39.7</td>
</tr>
</tbody>
</table>
2. One ml 5% silver nitrate (AgNO₃) was added to each.

3. Samples were mixed for 2 min on a vortex and centrifuged for 5 min at 2000 x g.

4. The supernatant was removed and the pellet washed by repeating steps 2 and 3.

5. 25 μl of the supernatant was pipetted into glass liquid scintillation vials containing 15 ml Aquasol-2, 200 μl of 30% H₂O₂ was added (decolorization) and then counted for 10 min for ³⁶ClO₃⁻ determination.

6. To one set of pellets, 2 ml concentrated NH₄OH was added and to the other set 2 ml of 2% sodium thiosulfate (Na₂S₂O₃) was added. Then both sets of samples were sonicated (20,000 cps) for 2 min. Tubes were capped to minimize the volatilization of NH₄OH and centrifuged for 5 min at 2000 x g.

7. 200 μl from the supernatant of the NH₄OH and Na₂S₂O₃ samples were counted for Cl⁻ + ClO₃⁻ and Cl⁻ alone, respectively.

8. Sample quench effects were corrected using the method of channel ratios. The supernatant volume was measured to correct for volatility during the sonication. Separation of ³⁶Cl compounds was according to the scheme:
The concentration of $^{36}\text{Cl}^-$ and $^{36}\text{ClO}_3^-$ were calculated from radioactivity present in supernatant 2 and 1, respectively. The difference in DPM between supernatant 2 and 3 gave $^{36}\text{ClO}_2^-$. 
Quantitation of $^{36}\text{Cl}$-Compounds in Biological Fluids

After the addition of $^{36}\text{Cl}$-compounds to plasma, 82.4% Cl$^-$ and no ClO$^-_2$ and ClO$^-_3$ was detected (Table 2). The addition of ClO$^-_3$ yielded no ClO$^-_2$, 81.8% ClO$^-_3$ and 10.85% Cl$^-$. Also, 80.2% ClO$^-_3$ and 95.8% Cl$^-$ were detected after the addition of combined ($^{36}\text{Cl}^-$/ $^{36}\text{ClO}_3^-$). It was noted that when using ($^{36}\text{ClO}_2^-$$/\text{Cl}^-$), 12.8% was lost as Cl$_2$ during the addition of ClO$_2^-$ and Cl$^-$ to the plasma; however, 100% Cl$^-$ was recovered and neither ClO$_3^-$ nor ClO$_2^-$ were detected. When ClO$_2^-$ in H$_2$O was tested, 7.1%, 21.46% and 53.87%, ClO$_3^-$, Cl$^-$ and ClO$_2^-$ were recovered, respectively.

D. CHLORINE DIOXIDE ABSORPTION, DISTRIBUTION AND ELIMINATION STUDIES

A group of 4 male Sprague-Dawley rats (200-220 gm) was administered 100 mg/l $^{36}\text{ClO}_2$ (0.07 µCi) orally. In another set of experiments, an identical group received 300 mg/l $^{36}\text{ClO}_2$ (0.7 µCi) orally, after drinking 100 mg/l ClO$_2$ for 2 weeks. Blood samples (heparinized) were collected at 5, 10, 20, 30, 60 min, 2, 4, 8, 24 and 48 hrs by orbital sinus puncture. The blood was centrifuged at 1000 x g for 15 min to separate the red blood cells from the plasma. At 72 hrs, rats were killed by decapitation and blood was collected in heparinized tubes. Tissue specimens of kidney, lung, stomach, small intestine, liver, spleen, thymus, bone marrow and testes were prepared for the determination of $^{36}$ chlorine content by liquid scintillation spectrometry.
**TABLE 2**

**Recovery of $^{36}$Cl Compounds**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>% Radioactivity *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ClO$_3^-$</td>
</tr>
<tr>
<td>$^{36}$Cl Compounds</td>
<td></td>
</tr>
<tr>
<td>Na$^{36}$Cl</td>
<td>-</td>
</tr>
<tr>
<td>K$^{36}$ClO$_3$</td>
<td>81.8</td>
</tr>
<tr>
<td>Na$^{36}$Cl + K$^{36}$ClO$_3$</td>
<td>80.2</td>
</tr>
<tr>
<td>K$^{36}$ClO$_2$ + Na$^{36}$Cl</td>
<td>-</td>
</tr>
<tr>
<td>K$^{36}$ClO$_2$ in H$_2$O</td>
<td>7.10</td>
</tr>
</tbody>
</table>

* Values represent % of original $^{36}$Cl compound added to plasma and K$^{36}$ClO$_2$ also added to H$_2$O as described in the methods.

— None present
Sample Preparation

a. Whole blood and packed cells, Mahin et al. (83)

An aliquot, up to 0.2 ml of whole blood or packed cells was dispensed into the bottom of each counting vial. An equal volume of 70% perchloric acid was added and the contents were swirled to mix. A volume of 30% hydrogen peroxide equal to twice the initial volume of the samples was then added and the contents were again swirled to mix. Caps were screwed tightly onto the vials to minimize evaporative loss of fluid during warming. Vials were placed in a shaking water bath at 70 to 80° for 60 min. Samples were clear and colorless after 60 min. A fine precipitate may remain, but it is soluble in the final solution (Aquasol-2, New England Nuclear Corp). Vials were cooled to room temperature and sample preparation completed by addition of 15 ml Aquasol-2.

b. Tissue and bone marrow

Samples of tissue weighing up to 200 mg (wet), and samples of bone marrow weighing up to 50 mg were placed on the bottom of individual glass counting vials and capped with teflon or polyethylene lined caps. Then 2 ml protosol was added to the vial and vial caps were secured to avoid loss of solvent. Samples were solubilized by heating overnight at 50°, then cooled to room temperature; afterwards 0.2 ml 30% H₂O₂ (decolorization) was added. The samples were then heated at 50°C for 30 min, cooled and 15 ml of Aquasol-2 was added.

Radioactivity was measured in the solubilized samples and plasma in a Packard Model 3255 liquid scintillation spectrometer. Sample
quench effects were corrected by using the method of channel ratios, and 
$^{36}\text{Cl}$ efficiency was higher than 80%.

E. CHLORINE DIOXIDE EXCRETION STUDIES

Male Sprague-Dawley rats (200-250 gm) were administered 100 mg/1 $^{36}\text{ClO}_2$ (0.7 μCi) orally and housed in modified Roth all glass metabolism chambers for the collection of expired air, fecal and urine samples at 12, 24, 48 and 72 hrs. Radioactivity was measured as described above.

F. IN VIVO METABOLISM STUDIES

Male Wistar rats (180-200 gm) were administered 100 mg/1 $^{36}\text{ClO}_2$ (0.7 μCi) orally, then were housed in metabolism cages. Urine was collected at 12, 24, 48 and 72 hrs. Rats were killed by exanguination at 72 hrs. Urine and plasma sample radioactivity was determined as well as the quantity and the quality of chlorine compounds. In another set of experiments, groups of 4 rats received either 10 mg/1, or 100 mg/1 $^{36}\text{ClO}_2$ orally. Animals were killed after 1/2 hr. The blood was centrifuged at 1000 x g for 15 min to separate the red blood cells from the plasma. The plasma and buffy coat were separated and the cells were washed twice with cold saline, then reconstituted with saline to the initial whole blood volume. Erythrocyte hemolysates were prepared for counting by the method of Mahin et al. 1966 (83); plasma and solubilized hemolysates were analyzed for chlorine compounds. Liver was used to determine subcellular localization of $^{36}\text{Cl}$-compounds.
G. EFFECT OF ClO₂, ClO⁻ and ClO₃⁻ ON RAT AND CHICKEN BLOOD

Male Sprague-Dawley rats (150-170 gm) and male white leghorn chickens (250-300 gm) were used in these experiments. The animals were housed in a controlled environment conditions (described above). Rats and chickens were fed ad libitum and given 0, 1, 10, 100, 1000 mg/l ClO₂ in double distilled water 20 hrs/day, 7 days/week for 12 months in the drinking water. In other experiments, identical groups of rats drank 10 and 100 mg/l ClO⁻ or ClO₃⁻ for the same time period. Blood was collected by cardiac puncture in syringes coated with heparin at 2, 4, 7 and 9 months from control and treatment group rats. Blood samples were kept on ice and determination of glutathione, methemoglobin, osmotic fragility, 2,3-diphosphoglycerate, hematologic analysis of blood cell compartment and erythrocytes scanning by electron microscopy were conducted.

a. Determination of Blood Glutathione, Beutler et al. (84)

Reagents

1. Precipitation solution consists of 1.67 gm glacial metaphosphoric acid, 0.2 gm EDTA and 30 gm of NaCl per 100 ml of distilled water.

2. Phosphate solution. A 0.3 M Na₂HPO₄ solution is prepared in distilled water.

3. DTNB reagent. 40 mg 5,5'dithiobis-2-nitrobenzoic acid per 100 ml of 1% sodium citrate.
Assay Procedure

Two-tenth milliliter of whole blood was added to 1.8 ml of distilled water. Three ml of the precipitating solution is mixed with the hemolysate. The mixture is centrifuged at 1000 g for 10 min. One ml of supernatant is added to 4 ml of the phosphate solution in a 13 x 100 mm test tube. One half ml of DTNB reagent is added and the absorption measured at 412 nm in a Varian Spectrophotometer Model 635.

b. Methemoglobin Determination, Evelyn K.A. et al. (85)

Reagents

Phosphate buffer, M/15, pH 6.6. Phosphate buffer M/60, pH 6.6 by dilution M/15 buffer 1:4. Neutralized cyanide solution, prepared within 1 hr of use by mixing equal volumes of 10% KCN and 12% acetic acid (V/V).

Assay Procedure

A 0.2 ml aliquot of whole blood was added to 10.0 ml M/60 phosphate buffer, allowed to stand 5 min and absorption read at 630 nm ($A_1$). Then one drop of neutralized cyanide solution was added to the same 10.0 ml, mixed, allowed to stand 2 min and absorption read at 630 nM ($A_2$). Methemoglobin was calculated as follows:

\[ \text{g methemoglobin } \% = 23.4 \times (A_1 - A_2) \]
c. Osmotic Fragility Determination

The semipermeable characteristics of the surface membrane of an erythrocyte make each cell an osmometer that changes volume with changes in the osmotic pressure of the external environment. The addition of blood to hypotonic solution was used to determine the osmotic fragility, Method of Dacie (86).

Reagent

Stock solution of buffered sodium chloride, dissolve 90 gm of NaCl, 13.66 gm of Na₂HPO₄ and 2.43 gm of NaH₂PO₄ in 1 liter double distilled water. This stock solution is osmotically equivalent to 10% NaCl. Working solution was prepared by dilution of the stock solution 1:10 to make up a solution equivalent to 1% NaCl. The working solution was diluted with double distilled water to prepare 0.85, 0.8, 0.65, 0.60, 0.50, 0.45, 0.4, 0.35, 0.3, 0.2 and 0.1% NaCl tubes.

Assay Procedure

Test tubes were set up, each containing 5 ml of the various working solutions. Fifty μl of blood was added to each tube and mixed well. After 30 min, tubes were centrifuged at 500 g for 5 min. The supernatant was read in Varian Model 635 spectrophotometer at 545 nm. The supernatant of 0.85% was
used as the blank and the 0.1% as 100% hemolysis. Osmotic fragility curve in rat and chicken was plotted as percent hemolysis against salt concentration (Fig. 2 and 3).

d. **2,3-diphosphoglycerate Determination, Fiske and Subbarow (87)**

2,3-diphosphoglyceric acid (2,3-DPG) is enzymatically hydrolyzed to 3-phosphoglyceric acid (3-PGA) and inorganic phosphorus (P). The enzyme which catalyzes this reaction is phosphoglycerate mutase (PGM) and is termed 2,3-DPG phosphatase. 2-phosphoglycolic acid is necessary to stimulate the action.

**Reagents**

- Triethanolamine buffer solution
- Phosphoglycerate Mutase (PGM), 2400 units/ml
- Phosphoglycolic acid
- Trichloroacetic acid 8% (W/V)
- Acid Molybdate (contains ammonium molybdate, 1.25 gm/100 ml 2.5 N sulfuric acid)
- Fiske and Subbarow Reducer 15% (contains 1-amino-2-naphthol-4-sulfonic acid, 0.8%, sodium sulfite and sodium bisulfite)

**Assay Procedure**

Two-tenth milliliter of heparinized blood was pipetted into 0.6 ml of cold TCA mix, a clear supernatant was obtained by centrifugation for 10 min at 2000 x g. Two tubes were labelled non-enzymatic
Blood was collected in heparinized tubes by cardiac puncture. Fifty μl of blood was added to 5 ml of various concentration of buffered sodium chloride and mixed well. After 30 min tubes were centrifuged at 500 x g for 5 min. The supernatant was read at 545 nm. The supernatant of 0.85% was used as the blank and the 0.1% as 100% hemolysis. The values reported represent the mean ± S.E. of 4 animals.
OSMOTIC FRAGILITY CURVE IN RAT BLOOD.

FIGURE 2
Osmotic Fragility Curve in Chicken Blood

Blood was collected in heparinized tubes by wing vein puncture. Osmotic fragility was determined as described in the legend to Figure 2. The values reported represent the mean ± S.E. from 4 chickens.
OSMOTIC FRAGILITY CURVE IN CHICKEN BLOOD

% Hemolysis vs. % NaCl

FIGURE 3
and enzymatic. The following were added to the respective tube:

<table>
<thead>
<tr>
<th></th>
<th>Non-Enzymatic</th>
<th>Enzymatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>Triethanolamine Buffer</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>PGM</td>
<td>10 μl</td>
<td>10 μl</td>
</tr>
<tr>
<td>Phosphoglycolic acid</td>
<td>---</td>
<td>40 μl</td>
</tr>
</tbody>
</table>

Tubes were placed into a 37° water bath for 20 min, removed from water bath and 1.0 ml TCA was added to stop the reaction. Forty μl of phosphoglycolic acid was added to the non-enzymatic tube. Then 0.4 ml of acid molybdate was added followed by the addition of 100 μl of Fiske and Subbarow solution. Tubes were measured after 10 min (to develop the color) at 660 nm.

The concentration of blood, 2,3-DPG was obtained by subtracting the concentration of non-enzymatic from the concentration of enzymatic. The 2,3-DPG values obtained can be used to calculate 2,3-DPG level on the basis of hemoglobin as follows:

\[
\text{Hemoglobin 2,3-DPG (μMoles/gm)} = \frac{\text{Blood 2,3-DPG (μMoles/ml)}}{\text{Hemoglobin (gm/100 ml)}} \times 100
\]
H. HEMATOLOGIC ANALYSIS OF BLOOD CELL COMPARTMENT

Coulter Model S was used to determine red blood cell count (RBC), hemoglobin % (Hg), hematocrit % (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). Fresh heparinized blood was used from rats treated by Cl-compounds after 2, 4, 6, 7 and 9 months.

I. ERYTHROCYTES MORPHOLOGY STUDIES

The study of the shape of red blood cells is of considerable importance for understanding pathologic states associated with abnormalities of shape. The morphology of rat and chicken red blood cells was studied by light microscope and scanning electron microscopy (SEM). Wright stain (0.3% polychrome methylene blue stain in methanol) was used for preparation of blood smears for light microscope.

Preparation of Red Blood Cells for SEM

Bessis and Weed (88) method was used to prepare the blood for SEM. Fresh blood was added slowly drop by drop into a tube containing a 1% solution of freshly distilled glutaraldehyde in Eagle's solution and mixed. The cells remained in the fixative for 30 min. The cells were then centrifuged at 500 x g for 10 min and post fixed in a 0.5% solution of osmium tetroxide in Eagle's solution for 60 min. The cells were washed in distilled water to remove all salt from the preparation three

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The hematologic determinations were conducted by Hematology Laboratory, Ohio State University Hospital.
times. The erythrocytes were dehydrated by washing progressively with alcohols of increasing concentration (50, 70, 80, 95, 100%) for 5 min in each alcohol, followed by washing the dehydrated cells in propylene oxide. A small drop of this cell suspension was deposited on a coverslip. The preparation was then coated by evaporation of a fine layer of gold palladium before examination with the scanning microscope.

J. THE EARLY EFFECT OF ACUTE ADMINISTRATION OF C1O$_2$ ON BLOOD GLUTATHIONE AND OSMOTIC FRAGILITY

Groups of 5 Sprague-Dawley male rats (250-270 gm) were administered 3 ml p.o. 0, 10, 20, 40 mg/l C1O$_2$ in distilled water. Blood was collected by cardiac puncture at 15, 30, 60, 120 min, glutathione, methemoglobin and osmotic fragility were determined for each time interval as described above.

K. EFFECT OF C1-COMPOUNDS ON WHOLE BLOOD IN VITRO STUDIES

Rats were sacrificed by decapitation and blood was collected in heparinized tubes. Vessels containing 3 ml of blood each were incubated in a water bath at 35°C after the addition of C1O$_2$, C1O$_2^-$ and C1O$_3^-$. The final concentration of C1O$_2$ was 500, 100, 50 and 5 mg/l, while 100 and 10 mg/l were used in both C1O$_2^-$ and C1O$_3^-$. Glutathione, osmotic fragility and methemoglobin were determined in blood at 30, 60, 120 and 240 min.

L. GLUTATHIONE RELATED ENZYMATIC ACTIVITY IN RAT AND CHICKEN AFTER C1-COMPOUNDS CHRONICALLY TREATED IN DRINKING WATER

Blood was taken by cardiac puncture from rat and by wing vein puncture from chicken into EDTA tubes after 6 and 10 months treatment
with Cl-compound respectively, glutathione peroxidase, glutathione reductase and catalase activity were determined. Whole blood hemoglobin concentration was determined by the cyanomethemoglobin method, Van Kampen and Zijlstra (89). Blood was washed once with 5 volumes of cold saline and then centrifuged at 1000 x g for 10 minutes. After removing the saline, plasma and buffy coat, the remaining cells were lysed in 3 volumes of double distilled water for determination of glutathione reductase and catalase, and in 6 volumes for glutathione peroxidase.

a. **Glutathione Peroxidase Assay, Hafeman (90)**

The enzyme assay tubes were incubated at 37°C and contained 1.0 ml of 2.0 mM GSH, 1.0 ml of 0.40 M sodium phosphate buffer (pH 7.0), also containing 4 x 10⁻⁴ M EDTA, 0.5 ml of 0.01 M NaN₃ (to inhibit catalase), 0.1 ml of the erythrocyte hemolysate and water to bring the total volume to 4.0 ml. After a 5 minute preincubation, 1.0 ml of 1.25 mM H₂O₂ was added. Thereafter, at 1, 3 and 6 minutes, 1 ml aliquots of the incubation mixture were removed and added to 4.0 ml metaphosphoric acid precipitation solution (described above). Glutathione in the protein-free filtrate was determined by mixing 0.5 ml of the filtrate with 2.0 ml of 0.4 M Na₂HPO₄ and 0.5 ml of DTNB reagent. Absorption at 412 nm was recorded 2 minutes after mixing. Since non-enzymatic GSH oxidation by H₂O₂ occurred during incubation, the non-enzymatic activity was determined by substituting H₂O for the enzyme source (hemolysate). Both enzymatic and non-enzymatic reactions proceeded at rates directly proportional to GSH concentration, since a plot of log (GSH) vs. reaction time was linear both with and without addition of enzyme source. One
enzyme unit of activity was defined as a decrease in the log (GSH) of 0.001 per minute after the decrease in log (GSH) per minute of the non-enzymatic reaction was subtracted.

b. **Glutathione Reductase Assay**

The enzyme assay tubes were incubated at 37°C and contained:

- 2.4 ml of 0.067M phosphate buffer (pH 6.6),
- 0.2 ml of 7.5 x 10^{-2} M oxidized glutathione (G-S-S-G),
- 0.4 ml of 0.01 M NaN₃,
- 0.3 ml of erythrocyte hemolysate.

The tubes were swirled and one minute later the reductase reaction was initiated by the addition of 0.5 ml NADPH (2 mM in 1% NaHCO₃). One ml aliquots were withdrawn at 1 minute intervals and pipetted into 4.0 ml of precipitation solution. Glutathione in the protein-free filtrate was determined as described in the glutathione peroxide assay. The unit of glutathione reductase activity was defined as an increase in the log (GSH) of 0.001 per minute.

c. **Catalase Assay, Aebi and Suter (91)**

The stock hemolysate was diluted 500-fold with 0.05 M phosphate buffer, pH 6.8. Hemolysate and reagents are pipetted in two quartz cuvettes as follows:

<table>
<thead>
<tr>
<th>Cuvette A (reference)</th>
<th>Cuvette B (assay sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 ml phosphate buffer</td>
<td>1.0 ml phosphate buffer</td>
</tr>
<tr>
<td>1.0 ml hemolysate</td>
<td>1.0 ml hemolysate</td>
</tr>
<tr>
<td>1.0 ml distilled water</td>
<td>1.0 ml H₂O₂ (0.03M)</td>
</tr>
</tbody>
</table>
The reaction is started by the addition of the $\text{H}_2\text{O}_2$ solution and the absorption at 240 nm was recorded at zero time ($E_1$) and after 30 seconds ($E_2$). The velocity constant ($K$) is given by the term:

$$K = \frac{2.3}{30} \log \frac{E_1}{E_2} \text{ (sec}^{-1}\text{)}$$

The specificity of enzyme assay was tested by adding NaN$_3$ (inhibit catalase) to cuvette A and B and no change in $E_1$ and $E_2$ was observed.

M. $^3\text{H}$-THYMIDINE INCORPORATION IN ORGANS OF RATS DRINKING Cl-COMPOUNDS

Animals were administered 0.5 $\mu$Ci/gm of body weight of methyl, $1',2'\text{H}$-thymidine (128 Ci/mmmole) intraperitoneally after 3 months treatment with 10 and 100 mg/l Cl$_2$, 10 and 100 mg/l ClO$_2^-$ and 10 mg/l ClO$_3^-$ in daily drinking water. An 8 hour labelling period was used in all animals, since the number of labelled mitoses reaches a peak at 6-8 hours in small intestine, Creamer (92). At the end of this time, rats were sacrificed by decapitation and the nuclei of liver, kidney, testes and mucosa of small intestine were prepared as follows. Organs were removed quickly, rinsed twice in a beaker of ice cold saline, gently blotted dry with gauze and weighed. The liver, kidney and testes were minced into fine pieces with scissors, while small intestinal mucosa was obtained by scraping the epithelial layer with a scalp blade. The minced pieces and mucosa were homogenized in 5 volumes (5 ml/g) of 0.25M STM buffer with 5 strokes in a Potter-Elvejem type glass tissue grinder equipped with a teflon pestle. The STM buffer consisted of 0.25 M sucrose,
50 mM tris HCl (pH 7.5), 5 mM Cl$_2$ and 0.1 mM Na$_2$H$_2$ EDTA. Homogenates were subsequently centrifuged for 10 minutes, 750 x g, 0°C in a Sorvall Model RC-2B ultracentrifuge equipped with an SS34 rotor.

The 750 x g pellet was resuspended in 0.34 M STM buffer, i.e., 0.34 M sucrose replacing 0.25 M sucrose in STM buffer, (10 ml/g tissue) using 3 strokes of a teflon-glass tissue grinder described above. This suspension was then centrifuged at 20,000 x g for 20 minutes in the Sorvali ultracentrifuge. The supernatant was discarded and the pellet taken up in 0.25 M STM buffer (0.5 ml/g tissue) using a hand held, ground glass tissue grinder. This fraction was designated "nuclei".

a. Assay of Thymidine Incorporation Activity

Two ml of ice cold 10% trichloroacetic acid, TCA (W/V) was added to 100 µl nuclei sample obtained from each organ. Samples were then vortexed and centrifuged at 1000 x g for 10 minutes in a Sorvall GLC-1 tabletop centrifuge equipped with a type HL-4 rotor. The supernatant was decanted and discarded. The pellet was washed twice by resuspending in 2 ml of ice cold 5% TCA (W/V) with a vortex mixer. After the final centrifugation, pellets were suspended in 2 ml of 5% TCA by sonication, filtered on a millipore (0.45 µm) glass fiber filter. Samples were drawn through the filter by a vacuum attachment, trapping DNA on the filter. Filters were washed twice with 2 ml aliquots of toluene to remove TCA. Filters were then removed from the filtration apparatus and air dried for 30 minutes on an absorbent pad. When completely dry, the filters were added to 10 ml of the toluene-PPO-POPOP cocktail. The toluene-PPO-POPOP cocktail was made by dissolving 100 g of PPO (Packard) and 1.25 g POPOP
(Packard) in 1000 ml toluene to make a concentrated solution. Forty ml of this concentrate was then dissolved in a liter of toluene prior to use. Radioactivity was determined in a Packard Tri-Carb #3255 liquid scintillation spectrometer. Counting efficiency was approximately 35% as determined by automatic external standardization. Final results were expressed as DPM/μg DNA.

b. DNA Determination

DNA was quantitated by the diphenylamine method of Burton (93) as modified by Giles and Myers (94). This was done as follows: 2 ml of 10% perchloric acid V/V added to an aliquot of the nuclei pellet (prepared as described above) in 13 x 100 mm test tubes. The samples were then placed in a hot water bath at 90°C for 15 minutes to hydrolyze the DNA. Two ml of a 4% diphenylamine solution (in glacial acetic acid) was added to the DNA sample solution. A 0.1 ml aliquot of a 16 mg/100 ml acetaldehyde solution was added to the mixture and the samples were allowed to stand overnight at 30°C. A blue color developed and the difference in absorbance at 595 and 700 nm was read using a Varian techtron UV-VIS spectrophotometer 635. Absorbance was linear to 160 μg DNA and as little as 5 μg was detected. The concentration of DNA obtained from each sample was used for calculation of dpm/μg DNA.

N. EFFECT OF DRINKING Cl-COMPOUND ON GROWTH OF RAT AND CHICKEN

Rats and chickens were given drinking water containing 1000, 100, 10 and 1 mg/l ClO₂ for 12 months. Another group of rats drank 10,
100 mg/l ClO\(_2^−\) or ClO\(_3^−\) for the same time period. Animals were housed in controlled environment conditions (described above) and were given food ad libitum. Daily water intake and bimonthly body weight were measured.
RESULTS

A. CHLORINE DIOXIDE ABSORPTION AND ELIMINATION FROM BLOOD

The absorption and elimination time course for chlorine dioxide in rat plasma is shown in Figure 4. A peak $^{36}$Cl plasma level (7 μg/ml) was reached at 1 hr following oral administration of 3 ml of 100 mg/l $^{36}$ClO$_2$. The half life for the elimination of $^{36}$Cl from the rat was 43.9 ± 2.3 hrs, corresponding to a rate constant of 0.016 hr$^{-1}$ (Fig. 4-B). When rats drinking 100 mg/l ClO$_2$ for 15 days were then given 3 ml of 300 mg/l $^{36}$ClO$_2$ orally, $^{36}$Cl plasma level reached a peak at 2 hr. The half life for the elimination of $^{36}$Cl from the rat was 31.0 ± 1.2 hrs, corresponding to a rate constant of 0.022 hrs$^{-1}$ (Fig. 4-A).

B. ClO$_2$ DISTRIBUTION

Figure 5 reveals that the distribution of $^{36}$Cl compounds 72 hrs after the administration of $^{36}$ClO$_2$ orally was the highest in plasma, 2.74 ± 0.30 followed by kidney 2.45 ± 0.46, lung 2.25 ± 0.44, stomach 2.15 ± 0.49, duodenum 1.66 ± 0.3, ileum 1.47 ± 0.28, liver 1.16 ± 0.26, spleen 0.76 ± 0.11, thymus 0.68 ± 0.07 and bone marrow 0.47 ± 0.09 μg/gm. The distribution of $^{36}$Cl compounds in rats drinking 100 mg/l ClO$_2$ for 15 days, then given $^{36}$ClO$_2$ orally was highest in plasma followed by lung, stomach, kidney, testes, skin, small intestine, thymus, spleen, carcass, liver and bone marrow (Table 3). In whole blood, when plasma was separated from
Blood samples (heparinized) were collected by orbital sinus puncture. The blood was centrifuged at 1000 x g for 15 min to separate the red blood cells from the plasma. $^{36}$Cl was determined at each time point.

Figure 4-A: Each time point represents the mean of 4 animals drinking 100 mg/l ClO$_2$ for 15 days, then given 300 mg/l $^{36}$ClO$_2$ orally at zero hours.

Figure 4-B: Each time point represents the mean of 5 animals given 100 mg/l $^{36}$ClO$_2$ orally at zero hours.
Time course of $^{36}$ClO$_2$ elimination from rat plasma

(A) 4 Rats drinking 100mg/L ClO$_2$ for 15 days, then given 300mg/L $^{36}$ClO$_2$ orally at zero hours.

(B) 4 Rats were given 100mg/L $^{36}$ClO$_2$ orally at zero hours.

$T_{1/2}$ = 31.0 ± 1.20 hrs.

$T_{1/2}$ = 43.9 ± 2.3 hrs.

FIGURE 4
Rats were administered 3 ml of 100 mg/1 $^{36}\text{ClO}_2$ (0.07 μCi) orally. At 72 hrs rats were sacrificed by decapitation and $^{36}\text{Cl}$ was determined in different organs after tissues were prepared as described in methods section. Values represent the mean ± S.E. as $^{36}\text{Cl}$ (μg/gm) from 4 rats.
TABLE 3

EFFECT OF REPEATED DOSES OF ClO$_2$ ON THE DISTRIBUTION OF $^{36}$Cl IN RAT.

<table>
<thead>
<tr>
<th></th>
<th>$100\text{mg/L}^{*}$</th>
<th></th>
<th>$300\text{mg/L}^{**}$</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu g/\text{ml or gm}$</td>
<td>% of doses/\text{(ml or gm)}</td>
<td>$\mu g/\text{ml or gm}$</td>
<td>% of doses/\text{(ml or gm)}</td>
</tr>
<tr>
<td>Plasma</td>
<td>2.21 ± 0.07</td>
<td>0.72 ± 0.02</td>
<td>3.40 ± 0.22</td>
<td>0.40 ± 0.03</td>
</tr>
<tr>
<td>Packed Cells</td>
<td>—</td>
<td>—</td>
<td>2.06 ± 0.04</td>
<td>0.24 ± 0.00</td>
</tr>
<tr>
<td>Whole Blood</td>
<td>—</td>
<td>—</td>
<td>3.01 ± 0.19</td>
<td>0.35 ± 0.02</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.45 ± 0.46</td>
<td>0.81 ± 0.15</td>
<td>2.14 ± 0.20</td>
<td>0.25 ± 0.02</td>
</tr>
<tr>
<td>Lung</td>
<td>2.25 ± 0.44</td>
<td>0.74 ± 0.15</td>
<td>2.24 ± 0.17</td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td>Stomach:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardia</td>
<td>2.15 ± 0.49</td>
<td>0.70 ± 0.15</td>
<td>2.22 ± 0.15</td>
<td>0.25 ± 0.02</td>
</tr>
<tr>
<td>Pylorus</td>
<td>2.15 ± 0.49</td>
<td>0.70 ± 0.16</td>
<td>2.16 ± 0.14</td>
<td>0.25 ± 0.02</td>
</tr>
<tr>
<td>Duodenum:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole</td>
<td>0.30 ± 0.22</td>
<td>0.29 ± 0.07</td>
<td>1.58 ± 0.14</td>
<td>0.18 ± 0.02</td>
</tr>
<tr>
<td>Mucosa</td>
<td>1.05 ± 0.15</td>
<td>0.35 ± 0.05</td>
<td>1.80 ± 0.04</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.79 ± 0.04</td>
<td>0.26 ± 0.01</td>
<td>1.48 ± 0.07</td>
<td>0.17 ± 0.00</td>
</tr>
<tr>
<td>Ileum</td>
<td>1.47 ± 0.28</td>
<td>0.48 ± 0.09</td>
<td>1.04 ± 0.03</td>
<td>0.12 ± 0.00</td>
</tr>
<tr>
<td>Liver</td>
<td>1.16 ± 0.26</td>
<td>0.38 ± 0.09</td>
<td>1.03 ± 0.06</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.76 ± 0.11</td>
<td>0.25 ± 0.04</td>
<td>1.29 ± 0.12</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>Thymus</td>
<td>0.68 ± 0.07</td>
<td>0.22 ± 0.02</td>
<td>1.38 ± 0.11</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>Bone Marrow</td>
<td>0.47 ± 0.09</td>
<td>0.16 ± 0.03</td>
<td>0.33 ± 0.06</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>Testes</td>
<td>—</td>
<td>—</td>
<td>2.12 ± 0.14</td>
<td>0.25 ± 0.02</td>
</tr>
<tr>
<td>Skin</td>
<td>—</td>
<td>—</td>
<td>2.01 ± 0.25</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td>Carcass</td>
<td>—</td>
<td>—</td>
<td>1.22 ± 0.06</td>
<td>0.14 ± 0.01</td>
</tr>
</tbody>
</table>

* Values represent mean ± S.E. from 4 rats given 100 mg/L $^{36}$ClO$_2$ orally. S.A. = 420 DPM/4g $^{36}$ClO$_2$.

** Values represent mean ± S.E. from 4 rats drinking 100 mg/L ClO$_2$ for 15 days, then given 300 mg/L $^{36}$ClO$_2$ orally. S.A. = 1800 DPM/4g $^{36}$ClO$_2$.

— Not Determined
packed cells, $^{36}\text{Cl}$ compounds were 3.40 ± 0.22 and 2.06 ± 0.04 for plasma and packed cells respectively, as compared to 3.01 ± 0.19 μg/ml for whole blood. The quantity of $^{36}\text{Cl}$ compounds in cardia and pylorus in rat stomach was the same, but the mucosa was higher than duodenum muscle (1.8 ± 0.04 and 1.48 ± 0.07 μg/gm respectively) (Table 3).

C. SUBCELLULAR DISTRIBUTION OF $^{36}\text{Cl}$ COMPOUNDS

Subcellular distribution of $^{36}\text{Cl}$ compounds in rat liver 1/2 hr following $^{36}\text{ClO}_2$ administration indicated that 2.19, 1.61, 0.19, 0.19 and 0.08% of initial dose were in whole homogenate, cytosol, microsomes, nuclei and mitochondria respectively (Figure 6). Approximately 75% of total radioactivity in whole homogenate was recovered in the trichloroacetic acid supernatant of the homogenate. These results indicate that about 25% of $^{36}\text{Cl}$ compounds were bound to the precipitated protein fraction.

D. EXCRETION STUDIES

Four rats were administered 100 mg/l $^{36}\text{ClO}_2$ orally. Collections of urine, expired air and feces were obtained over a 3 day period as described in the method section. The $^{36}\text{Cl}$ compounds recovered from rats by pulmonary, urinary and intestinal routes of excretion are summarized in Table 4. Values represent data from two separate experiments (2 treated rats/experiment) expressed as percentage of the total initial dose; 39 and 43% of $^{36}\text{Cl}$ was recovered in the 72 hour period in experiments 1 and 2, respectively. In the first 24 hrs 25.20% and 11.20% were excreted in the urine, 2.83% and 6.20% in feces. During the second 24 hours 4.38%
FIGURE 6

Subcellular Distribution of $^{36}\text{Cl}$ Compounds in Rat Liver Following $^{36}\text{ClO}_2$ Administration

Rats were administered 3 ml of 100 mg/1 $^{36}\text{ClO}_2$ (0.7 μCi) orally. At 1/2 hrs rats were sacrificed and $^{36}\text{Cl}$ was determined in each subcellular fraction as percent of initial dose.
% $^{36}$Cl in each subcellular fraction

36ClO$_2$ ADMINISTRATION
LIVER FOLLOWING OR $^{36}$Cl COMPOUNDS IN RAT
SUBCELLULAR DISTRIBUTION
### TABLE 4

**Excretion of $^{36}$ClO$_2$ in the Rat**

<table>
<thead>
<tr>
<th>Collection Period (hrs.)</th>
<th>Experiment No. 1</th>
<th></th>
<th></th>
<th>Experiment No. 2</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urine</td>
<td>Feces</td>
<td>Total</td>
<td>Urine</td>
<td>Feces</td>
<td>Total</td>
</tr>
<tr>
<td>0—12</td>
<td>13.2</td>
<td></td>
<td></td>
<td>1.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12—24</td>
<td>12.0</td>
<td></td>
<td></td>
<td>9.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0—24</td>
<td>25.20</td>
<td>2.83</td>
<td>28.03</td>
<td>11.20</td>
<td>6.20</td>
<td>17.4</td>
</tr>
<tr>
<td>24—48</td>
<td>4.38</td>
<td>1.97</td>
<td>6.35</td>
<td>19.66</td>
<td>3.23</td>
<td>22.89</td>
</tr>
<tr>
<td>48—72</td>
<td>0.47</td>
<td>3.62</td>
<td>4.09</td>
<td>0.71</td>
<td>2.34</td>
<td>3.05</td>
</tr>
<tr>
<td>0—72</td>
<td>30.05</td>
<td>8.42</td>
<td>38.47</td>
<td>31.57</td>
<td>11.77</td>
<td>43.34</td>
</tr>
</tbody>
</table>

*Values represent data from 2 treated rats/experiment expressed as percentage of the total initial dose. $^{36}$Cl was not detected in expired air throughout the 72 hrs. studied.*
and 19.66% were obtained via urinary routes, while 1.97% and 3.23% were obtained in feces from experiments 1 and 2 respectively. At the end of the 3 day collection period, the total fraction of the initial dose eliminated by urinary routes were 30.05% and 31.57%, while 8.42% and 11.77% were obtained in feces. $^{36}\text{Cl}$ was not detected in the expired air throughout the 72 hrs studied. The urinary and fecal excretion varied during the first 24 hr in both experiments; however, at the end of the 3 day collection period the total fraction of excreted $^{36}\text{Cl}$ compounds were similar.

E. METABOLISM OF ClO$_2$ IN RAT

Rat urine was collected at 12, 24, 48 and 72 hrs after the oral administration of 3 ml of 100 mg/l $^{36}\text{ClO}_2$ (0.7 μCi). The analysis of chlorine compounds is summarized in Table 5, as percentage of the initial dose. 6.09 ± 4.7, 8.06 ± 1.82, 12.33 ± 7.99 and 0.46 ± 0.13 were detected as the Cl$^-$ form at 12, 24, 48 and 72 hrs, respectively. For the same time periods ClO$_2^-$ values were 1.22 ± 0.82, 1.63 ± 0.64, 0.48 ± 0.48 and 0.13 ± 0.01. While ClO$_3^-$ was 0.73 ± 0.73 at 24 hrs only. At 72 hrs, Cl$^-$ and ClO$_2^-$ in plasma was 0.82 ± 0.11 and 0.22 ± 0.07, respectively, as percentage of the initial dose in one ml.

Concentration of Chlorine Compounds in Rat Blood

Rats were sacrificed 1/2 hr after the administration of 3 ml of either 100 or 10 mg/l $^{36}\text{ClO}_2$. Plasma and packed cells, with and without cold saline wash, were analyzed for chlorine compounds. As shown in Table 6, the total chlorine compounds as percentage of the initial dose
### TABLE 5

**Metabolism of ClO₂ in Rat**

<table>
<thead>
<tr>
<th>Collection Period (hrs.)</th>
<th>Urine</th>
<th>Plasma 72 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cl⁻</td>
<td>ClO₂⁻</td>
</tr>
<tr>
<td>0 – 12</td>
<td>6.09 ± 4.70</td>
<td>1.22 ± 0.82</td>
</tr>
<tr>
<td>12 – 24</td>
<td>8.06 ± 1.82</td>
<td>1.63 ± 0.64</td>
</tr>
<tr>
<td>24 – 48</td>
<td>12.33 ± 7.99</td>
<td>0.48 ± 0.48</td>
</tr>
<tr>
<td>48 – 72</td>
<td>0.46 ± 0.13</td>
<td>0.13 ± 0.01</td>
</tr>
</tbody>
</table>

**Values** represent the Mean ± S.E. of data from 4 rats as percentage of the total initial dose.

— None detected
TABLE 6

Content of $^{36}$Cl-Compounds in Rat Blood After $^{36}$ClO$_2$ Treatment

<table>
<thead>
<tr>
<th></th>
<th>100 mg/L</th>
<th>10 mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PLASMA</td>
<td>PACKED CELLS**</td>
</tr>
<tr>
<td>Total $^{36}$Cl</td>
<td>$1.42 \pm 0.05$</td>
<td>$0.47 \pm 0.02$</td>
</tr>
<tr>
<td>$^{36}$Cl$^-$</td>
<td>$1.17 \pm 0.04$</td>
<td>—</td>
</tr>
<tr>
<td>$^{36}$ClO$_2^-$</td>
<td>$0.05 \pm 0.04$</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>$2.22 \pm 0.13$</td>
<td>$0.86 \pm 0.03$</td>
</tr>
</tbody>
</table>

* PACKED CELLS WERE RECONSTITUTED WITH SALINE TO THE INITIAL WHOLE BLOOD VOLUME.

** WASHED CELLS WERE WASHED TWICE WITH SALINE, THEN RECONSTITUTED WITH SALINE TO THE INITIAL WHOLE BLOOD VOLUME.

— NOT DETERMINED

$^a$ VALUES REPRESENT THE MEAN $\pm$ S.E. OF DATA FROM 4 RATS AS PERCENTAGE OF THE INITIAL DOSE/ml PLASMA OR PACKED CELL HEMOLYSATE.
in one ml of plasma or erythrocyte hemolysates were 1.42 ± 0.05 and
0.47 ± 0.02 respectively, after a 100 mg/l dose; however, 2.22 ± 0.13 and
0.86 ± 0.03 respectively, were detected after a 10 mg/l dose. The con-
ccentration in washed packed cells determined as described in methods was
0.15 ± 0.01 and 0.18 ± 0.00 in 100 and 10 mg/l doses, respectively. The
$^{36}$Cl metabolites in plasma were determined; $^{36}$Cl$^-$ was 1.17 ± 0.04 and
0.91 ± 0.24 and $^{36}$ClO$_2^-$ was 0.05 ± 0.04 and 0.92 ± 0.1 (as percentage of
initial doses) for the 100 and 10 mg/l doses, respectively.

F. EFFECT OF CL-COMPOUNDS IN DRINKING WATER ON RAT BLOOD, GLUTATHIONE,
OSMOTIC FRAGILITY AND METHEMOGLOBIN

Blood was collected in heparinized tubes by cardiac puncture at 2,
4, 7 and 9 months from groups of rats drinking ClO$_2$, ClO$_2^-$, and ClO$_3^-$. 
Blood glutathione, osmotic fragility and methemoglobin were determined
as described in methods section.

The results of blood glutathione determinations are shown in Table
7. At 2 months, glutathione content decreased significantly in all
treatment groups except the 100 mg/l ClO$_2$ group. At 4 months, glutathione
content decreased only in 10, 1 mg/l ClO$_2$ and 100 mg/l ClO$_2^-$ groups. At
9 months in both the ClO$_2^-$ and ClO$_3^-$ groups decreased glutathione was
observed while the 100 mg/l ClO$_2$ group was significantly increased. The
fluctuation of blood glutathione concentration is most likely due to the
redox state of the compound as influenced by the Cl-compounds oxidative
stress.
**TABLE 7**

**EFFECT OF CI-COMPOUNDS IN DRINKING WATER ON RAT BLOOD GLUTATHIONE.**

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>2</th>
<th>4</th>
<th>7</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>44.00 ± 2.10</td>
<td>60.80 ± 2.50</td>
<td>62.70 ± 2.86</td>
<td>52.29 ± 1.75</td>
</tr>
<tr>
<td>ClO₂ (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>38.00 ± 1.59*</td>
<td>60.70 ± 2.50</td>
<td>60.30 ± 3.40</td>
<td>51.04 ± 2.48</td>
</tr>
<tr>
<td>100</td>
<td>38.65 ± 3.80</td>
<td>64.50 ± 1.30</td>
<td>55.15 ± 3.50</td>
<td>57.25 ± 1.97**</td>
</tr>
<tr>
<td>10</td>
<td>36.30 ± 1.98*</td>
<td>47.60 ± 3.60*</td>
<td>55.30 ± 5.30</td>
<td>49.80 ± 2.80</td>
</tr>
<tr>
<td>1</td>
<td>38.30 ± 0.65**</td>
<td>51.50 ± 2.70*</td>
<td>57.10 ± 2.30*</td>
<td>45.66 ± 3.72</td>
</tr>
<tr>
<td>ClO₂⁻ (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>19.65 ± 0.99**</td>
<td>42.80 ± 1.80**</td>
<td>44.80 ± 2.90*</td>
<td>32.56 ± 2.14****</td>
</tr>
<tr>
<td>10</td>
<td>34.30 ± 3.90*</td>
<td>58.20 ± 2.60</td>
<td>56.30 ± 3.70</td>
<td>42.68 ± 1.70****</td>
</tr>
<tr>
<td>ClO₃⁻ (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>26.70 ± 1.30*</td>
<td>58.00 ± 3.40</td>
<td>48.20 ± 3.80*</td>
<td>34.49 ± 1.07****</td>
</tr>
<tr>
<td>10</td>
<td>30.30 ± 0.64*</td>
<td>56.80 ± 3.60</td>
<td>54.50 ± 4.90</td>
<td>39.04 ± 1.27****</td>
</tr>
</tbody>
</table>

*Values represent mean ± S.E. (mg%) from 4 rats/group.

** and *** Significantly different from Control, student t-test, p<0.05 and p<0.01 respectively.
Blood osmotic fragility results are summarized in Table 8. Decreased osmotic fragility was observed in all groups after 2, 4, 7 and 9 months treatment. The intensity of decreased osmotic fragility produced after 9 months of treatment was as follows: \( \text{ClO}_2^- > \text{ClO}_3^- > \text{ClO}_2 \). The results of osmotic fragility could actually represent an increase in osmotic fragility, because in the determination of osmotic fragility hemoglobin could interact with the red cell membrane and be precipitated, then less free hemoglobin would be available for measurement under testing conditions (as described in methods section). This may explain why the net result would be an apparent decrease in hemolysis.

G. EFFECT OF \text{ClO}_2, IN DRINKING WATER ON CHICKEN BLOOD, GLUTATHIONE, OSMOTIC FRAGILITY AND METHEMOGLOBIN

In experiments with groups of chickens, the blood glutathione after 2 and 4 months was significantly depressed in the 100 mg/l \text{ClO}_2 group and increased in the 1000 mg/l group at 4 months. No significant change in blood glutathione at 6 months treatment was observed (Table 9). Hemolysis was decreased after 2 months treatment in all groups. At 4 and 6 months, no significant change in hemolysis was seen (Table 9). Methemoglobin was not detected in all blood samples. The data from these experiments suggest that chicken erythrocytes gradually adapted to \text{ClO}_2 oxidation stress with increased treatment time.

H. EFFECT OF Cl-COMPOUNDS ON RAT BLOOD CELL COMPARTMENT AFTER CHRONIC Cl-COMPOUNDS IN DRINKING WATER

The Coulter counter was used to determine the values for the rat red blood cell compartment at 2, 4, 6, 7 and 9 months of chronic
TABLE 8

EFFECT OF CI-COMPOUNDS IN DRINKING WATER ON RAT BLOOD OSMOTIC FRAGILITY.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>2</th>
<th>4</th>
<th>7</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>69.0 ± 10.80a</td>
<td>61.70 ± 16.20</td>
<td>71.20 ± 6.60</td>
<td>59.20 ± 9.10</td>
</tr>
<tr>
<td>ClO₂ (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>56.70 ± 2.50k</td>
<td>58.50 ± 7.80</td>
<td>41.69 ± 7.70k</td>
<td>39.68 ± 3.04kk</td>
</tr>
<tr>
<td>100</td>
<td>44.00 ± 3.60k</td>
<td>28.20 ± 7.90k</td>
<td>36.60 ± 12.10k</td>
<td>23.08 ± 5.89kk</td>
</tr>
<tr>
<td>10</td>
<td>57.40 ± 8.10</td>
<td>51.10 ± 9.60</td>
<td>56.30 ± 5.60</td>
<td>24.35 ± 5.50kk</td>
</tr>
<tr>
<td>1</td>
<td>59.80 ± 8.20</td>
<td>62.10 ± 5.80</td>
<td>37.60 ± 5.70kk</td>
<td>49.98 ± 12.40</td>
</tr>
<tr>
<td>ClO₂⁻ (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>36.90 ± 10.60k</td>
<td>54.90 ± 11.70</td>
<td>50.40 ± 5.40k</td>
<td>14.88 ± 3.70kk</td>
</tr>
<tr>
<td>10</td>
<td>54.00 ± 9.80</td>
<td>48.90 ± 9.90</td>
<td>46.10 ± 5.80kk</td>
<td>24.90 ± 7.90k</td>
</tr>
<tr>
<td>ClO₃⁻ (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>54.90 ± 6.50</td>
<td>34.30 ± 2.50kk</td>
<td>39.50 ± 9.00k</td>
<td>22.33 ± 9.04k</td>
</tr>
<tr>
<td>10</td>
<td>68.00 ± 2.40</td>
<td>62.90 ± 4.00</td>
<td>32.50 ± 8.00kk</td>
<td>44.60 ± 4.70kk</td>
</tr>
</tbody>
</table>

q Values represent mean ± S.E. (% Hemolysis) from 4 rats/group.

k, kk Significantly different from Control, Student t-test; p<0.05 and p<0.01 respectively.
### TABLE 9

**EFFECT OF ClO₂ ON CHICKEN BLOOD GLUTATHIONE AND OSMOTIC FRAGILITY.**

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>2 MONTHS</th>
<th>4 MONTHS</th>
<th>6 MONTHS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GSH mg %</td>
<td>Hemolysis %</td>
<td>GSH mg %</td>
</tr>
<tr>
<td>CONTROL ClO₂ (mg/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>88.1 ± 4.1</td>
<td>44.75 ± 3.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>118.2 ± 2.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>100</td>
<td>74.7 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.2 ± 10.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>85.3 ± 1.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>89.0 ± 2.6</td>
<td>37.6 ± 8.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>99.0 ± 4.7</td>
</tr>
<tr>
<td></td>
<td>90.5 ± 5.2</td>
<td>60.1 ± 5.0</td>
<td>105.0 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>93.5 ± 4.8</td>
<td>56.67 ± 16.1</td>
<td></td>
</tr>
</tbody>
</table>

* Methemoglobin was not detected in all blood samples.

<sup>a</sup> Significantly different from Control, Student t-test; p<0.05 and n= 4 for all groups.
exposure to Cl-compounds in drinking water. At 2, 4 and 6 months no significant changes were noted in treated rats compared to control in red blood cell count (RBC), hemoglobin % (HGB), hematocrit % (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). However, after 7 months treatment, the RBC counts were significantly increased in the 100 mg/l ClO₂ group, while the 10 mg/l ClO₃⁻ group was decreased. Hematocrit was increased in 1000 and 10 mg/l ClO₂ treatment groups and decreased in 10 mg/l ClO₃⁻. The mean corpuscular hemoglobin concentration was increased in 10 mg/l ClO₂, 100 and 10 mg/l ClO₂ groups, Table 10.

Results at 9 months treatment are summarized in Table 11. The RBC counts were decreased in all treatment groups; however, significant differences from control were observed only in 1000 and 1 mg/l ClO₂, 100 mg/l ClO₂ and in both ClO₃⁻ treatment groups. The HCT and HGB were significantly decreased in all treatment groups except the 100 mg/l ClO₂ group, while the MCHC was increased significantly in 1000 and 100 mg/l ClO₂ groups only.

I. EFFECT OF Cl-COMPOUNDS ON 2,3-DIPHOSPHOGLYCERATE IN RAT BLOOD

Blood was collected in heparinized tubes by cardiac puncture from rats treated with Cl-compounds for 5 months. Erythrocyte 2,3-diphosphoglycerate was determined as described in methods section. The results are summarized in Figure 7.

Dose-related increases in RBC 2,3-diphosphoglycerate (2,3-DPG) were observed in animals consuming 10 and 100 mg/l ClO₂⁻ in drinking water,
### TABLE 10

**EFFECT OF CI-COMPOUNDS ON RAT BLOOD CELL COMPARTMENT AFTER 7 MONTHS TREATMENT.**

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>RBC $10^6$/mm$^3$</th>
<th>Hct %</th>
<th>MCHC %</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>8.30 ± 0.07</td>
<td>46.23 ± 0.20</td>
<td>34.63 ± 0.33</td>
</tr>
<tr>
<td>ClO$_2$ (mg/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>8.60 ± 0.18</td>
<td>48.13 ± 0.64*</td>
<td>34.56 ± 0.14</td>
</tr>
<tr>
<td>100</td>
<td>8.93 ± 0.26*</td>
<td>48.37 ± 0.78*</td>
<td>35.20 ± 0.42</td>
</tr>
<tr>
<td>10</td>
<td>8.45 ± 0.08</td>
<td>46.50 ± 1.01</td>
<td>35.20 ± 0.08**</td>
</tr>
<tr>
<td>1</td>
<td>8.25 ± 0.23</td>
<td>45.80 ± 1.17</td>
<td>36.03 ± 1.07</td>
</tr>
<tr>
<td>ClO$_2^-$ (mg/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>8.23 ± 0.08</td>
<td>46.10 ± 0.86</td>
<td>35.60 ± 0.04**</td>
</tr>
<tr>
<td>10</td>
<td>8.40 ± 0.15</td>
<td>47.07 ± 1.37</td>
<td>35.73 ± 0.13**</td>
</tr>
<tr>
<td>ClO$_3^-$ (mg/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>8.23 ± 0.17</td>
<td>44.28 ± 1.15</td>
<td>35.23 ± 0.14</td>
</tr>
<tr>
<td>10</td>
<td>8.08 ± 0.08*</td>
<td>44.17 ± 0.82*</td>
<td>35.60 ± 0.26</td>
</tr>
</tbody>
</table>

*Significantly different from Control, Student t-test; p<0.05 and p<0.01 respectively and n=4 for all groups.
### Table 11

**EFFECT OF CI- COMPOUNDS ON RAT BLOOD CELL COMPARTMENT AFTER 9 MONTHS TREATMENT.**

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>RBC $10^6$/mm$^3$</th>
<th>Hct %</th>
<th>Hb gm%</th>
<th>MCHC %</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>8.89 ± 0.28</td>
<td>48.88 ± 0.74</td>
<td>16.55 ± 0.26</td>
<td>33.78 ± 0.49</td>
</tr>
<tr>
<td>ClO$_2$ (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>8.17 ± 0.03$^{**}$</td>
<td>44.03 ± 0.71$^{**}$</td>
<td>15.28 ± 0.29$^{**}$</td>
<td>34.60 ± 0.15$^{**}$</td>
</tr>
<tr>
<td>100</td>
<td>8.25 ± 0.32</td>
<td>43.45 ± 0.97$^{**}$</td>
<td>15.15 ± 0.32$^{**}$</td>
<td>34.93 ± 0.11$^{**}$</td>
</tr>
<tr>
<td>10</td>
<td>8.47 ± 0.35</td>
<td>45.33 ± 0.64$^{**}$</td>
<td>15.43 ± 0.22$^{**}$</td>
<td>33.95 ± 0.27</td>
</tr>
<tr>
<td>1</td>
<td>8.16 ± 0.15$^{**}$</td>
<td>46.28 ± 1.03$^{**}$</td>
<td>15.48 ± 0.45$^{**}$</td>
<td>33.70 ± 0.42</td>
</tr>
<tr>
<td>ClO$_2$ (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>8.29 ± 0.28$^*$</td>
<td>46.58 ± 1.15</td>
<td>15.90 ± 0.44</td>
<td>33.93 ± 0.23</td>
</tr>
<tr>
<td>10</td>
<td>8.36 ± 0.33</td>
<td>44.95 ± 0.51</td>
<td>15.58 ± 0.23$^*$</td>
<td>34.58 ± 0.49</td>
</tr>
<tr>
<td>ClO$_3$ (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>6.42 ± 0.59$^{**}$</td>
<td>40.90 ± 1.35$^{**}$</td>
<td>13.78 ± 1.17$^{**}$</td>
<td>33.40 ± 1.81</td>
</tr>
<tr>
<td>10</td>
<td>7.72 ± 0.41$^*$</td>
<td>44.60 ± 1.38$^*$</td>
<td>15.58 ± 0.41$^*$</td>
<td>34.78 ± 0.52</td>
</tr>
</tbody>
</table>

$^*$, $^{**}$ Significantly different from Control, Student t-test; p<0.05 and <0.01 respectively and n=4 for all groups.
FIGURE 7

Effect of Cl-Compounds on 2,3-Diphosphoglycerate in Rat Blood

Blood was collected in heparinized tubes by cardiac puncture from 4 rats/group treated with different concentration of ClO₂, ClO₇ and ClO₃ for 5 months. Values represent the mean ± S.E. as μmoles/gm HGB.
EFFECT OF CI- COMPOUNDS ON 2,3-DIPHOSPHOGLYCERATE IN RAT BLOOD.

FIGURE 7
while the drinking of 10 and 100 mg/l ClO$_3^-$ caused dose-related decreases in 2,3-DPG. The consumption of ClO$_2^-$ significantly decreased 2,3-DPG in the 10 mg/l ClO$_2^-$ group only.

J. ERYTHROCYTE MORPHOLOGY

Blood smears (Wright stain) and scanning electron micrographs of red cells were prepared from rats and chickens drinking ClO$_2^-$, ClO$_2^+$ and ClO$_3^-$ daily for four months. Figure 8 depicts abnormalities in rat erythrocyte morphology. Alterations of the red cell membrane were also observed in all ClO$_2^+$ rat treatment groups. Some red cell changes from the normal biconcave disk-shaped (discocyte) to crenated disk and crenated sphere (echinocyte) were observed. Besides the crenated cell, marked distortions in the middle of erythrocytes of 100 mg/l ClO$_2^-$ treated group were present (Figure 9). In ClO$_3^-$ treatment groups, the codocyte (mexican hat) and echinocytes were observed (Figure 9). Figure 10 shows dacrocytes (teardrop-shaped) is the characteristic abnormality of chicken erythrocyte in 100 and 1000 mg/l ClO$_2^+$ treatments only (none were noted at 10 mg/l). Also, a teardrop-shaped was observed in both ends of the erythrocyte (football-shape).

K. EARLY EFFECTS OF ClO$_2^+$ ON RAT BLOOD GLUTATHIONE AND HEMOLYSIS

Four groups of 5 rats each were administered 3 ml of 0, 10, 20 and 40 mg/l ClO$_2^+$ orally. Blood was collected as described in methods section. Glutathione, osmotic fragility and methemoglobin were determined at 15, 30, 60 and 120 min following ClO$_2^+$ administration. In the first 15 min, blood glutathione values were 62.4 ± 2.7, 55.0 ± 1.10,
FIGURE 8

Effect of C1O₂ on Rat Erythrocyte Morphology

Blood was collected by cardiac puncture from rats drinking the indicated concentration of C1O₂ for 4 months. Blood was added to 1% solution of glutaraldehyde in Eagle's solution, mixed and cells remained in the fixative for 30 min. A 0.5% solution of osmium tetroxide in Eagle's solution was used as post fix for 60 min. The cells were washed in distilled water, then dehydrated by washing the dehydrated cells in propylene oxide. A small drop of this cell suspension was prepared for SEM as described in methods section. Wright stain was used for preparation of blood smears for light microscope.
Effect of ClO₂ on Rat Erythrocyte Morphology

<table>
<thead>
<tr>
<th></th>
<th>Wright's Stain</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td><img src="image" alt="Control" /></td>
<td><img src="image" alt="Control" /></td>
</tr>
<tr>
<td>1000 mg/L</td>
<td><img src="image" alt="1000 mg/L" /></td>
<td><img src="image" alt="1000 mg/L" /></td>
</tr>
<tr>
<td>100 mg/L</td>
<td><img src="image" alt="100 mg/L" /></td>
<td><img src="image" alt="100 mg/L" /></td>
</tr>
<tr>
<td>10 mg/L</td>
<td><img src="image" alt="10 mg/L" /></td>
<td><img src="image" alt="10 mg/L" /></td>
</tr>
<tr>
<td>1 mg/L</td>
<td><img src="image" alt="1 mg/L" /></td>
<td><img src="image" alt="1 mg/L" /></td>
</tr>
</tbody>
</table>

**Figure 8**
FIGURE 9

Effect of $\text{ClO}_2^-$ and $\text{ClO}_3^-$ on Rat Erythrocyte Morphology

Blood was collected by cardiac puncture from rats drinking the indicated concentrations of $\text{ClO}_2^-$ and $\text{ClO}_3^-$ for 4 months. Blood was prepared for SEM and light microscope as described in the legend to Figure 8.
### Effect of $\text{ClO}_2$ & $\text{ClO}_3^-$ on Rat Erythrocyte Morphology

<table>
<thead>
<tr>
<th></th>
<th>Wright's Stain</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td><img src="image" alt="Control" /></td>
<td><img src="image" alt="Control" /></td>
</tr>
<tr>
<td>$\text{ClO}_2^-$ 100 mg/L</td>
<td><img src="image" alt="ClO2 100mg/L" /></td>
<td><img src="image" alt="ClO2 100mg/L" /></td>
</tr>
<tr>
<td>$\text{ClO}_2^-$ 10 mg/L</td>
<td><img src="image" alt="ClO2 10mg/L" /></td>
<td><img src="image" alt="ClO2 10mg/L" /></td>
</tr>
<tr>
<td>$\text{ClO}_3^-$ 100 mg/L</td>
<td><img src="image" alt="ClO3 100mg/L" /></td>
<td><img src="image" alt="ClO3 100mg/L" /></td>
</tr>
<tr>
<td>$\text{ClO}_3^-$ 10 mg/L</td>
<td><img src="image" alt="ClO3 10mg/L" /></td>
<td><img src="image" alt="ClO3 10mg/L" /></td>
</tr>
</tbody>
</table>

**FIGURE 9**
FIGURE 10

Effect of ClO$_2$ on Chicken Erythrocyte Morphology

Blood was collected by wing vein puncture from chickens drinking the indicated concentration of ClO$_2$ for 4 months. Blood was prepared for SEM and light microscope as described in the legend to Figure 8.
Effect of ClO₂ on Chicken Erythrocyte Morphology

<table>
<thead>
<tr>
<th></th>
<th>Wright's Stain</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td><img src="image1" alt="Wright's Stain" /></td>
<td><img src="image2" alt="SEM" /></td>
</tr>
<tr>
<td>1000 mg/L</td>
<td><img src="image3" alt="Wright's Stain" /></td>
<td><img src="image4" alt="SEM" /></td>
</tr>
<tr>
<td>100 mg/L</td>
<td><img src="image5" alt="Wright's Stain" /></td>
<td><img src="image6" alt="SEM" /></td>
</tr>
<tr>
<td>10 mg/L</td>
<td><img src="image7" alt="Wright's Stain" /></td>
<td><img src="image8" alt="SEM" /></td>
</tr>
</tbody>
</table>

**FIGURE 10**
57.9 ± 2.7 and 43.6 ± 1.98 mg% in control, 10, 20 and 40 mg/l groups, respectively. At the end of 30 min in the same groups, values were 62.0 ± 3.0, 53.6 ± 1.14, 55.40 ± 0.87 and 50.0 ± 1.96 mg%, respectively. After 60 min, the blood glutathione in control, 12, 20 and 40 mg/l ClO\textsubscript{2} groups was 60.0 ± 3.5, 46.0 ± 1.94, 47.4 ± 1.2 and 45.8 ± 2.37 mg%, respectively. At the end of the two hours values were 60.0 ± 3.0, 46.1 ± 2.55 and 45.8 ± 1.75 mg% in control, 10 and 40 mg/l ClO\textsubscript{2}, respectively. (Figure 11).

Blood hemolysis was determined at the indicated times for all treatment groups. The control values at 12 and 120 min were 58.36 ± 7.6 and 58.0 ± 8.0, respectively. In the 10 mg/l ClO\textsubscript{2} groups at 15, 30, 60 and 120 min, values were 69.88 ± 6.5, 59.05 ± 3.5, 52.6 ± 4.97 and 50.4 ± 2.8, respectively. In the 20 mg/l ClO\textsubscript{2} group, hemolysis was 56.95 ± 10.7, 54.3 ± 6.5 and 42.12 ± 7.1% at 15, 30 and 60 min, respectively. Values in the 40 mg/l ClO\textsubscript{2} group were 58.5 ± 9.7, 53.6 ± 7.4, 48.78 ± 5.7 and 38.7 ± 3.0% at 15, 30 60 and 120 min, respectively (Table 12).

L. \textit{IN VITRO} STUDIES

In a series of \textit{in vitro} experiments, heparinized rat blood was incubated with final concentrations of 500, 100, 50 and 5 mg/l ClO\textsubscript{2} and also with 10 mg/l ClO\textsubscript{2} and ClO\textsubscript{3}. Blood glutathione, osmotic fragility and methemoglobin were determined at various times as described in methods section.
Rats were administered 3 ml of 10, 20, 40 mg/l ClO\textsubscript{2} and 3 ml of H\textsubscript{2}O orally. Blood was collected by cardiac puncture at 15, 30, 60 and 120 min. Glutathione was determined for each time interval. Values represent the mean ± S.E. from 5 rats/group.
THE EARLY EFFECT OF CI\textsubscript{2} ON RAT BLOOD GLUTATHIONE AFTER ACUTE ADMINISTRATION

- Control
- 10 mg/L
- 20 mg/L
- 40 mg/L

\* \* = p < 0.01
n = 5 Animals

FIGURE 11
# TABLE 12

THE EARLY EFFECT OF CI\textsubscript{2}O\textsubscript{2} ON RAT BLOOD HEMOLYSIS AFTER ACUTE EXPOSURE.

<table>
<thead>
<tr>
<th>CI\textsubscript{2}O\textsubscript{2} (conc.)</th>
<th>TIME (min.)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>15</td>
<td>30</td>
<td>60</td>
<td>120</td>
</tr>
<tr>
<td>10 mg/L</td>
<td>58.36 ± 7.6 (^a)</td>
<td>—</td>
<td>—</td>
<td>58.0 ± 8.0</td>
</tr>
<tr>
<td>20 mg/L</td>
<td>69.88 ± 6.5</td>
<td>59.05 ± 3.5</td>
<td>52.6 ± 4.97</td>
<td>50.4 ± 2.8</td>
</tr>
<tr>
<td>40 mg/L</td>
<td>56.95 ± 10.7</td>
<td>54.30 ± 6.5</td>
<td>42.12 ± 7.10</td>
<td>—</td>
</tr>
</tbody>
</table>

\(^a\) Values represent mean ± S.E. \% Hemolysis from 5 rats/group given 10, 20, and 40 mg/L CI\textsubscript{2}O\textsubscript{2} orally.

— Not Determined.
The glutathione content expressed as percentage of controls is shown in Table 13. In ClO$_2$ treatment, blood glutathione was decreased with incubation time. Slight fluctuation was observed but the values never reached the control values, even after 4 hrs incubation. Also, ClO$_2^-$ and ClO$_3^-$ significantly decreased glutathione content at 30 and 60 min, but slight increases were shown at 120 min compared to 30 and 60 min values. At 4 hrs incubation the level of glutathione decreased to the same values of 30 min incubation or lower.

The percent hemolysis relative to controls is summarized in Table 14. The osmotic fragility was decreased significantly (P < 0.05) in rat blood after 30, 60 or 120 min with final concentration 500 mg/l ClO$_2$. In 100 mg/l ClO$_2$, the decrease of osmotic fragility was detected at 60 and 120 min (P < 0.05), while 50 or 5 mg/l ClO$_2$ decreased the osmotic fragility after 120 min incubation time. Although both concentrations of ClO$_2^-$ and ClO$_3^-$ decreased the osmotic fragility at 30, 60 and 120 min incubation time, the lower concentration of ClO$_2^-$ and ClO$_3^-$ reached the control value by 4 hrs incubation time. The decreased glutathione content and osmotic fragility \textit{in vitro} is in agreement with \textit{in vivo} studies.

a. \textbf{Effect of Exogenous Glutathione and ClO$_2$ on Osmotic Fragility of Rat Blood}

To test the assumption (described above) that oxidative stress of Cl-compounds causes the formation of disulfide bonds between membrane elements and hemoglobin causing precipitation of hemoglobin, reduced glutathione was added to rat blood directly before the addition of ClO$_2$ and
TABLE 13

EFFECT OF CI-COMPOUNDS ON RAT BLOOD GLUTATHIONE IN VITRO

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>30</th>
<th>60</th>
<th>120</th>
<th>240</th>
</tr>
</thead>
<tbody>
<tr>
<td>ClO₂ (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>50.60 ± 1.20*</td>
<td>52.40 ± 2.33*</td>
<td>62.30 ± 1.90*</td>
<td>66.10 ± 3.50*</td>
</tr>
<tr>
<td>100</td>
<td>59.10 ± 3.50*</td>
<td>60.20 ± 2.10*</td>
<td>71.10 ± 3.69*</td>
<td>88.30 ± 0.80*</td>
</tr>
<tr>
<td>50</td>
<td>77.50 ± 2.50*</td>
<td>94.00 ± 3.00</td>
<td>78.30 ± 1.80*</td>
<td>78.30 ± 6.00</td>
</tr>
<tr>
<td>5</td>
<td>87.00 ± 6.00</td>
<td>88.30 ± 7.00</td>
<td>80.80 ± 2.70*</td>
<td>—</td>
</tr>
<tr>
<td>ClO₃⁻(mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>46.10 ± 4.10*</td>
<td>30.90 ± 1.60*</td>
<td>54.70 ± 8.30*</td>
<td>42.30 ± 5.10*</td>
</tr>
<tr>
<td>10</td>
<td>75.60 ± 1.90*</td>
<td>55.60 ± 1.60*</td>
<td>87.00 ± 5.80</td>
<td>60.30 ± 3.00*</td>
</tr>
<tr>
<td>ClO₃ (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>71.70 ± 6.80*</td>
<td>72.70 ± 1.30*</td>
<td>86.05 ± 5.20</td>
<td>73.97 ± 2.60*</td>
</tr>
<tr>
<td>10</td>
<td>77.70 ± 5.80*</td>
<td>69.30 ± 1.90*</td>
<td>93.49 ± 6.30</td>
<td>77.13 ± 1.30*</td>
</tr>
</tbody>
</table>

*Values (mean ± S.E.) represent % Glutathione of Control values.
- Non determined

*Significantly different from Control, student t-Test, p<0.01
TABLE 14

**EFFECT OF CI- COMPOUNDS ON RAT BLOOD OSMOTIC FRAGILITY IN VITRO.**

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>Incubation Time (min.)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
</tr>
<tr>
<td><strong>ClO₂ (mg/L)</strong></td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>56.90 ± 11.90*</td>
</tr>
<tr>
<td>100</td>
<td>78.90 ± 9.10</td>
</tr>
<tr>
<td>50</td>
<td>80.00 ± 5.00</td>
</tr>
<tr>
<td>5</td>
<td>80.90 ± 3.00</td>
</tr>
<tr>
<td><strong>ClO₂⁻ (mg/L)</strong></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>40.80 ± 4.70*</td>
</tr>
<tr>
<td>10</td>
<td>83.02 ± 11.10</td>
</tr>
<tr>
<td><strong>ClO₃⁻ (mg/L)</strong></td>
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</tr>
<tr>
<td>100</td>
<td>75.10 ± 9.60</td>
</tr>
<tr>
<td>10</td>
<td>80.30± 15.10</td>
</tr>
</tbody>
</table>

*Values (mean ± S.E.) represent % hemolysis of Control values.

— Non determined

*,**,*** Significantly different from Control, student t-test, p<0.01
osmotic fragility was determined at 1 and 2 hrs. The data from these experiments are presented in Figure 12.

As shown earlier ClO\textsubscript{2} alone \textit{in vivo} and \textit{in vitro} decreased osmotic fragility (Tables 8, 14). The decrease of osmotic fragility in these experiments was similar to that obtained in the previous experiments.

When GSH was added to the blood alone hemolysis was decreased significantly at 1 hr incubation time when compared to control. This change is related to the stabilization of the red blood cell membrane and protects hemoglobin from being released in hypotonic solution. This protection lasted for only 1 hr incubation time, and hemolysis reached that of controls by 2 hrs incubation time. When ClO\textsubscript{2} was added with GSH to the incubation vessels, no effect on hemolysis was observed when compared to control or to GSH alone at 2 hrs period, but were significantly different from the ClO\textsubscript{2} alone (Figure 12).

These results indicate that exogenous GSH can alleviate the formation of disulfide bonds formed between hemoglobin and cell membrane elements by providing reduced power for the erythrocytes while under oxidative stress of Cl-compounds, and thus prevents the precipitation of hemoglobin in hypotonic solution (i.e., the 2 hr ClO\textsubscript{2} alone, Figure 12, is an "apparent resistance to hemolysis").
FIGURE 12

Effect of Exogenous Glutathione and ClO₂ on Osmotic Fragility of Rat Blood In Vitro

Reduced glutathione (50 mg%) was added to rat blood directly before the addition of 100 mg/l ClO₂. Blood was incubated at 37°C and osmotic fragility was determined at 1 and 2 hrs in each treatment. The values represent the mean of % hemolysis ± S.E. of 4 rats in each treatment.
EFFECT OF EXOGENOUS GLUTATHIONE AND \( \text{ClO}_2 \) ON OSMOTIC FRAGILITY OF RAT BLOOD \text{IN VITRO}
b. **Effect of Glutathione Reductase, ClO₂ and ClO²⁻ on Osmotic Fragility of Rat Blood In Vitro**

ClO₂ (100 mg/l) or ClO²⁻ (10 mg/l) were added to rat blood; afterwards 17 units of glutathione reductase and 20 μl NADPH (2mM) were added. Tubes were incubated at 37°C and 50 μl aliquots withdrawn at one hour to determine osmotic fragility. The results after 1 hr incubation are summarized in Figure 13.

NADPH alone prevented ClO₂ and ClO²⁻ from exhibiting hemolysis resistance. Glutathione reductase and his cofactor (NADPH) increased the hemolysis about 35%. Removing the enzyme only results in increased resistance with both ClO₂ and ClO²⁻. These data indicate, that the decreased hemolysis in blood treated with ClO²⁻ was about 20 fold compared to ClO₂ treatment.

M. **EFFECT OF Cl-COMPOUNDS ON THE ACTIVITY OF GLUTATHIONE REDUCTASE, GLUTATHIONE PEROXIDASE AND CATALASE IN RAT AND CHICKEN BLOOD**

Considerable information has accumulated on glutathione reductase (G-R), glutathione peroxidase (G-Px), and catalase (C) in blood catalyzing the redox transitions of the glutathione couple, 2 GSH/G-S-S-G Nicholls (69) and Aebi (96). The activity of these enzymes and the level of reduced glutathione (GSH) were studied in the blood of rats after drinking the indicated concentrations of Cl-compounds daily for 6 months (Table 15).

There was an increase of about 60% in the activity of glutathione reductase in the 1000, 100 and 10 mg/l ClO₂ treatments. A similar
FIGURE 13

Effect of Glutathione Reductase, C1O₂ and C1O₂⁻ on Osmotic Fragility of Rat Blood In Vitro

C1O₂ (100 mg/l) or C1O₂⁻ (10 mg/l) were added to rat blood; afterwards, 17 units of glutathione reductase and 20 µl NADPH (2 mM) were added. Blood was incubated at 37°C and osmotic fragility was determined at 1 hr in each treatment. The values reported represent the mean of % hemolysis ± S.E. from 4 rats in each treatment.
EFFECT OF GLUTATHIONE REDUCTASE, 
\(\text{ClO}_2\) AND \(\text{ClO}_2^-\) ON OSMOTIC FRAGILITY 
OF RAT BLOOD \textit{IN VITRO}

![Graph showing hemolysis values](image)

Values (mean ± S.E.) represent % Hemolysis after 
1 hr. Incubation, and \(n = 4\) for all groups.

\(* p < 0.01\)

FIGURE 13
TABLE 15

EFFECT OF Cl—COMPOUNDS ON GLUTATHIONE REDUCTASE, PEROXIDASE, AND CATALASE IN RAT BLOOD.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>G—R U/mg Hb</th>
<th>G—P X U/mg Hb</th>
<th>C K/gm Hb</th>
<th>GSH mg%</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>1.59 ± 0.14</td>
<td>38.60 ± 0.95</td>
<td>42.01 ± 1.31</td>
<td>71.20 ± 4.50</td>
</tr>
<tr>
<td>ClO₂ (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>2.52 ± 0.29*</td>
<td>39.53 ± 1.36</td>
<td>54.55 ± 2.93**</td>
<td>73.20 ± 2.31</td>
</tr>
<tr>
<td>100</td>
<td>2.68 ± 0.16**</td>
<td>37.20 ± 3.60</td>
<td>40.75 ± 3.33</td>
<td>59.57 ± 1.65**</td>
</tr>
<tr>
<td>10</td>
<td>2.68 ± 0.14**</td>
<td>34.03 ± 3.07</td>
<td>37.11 ± 1.59*</td>
<td>63.75 ± 2.22**</td>
</tr>
<tr>
<td>1</td>
<td>1.41 ± 0.24</td>
<td>39.20 ± 1.10</td>
<td>33.33 ± 2.75**</td>
<td>66.25 ± 1.53**</td>
</tr>
<tr>
<td>ClO₂⁻ (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>2.53 ± 0.19**</td>
<td>43.00 ± 2.03</td>
<td>30.20 ± 4.34**</td>
<td>46.60 ± 1.65**</td>
</tr>
<tr>
<td>10</td>
<td>2.13 ± 0.03**</td>
<td>39.80 ± 1.06</td>
<td>29.11 ± 0.53**</td>
<td>58.50 ± 2.7**</td>
</tr>
<tr>
<td>ClO₃⁻(mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>1.64 ± 0.15</td>
<td>42.73 ± 1.58**</td>
<td>24.05 ± 1.05**</td>
<td>61.36 ± 1.36**</td>
</tr>
<tr>
<td>10</td>
<td>1.72 ± 0.09</td>
<td>40.00 ± 1.70</td>
<td>43.50 ± 2.73</td>
<td>61.10 ± 2.07*</td>
</tr>
</tbody>
</table>

* Values represent mean ± S.E. from 4 rats/group drinking the indicated Cl—Compounds for 6 months.
** Significantly different from Control, Student t-test, p<0.05 and p<0.01 respectively.
increase in activity was shown in the 100 mg/l ClO\textsubscript{2} treatment; while in the 1 mg/l ClO\textsubscript{2} and in both ClO\textsubscript{3} groups, no changes in activity were observed. The glutathione-peroxidase activity was significantly increased only in the 100 mg/l ClO\textsubscript{3} group.

The activity of catalase (C) in the ClO\textsubscript{2} treatment groups was significantly higher in the 1000 mg/l group (P < 0.01), while in the 1 and 10 mg/l groups, the activity was decreased (P < 0.05). Also, catalase activity was decreased in both ClO\textsubscript{2} and 100 mg/l ClO\textsubscript{3} groups.

Catalse appears to play an important role in the oxidative stress of Cl-compounds, since the increased activities of catalase and GSH reductase maintained the blood glutathione level in the 1000 mg/l ClO\textsubscript{2} group (73 vs 71 mg%). Also the decrease in catalase in the 100 mg/l ClO\textsubscript{2} group was accompanied by lowered glutathione (about 35%) even in the presence of increased glutathione reductase activity.

The results of similar experiments conducted using chickens to investigate the activity of glutathione reductase, glutathione peroxidase and catalase are summarized in Table 16. There was an increase of about 70% in the activity of glutathione reductase in chickens treated with 10, 100 and 1000 mg/l ClO\textsubscript{2} in drinking water for 10 months. Glutathione peroxidase was significantly decreased (P < 0.01) in the 1000 mg/l group; however, catalase was increased (P < 0.01) in the same group. All ClO\textsubscript{2} groups showed elevated G-R and catalase activities. Glutathione peroxidase activity varied inversely with ClO\textsubscript{2} drinking water concentrations. The GSH content was not different from controls in the high ClO\textsubscript{2} group, but was significantly elevated in the lower ClO\textsubscript{2} groups.
TABLE 16

EFFECT OF ClO₂ IN DRINKING WATER ON GLUTATHIONE REDUCTASE, PEROXIDASE, AND CATALASE IN CHICKEN BLOOD.⁰

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>G—R U/mg Hb</th>
<th>G—Pₓ U/mg Hb</th>
<th>C K/gm Hb</th>
<th>GSH mg %</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>1.00 ± 0.02</td>
<td>6.55 ± 0.77</td>
<td>3.25 ± 0.46</td>
<td>98.6 ± 3.40</td>
</tr>
<tr>
<td>ClO₂ (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>1.54 ± 0.14 ⁵</td>
<td>2.30 ± 0.25 ⁶</td>
<td>5.87 ± 0.02 ⁷</td>
<td>100.9 ± 1.00</td>
</tr>
<tr>
<td>100</td>
<td>1.83 ± 0.11 ¹⁰</td>
<td>7.52 ± 1.27</td>
<td>4.17 ± 0.05 ¹⁰</td>
<td>103.0 ± 0.40 ²²</td>
</tr>
<tr>
<td>10</td>
<td>1.72 ± 0.24 ⁵</td>
<td>10.16 ± 0.49 ⁶</td>
<td>4.07 ± 0.06 ⁷</td>
<td>106.3 ± 1.70 ⁹</td>
</tr>
</tbody>
</table>

⁰ Values represent mean ± S.E. from 4 chickens/group after drinking the indicated Cl-Compounds for 10 months.

* Significantly different from Control, student t-test, p<0.05 and p<0.01 respectively.
Again, the results in the chicken experiments are in agreement with the data obtained from rat experiments emphasizing the important role of catalase in response to the oxidative stress from Cl-compounds.

N. EFFECT OF CL-COMPoudS ON $^3$H-THYMIDINE INCORPORATION INTO NUCLEI OF RAT ORGANS

In mammals, the small intestinal mucosa has evolved into a highly specialized organ. Some organs of the body, like the kidney are composed of almost permanent cells, whereas others, such as gastrointestinal tract epithelium, work on the principle of rapid replacement. The most dynamic of these is the small intestinal mucosa. To examine the effect of Cl-compounds in drinking water on the turnover of rat intestinal mucosa, liver, kidney and testes, rats were injected $^3$H-thymidine (0.5 μCi/gm body weight) intraperitoneally after drinking Cl-compounds for 3 months. Animals were sacrificed 8 hours later and $^3$H-thymidine incorporation into nuclei of these organs was determined (Table 17).

The incorporation of $^3$H-thymidine in liver was decreased significantly ($P < 0.01$) only in the 100 and 10 mg/l ClO$_2$ groups, while the incorporation in kidney decreased significantly ($P < 0.01$) in the 100 mg/l ClO$_2$ group only. The results in testes reveal that ClO$_2$, ClO$_2^-$ and ClO$_3^-$ diminished the incorporation of $^3$H-thymidine in all groups. In intestinal mucosa, about a two-fold increase of thymidine incorporation (compared to control) was observed in the 10 mg/l ClO$_2$ group. While about a 30% increase in incorporation occurred in both the 100 mg/l ClO$_2$ and 10 mg/l ClO$_2^-$ groups; the ClO$_3^-$ group was without change.
### TABLE 17

**EFFECT OF CI-COMPOUNDS ON $^3$H-THYMIDINE INCORPORATION INTO NUCLEI OF RAT LIVER, KIDNEY, TESTES, AND INTESTINAL MUCOSA.**

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>Liver</th>
<th>Kidney</th>
<th>Testes</th>
<th>Intestinal Mucosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>7.61 ± 0.73</td>
<td>0.15 ± 0.015</td>
<td>4.70 ± 0.82</td>
<td>61.30 ± 2.99</td>
</tr>
<tr>
<td>ClO$_2$ (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>7.02 ± 0.87</td>
<td>0.05 ± 0.006$^{**}$</td>
<td>1.80 ± 0.29$^{**}$</td>
<td>88.32 ± 19.50$^{*}$</td>
</tr>
<tr>
<td>10</td>
<td>8.78 ± 0.55</td>
<td>0.13 ± 0.028</td>
<td>3.05 ± 0.39$^{**}$</td>
<td>126.4 ± 16.13$^{**}$</td>
</tr>
<tr>
<td>ClO$_2^-$ (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>2.87 ± 0.37$^{**}$</td>
<td>0.10 ± 0.024</td>
<td>2.11 ± 0.23$^{**}$</td>
<td>40.6 ± 5.40$^{**}$</td>
</tr>
<tr>
<td>10</td>
<td>4.79 ± 0.31$^{**}$</td>
<td>0.12 ± 0.04</td>
<td>2.20 ± 0.34$^{**}$</td>
<td>87.40 ± 11.96$^{**}$</td>
</tr>
<tr>
<td>ClO$_3$ (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>6.29 ± 1.11</td>
<td>0.20 ± 0.034</td>
<td>2.45 ± 0.05$^{**}$</td>
<td>63.3 ± 14.30</td>
</tr>
</tbody>
</table>

$^0$Values represent mean ± S.E. as DPM/µg DNA from 4 rats/group given $^3$H-Thymidine (0.5ucI/g Body Weight) after drinking CI-Compounds for 3 months.

$^*-$Significantly different from Control, Student t-test; $p<0.05$

$^{**}$Significantly different from Control, Student t-test; $p<0.01$
The weights of rats drinking varied concentrations of Cl-compounds daily for 11 months are given in Table 18. As early as 2 months treatment with 1000 mg/l ClO₂ and both concentrations of ClO₃⁻ (10 and 100 mg/l) resulted in a significant decrease in body weight in these groups compared to controls. This decrease of body weight persisted throughout the treatment period. The other treatment groups (100, 10 and 1 mg/l ClO₂ and 10 mg/l ClO₂) had significantly decreased body weights at 10 and 11 months.

Chicken weight was significantly decreased from control at 9 and 10 months drinking 100 mg/l ClO₂; however, the weight of 10 mg/l group was increased (P < 0.05) at 4 and 7 months. The body weight of the 100 mg/l ClO₂ group was without change when compared to controls throughout the 11 months studied (Table 19).
### TABLE 18

**EFFECT OF CI-/COUMPOUNDS IN DRINKING WATER ON RAT BODY WEIGHT**

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>2</th>
<th>5</th>
<th>7</th>
<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>380.3 ± 8.0</td>
<td>474.7 ± 7.8</td>
<td>520.5 ± 11.6</td>
<td>553.0 ± 14.4</td>
<td>542.5 ± 14.6</td>
</tr>
<tr>
<td>ClO₂ (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>349.1 ± 5.8**</td>
<td>437.2 ± 5.4**</td>
<td>469.3 ± 6.0**</td>
<td>484.5 ± 6.7**</td>
<td>470.4 ± 6.8**</td>
</tr>
<tr>
<td>100</td>
<td>370.0 ± 7.7</td>
<td>470.0 ± 11.6</td>
<td>491.9 ± 12.2</td>
<td>497.7 ± 18.6*</td>
<td>491.3 ± 19.2*</td>
</tr>
<tr>
<td>10</td>
<td>371.0 ± 7.2</td>
<td>454.2 ± 13.8</td>
<td>509.6 ± 9.2</td>
<td>523.9 ± 11.5*</td>
<td>501.9 ± 12.4*</td>
</tr>
<tr>
<td>1</td>
<td>372.0 ± 11.5</td>
<td>460.7 ± 14.9</td>
<td>511.0 ± 15.7</td>
<td>479.1 ± 12.4**</td>
<td>445.9 ± 11.7**</td>
</tr>
<tr>
<td>ClO₂⁻ (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>355.9 ± 5.7*</td>
<td>450.0 ± 8.9*</td>
<td>501.0 ± 10.0</td>
<td>512.9 ± 8.2**</td>
<td>502.0 ± 11.2**</td>
</tr>
<tr>
<td>10</td>
<td>377.4 ± 1.0</td>
<td>478.9 ± 11.4</td>
<td>505.5 ± 14.2</td>
<td>449.1 ± 8.7**</td>
<td>455.2 ± 8.2**</td>
</tr>
<tr>
<td>ClO₃⁻ (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>342.0 ± 10.0**</td>
<td>432.7 ± 12.1**</td>
<td>478.9 ± 14.5**</td>
<td>438.9 ± 14.5**</td>
<td>434.2 ± 14.6**</td>
</tr>
<tr>
<td>10</td>
<td>347.6 ± 6.0**</td>
<td>438.3 ± 7.9**</td>
<td>470.0 ± 10.6*</td>
<td>473.8 ± 10.7*</td>
<td>449.7 ± 11.7**</td>
</tr>
</tbody>
</table>

* and ** p < 0.05 and p < 0.01 respectively.

n = 10 animals for all groups except 10 mg/L ClO₂⁻ and ClO₃⁻, where n = 6 and 7 respectively.
TABLE 19

EFFECT OF ClO₂ IN DRINKING WATER ON CHICKEN BODY WEIGHT.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>MONTHS</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>4</td>
<td>7</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>CONTROL</td>
<td>978.8 ± 55.4</td>
<td>1231.9 ± 53.7</td>
<td>1943.7 ± 48.0</td>
<td>2086.2 ± 52.4</td>
<td>2069.8 ± 69.0</td>
<td></td>
</tr>
<tr>
<td>ClO₂ (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>888.7 ± 56.4</td>
<td>1298.9 ± 76.3</td>
<td>1889.7 ± 46.4</td>
<td>1952.69 ± 69.0*</td>
<td>1917.5 ± 66.0**</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>952.5 ± 45.6</td>
<td>1229.8 ± 92.6</td>
<td>1964.5 ± 84.3</td>
<td>1988.10 ± 126.0</td>
<td>1921.1 ± 112.0</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>974.3 ± 29.5</td>
<td>1389.4 ± 45.6*</td>
<td>2068.0 ± 45.2*</td>
<td>2133.2 ± 68.5</td>
<td>1991.5 ± 124.0</td>
<td></td>
</tr>
</tbody>
</table>

* = p<0.05
n = 8 animals for all groups
DISCUSSION

The Recovery of KClO₃ Synthesis

Neutron activation analysis following the synthesis of KClO₃ was conducted to demonstrate that KCl was converted completely to KClO₃ during the electrolysis. The determination of the percent of O₂ in KClO₃ indicated that 95% of the synthesized compound was in the form of KClO₃ (Table 2). K₃⁶ClO₃ is an essential intermediate step to the production of chlorine dioxide (³⁶ClO₂). K₃⁶ClO₃ is not commercially available at this time. It was therefore synthesized from the commercially available ³⁶ chlorine labelled HC1 purchased from New England Nuclear, Inc.

The Kinetics of ³⁶ClO₂ in Rat

Rats were given ³⁶ClO₂ in acute or chronic experiments (Fig. 4). The calculated kinetic constants derived from these studies were as follow:

<table>
<thead>
<tr>
<th></th>
<th>Acute</th>
<th>Chronic (15 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hrs⁻¹</td>
<td>hrs⁻¹</td>
</tr>
<tr>
<td>Absorption rate constant</td>
<td>3.77 ± 0.24</td>
<td>3.16 ± 0.42</td>
</tr>
<tr>
<td>Absorption half-life</td>
<td>0.18 ± 0.01</td>
<td>0.22 ± 0.03</td>
</tr>
<tr>
<td>Elimination rate constant</td>
<td>0.0158 ± 0.0008</td>
<td>0.0225 ± 0.0009</td>
</tr>
<tr>
<td>Elimination half-life</td>
<td>43.9 ± 2.3</td>
<td>31.0 ± 1.2</td>
</tr>
</tbody>
</table>

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These data indicate that no significant difference exists in the rate of absorption between the acute administration and the chronic pretreatment followed by $^{36}$ClO$_2$ administration. However, the rate of the elimination increased and the elimination half life decreased in the chronic administration group.

Approximately 25% of $^{36}$Cl-compounds in the liver subcellular fraction was bound to protein at 72 hrs after the administration of a single dose of $^{36}$ClO$_2$. It would appear that the saturation of protein binding may explain the increase in the elimination rate and the decrease in the elimination half life of $^{36}$Cl-compounds in the chronic group. Further evidence of binding of $^{36}$Cl-compounds to protein (erythrocyte membrane) has been observed.

The elimination of $^{36}$ClO$_2$ from the body by kidney and intestine has been demonstrated. ClO$_2$ is eliminated mainly as Cl$^-$ and ClO$_2^-$, and only a small amount of ClO$_3^-$ and no ClO$_2$ was present in rat urine. After a single dose of ClO$_2$, 43% of the total initial dose was excreted through urinary and fecal routes; the total recovery of $^{36}$Cl from organs, skin, carcass and excretion was 95% at 72 hrs.

The distribution studies revealed that $^{36}$Cl-compounds were high in stomach and small intestine even 72 hrs after the administration of $^{36}$ClO$_2$ by gavage. The prolonged and high concentration of ClO$_2$ metabolites in GI tract (site of administration) and in testes as demonstrated in Table 3 suggest possible pharmacological actions at these sites.
Metabolism studies revealed that ClO$_2$ is converted to Cl$^-$, CIO$^-_2$ and CIO$^-_3$ in the rat. The ratio of occurrence of urinary CIO$^-_2$ to Cl$^-$ is 1:5 at 12 and 24 hrs from the time of the administration of CIO$_2$; however, at 72 hrs the ratio in urine and plasma was 1:4. CIO$_3^-$ was detected only in the 12 to 24 hr urine.

The decrease of total $^{36}$Cl after washing packed red blood cells with cold saline suggested that a high percentage of total $^{36}$Cl was loosely bound to the erythrocyte membrane and/or readily exchanged with chloride of saline.

Oxidative Stress of Cl-Compounds

Previous reports of methemoglobin formation were observed only after bolus doses of CIO$_2^-$ were administered either orally (tablets of NaClO$_2$) or intraperitoneally, Hefferman et al. (66) and Musil et al. (45). Methemoglobin concentration in blood is a function of the rate at which it is produced and the rate at which it is reduced by methemoglobin reductase enzymes, Kiese (96). The complete depletion of blood glutathione is followed by the formation of methemoglobin. This is consistent with the protective role of reduced glutathione in damage by oxidants, Hill et al. (97); Cohen and Hochstein (98). The gradual intake of CIO$_2$ and CIO$_2$ metabolites in drinking water in this study, at all times and dosages indicated, produced no signs of methemoglobin. Thus, methemoglobinemia does not appear to be a hazard from CIO$_2$, CIO$_2^-$ and CIO$_3^-$ in drinking water.
The present investigation revealed that blood glutathione (GSH) was decreased after being exposed to Cl-compounds \textit{in vivo} and \textit{in vitro} studies. Low GSH levels are produced by the effect of these agents on GSH. In a number of cases, the reaction which lowers the GSH level can be identified as an oxidation of GSH to GSSG. It has been demonstrated that ClO$_2$ elicits the production of hydrogen peroxide, Cohen and Hochstein (99); Kiese (96) and Heffernan et al. (66). The hydrogen peroxide can oxidize GSH via the glutathione peroxidase system. The studies of Beutler et al. (76) have established an association between intracellular reduced glutathione level and the susceptibility \textit{in vitro} and \textit{in vivo} of red cells to injury by oxidant chemicals; however, Kosower (80) reported that the damage to red cells occurs only when chemically reactive agents are generated at times when GSH concentration is very low. This may explain the moderate abnormalities (echinocyte) seen in rat erythrocyte morphology after ClO$_2$ and low concentration of ClO$_2^-$ treatment for 4 months. At the same time, when animals were treated with high concentration of ClO$_2^-$, blood glutathione was significantly decreased and marked distortions in the middle of erythrocytes were observed (Fig. 9).

The present data show that ClO$_2$, ClO$_2^-$ and ClO$_3^-$ decrease erythrocytic fragility. The apparent decreased osmotic fragility is probably related to the formation of disulfide bonds between membrane elements and hemoglobin, causing precipitation of hemoglobin, Allen et al. (100). The Cl-compounds produced a decrease in erythrocytic osmotic fragility in the absence of any methemoglobin formation. This is in agreement with studies of Beutler et al. (101) reporting hemoglobin breakdown in the
absence of methemoglobin formation. The oxidation of the first two sulf-hydryl groups in globin caused denatured hemoglobin and the addition of glutathione reversed this denaturation, Allen (100). This explains the decreased hemoglobin precipitation (increased hemolysis) after 2 hours upon the addition of \( ClO_2 \) and exogenous GSH to rat blood compared to the addition of \( ClO_2 \) alone (Fig. 12). These findings are consistent with the protective role of GSH towards hemoglobin and other thiol-containing proteins against irreversible oxidative denaturation, Allen (100) and Beutler et al. (76).

**Effects of Cl-Compounds on Blood Compartment**

Data obtained in the present study demonstrated that rats drinking \( ClO_2, ClO^- \) and \( ClO_3^- \) daily for 9 months exhibited depressed red blood cell counts, hemoglobin concentration and packed cell volumes (Table 11). This was also found with \( ClO^- \) given parenterally or orally in tablet form, Heffernan et al. (66). The values for erythrocyte count, hemoglobin concentration and hematocrit can be used to obtain certain erythrocyte indices that define the size and hemoglobin content of the erythrocyte. Mean corpuscular volume (MCV) or erythrocyte size, and mean corpuscular hemoglobin (MCH) or hemoglobin weight per erythrocyte, were without significant change; however, the mean corpuscular hemoglobin concentration (MCHC) was significantly higher in the 1000 and 100 mg/l \( ClO_2 \) groups. A higher MCHC indicates some degree of damage to the erythrocytes (spherocytosis) which lack the normal disk shape and appear darker than normal, not because of a high hemoglobin content, but because the cell is spherical.
Protective Function of Reduced Glutathione Against the Effect of Cl⁻ Compound in Rat and Chicken Blood

Glutathione protects hemoglobin from oxidative breakdown by acting as a hydrogen donor in the peroxidative destruction of hydrogen peroxide. The necessity for regenerating GSH in such a mechanism would explain the susceptibility to the action of oxidant compounds on red blood cells. At present, a controversy exists as to whether GSH-peroxidase or catalase is more important as a protective enzyme in the erythrocyte. These studies support the view that erythrocytic catalase is a first line of defense against oxidative stress in rat and chickens drinking varying concentrations of ClO₂ for 6 and 10 months, respectively (Tables 15, 16). Studies by Jacob et al. (102) also support catalase as the major protective enzyme in erythrocytes. However, in the chicken, glutathione peroxidase activity was decreased in 1000 mg/l ClO₂ and increased in the 10 mg/l groups. Blood glutathione was increased and may be the result of the elevated glutathione reductase activity (Table 16), which has the capacity to reduce GSSG as well as other disulfides, including mixed disulfides of glutathione and protein or hemoglobin.

Effect of Glutathione Reductase, ClO₂ and ClO⁻ on Osmotic Fragility In Vitro

In oxidative damage of hemoglobin in solution or in red cells, thiol groups play a key role. Whether oxidation is spontaneous or accelerated by such oxidizing agents as ClO₂ and ClO⁻ the following steps most likely exist:
1. Reduced glutathione (GSH) is oxidized to GSSG. In being oxidized a portion of the GSH becomes bound to hemoglobin by forming mixed disulfides with globin sulfhydryl groups.

2. Oxidation of thiol groups, especially the reactive ones, results in the loss of the normal configuration of the hemoglobin molecule.

3. Hemoglobin is precipitated under oxidative stress. The oxidation or blockade of more than two sulfhydryl groups in the presence of an oxidant produces an accelerated precipitation of hemoglobin, Allen and Jandle (100).

To test the assumption that oxidative stress of ClO₂ and ClO₂⁻ caused the precipitation of hemoglobin by the formation of disulfide bonds between membrane elements and hemoglobin. NADPH, as cofactor to endogenous glutathione reductase, was added alone, or, with exogenous glutathione reductase to rat blood incubated (1 hr) with ClO₂ or ClO₂⁻. Afterwards osmotic fragility was determined. The present investigations reveal that membrane sulfhydryl groups that bound rat hemoglobin after exposure to ClO₂ (and ClO₂⁻) were then allowed the rapid release of hemoglobin by the presence of NADPH alone (enhancement of endogenous glutathione reductase). Also, the addition of endogenous glutathione reductase (and NADPH) allowed further release of hemoglobin (Fig. 13). These findings are consistent with the enzymatic reduction of disulfide bonds in erythrocytic membranes, which in turn, would release hemoglobin to the
hypotonic media giving a more valid estimate of hemolysis. Further evidence in support of this interpretation are the studies of Srivastava and Beutler (103) wherein glutathione reductase was shown to reduce erythrocytic membrane disulfide bonds, an event in agreement with the hemolysis data of this study.

3H-Thymidine Turnover in Organs of the Rat

3H-thymidine incorporation into nuclei was used to test the activity of DNA synthesis in rats drinking water containing ClO\textsubscript{2}, ClO\textsuperscript{-} and ClO\textsubscript{3} for 3 months. 3H-thymidine is usually cleared from circulation some minutes after ip. administration. Subsequently it is taken up by all cells in the synthetic phase of the cell cycle, and incorporated into deoxyribonucleic acid (DNA). In cell division, thymidine is shared equally between the daughter cells; and once division is finished, the label of thymidine remains within the nucleus throughout the life of the cell (92).

These studies have shown that ClO\textsubscript{2}, ClO\textsuperscript{-} and ClO\textsubscript{3} inhibit DNA synthesis in testes (Table 17). Increased DNA synthesis in intestinal mucosa in rats drinking ClO\textsubscript{2} or 10 mg/l ClO\textsuperscript{-}, was observed; however, thymidine incorporation decreased in the 100 mg/l ClO\textsuperscript{-} groups. In liver nuclei, ClO\textsuperscript{-} decreased DNA synthesis. One explanation for these data is the role glutathione plays in biological systems. The interior of living cells normally contains a substantial concentration of glutathione (GSH 1-50 x 10\textsuperscript{-4} M), Tietze (194). Fredborg and Lindahl (105) reported that alterations in the GSSG content during cell cycle plays a role in
the regulation of protein synthesis. Also, Kosower et al. (80) described that a small increase in GSSG leads to an inhibition of the initiation of protein synthesis. The increase of GSSG involves the conversion of thiols to disulfides and mixed disulfides which seem to result from lowering the GSH content. The reduction in DNA synthesis in liver and testes observed in this study may be related to the depression in overall protein synthesis. The major stages in protein synthesis are generally recognized as: initiation of polypeptide chains, polypeptide chain elongation followed by termination and release. None of these stages operate at the time when GSH concentration is very low. As GSH concentration increases slightly the restriction on elongation is relieved; while initiation remains blocked, Kosower et al. (80).

On the other hand, the enhancement of DNA synthesis in small intestine may be explained by the direct effect of ClO₂ and ClO₂⁻ treatment on the mucosa of small intestine. Fridlyand et al. (59) reported that 30% of the initial oral dose of ClO₂ is fixed in the mouth and 70% may actually enter the stomach. In the present investigation, a high percentage of ³⁶Cl was present in the small intestine 72 hrs after the administration of ³⁶ClO₂ by gavage. The high concentration of ClO₂ and ClO₂⁻ remaining in the small intestine, results in high fixation of these compounds and possible stimulation of protein synthesis. The increased protein synthesis could then result in observable increases in DNA synthesis.
An apparent discrepancy exists between increased DNA synthesis in intestinal mucosa with decreases in liver, kidney and testes. In the case of decreased turnover, possible explanations include:

1. Primarily, the effect of the redox state of GSH (as described above)

2. Secondarily, the presence of higher concentration of Cl\(^-\) than usually found in cytoplasm. Cl\(^-\) is the major metabolite of ClO\(_2\) found in blood and urine. Over 80\% of Cl\(^-\) present in liver cell is in the soluble cytoplasmic fraction (Fig. 6). Weber et al. (106) reported that high Cl\(^-\) inhibits initiation, which occurs after the binding of met-tRNA to 40S ribosomal subunits; but Cl\(^-\) is necessary for the binding of m-RNA to ribosome.

From the previous experiments, it appears that the increased DNA synthesis observed in the small intestine may be explained by its direct exposure to ClO\(_2\) and ClO\(_2\)\(^-\). Liver, kidney and testes are not directly exposed to ClO\(_2\), but instead are exposed to the metabolites in the circulation. Exposure to these metabolites and change in the GSH/GSSG ratio could account for the decreased DNA synthesis.
SUMMARY

1. Since $K^{36}ClO_3$ is an essential intermediate step to the production of $^{36}ClO_2$, an assay was developed to synthesize $K^{36}ClO_3$ from available $H^{36}Cl$ and KOH. In addition, a new assay was developed to qualitatively and quantitatively measure the metabolites of $^{36}ClO_2$ in biological fluids.

2. $^{36}ClO_2$ absorption and elimination time course in rat plasma after oral administration exhibited a $t_{1/2}$ of 43.9 hrs elimination. The distribution studies revealed that $^{36}Cl$ was highest in plasma followed by kidney, lung, stomach, small intestine, liver, spleen, thymus and bone marrow.

3. The excretion and elimination of $^{36}Cl$-compounds in rat 72 hours after oral administration was urine > feces and no $^{36}Cl$-compounds were detected in expired air. $Cl^-$ and $ClO_2^-$ were detected in rat plasma as $ClO_2$ metabolites; however, in rat urine $Cl^-$, $ClO_2^-$ and a small quantity of $ClO_3^-$ were determined. About 80% of the total $^{36}Cl$-compounds in liver was detected in cytosol. Twenty-five percent of total activity in liver whole homogenate was bound to the precipitated protein fraction.

4. $ClO_2$, $ClO_2^-$ and $ClO_3^-$ in drinking water decreased blood glutathione in rat after 2 months treatment; however, some groups gradually adapted.
to Cl-compound stress with increased treatment time, as noticed in the 100 mg/l ClO₂ after 4 and 9 months chronically treatment. Also, decreased osmotic fragility was observed in all groups after 2, 4, 7 and 9 months treatment. The results of osmotic fragility are most likely related to the interaction between hemoglobin with the red cell membrane causing the precipitation of hemoglobin, resulting in less free hemoglobin available for measurement. This result was confirmed by the addition of the glutathione or glutathione reductase and NADPH to rat blood treated with ClO₂ or ClO₂⁻ in vitro. Methemoglobin was not detected in all treatment groups throughout the study.

5. The decrease of blood glutathione and hemolysis in rat after one acute dose of ClO₂ were observed as early as 15 and 60 min respectively and no methemoglobin was detected throughout 2 hrs from the administration.

6. After 9 months treatment, RBC counts, HCT% and HG% were decreased in all rat treatment groups. Alterations in some red cell morphology from the normal shape to echinocyte and marked distortion in the middle of erythrocytes were observed. In chicken red cell the tear drop-shaped and football-shape were the characteristic abnormalities.

7. The study of glutathione reductase, glutathione peroxidase and catalase activity indicated that glutathione reductase in rat and chicken was increased after Cl-compound treatment. This may be explained by the system's adaptation to reduced G-S-S-G as well as to various other reduced disulfides that resulted from the oxidation stress from the treatment. Catalase played an important role in this oxidative stress in both species.
8. ClO$_2$, ClO$_2^-$ and ClO$_3^-$ administered chronically in drinking water for 3 months inhibited the incorporation of $^3$H-thymidine into nuclei of rat testes. Also, this inhibition was observed in the liver of ClO$_2^-$ groups and in the kidney of 100 mg/l ClO$_2$ treatment. The incorporation in small intestine nuclei was increased in both the treatment of ClO$_2$ and in 10 mg/l ClO$_2^-$.

9. The treatment with Cl-compounds in drinking water decreased rat body weight in all groups after 10 and 11 months treatment. However, the decreased body weight in chicken was observed only in the 1000 mg/l ClO$_2$ group.


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