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# THE EFFECTS OF EXPOSURE TO LOW CONCENTRATIONS OF CARBON MONOXIDE AS REFLECTED BY TRACE METAL ALTERATIONS

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# THE EFFECTS OF EXPOSURE TO LOW CONCENTRATIONS OF CARBON MONOXIDE AS REFLECTED BY TRACE METAL ALTERATIONS

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## CHAPTER I

## INTRODUCTION AND LITERATURE REVIEW

> This goodly frame, the earth, seems to me a sterile promontory; this most excellent canopy, the air, this brave o'er hanging firmament; this majestic roof . . . , why, it appears no other thing to me than a foul and pestilent congregation of vapors (1).

Carbon monoxide (CO), ubiquitous and notorious, has long been an enigma within a riddle to scientists. Although the acute form of carbon monoxide poisoning has long been recognized and well documented in the literature, and the metabolism of CO and the signs and symptoms well studied both in animals and humans (2, 3, 4, 5, 6, 7, 8, 9, 10), the slower and insidious form of intoxications, with intermittent symptoms and vague relations to the sources of the poisoned atmosphere, is being brought into prominence (3, 11-21). Indeed, in most textbooks of neurology, no mention is made of chronic CO poisoning (15). The basic facts have mostly been gathered in Europe; and in 1949, they were summarized in a Danish monograph by Grut (4).

Chronic CO intoxication is believed by many investigators to

be a clinical entity (5, 6, 14, 15, 21). Others argue that the concept of chronic CO intoxication is nonexistent (7, 16, 17, 19).

Because of the obscurity concerning chronic CO intoxication in research (16-19), there exists a need for exploration of the possible effects of chronic CO exposure at the cellular and subcellular levels (22, 23). Current trends in air pollution research, after reevaluation of older data and presentation of newer data, have shifted toward the problem of chronic diseases and pollution in the total or mundane environment (18, 22-25).

Evidences of concern or interest in CO as an air pollutant are the additions of the automobile exhaust control legislation to the Clean Air act by the 89th Congress (12), the formation of the Laboratory of Medical and Biological Sciences in the Division of Air Pollution (26), and the inception of the Fried Laboratory (27), which selected CO as the primary contaminant to be studied by behavioral methods. Other behavioral studies using small doses of CO have recently been carried out by Schulte (11), Beard and Wertheim (28), and Goldberg and Chappell (27). The reason for behavioral analysis is that it is felt that the current physiological and toxicological methods are inadequately analyzing the subtle effects of chronic environmental pollutants (27, 28).

Further evidence of the significance of chronic CO as a health hazard may be indicated by the action of the Committee of Threshold Limits of the American Conference of Governmental Industrial Hygienists, which in 1964, recommended that the Threshold Limit Value (TLV) for CO in workroom air be lowered to 50 parts per million (ppm) by volume, from

the figure of 100 ppm which it had sustained for many years (12). The California State Board of Public Health in 1959, adopted standards for ambient air quality, and stipulated as a "serious level," 30 ppm of CO for 8 hr or 120 ppm for 1 hr (29). It is reasonable to assume that if chronic CO intoxication was not a problem there would not be such emphasis on lowering the TLV.

From accumulated knowledge, a limit of 10 per cent carboxyhemoglobin (HbCO) has been set as a level significant in affecting oxygen ($O_2$) transport; yet, 30 ppm ties-up approximately 5 per cent of the HbCO (10). McFarland et al. (30), investigating physiological abilities, demonstrated decrease in visual discrimination at a threshold of 4 per cent HbCO, a level maintained frequently by smokers. According to Giever (12), Eysenck reported observing impairment in control precision and multiple limb coordination when HbCO exceeded 5 per cent, and Schulte (11) found impairment of functions detectable at HbCO levels as low as 5 per cent. Beard and Wertheim (28) found that early in the course of CO exposure at 50 ppm, the animals showed derangement of time sense and their response patterns became temporarily irregular.

Giever (12) has indicated that toxicity of CO exposures below the levels of subjective symptoms has yet to be more thoroughly explored, although some of the research data have indicated that such toxic exposure does exist, and that continued research into the significance of detectable changes at low concentrations is imperative to retain the proper perspective in regard to the relationship of concentration to damage or impairment of bodily functions. This approach was

further substantiated by Grut (4), Bartless (18), and Heiman (22).

The development and intensification of the air pollution problem, and its intrinsically related CO problem, have received much impetus because of the following factors: the Industrial Revolution and its concomitant urbanization of agrarian societies; the invention of the internal combustion engine, the automobile, elevator, the Bessemer Process, and mass production; the discovery of oil, coal, and gas; and finally, the population explosion.

Motor vehicles are the major source of CO pollution (12, 31). In 1900 there were very few automobiles, in 1962 there were 75 million motor vehicles registered in the United States (32), and in 1967 there were in excess of 70 million private motor cars (33).

Nationally, 70 million automobiles consume 600,000 tons of gasoline per day while discharging more than 270,000 tons of CO, unburned hydrocarbons, nitrogen oxides, and organic lead into the environment (33). In 1962 motor fuel was consumed at the rate of approximately 60 billion gallons per year (32). In 1953 an estimated 60 million tons of CO were discharged into the United States atmosphere (14). The severity of the problem can further be exemplified on a city-wide basis. In 1963 in Los Angeles, motor vehicles emitted an estimated 9,000 tons of CO per day or about 3.4 million tons per year, but distribution was not uniform over time or area of the county (14). New York City automobile traffic produced approximately 4,150 tons of CO each day.

Because of the seriousness of the automobile emissions problem, and the ultimate urban sprawl, with its increase in motor vehicles, a national control program was initiated with the introduction of the 1968

model cars. The federal regulations for all 1968 models, with engines greater than 140 cubic inches in displacement restrict CO emission concentrations to 1.5 per cent. Many people have developed complacency and a false sense of security due to these regulations. They are not a panacea. Heller (33) pointed out that in December of 1966, the California Motor Vehicle Board indicated that there was a high deterioration rate of the automobile pollution control devices that had been mandatory in the State since 1966. From this very fact alone, it is possible to surmise that this problem will exist and intensify on a national basis after 1968. The number of motor vehicles and population are also rapidly increasing in an already crowded environment, thereby intensifying an already acute pollution problem.

Emphasis has been placed on the production of a feasible electric car, but technical problems have kept it out of mass production. Also, the nation is geared to the internal combustion engine and the gas and oil industries. Therefore an abrupt change in economic factors would disrupt the stability of the nation.

According to the view of the participants in the Carbon Monoxide Conference, Riverside, California, 11 April 1967, the effects of CO on man's health are a most important and nearly neglected public health problem. The 35 scientists present agreed that CO, in most areas, was the most important constituent to be controlled in the exhaust of motor vehicles. It was further felt that it may be the CO content of cigarette smoke which is the agent responsible for excesses in mortality from cardiovascular diseases and possibly even from emphysema

and lung cancer (34). According to Giever (12) Drinker pointed out that the effect of CO may be increased by preexisting physiological factors, by existing impairment of circulation, heart disease, anemia, asthma, lung disorders, or by any condition that speeds metabolism, such as increased activity, high temperature, or high altitude.

There exists a correlation of high CO levels in community ambient air to cigarette smoking. Cigarette smokers commonly have 5 to 10 per cent HbCO, and values as high as 18 per cent (10). It seems likely that the addition of 5 per cent HbCO by community air pollution to that caused by other exposures may be sufficient to pose a substantial risk to the health of sensitive persons. It is the impaired individual, not the healthy person, that must be given first consideration in the protection against the potential effects of CO. Such levels might be produced by exposure to 30 ppm of CO in polluted air from 4 to 6 hrs, or exposure to 120 ppm for 1 hr (35).

Measures are needed which will identify the person with some chronic physiologic defect that makes him a susceptible candidate for chronic CO intoxication. According to Hofreuter (36), the mere determination of HbCO levels or the recording of subjective symptoms will not provide a definitive answer to the chronic CO intoxication question. Additional criteria, i.e., behavioral changes, serum enzyme changes, and changes at the organ and cellular-subcellular levels must be developed to assess early tissue damage related to low concentrations of CO.

Another group of important environmental contaminants has gained national prominence in recent years. The Task Force on En-

vironmental Health and Related Problems (37) reported that trace metals which are known or suspected to be harmful to humans are being discharged into the environment at an increasing rate.

Unlike other macro- and micronutrients, trace metals or elements cannot be synthesized by biological processes. They must all come from soil or sea, or in recent history, by man's own unremitting efforts, from mines and air (38, 39). Little is known about the adverse effects of these substances with regard to allergies, long-term genetic changes, or chronic diseases.

The Task Force on Environmental Health and Related Problems (37) has recommended that an effort be made to establish, by 1970, human safety levels for synthetic materials, trace metals, and chemicals currently in use; and prohibit after 1970, general use of any new synthetic material, trace metal, or chemical until approved by the Department of Health, Education, and Welfare.

According to Leddicotte (38), biological and medical research advances during the last few years have brought a new appreciation of the role of elemental species (trace metals) in living systems. At least sixty elements in low concentrations (trace: 0.01 to 0.001 per cent), have been discovered in fungi, bacteria, higher plants, animals, and humans.

The need for understanding trace metal functions has already stimulated medical researchers to speculate and hypothesize about the role of trace metals in the causes of, and in the therapy for, many chronic diseases (40, 38).

The development and commercialization of gas chromatography, polarography, emission spectrometry, atomic absorption spectroscopy, mass spectrometry, x-ray spectrometry, and activation analysis have greatly aided and extended the scientist's capability in determining elements and compounds in concentration ranges below 0.1 per cent (41).

Trace metals have been implicated as causative factors in a few chronic diseases (40). There is speculation that a metal accumulating in an organ from the modern environment could so modify an enzymatic reaction that eventual breakdown of a metabolic pathway would result in a chronic disease (42).

According to D'Alonzo et al. (43), in their study of trace metals and heart disease, Schroeder related water hardness to heart disease, and Strain observed large geographic variations in death rates from cardiovascular disease, possibly associated with minor element deficiencies, especially vanadium and zinc.

There are virtually no data available from exploration of the possible effects of chronic CO exposure to biological systems and trace metal alterations within these systems. The literature reveals little on the possibility that a chronic environmental pollutant, such as CO, could cause accumulations and/or shifts of trace metals at the organ, cellular, and subcellular levels, and disrupt major metabolic pathways as referred to by Schroder (40, 42), causing a chronic disease.

It has been stated that enzymatic oxidations are highly sensitive to traces of the inhibitors which are known to inhibit radical reaction chains, e.g., malonic acids, organic iodides, sulphites, cyanides, and CO (44); furthermore, CO is known to inhibit many respiratory

enzymes which contain iron or copper atoms as an essential part of their catalytic mechanism.

According to Mahler and Cordes (44) carbon monoxide's postulated mode of action on metal complexing agents is the formation of complexes with metalloenzymes, especially metalloporphyrins and copper enzymes. Carbon monoxide is also known to react with microsome $B_{420}$, perhaps the terminal oxidase for microsomal electron transport with either cytochrome $b_5$ (liver mitochondrial microsomes) or microsomal hydroxylase functioning as an electron donor (44). Conn and Stumpf (45) postulated that it was the combination of CO with ferrous iron of cytochrome $a_3$ (cytochrome oxidase) which accounted for the extreme toxicity of this compound to biological organisms.

Mahler and Cordes (44) indicated that CO also acts on cuproproteins. Gallagher's (46) work on copper deficiency in rats and chickens showed that cytochrome oxidase activity was greatly reduced, and that tissue respiration failure was due to the progressive depletion of cytochrome oxidase, which in all probability was the immediate cause of death. Gallagher (46) also indicated that copper deficiency in the lamb, may produce demyelination of the central nervous system (CNS) by depletion of cytochrome oxidase activity leading to inhibition of aerobic metabolism. Such was not the case for copper deficient rats. Butcher and Fox (47) have recently shown that copper may have a significant and specific function in the metabolism of the caudate nucleus of the cat.

Datsenko (48) showed that chronic CO intoxication in rats

resulted in a decrease of activity in cholinesterase in blood serum, which can be used as a sensitive index of the degree of intoxication, and that changes in the organism under chronic CO intoxication are persistent, as demonstrated by the incomplete restoration of the activity of cholinesterase for three months after the inspiration of CO stopped.

Changes in several physiological and biochemical indices in man after exposure to small concentrations of CO were noted by Kustov et al. (49). The effects were not entirely due to CO hypoxemia, but tissue effects of CO were considered a prime factor. Carbon monoxide also caused a pronounced disorder in the blood circulation, dystrophic albumin exchanges, and total increase in the size of nuclei of liver cells (50).

In summation, there is a critical need to explore chronic health problems and diseases, as exemplified by recent emphasis on the environment by the federal government (12, 26). Chronic contamination of the environment by various chemicals, gases, heat, radioactive materials, noise, solid waste, sewage, and trace metals throughout the world has opened up many new areas of research. Bowen (51) stated that CO, a global pollutant, has a mean residence time in the atmosphere of a few months, and that it is not known how the gas is removed from the atmosphere. He further stated that CO is known to inhibit metalloenzymes at high concentrations in plants. Swinnerton et al. (52) recently reported that industrialization has raised the CO level in the atmosphere in the last 50 years, and since CO and oxygen have about the same solubility in water, the oceans, lakes, and

rivers may accumulate the gas and create a threat to aquatic life. Since aquatic organisms are part of man's food chain, it is important to know if chronic CO exposure has an adverse effect on these organisms, and if the gas has an effect on trace metals within the organism's metabolic systems. Carbon monoxide has been considered an important factor in the genesis of lung cancer, and in increasing the significance and severity of angina pectoris, and vascular diseases (34). Carbon monoxide has been linked, as a causative agent, to myocardial infarction and arteriosclerosis (53-56). Trace metals have also been implicated as a causative factor in these same diseases (40, 42, 43, 57, 58).

This investigator feels that any significant trace metal alterations in rats exposed to CO during the chronic CO-trace metal study would be important because similar trace metal alterations might be more extreme in humans. Goldberg and Chappell (27) stated that CO is excreted from the blood of rats more rapidly than the blood of humans; therefore, any given exposure should have less effect on rats than on humans. Little is known about chronic CO intoxication, and its relationship to trace metals within the body at the cellular and subcellular levels. This fact, along with recent developments in computer systems, statistical model analysis, and trace metal instrumentation for rapid quantitative and qualitative analyses, has opened the door for comprehensive research in this area (16-20, 22, 23, 37, 38, 40, 41, 49, 59).

## Acute Effects

Carbon monoxide (CO), formula weight 28.01, is a colorless,

odorless, explosive, toxic gas, produced by incomplete oxidation of carbonaceous material (2, 3, 7, 10, 13, 60, 61). It is a principal constituent of manufactured fuel gas, such as producer gas and blue water gas, and it is also used for the synthesis of organic compounds, including acids, alcohols, and hydrocarbons.

Historically, CO was discovered by Priestly (13) in 1799, and the composition of CO was first established by Clement and Desormes in 1801. The Greeks knew of the effects of CO, and the Romans used it for punishment of criminals and for suicides (3). Today, CO is one of the major chronic pollutants of urban ambient air and closed environmental systems (10, 14, 25, 32, 35, 36, 62-64). Carbon monoxide is currently the most important gaseous poison which confronts physicians, and it causes more deaths than all other toxic gases combined (11, 64). According to Du Bois and Geiling (61), CO is responsible for 95 per cent of the deaths due to toxic gases in the United States, and 2,000 accidental deaths annually.

The toxic action of CO is related primarily to its affinity for hemoglobin (Hb), the oxygen-carrying component of blood, combining to form carboxyhemoglobin (HbCO), and also combining with some of the constituents of the cytochrome system and myoglobin (19, 44, 45, 65, 66). Affinity of CO for Hb is approximately 200 to 300 times that of oxygen (66). A relatively small concentration of CO in the inhaled air can tie up significant quantities of Hb as HbCO.

A CO concentration at equilibrium of 0.01 per cent (100 ppm) leads to 17 per cent HbCO, and 30 ppm ties up 5 per cent HbCO (8, 10). Hemoglobin bound with CO is then unavailable for the transport of

oxygen to the various tissues of the body. Secondarily, and less important, CO effects indirectly result from HbCO inhibiting the dissociation of oxyhemoglobin ($HbO_2$). This further reduces the oxygen supply to the body. Carbon monoxide in the blood also reduces the partial pressure of oxygen, and lessens oxygen diffusion into the tissues.

The amount of CO within the body is related to both its concentration in the air and length of exposure to the individual. Unless the concentration in the air is sufficient to bring about death, an equilibrium is established between inspired air and HbCO in the blood. The lower the CO air level (10), the longer the time to reach equilibrium, and at normal breathing rates, 7 to 8 hrs are required to reach blood saturation at CO levels of from 50 to 100 ppm.

Previously, it was thought that death did not supervene until 60 to 80 per cent of the Hb was combined with CO (66), but contrary to the accepted belief that only a very high HbCO content causes death, subsequent findings have definitely established that in some fatal CO asphyxiations, the blood CO saturation may be lower than 60 per cent, and in exceptional cases, as low as 30 to 40 per cent.

The CO exposure time factor is important. The time factor, according to Bour and Ledingham (13) does not increase the poisoning coefficient, but prolongs the anoxemia induced by neutralization of the Hb. Furthermore, the resulting asphyxia produces anatomical and functional disorders of the tissues and cells, which disorders are secondary to the oxygen deficiency and the functioning of the organism under conditions of anaerobiosis. These conditions give rise to cardiac

and respiratory reactions, particularly to hyperventilation accompanied by respiratory alkalosis, to irreversible acidosis as a result of incomplete combustion and to the liberation of acid metabolites.

Two tissues in the organism are particularly sensitive to hypoxia or anoxia, and their sensitivity increases with the period for which the hypoxia or anoxia continues to be present. The changes which cause a large proportion of the symptoms to be observed are found in the central nervous system (CNS). Diffuse degenerative lesions of the CNS during CO poisoning are extremely common and severe. In addition, asphyxia gives rise to symptoms of edema and vascular stasis in the brain, and an oxygen deficiency continuing beyond 6 min is known to be fatal to brain cells (13). The myocardium, also frequently shows symptoms of being affected during carbon monoxide intoxication.

The toxic action of CO is essentially an asphyxia caused by blocking of the oxygen transport mechanism of the erythrocytes, and it also acts on tissue cells by inhibiting the oxydo-reduction enzyme system (13, 44, 65, 66). Serious respiratory manifestations of the obstructive type and frequently cardiovascular manifestations, especially collapse, occurring during the course of CO intoxication, increase and prolong the asphyxia, thus instituting further disorders of the CNS (13).

According to Bour and Ledingham (13) neuropathological problems presented by intoxication with CO are far from being resolved. The general mechanism of causation of the lesions is elusive and difficult to explain on the basis of the physiopathology of anoxia alone. The alteration in the white matter and determinism of fundamental pro-

cesses have not been explained completely.

In 1920 Barcroft (67) classified four main types of anoxia:

1. Anoxic or asphyxial anoxia, caused by lack of oxygen in the air inhaled or respiratory insufficiency.

2. Anemic anoxia, due to the decreased number of red corpuscles or to the diminished production rate of Hb.

3. Stagnant anoxia, which occurs during an arrest or lowering of the blood flow (oligaemia, ischaemia).

4. Metabolic or toxic anoxia, which is caused not by the lack of oxygen, but rather by factors interfering with the consumption of oxygen (cyanide poisoning, hypoglycemia), or which have a direct effect on the cells.

Acute CO poisoning, according to Bour and Ledingham (13) should be classified among those anoxic conditions which are becoming increasingly frequent, presenting progressively more complex problems, and stimulating numerous studies, i.e., problems of sea-divers, open-heart surgery, prolonged anesthesia, altitude exposure, etc.

The brain is the most sensitive organ to anoxia, and it consumes the largest proportion of oxygen of the whole organism. The adult brain consumes about a quarter of the inspired oxygen. In nursing infants and children up to age four, consumption of oxygen by the brain rises to a third.

According to Bour and Ledingham (13), Bernard discovered the affinity of CO for Hb, and indicated the disturbance in oxygen transport which occurs when HbCO is formed. Haldane (68) in 1895, showed that HbCO formation was an equilibrium reaction which depended upon the relative partial pressures of CO and oxygen in inhaled air.

The dissociation of HbCO follows the same laws as the dissociation of $HbO_2$, and the HbCO dissociation curve is governed by the same factors of pH, salt content, and temperature as the $HbO_2$ curve (13). Many literature reviews cover this subject. Patty (2) gave a lucid presentation of the dissociation curves for $HbO_2$ and HbCO.

Carbon monoxide is eliminated from the body via the lungs. The reaction is a reversible one, and the rate of elimination is governed by the same factors that affect its absorption (66). Bartlett (18) showed that CO reversibly combined with Hb and myoglobin (Mb) in the following reactions:

(1) $CO + Hb \rightleftharpoons HbCO$

(2) $CO + Mb \rightleftharpoons MbCO$

The reaction of CO with Mb is biologically significant, but less important than CO combining with Hb.

According to Bour and Ledingham (13), Smith and Sharp described in 1960, the first clinical application of the treatment of CO poisoning by administration of oxygen at high atmospheric pressure. The results showed that the period of hypoxia was terminated as soon as the amount of oxygen dissolved in the plasma was sufficient to adequately oxygenate the tissues. The elimination of CO took place more rapidly, resulting in increased alveolar oxygen tension.

Many CO experiments have been carried out on animals. Chornyak and Sayers (69) fatally exposed dogs to 600 ppm CO in air by volume from 20 to 30 min. A diffuse degenerative change throughout the brain was noted. The effects produced were edema of the dorsal

motor nucleus of the vagus and medulla oblongata.

De Boer and Carroll (70) showed that spleen volume decreased when cats were poisoned with CO; furthermore, no vaso-construction in the spleen was noted. The function of the splenic contraction was to expel unpoisoned red cells into the blood, and thereby reduce the ratio of HbCO to $HbO_2$ in the general circulation (70). Ehrich et al. (71) produced acute CO poisoning in some dogs by inhalation of CO, and in others by intravenous introduction of erythrocytes saturated with CO. Morphological changes were hemorrhages and myocardium necroses and degenerative changes of individual muscle fibers. It was noted that electrocardiographic and morphologic changes of the heart in CO poisoning resembled closely those seen in anoxia due to other causes.

Gorbatow and Noro (72) exposed mice and rats daily to CO in amounts of 250, 400, 500, and 1,000 ppm. The exposed animals became acclimatized to CO in doses of 250 to 500 ppm, and the tolerance against CO increased 2 to 4 times its original value in 8 to 15 days. Only slight acclimatization was observed at the 1,000 ppm level. Polycythemia was observed during acclimatization, but disappeared later. No HbCO decrease was observed. Rabbits were used by Jerzykowski and Nowak (73) to show the effect of acute CO poisoning on riboflavin level in blood and tissues. He observed that riboflavin was released to the blood via the muscles probably from protein decomposition, and that the differences between exposed and controlled groups were statistically significant.

Spencer (74), in his experiment showing the effects of CO on

man and canaries, found that even though a canary is useful as an indicator of CO in mines, at lower levels of CO the canary does not become affected as soon as man; therefore, the canary will not only be useless as an indicator, but also dangerous because of the false confidence it creates.

In relationship to the effects of acute CO exposure on humans, Abt and Witt (75) showed that a five year old male became totally blind after acute CO poisoning. Adler (76) presented a case in which CO fumes were related to the disintegration of optic recognition in visual agnosia.

According to Burck and Portwich (77) renal failure as a consequence of acute CO intoxication produced vacuolation of the large epithelia, and flattening of the epithelium in the kidney tubules. Chalupa (78) indicated that memory is affected following acute CO intoxication. Carbon monoxide poisoning affected hearing by the loss of sensitivity in middle range frequencies according to Taniewski and Kubler (79). The occurrence of Parkinsonism following acute CO poisoning is well authenticated (80), and hypoglycemia is now a classic symptom of intoxication due to CO (13). Carbon monoxide intoxication has been shown to reduce the activity of cholinesterase in blood serum (48), and act directly on metabolism of porphyrinic pigments (81).

Sjostrand (82) has recently shown that CO is produced both pathologically and physiologically by the breakdown of Hb and that the expired air CO concentration reflected the rate of Hb destruction. It was shown also that endogenous CO formation increased considerably in

certain pathological conditions with abnormal decomposition of erythrocytes (82). Sjostrand (83) has postulated that a molecule of CO is liberated when each porphyrin ring splits in the Hb molecule.

Hayes and Hall (53) and Shafer et al. (54), have indicated that CO is toxic to the myocardium. Litzer (55) discussed the effect of CO poisoning on capillaries and "secondary sickness phenomena-damage" to circulation and the heart.

Much research is currently being carried out on the relationship between smoking and cardiovascular diseases. According to Mills and Porter (84), there exists a relationship between tobacco smoking and automobile driving stress, and cardiac and vascular anomalies. In regard to heavy cigarette smoking, the risk ratio prevailed for both myocardial infarction and angina pectoris, and coronary heart disease was found to occur twice as often among male cigarette smokers as among nonsmokers (85). Bokhoven and Niessen (86) in evaluating health effects and smoking indicated there is a possibility of synergism, caused by the simultaneous presence of CO and oxides of nitrogen in cigarette smoke. Evidence also exists that smokers are more likely to die of coronary heart disease and other cardiovascular conditions, chronic bronchitis, emphysema, and cancer of the urinary bladder, according to a recent article in the American Journal of Public Health (87).

Cigarette smoking is associated with a 70 per cent increase in the age-specific death rates of males, and to a lesser extent with increased death rates in females, according to the 1964 Surgeon General's

Report (88). Carbon monoxide is a major constituent of cigarette smoke. One cigarette smoked involves a short time exposure of about 475 ppm CO, and deBruin (89) has stated that 3 to 5 per cent HbCO may already give rise to psychoneurological symptoms, manifested in psychometric tests.

It has been shown that the CO dose from cigarette smoke is high and short, relative to that from community air pollution (10). Cigarette smoking ties up about 3 to 5 per cent Hb as HbCO (89). Combined smoking and ambient air levels of 30 ppm CO may tie up over 10 per cent Hb (10).

According to Dunlap (90) and data previously stated (14, 32), every year motor vehicles pour out about 169 billion pounds of CO into the air of urban America. Each urban dweller's share amounts to about 30 pounds of CO per 24 hrs. There is concern about the relationship of auto accidents, cigarette smoking, and high CO ambient urban air levels, and over-all CO exposure to people with chronic diseases (10, 14, 90).

Ringold et al. (91) have developed a valid method for determining HbCO levels in the body using an infrared analysis, and Furlong (92) has developed a relatively accurate disposable blood-CO analyzer. Various methods are available and are recorded in the literature. Allen and Root (93) have improved the palladium chloride method for colorimetric determination of CO in air. Recent developments in gas chromatography offer an excellent method of both qualitative and quantitative analysis of CO (94). The nondispersing infrared CO analyzer is

currently used for continuous analysis of urban air (95). Haldane (96) was one of the first investigators to develop a usable method for CO detection. Silverman and Gardner (97) described an improved method for directly measuring low concentrations of CO in air. They stated that CO reacted with potassium pallado sulfite impregnated on a chemically inert gel contained in sealed glass tubes. The length of discoloration caused by conversion of yellow sulfite to dark brown palladium or its oxides is an expotential function of CO concentration. Breysse et al. (98) used an expired air technique for estimating the percentage of HbCO and periodically checked the reliability of these techniques by drawing and examining blood specimens for percentage of HbCO. They compared the results, from a Mine Safety Appliance (MSA) Carbon Monoxide Poison Kit and a gas chromatograph (GC) of expired air from men exposed to CO, against a modification of the microdiffusion procedure described by Feldstein and Klendshoj for blood samples. The MSA and GC methods correlated significantly with the results obtained from the blood specimens, and the correlation of the MSA method was greater than the GC method.

## Chronic Effects

There are more than 3,000 bibliographic references on CO according to Finck (8), but very few are concerned with the problem of chronic CO intoxication (4, 11, 12, 15, 17-21, 48, 63, 64, 66, 99-101). As previously stated, there is strong evidence, especially from European investigators (4, 48, 62) that chronic CO intoxication is a serious public health problem in major urban areas and closed environ-

mental systems.

Pecora et al. (102) have indicated in their study on the binding of CO in the blood in acute and chronic CO poisoning, that the more prolonged the chronic poisoning, the less was the quantity of CO freed from the erythrocytes or plasma after exposure to air, with an ultimate increase in globulin and plasma carboxyemia. Datsenko (48) showed that chronic intoxication of animals with CO resulted in reduced cholinesterase activity in blood serum, with three months needed for activity restoration after CO had been stopped.

A subacute CO poisoning case with cerebral myelinopathy and multiple myocardial necroses was reported in 1945 by Neubuerger and Clarke (103). Grudzinska (104) concluded that chronic exposure to low concentrations of CO may not cause distinct changes in the nervous system, but may have a certain inhibitory effect on the bioelectric activity of the brain. Malorney et al.(105) noted complete loss of reflexes after 10 weeks of exposure to 140 to 160 ppm of CO in rats.

In a chronic industrial CO poisoning study, asthenia, intellectual apathy, depression, muscular fatigue, speech slowness, impaired memory, and sexual impotency were frequently observed according to Duvoir and Gaultier (106). Datsenko (107) in 1965, reported that chronic poisoning concentrations of CO in inhaled air produced changes in the glycemic curves.

In a series of carefully observed slow CO asphyxiation cases, Beck (100) noted a definite clinicopathologic entity despite views held to the contrary, especially in the heart and CNS. Campbell (108)

in a study of hypertrophy of the heart in acclimatization to chronic CO intoxication showed that the average weight of mice hearts exposed to CO was considerably greater than the mice hearts of the control and oxygen exposed groups.

Lewey and Drabkin (109) exposed dogs to 100 ppm CO for 11 weeks, 6 days per week, 5-1/2 hours per day, and found consistent disturbances of gait and of postural and position reflexes. They concluded that chronic CO intoxication may occur in dogs at CO concentrations which have been considered within the safe limits for man. On the other hand, Musselman et al. (110) ran a similar experiment using rats, dogs, and rabbits, and found the absence of toxic signs and only slight blood changes in these animals at the 50 ppm CO level of exposure.

In a recent study of enzyme changes from exposure to chronic CO intoxication, Rozera and Fati (111) observed early a marked decrease in erythrocytic phosphatase activity, and with continued exposure, a less marked decrease in erythrocyte alkaline phosphatase and serum phosphatase.

Because of psychiatric and neurological symptomology, chronic CO intoxication is frequently diagnosed as epilepsy (3). Recent investigations have revealed that the development of atherosclerosis may be accelerated by CO hypoxemia, and that high community CO levels could conceivable increase the incidence of atherosclerosis (10).

During experimental animal studies at the University of Copenhagen recently, very small amounts of CO inhaled over a period of time were believed to cause arteriosclerosis and related coronary

artery diseases (56).

Halperin et al. (21) used visual sensitivity to differences in light intensity as an index of the effects of CO. They showed the persistence of these effects during elimination of CO following inspiration of air, oxygen, and carbogen mixtures. It was shown that recovery from the effects of CO on visual function lags well behind the elimination of CO from the blood. This suggested the existence of some enzyme that competitively combines with oxygen and CO in the CNS and the peripheral visual system (19).

Roughton and Root (112) have estimated that 30 to 40 per cent of the CO entering the blood stream combines reversibly with the Hb-like pigments outside the main blood stream. Five per cent of this CO was estimated to combine with myoglobin. This left 25 to 35 per cent of the CO outside the blood stream unaccounted for.

According to Pfrender (19) certain experiments on the distribution of CO in normal men have suggested the existence of an extracirculatory substance in the liver with a greater affinity for CO than has blood Hb. Tobias et al. (113) have postulated that this substance was related to or identical with the pseudohemoglobin of Barkan. Breysse (16), stated that Kamei has shown that urinary thiamine excretions in rats increased with prolonged exposure to CO. The impairment of phosphorylation of thiamine in chronic CO intoxication was suggested. He indicated that prolonged and repeated inhalation of CO reduced the riboflavin, nicotinic acid, flavin adenine-dinucleotide and flavin mononucleotide content of various organs in the rat, and

that the long-term inhalation of CO may destroy the respiratory enzymes (16). Furthermore, the vitamins necessary for the composition of the respiratory enzymes may also be reduced by chronic CO intoxication. Komatsu et al., as reported by Breysse (16), studied the influence of prolonged and repeated exposure of rabbits to CO on tissue metabolism, and found that Hb content and oxygen capacity of the blood were continually changing. The oxygen conveying efficiency of Hb was also variable. The oxygen consumption was decreased in the heart and kidney homogenates, but was unchanged in the liver and brain. They suggested that the respiratory enzymes of the kidney and heart were extremely sensitive to anoxia.

Warburg was one of the first to observe respiratory inhibition by CO in vitro according to Keilin (114). It was found that the iron moiety of cytochrome oxidase reacted with CO to inhibit electron transport and tissue respiration (44, 45, 65). According to Pfrender (19) Bandu and Kiese have blocked the respiratory system in isolated mitochondria at relatively low CO tensions such as have been seen in vivo.

It is well documented (44, 45, 65) that CO acts upon the iron moiety in Hb and in the cytochrome system of mitochondria. Wainio and Greenless (115) have suggested that the copper of cytochrome oxidase is the CO-binding site and not the heme moiety as was previously supposed.

According to Pfrender (19) CO combines with substances other than Hb, notably cytochrome oxidase and myoglobin, and recently the copper of cytochrome oxidase has been implicated as a binding site.

## Trace Metals

Trace metals occupy an important niche in man's biological exogenous-endogenous ecosystem. This dynamic ecosystem comprises the interacting, living and nonliving elements in a particular habitat (25). There exists a relationship between plant cover, the animal kingdom, and various soils with respect to trace metal metabolism (116).

The essential trace metals are more important in the nutrition of living things than are their organic micronutrient counterparts, the vitamins (39). Trace metals cannot be synthesized by biological processes (38), as can vitamins, but they can, and often do, change their valences (39). Trace metals are present in the environment within a narrow range of concentration, and their only sources are the soil, sea, and air; without trace metals, life would cease to exist (38, 39, 57, 58, 116).

By definition, essential trace metals are those without which the organism cannot maintain optimum growth, health, and longevity, and which perform physiological functions desirable for normal metabolism, and without which disease occurs (39). According to Schroeder (42) the role of essential trace metals in the nutrition of plants has long been studied, but the knowledge of their precise functions in mammalian metabolism shows vast gaps.

Both deficiencies and excesses of trace metals produce adverse effects, and can kill (2, 39, 57, 101), and the possibility of one or more chronic diseases being produced also in part by gradual accumulation of certain abnormal metals is real (42).

Trace metals are intimately related with enzyme action and act as metallic cofactors and catalysts. Biological activity can be lost in living cells when the metalloenzyme's protein residue contained in the cells is disrupted and split. Iron, molybdenum, and copper, have established roles in oxidation-reduction physiology of cellular metabolism, as intrinsic parts of enzymes containing flavoproteins. Copper has been shown to be related to ceruloplasmin activity (117). Manganese or cobalt act only as catalytic co-factors, and appear to be an essential part of the enzyme molecule as ascorbic acid is synthesized in the liver. Elevated trace manganese appears to be essential for cytoplasmic metabolism in mitochondrial cells (38). Zinc has been shown to affect the rate of wound healing (118), and low concentrations of zinc have been related to cardiovascular disease (43). Magnesium is known to activate all enzymes transferring phosphate from ATP or ADP (119).

Recent research in trace metals concerns the following areas (32):

1. The role of trace metals in arteriosclerosis.
2. The role of copper and manganese in Wilson's disease.
3. Magnesium deficiency in chronic alcoholism.
4. The assay and correlation of enzymes patterns of aerospace candidates.

The present investigation is directed toward the study of copper, cobalt, magnesium, iron, and zinc in rats exposed to chronic $CO_2$. These particular metals were chosen because of their relationship to gross and cellular respiration.

Copper is widely distributed in nature, and has diverse functions in animals and plants. It is an essential trace metal for man and animals because it is required for the formation of erythrocytes and hemoglobin, as well as oxidative enzymes such as catalase, peroxidase, cytochrome oxidase, and many others. In chronic copper exposure the liver, kidneys, and spleen may be injured and anemia may develop (2).

Copper is an essential metal in tryosinase, ceruloplasmin, hematocuperin, hemocuprein, hemocyanins, and acyl CoA dehydrogenase, uricase, ascorbic acid oxidase and δ-aminoevulinic acid (57, 120).

In the relationship between serum metal concentration and disease, reduced copper levels are involved in nephrosis and Wilson's disease, while elevated copper levels are apparent in Hodgkin's disease and in acute leukemia (57, 58). Deficiency in copper has been shown by Gallagher (46) to progressively reduce cytochrome oxidase, increase susceptibility of mitochondria to aging, and decrease the rate of phospholipid production. It was previously stated (44, 65) that CO blocks cytochrome oxidase in the mitochondrial respiration system. A possible relationship between copper and CO might exist, but has not yet been shown to do so.

Copper has been postulated to have an effect on the caudate nucleus of the cat according to Butcher and Fox (47).

Cobalt was discovered by Georg Brandt, a Swedish scientist in 1735. Cobalt closely followed the distribution of nickel in the organs of man and animals. Liver, pancreas, spleen and kidneys have

high concentrations of cobalt (57, 58). In intracellular distribution studies of radioactive cobalt, some radiocobalt was always found in the nuclear fraction, especially in liver and tumor tissue, but the cytoplasmic concentration of the metal was usually higher than the nuclear (121). Normal human plasma contained 0.85 plus or minus 0.47 ug cobalt per 100 ml, and red cells 1.20 plus or minus 0.32 ug cobalt per 100 ml. Urinary output per 24 hrs was 1.6 plus or minus 1.05 ug (57, 58, 121). An increase in cobalt was noted in leukemias. A decrease in the red cell content of cobalt was found in polycythemia vera and in some hypochromic anemias (42, 121).

According to Carlberger (121), Faulkner and Blood in 1957 studied the myoglobin concentration in rats which had become polycythemic owing to cobalt administration. They assumed that, if the theory of cobalt interference with the respiratory enzymes was true, the myoglobin, lying between the respiratory enzymes and the erythrocytes, might increase in cobalt-induced polycythemia. It was also strongly indicated that cobalt produced polycythemia by increasing erythropoetic stimulating factor. Chronic CO intoxication is known to produce polycythemia (2). Injected cobalt also produces a polycythemia (57, 58, 121). The possibility exists that CO reacts with cobalt causing a polycythemia, but this speculation has never been proven.

Cobalt absorbed from the intestine is excreted rapidly via the urine. The liver, pancreas, and kidneys contain the highest concentrations (57, 121). In sheep and cattle cobalt deficiency causes wasting disease. In high doses, cobalt causes impaired growth, ne-

croses of the pancreatic alpha cells, reduced blood pressure, hyperglycemia, and polycythemia, probably caused by cellular hypoxia. The effects of cobalt were reduced by administration of amino acids containing sulfhydryl groups (121). Carbon monoxide is also known to produce hyperglycemia (13). Cobalt is known to produce enzootic marasmus (116).

Cobalt is an essential trace metal for man and animal (2, 57, 121). It is an important constituent of vitamin $B_{12}$ and certain enzymes, and is associated with the production of erythropoietin, the red cell stimulant. Chronic CO intoxication is thought to stimulate this system (122).

Magnesium, next to potassium, is the most abundant intracellular cation in the normal adult body, comprising 6 to 20 mEq per liter compared to about 120 mEq per liter for potassium (2). At ordinary levels of intake and absorption, renal conservation of magnesium is high; therefore, plasma magnesium levels usually bear little resemblance to intracellular levels (2).

The average adult human body contains about 21 g of magnesium, representing a concentration of approximately 0.36 g (30 mEq) per kilogram (119). The skeleton contains 11 g, the cells, 9.5 g, and 0.5 g are found in extracellular water which corresponds to about 2 mEq per liter of plasma (2). The normal muscle content is approximately 0.24 g (20 mEq) per kilogram. Magnesium concentration in serum or plasma has a mean value of approximately 1.67 mEq per liter in healthy adults (119). Approximately 36 per cent of the magnesium in plasma is

bound to protein. Red blood cells contain about 4.2 mEq per liter of magnesium (2, 119).

A relationship between cardiovascular disease and low magnesium values in drinking water has been postulated (123). According to Comar and Bronner (58), Vallee stated that manganese will substitute for magnesium in many reactions. D'Alonzo and Pell (124) reported that Schroeder found magnesium and calcium to be the cations most closely related to death rates from coronary heart disease. Magnesium deficiency in chronic alcoholism is currently under study (38). Recent studies are also being carried out on the relationship of magnesium and the disease, Kwashiorkor.

It is known that low concentrations of magnesium lower the excitatory thresholds of the presynaptic nerve and of the muscle membrane, but increase the liberation of acetylcholine. The effect of magnesium deficiency on neuromuscular function is manifest in clinical circumstances by irritability, nervousness and convulsions (119).

In relationship between serum magnesium concentrations and disease, reduced magnesium was reflected by acute or chronic renal disease, while elevated magnesium was reflected by renal insufficiency with oliguria.

Magnesium is fundamental in enzyme systems, especially in the activation of most transphosphorylation reactions involving ATP and ADP and those which are associated with transport mechanisms. Magnesium is capable of crossing the cell membrane (44) and is maintained at its high intracellular concentration by an active metabolic process.

Mitochondria may be responsible for active transport in the cell (119).

Krehl (119) indicated that the associated potassium deficiency seen in experimental magnesium deficiency in rats may be due to a defect in ion transport secondary to the effect of magnesium deficiency on mitochondrial function. Bourne (125) stated that in the cell "template hypothesis," such structures as Nissl bodies, which are composed chiefly of ribonucleoprotein, are also rich in calcium and magnesium. Some phosphate transferring enzymes activated by magnesium are: hexokinase, fructokinase, creatinine transphosphorylase, phosphopyruvic transphosphorylase, phosphoglyceric transphosphorylase, acetyltransphosphorylase, diphosphopyridine nucleotide phosphorylase, and phosphoglucomutase (119).

Any blockage of cytochrome oxidase by CO should be ultimately reflected by an overall reduction of ATP production in cellular respiration.

According to Underwood (57), Keilin and others reported that the biological significance of iron was attributed to its role in oxygen transport, as part of the Hb molecule, and its relationship to the oxidative mechanism of all cells. Most of the body-iron is present in circulating Hb, a small fraction is incorporated in bone marrow Hb, myoglobin, and respiratory enzymes, and the remainder is stored in tissues as ferritin or hemosiderin (126). Putnam (127) reported that iron has been known to occur in plasma as protein-bound iron (acid soluble iron) and plasma Hb iron. The major part of plasma iron consists of iron bound to the specific iron-binding protein, transferrin,

and a small amount of iron in Hb firmly bound to haptoglobin, the normal Hb-binding plasma protein (127). Iron functions in the transport of oxygen by Hb in blood, in muscle myoglobin, where it delivers its oxygen to the cytochrome system of the cells, which contain a 4-Fe-porphyrin-containing complex of cytochrome oxidase, and cytochromes a, b, and c. Catalase and peroxidase are two other Fe-porphyrin enzymes present in nearly all tissues for the metabolism of peroxide oxygen (2). Carbon monoxide has been shown to block cytochrome oxidase and various microsomal systems (10, 44, 45, 65, 101).

Hemoglobin and myoglobin are heme proteins. The heme enzymes include cytochromes, catalases, and peroxidases. The characteristic reaction of any heme protein is mediated by one or several iron atoms in each molecule, with the iron moiety located at the center of the porphyrin ring, and bound to four pyrrole nitrogens by ionic bonds (128).

Hemoglobin is a complex of the basic protein, globulin, and four ferroprotoporphyrin or "heme" moieties, and was first synthesized by Fischer and Beile in 1929 according to Underwood (57). Hemoglobin contains three-fourths of the body iron. It contains about 0.34 per cent by weight of iron, has a molecular weight of 67,000, and contains four iron atoms (128). Hemoglobin iron atoms oxidized to the ferric state lose their capacity of transport oxygen (128). The mechanism of globin synthesis is not known, but heme synthesis and its attachment to globin takes place in the later stages of red cell development in the bone marrow. There is evidence that production of the two parts of the molecule occur together. The series of steps established in heme bio-

synthesis include the condensation of two molecules of $\delta$-aminolevulinic acid to form the substituted pyrrolic substance, porphobilinogen (57). Iodice et al. (129) reported that ALA-dehydrase, a copper containing enzyme, was involved in heme biosynthesis.

Myoglobin is the oxygen-carrying protein of mammalian muscle. It consists of a single chain of 153 amino acid residues and one iron-containing heme group, and it has a molecular weight of 17,500 (44). Because of its greater affinity for oxygen, myoglobin can accept oxygen released by Hb in the tissue, and can serve as an oxygen reservoir (128). Mahler and Cordes (44) reported that myoglobin was an atypical protein in two respects; possession of a relatively high content of $\alpha$-helix, and a complete lack of disulfide bridges or free sulfhydryl groups. White et al. (130) indicated that the glomerular capillaries are permeable to molecules the size of myoglobin, and if myoglobin is released from muscles it is filtered by the glomeruli. They further pointed out that myoglobin release occurred when muscles were injured extensively, and in the "crush syndrome," the renal tubules may become plugged by precipitated myoglobin.

The peroxidases and catalases are heme enzymes which liberate oxygen from peroxides, with iron being in the ferric state in both these groups. Peroxidases of animal origin are verdoperoxidase from white cells, and lactoperoxidase from milk. Hemoglobin has a peroxidase action, which is intensified by haptoglobin, the Hb-binding protein of plasma (128). According to Moore and Dubach (128) catalase was crystallized from beef liver by Sumner and Dounce, and has a molecular

weight of 25,000. They reported that it contains 0.09 per cent iron and has 4 iron atoms per molecule, in contrast to peroxidases, which have only one. Loftfield and Bonnichsen (131) measured the uptake of $Fe^{59}$ by liver catalase and cytochromes in the guinea pig, and concluded from the rate of turnover, that cytochrome b might be a precursor of catalase.

Mahler and Cordes (44) and Moore and Dubach (128) reported cytochromes as being heme enzymes that occurred in the mitochondria of cells, and that they provided a system of electron transport through the capacity of the iron atom to undergo oxidation and reduction. Theorell and Akeson (132) isolated cytochrome c from heart muscle and purified it to an iron content of 0.43 per cent. It has a molecular weight of about 12,000 and contains one heme per molecule according to Moore and Dubach (128), with two imidazole rings of histidine residues participating in the porphyrin iron linkage to the protein. Theorell et al. (133) noted, when they injected radioiron into guinea pigs intraperitoneally, cytochrome c of skeletal muscle and heart took up tracer iron distinctly faster than did myoglobin. Hemorrhage is known to stimulate the turnover of iron in cytochrome c (128).

Moore and Dubach (154) stated that xanthine oxidase, succinic dehydrogenase, and DPNH-cytochrome reductase are known to contain iron as an integral part of their enzyme molecule. They reported that the exact locus of attachment of the metal and its mode of action have not yet been established. Myosin and actomysin, muscle proteins, have been shown to contain small amounts of iron, the function of which is

not known (128). Dreyfus (134) reported that the iron content of actomysin and myosin diminished in muscular dystrophy.

The constant presence of nonhemoglobin iron in plasma has been known for many years, but it was not until 1937 that it became evident that plasma iron is transport iron (57). Transferrin (Tr), or siderophilin, a plasma protein, selectively binds and transports iron (128). It is a $\beta_1$-globulin with a molecular weight of 90,000. Transferrin binds two atoms of ferric iron per molecule and appears to serve as a true carrier for iron in the same manner that Hb acts as a carrier for oxygen (57). According to Fiala and Burk (135) the iron-protein linkage of Tr is probably through a hydroxamic acid group. In normal individuals of most species, only 30 to 40 per cent of the Tr carries iron, the remainder being known as the latent iron-binding capacity (57). Normal plasma contains about 0.24 to 0.28 g of Tr per 100 ml, with the concentration ordinarily expressed in terms of the "iron-binding capacity," which amounts to about 270 to 400 ug of iron per 100 ml (128).

Transferrin in plasma prevents $Fe^{+++}$ from rising to a toxic concentration on rapid release of iron in the organism. Transferrin acts as a buffer substance and effectively minimizes changes in Fe+++ activity in plasma (127). According to Putnam (127) Tr acts as the primary buffer against threatening acute iron intoxication, with the apoferritin-ferritin and hemosiderin systems functioning as the predominant buffer system in intermediary iron metabolism (127). He also indicated that the FeTr complex may be regarded as a small, readily available, atoxic, circulating iron depot. Cartwright and Wintrobe

(137) reported that Tr and iron disappear from plasma at different rates.

Underwood (57) reported that there were actually three different transferrins, genetically controlled at a single locus, while Turnbull and Gilbert (136) in 1961, identified eight genetically different transferrins, all of which bind iron equally *in vitro* and transport it *in vivo*.

Storage iron is divided into four sections (2). The normal human body contains about 4.5 g of iron or between 0.006 and 0.007 per cent of the entire body weight, and in the adult rat, 0.005 per cent (117). Hemoglobin iron comprises 72.9 per cent of the total iron; myoglobin iron, 3.3 per cent; parenchymal iron, 0.2 per cent; and storage iron as ferritin, hemosiderin, and unaccounted iron, 23.5 per cent (2). Ferritin, an iron-protein complex, occurs in significant amounts in the spleen, bone marrow, and liver, with smaller amounts found in other tissues. It contains over 20 per cent by weight of iron consisting of ferric hydroxide micelles attached to an apoferritin through an unknown chemical linkage (128). Hahn *et al.* (138) confirmed the storage function of ferritin by tracer experiments on dogs. Green *et al.* (139) investigated the mechanism of release of Fe++ from ferritin, and attributed the splitting off of iron from ferritin in the liver, to reduced xanthine oxidase. Mazur *et al.* (140) studied the release of ferrous iron from ferritin in the presence of glutathione, cysteine, ascorbic acid and liver hypoxia. According to Moore and Dubach (128), when liver cells were damaged, as by acute

liver hypoxia or by a toxic agent, there was a tremendous outpouring of storage iron into the plasma. According to Mazur et al. (140) ferritin, besides having the function of iron storage, has an antidiuretic effect and a vasodepressor action. Moore and Dubach (128) reported that in normal aerobic liver, ferritin was inactive, but when the liver tissue became anaerobic, ferritin transformed into a form which exerts the antidiuretic effect and vasodepressor action.

Hemosiderin is a relatively amorphous compound, which may contain up to 35 per cent iron, consisting mainly of ferric hydroxide condensed into an essentially protein-free aggregate (57). Greenburg (141) reported that a significant amount of hemosiderin consisted of iron-rich ferritin molecules. According to Moore and Dubach (128) Richter produced a severe hemosiderosis of the liver in rats fed a diet containing DL-ethionine. Hemosiderin particles have a mean diameter of 55 Å and were often located inside discrete cytoplasmic organelles that were boarded by membranes and sometimes contained cristae. These specialized cytoplasmic structures (siderosomes) may be derivatives of mitochondria and apparently play a part in the formation of hemosiderin (128). Shoden et al. (142) reported that in the spleen and liver of normal animals, a slight preponderance of ferritin over hemosiderin iron was noted.

The daily amount of iron used by an adult for Hb synthesis is 26 to 27 mg. Ferrous iron is generally absorbed more easily from the gastrointestinal tract than is ferric iron, probably due to the greater solubility of ferrous iron. Regulation of the absorption of

intestinal iron is not completely understood, but it appears to depend on the body stores and requirements (2, 57, 58). Ferrous iron passes into the intestinal mucosal cell, where it is converted to ferric iron in ferritin. No iron absorption as ferritin occurs until the cell is physiologically depleted. However, iron is withdrawn from ferritin as ferrous iron, when needed. Iron directly released into the blood stream is oxidized rapidly by dissolved oxygen, to ferric iron, which then complexes with Tr, the iron transport moiety (2, 44, 57, 58). According to Moore and Dubach (128) iron absorption can occur from the stomach or from any other section of the intestinal tract, but the greatest absorption appears to be the greatest in the duodenum, and progressively decreases in the more distal segments (128). Moore and Dubach (143) indicated that when ascorbic acid was administered with ferric iron, absorption was enhanced significantly. In low phosphate diets assimilation of iron was shown to increase (128). Sharpe et al. (144) indicated that the formation of insoluble iron salts, such as phosphates or phytates, can interfere with the absorption of iron. Underwood (57) suggested that the main factors controlling iron absorption, apart from dietary and local factors within the intestine, are the rate of erythropoiesis and the state of iron stores. Absorption was greater than normal not only in iron deficiency, but in many other conditions in which erythropoiesis was accelerated, e.g., after phlebotomy or acute hemorrhage, in hemolytic anemias and secondary polycythemia, after ascent to high altitudes, and as a result of erythropoietic stimulation by cobalt (128).

Animals have little capacity to excrete iron, and experiments have demonstrated that iron was avidly conserved and utilized by the body (128). Cruz et al. (145) demonstrated with radioactive techniques the relative completeness of conservation of Hb iron, but according to Moore and Dubach (128) no one has devised techniques capable of demonstrating the reutilization of myoglobin or tissue iron.

In all species iron deficiency resulted in an anemia of the hypochromic, microcytic type, accompanied by a normoblastic, hyperplastic bone marrow that contains little or no hemosiderin (57). Mild iron deficiency exists when the total iron is reduced but there is sufficient iron to provide a normal mass of hemoglobin. In various studies of infection, acute processes cause decreased iron absorption. Equivocal iron changes were observed during chronic diseases (146).

According to Putnam (127) higher Tr values are found during pregnancy, in chronic iron deficiency, and in the initial phase of acute hepatitis; furthermore, in active diseases, more plasma iron was retained by the reticulo-endothelial cells and less absorbed by the bone marrow, which itself normally utilizes 70 to 80 per cent of the daily plasma iron turnover. In severe hemolysis unbound Hb, Hb loosely bound to plasma proteins, methemalbumin, and a complex between hemin and an $\alpha_2$-globulin have been demonstrated (128). In severe hepatocellular damage, the plasma sometimes contains ferritin, the normal intracellular iron-apoferritin complex (127).

While reduced iron levels in serum reflected a deficiency anemia in man, elevated iron levels in serum manifested aplastic anemia, acute hepatitis, hemochromatosis, and some malignant tumor types.

Patients with iron-overload and hemolytic anemias may have hemosiderin granules in their urinary sediment. Moore and Dubach (128) reported that excessive accumulation of iron in man has been observed in idiopathic hemochromatosis, transfusion hemosiderosis, after prolonged iron therapy, and among Bantu natives who consume diets containing as much as 200 mg of iron per day. Finch and Finch (147) suggested that large amounts of tissue iron were not toxic as long as the iron remained mainly in the reticulo-endothelial cells. Richter (148) reported in 1960 that ferritin was enclosed in hemosiderin granules in liver of patients with transfusional hemosiderosis and hemochromatosis, and attribute the pathogenesis of these diseases to an abnormal cellular metabolic pathway of ferritin.

The presence of zinc in human tissues was first reported in 1877 by Raoult and Breton. It had previously been reported by Raulin in 1869 to be necessary for the growth of Aspergillus niger (58). Lutz (149) first reported that the total body composition of zinc for an adult 70 kilogram man was 2.2 g.

The normal zinc content of human tissues varies from 10 to 200 µg per g of fresh tissue, and most organs including the pancreas, contain around 20 to 30 µg per g, with liver, bone, and voluntary muscle containing from 60 to 180 µg of zinc per g of tissue (2). Human whole blood contains about 700 to 800 µg of zinc per 100 ml, with an average of 120 µg per 100 ml in the plasma (150). Established dietary zinc intake of normal man is about 10 to 15 mg per day (2, 151, 150).

Zinc occurs in the body in two different protein combinations: as a metalloenzyme in which zinc is an integral part of an important enzyme system, such as carbonic anhydrase for regulation of carbon dioxide exchange, and as a metal-protein complex in which zinc is loosely bound to a protein which acts as its carrier and transport mechanism in the body (2).

Approximately 34 per cent of the plasma zinc is firmly bound to a globulin, while the remaining 66 per cent is more loosely bound to a plasma protein and probably represents the transport fraction (150). Wohl and Goodhart (150) have stated that normal red corpuscles contain approximately 1.2 to 1.3 µg of zinc per ml of packed calls, most of which is carbonic anhydrase.

High concentrations of zinc have been noted in the choroid of the eye, prostate gland, skin, and testes, while the pancreatic content of zinc is double the concentration of most other tissues in man (43, 152).

Zinc has an important role in skin metabolism. One of the striking features of zinc deficiency is abnormal keratinization in swine and cattle. Wohl and Goodhart (150) reported that rats and mice fed a diet containing 1.6 ppm or less of zinc gained weight poorly and developed alopecia about the neck and shoulders. They further showed that zinc-deficient animals exhibited extreme parakeratosis with a thick layer of partially keratinized cells in the exophagus.

The importance of zinc in the integument is emphasized by the effect of zinc deficiency on wound healing. Studies in rats in-

dicated that the tensile strength of surgical incisions was decreased when there was a deficiency of zinc (152). Strain (153) noted that wounds and burns healed faster in rats on a zinc supplement diet than in control rats. According to Lichti (118) in his study of the transport of zinc to surgically inflicted wound sites in experimental animals, there was an elevation in the zinc level of plasma and wound fluid within a few hours. He suggested that zinc exerted its most important function biologically by activating the enzymes concerned with protein synthesis and carbohydrate metabolism, and acting in such a manner, appeared to be essential to tissue growth and repair.

Reduced zinc levels in blood serum are associated with acute and chronic infections, Laenneck's cirrhosis, malignant tumors and pernicious anemia. Elevated zinc levels in blood serum are indicative of hypertension and hyperthyroidism.

The identification of zinc-containing dehydrogenases in the liver of certain animals has prompted a study of the zinc content of the blood and liver of patients with post-alcoholic hepatic cirrhosis. According to Wohl and Goodhart (150) Vallee and his associates regard the reduced zinc serum concentrations and elevated zinc urinary excretions in patients with postalcoholic hepatic cirrhosis as a conditional zinc deficiency. Vallee et al. (154) also reported in a study of alcoholics with Laennec's cirrhosis that the hepatic zinc levels were low, along with variable iron levels. They also reported large quantities of zinc excreted in the urine of these patients.

Prasad et al. (155) demonstrated a syndrome in men which

included hypogonadism, dwarfism, and apparent zinc deficiency. Wohl and Goodhart (150) observed elevated RBC zinc concentrations in patients with pernicious anemia and sickle cell anemia. The zinc content in leukocytes of patients with chronic and acute leukemias was considerably lower than normal.

Wacker et al. (156) have postulated that an interrelationship between atherosclerosis, myocardial infarction, and zinc concentration exists. They found patients with significantly lower plasma zinc than normal. A statistically significant decrease in serum zinc has also been observed in myocardial infarction by D'Alonzo and Pell (43), with accompaning changes in a variety of enzyme activities and an increase in copper concentration.

Gunter (157) has reported that intravenous administration of Escherichia coli endotoxin, 0.4 mg per kg of body weight, resulted in a LD/80 for experimental adult mongrel dogs. He reported also in these experimental animals, a significant decrease in plasma zinc and total plasma protein concentrations, and increases in hematocrit values and total zinc concentrations.

It has been reported in the literature that zinc ingestion in experimental animals in amounts slightly lower than 1 per cent has reduced liver cytochrome oxidase and liver catalase activity (158).

According to Sandstead (152) tissue levels of zinc were altered in certain types of cancer, and prostatic carcinomas were reported to have lower concentrations of zinc than normal prostatic tissue; in contrast, thyroid cancer and renal tumors may have elevated zinc levels.

Zinc excretion is augmented in a variety of illnesses. Peters (159) suggested that excretion of coproporphyrin, uroporphyrin, porphobilinogen and δ-aminolevulinic acid in acute porphyrinuria resulted in increased urinary zinc due to chelation of the metal by the porphyrin molecule; therefore, zinc excretion may be increased in acute intermittent porphyria, hepatic porphyria, lead poisoning, and acute rheumatic fever. Excess zinc has been reported in urine of patients afflicted with nephrosis and severe liver disease.

So far, over twenty zinc metalloenzymes have been identified. Peters and co-workers (159) have reviewed the essential role of zinc ions in following metalloenzymes: carbonic anhydrase, lactic dehydrogenase, uricase, catalase, carboxypeptidase, peroxidase, and alcohol dehydrogenase. They postulated that an excess of zinc inhibits the enzymatic activity of lactic dehydrogenase, insulin and equine gonadotropin activity. Keilin and Mann (160) isolated and purified carbonic anhydrase, which contained as part of its molecule, 0.33 per cent zinc. Carbonic anhydrase catalyzes the dehydration of carbonic acid and aids in the elimination of carbon dioxide from the lungs, and in the incorporation of this gas at the tissue site (58, 117).

In addition to its function as an activator of certain enzymes, zinc, along with magnesium, manganese, calcium, and iron participates in ribonucleic acid (RNA) metabolism, which is still not clearly understood, but zinc appears to be an integral part of the RNA molecule of a number of species and is thought to help maintain the stability of the molecule's configuration (44, 152). In further studies

of the cellular activity of zinc, it was found that cell growth and protein synthesis of certain microorganisms were severely impaired by zinc deficiencies (152).

Prasad (161) indicated that zinc, iron, and copper might act as competitors. Later evidence indicated that this competition resulted in anemia, and was caused by a three-way competition between zinc and copper, with iron utilization depressed because of the interference with copper metabolism by zinc.

Yunice et al. (162) in a recent experiment investigating the effect of desferrioxamine on trace metals in rat organs, postulated that zinc replaces iron, i.e., some substances which normally chelate iron will, in its absence, accept zinc. He further stated that when zinc levels in the testes of zinc-deficient rats decreased, iron levels rose; when zinc levels increased following zinc repletion, iron levels fell.

Zinc and copper were shown to increase significantly after exposure to 3 ppm of ozone in rat lungs, and metal shifts in the liver were also observed by Dixon et al.(163). Ganong (164) reported that insulin has an affinity for zinc, and this metal was found in the islet tissue of the pancreas.

In recent studies at the subcellular level, Brierly and Knight (165) reported that the addition of low concentrations of $Zn^{++}$ to suspensions of heart mitochondria oxidizing ascorbate and N,N,N',N'-tetramethylphenylenediamine (TMPD) resulted in marked activation of the energy-linked accumulation of $Mg^{++}$.

Bourne (166) recognized three fundamental properties of mitochondria:

1. Citric acid oxidation.
2. Oxidative phosphorylation with the liberation of energy and its transport by adenosine triphosphate (ATP).
3. Electron transport.

White et al. (130) showed agreement with Bourne by stating that the enzymes of the Krebs Cycle and those required for electron transport from phridine nucleotides and flavoproteins to the cytochrome system and attendant phosphorylation are located in the mitochondria.

The liver, the largest gland in the body and rich in mitochondria, has many complex functions. These functions include carbohydrate storage, bile formation, ketone body formation, reduction and conjugation of adrenal and gonadal steroid hormones, detoxification of many drugs and toxins, manufacture of plasma proteins, inactivation of polypeptide hormones, urea formation, fat metabolism, and trace metal storage (44, 45, 65, 130, 164, 166). Liver subcellular organelles (nuclei, mitochondrial, microsomes) have been isolated into fractions by homogenization and differential centrifugation (59, 166-168). The fractions obtained may be analyzed for trace metals by atomic absorption spectroscopy.

Sanui and Pace (169) used atomic absorption spectroscopy in the analysis of biological membrane materials for micromolar levels of physiologically important cations. Their results were highly satis-

factory, and demonstrated that atomic absorption spectroscopy affords biologists a powerful and sensitive tool for the study of the important role played by trace metals in living systems (169).

Slavin (170) analyzed biological materials for the determination of trace metals in tissue, urine, and blood by atomic absorption spectroscopy. Excellent results were obtained by this method for copper, iron, cobalt, magnesium, and zinc.

Adelstein and Vallee (171) reported that feeding excessive dietary zinc to rats markedly reduced liver cytochrome oxidase and catalase activity, and that the administration of small amounts of copper sulfate restores the enzymatic levels to normal. There apparently exists an antagonism between copper and zinc. According to Rouiller (172) trace metals occur mainly in periportal cells of human liver, with copper located in the parenchymal cells. Iron is stored intracellularly in the liver as hemosiderin and ferritin as previously reported (128). Xanthine oxidase, aldehyde dehydrogenase, choline dehydrogenase, and other metalloflavoproteins are found in the liver (44), Loewy and Siekevitz (173) reported that the liver is rich in mitochondria. Lehninger (174) stated that the cytochromes, located in the mitochondria, catalyze the transfer of electrons and hydrogen to oxygen to produce carbon dioxide and water, and yield energy in the form of ATP to drive metabolic reactions.

At the subcellular level between 0.1 and 0.6 equivalents of magnesium are bound to the ribosome per mole of ribonucleic acid (RNA) phosphorus. Magnesium may also be involved in binding template-

RNA and transfer-RNA to the ribosome. The ribosomes (microsomes) are found in the supernatant fraction (44, 51, 59, 130). According to Bowen (51) the elements are distributed within the cells as follows: calcium, manganese, magnesium, and possibly cobalt were concentrated in the nucleus; zinc appeared to be the lowest in the mitochondria, and iron was lowest in the nuclei. The distribution of 17 elements in subcellular particles from mammalian heart, isolated by centrifugation in sucrose solution, has been reported by Webster according to Bowen (51). Zinc and to a lesser extent cobalt, were concentrated in the nuclear fraction, while copper and iron were concentrated in the mitochondria. Mahler and Cordes (44) reported magnesium in the mitochondria. Bowen (51) reported copper in nuclear deoxyribonucleic acid (DNA) and mitochondria, iron in nuclear DNA and mitochondria, magnesium in mitochondria and nuclei, and zinc in mitochondria.

## CHAPTER II

## PURPOSE AND SCOPE

The purpose of the present investigation was to accumulate, study, elucidate, and amplify data concerning the effects of exposure of rats to low concentrations of CO as reflected by trace metal alterations.

This study was intended primarily to investigate changes, migrations, and pooling of trace metals (zinc, copper, cobalt, iron, magnesium) in various organs, liver fractions, urine, and blood serum and red blood cells of rats exposed over varying periods of time to low levels (50 ppm) of CO.

Control and experimental rat groups were handled alike as far as possible, except that the experimental group was exposed to controlled amounts of CO. Urine was collected from both groups regularly. At specified times, sub-groups were sacrificed for analysis of blood and organ systems.

The trace metal content of biological material obtained from experimental and control animals was measured on a Jarrell-Ash Atomic Absorption-Flame Emission Spectrophotometer. These data provided the basis for analysis.

The research project will provide:

1. Baseline data on the trace metal status of untreated rats.

2. Data to evaluate the effect of varying periods of exposure to low levels of CO on the trace metal status of rats. These data are not presently available in the literature and are necessary for the general study of trace metal metabolism.

A statistical model was developed to implement and expedite analysis of small changes in large amounts of data. The results from this investigation will be used to facilitate further studies in trace metal metabolism by the Institute of Environmental Health, School of Health, The University of Oklahoma Medical Center, Oklahoma City.

# CHAPTER III

## EQUIPMENT, METHODS AND PROCEDURE

The 58 animals used in this chronic CO exposure experiment consisted of young male Holtzman Strain albino rats, ranging in weight from 160 to 180 g each, at the inception of the study. They were purchased from the Cheek Jones Company of Houston, Texas, and were received in excellent health. They maintained this condition throughout the 12-week study, which started in mid-January and continued to May of 1968.

The rats were randomly placed in individual metabolic cages and acclimatized to the laboratory environment for a 2-week period. During this period they were examined for overt signs of illness.

After the 2-week acclimatization period, 23 rats were randomly selected as the control group, and 35 rats were similarly selected as the exposed group. All animals received Rockland Mouse Breeder diet and water ad libitum throughout the experiment. Each rat was weighed at the initiation of the study, every two weeks, and at sacrifice.

The chronically exposed rats were subjected to 50 ppm CO for 5 days a week, 5 hrs per day. Approximately 9 exposed and 6

control rats were sacrificed at the end of each 3-week period, until the experiment had terminated at the end of 12 weeks.

The exposure chambers consisted of three air tight tanks each having the dimensions of 5 x 3-1/2 x 3 ft. Each tank was operated at a slightly negative pressure and was capable of holding 12 metabolic cages without crowding. Oil-free compressed air and CO were premixed in a laboratory fabricated mixing chamber containing small glass beads, and this CO/Air mixture was diffused throughout the chambers through small holes in plastic pipe within each chamber.

To make certain that the tanks had proper air movement to maintain a stable flow of gas, each tank was equipped with a small electrical motor which functioned as an exhaust fan for the incoming gas. The tanks were balanced to maintain the 50 ppm CO level for 5 hrs. Approximately 0.125 cu ft per day of CO was required to maintain the 50 ppm level in each tank. Commercial grade CO having 98 per cent purity was purchased from the Matheson Scientific Co. and used as the exposure gas. Positive CO pressure was maintained by a single stage regulator. A Mine Safety Appliance (MSA) Carbon Monoxide tester (portable, colorimetric) in the 10 to 1,000 ppm range was used each hr to check the tank CO concentration. For a back-up method, a Komoyo 100 ml syringe with Kitagawa detector tubes was used. The Kitagawa Carbon Monoxide Detector tubes provided rapid accurate measurements of CO concentrations in the range of 25 to 6,000 ppm. The MSA and Kitagawa tester's CO gas concentrated results were within plus or minus 10 per cent of each other. Both methods operate on a

positive colorimetric detection principle. The drawn air sample contacts a chemical (palladium chloride) impregnated on silica gel in the detector tube. If CO is present in the air sample, an oxidation reaction takes place with the chemical changing color immediately. The color intensity indicates the CO concentration in the air sample. A revolving color scale beside the detector tube allows various shades of color to be brought into position for direct comparison with detector tube discoloration. A table supplied with the CO tester relates color intensity to gas concentration.

At the end of the 3rd week and each consecutive 3 weeks thereafter, approximately 9 exposed and 6 control rats were sacrificed by anesthetizing with diethyl ether. The abdominal cavity was opened, and the rat was exsanguinated by the insertion of a needle attached to a syringe into the abdominal aorta dorsal to the iliac arch. Blood volume, ranging from 5 to 11 ml per rat was then transferred to a test tube. All blood collecting equipment and centrifuge tubes were pretreated with heparin.

After the animals had been bled, the liver, brain, heart, kidneys, lungs, and spleen were removed and immediately perfused first with ice-cold 0.145 M NaCl, followed by perfusion with ice-cold 0.25 M sucrose. This technique removed most of the residual organ blood. The liver was weighed and then homogenized in cold 0.25 M sucrose in a Potter-Elvehjem type homogenizer with a Teflon pestle at 4°C. Cellular debris and nuclei were spun down in a Servall RC-2 type refrigerated differential centrifuge at 600 x g for 20 min at 4°C.

The supernatant was centrifuged at 10,000 x g for 20 min to sediment the mitochondria. The mitochondria so centrifuged were washed twice in cold 0.25 M sucrose, sedimenting at 10,000 x g after each wash. The final mitochondrial pellet was suspended in cold 0.25 M sucrose and refrigerated. The liver homogenate was obtained by adding 3 ml of cold 0.25 M sucrose per each gram of liver tissue and homogenizing for 1 min. This homogenizing procedure was a modification of the fractionation of animal tissue cells procedure found in the 1963 Biochemist's Handbook (59) and by O'Brien (168).

After removal and perfusion by cold 0.145 M NaCl and cold 0.25 M sucrose, the brain, heart, lungs, kidneys, and spleen were blotted dry, placed in tared beakers, and the wet weight was determined. The tissues were then dried in an oven for 16 hrs at 103.5°C, placed in a dessicator over night, and the dry and ash weights determined. The nuclear, mitochondrial, and supernatant liver fractions obtained from homogenization and centrifugation were also dried for 16 hrs at 103.5°C, and dry weight determined. The tissue and liver fractions were then ashed for 8 hrs at 500°C in an electric furnace. Care was taken to ash separately the control ~~ex~~ exposed materials, in order to eliminate cross contamination by trace metals. The ash weight was determined following overnight dessication. After determining the ash weight 0.3 N HCl was added to each container in the following ratios of mg/ash to ml/acid: brain, 3:1; lungs, 3:1; kidneys, 3:1; heart, 1:1; spleen, 1:1. Liver fraction ratios were: nuclear, 5:1; mitochondrial, 1:1; supernatant, 6:1. The acidified

containers containing ash were allowed to stand at least 24 hrs before trace metal analysis.

Urine was collected separately from each rat during the first 15 days of CO exposure for an individual rat urine study. After the 3rd week, each rat's urine was pooled for every 5 days of exposure and a pooled urine study was made on urine so collected, from the 3rd to 12th week. The urine was analyzed directly without any dilution.

The hematocrit ratio for each animal in each group was determined in capillary tubes centrifuged at 15,000 x g for 5 min. The remaining blood sample was separated into serum and red blood cells (RBC) by centrifugation at 5,000 x g for 15 min. One ml of serum was withdrawn and diluted with 5 parts of 0.3 N HCl, and 1 ml of the packed RBC was diluted with 35 parts of 0.3 N HCl. The acidified solutions were refrigerated, and the concentrated solutions were frozen.

By preparing blood fractions and tissues in this manner, direct aspiration of the samples into the burner of the atomic absorption unit, was easily facilitated, and resulted in more reproducible results with fewer mechanical difficulties than would have been encountered in analysis by other methods.

A set of standard solutions containing 10,000 µg/ml of zinc, copper, cobalt, iron, and magnesium respectively (Fisher Scientific Company, Fairlawn, New Jersey), were serially diluted with 0.3 N HCl in order to obtain standards in the concentration ranges anticipated. These dilutions were used to prepare standard curves covering the

indicated ranges: zinc, 0.312 to 2.5 µg/ml; copper, 0.156 to 1.25 µg/ml; cobalt, 0.312 to 2.5 µg/ml; iron, 0.625 to 25.0 µg/ml; and magnesium, 0.312 to 20 µg/ml. No strontium chloride was needed to overcome phosphate bonding.

All samples from the experimental animals (urine, brain, heart, kidneys, lungs, spleen, blood serum and RBC, and liver fractions) were analyzed for zinc, copper, cobalt, iron, and magnesium in that sequence.

These analyses were accomplished by utilizing a Model 82-362 Jarrell-Ash Atomic Absorption Flame Emission Spectrophotometer using a 0.5 meter Ebert monochrometer having a grating of 30,000 lines per inch in the ultraviolet range.

Hollow cathode tubes supplied the characteristic photons for each metal at the following amperages: zinc, 9.0; copper, 3.0; cobalt, 9.0; iron, 13.0; and magnesium, 5.0. An RCA photomultiplier tube Model IR 106 was operated at the following wavelengths and voltages: zinc, 2139 Å and 580; copper, 3246 Å and 500; cobalt, 2408 Å and 680; iron, 2483 Å and 580; and magnesium, 2851 Å and 500. Energy necessary for placing the atoms in a neutral state was furnished by a direct aspiration Hecto burner using hydrogen and air at the following pressures: zinc, hydrogen 15 psi and air 24 psi; copper, hydrogen 15 psi and air 24 psi; cobalt, hydrogen 17 psi and air 27 psi; iron, hydrogen 21 psi and air 21 psi; and magnesium, hydrogen 5 psi and air 20 psi.

A Beckman Model 1005 10 inch Recorder and Scale Expander

were used to record all trace metal per cent absorption signals from the Jarrell-Ash Atomic Absorption Flame Emission Spectrophotometer during analyses of trace metals in this investigation. Recorder and scale expansion parameters were as follows: power range, 1 mv; chart speed, 0.5 inches per min; linear/log function switch, linear position; and scale expansion, 1 x and 10 x.

The data were subjected to analysis of variance, and the various treatment groups and parameters were compared by using Duncan's New Multiple Range Test (175). A 0.05 statistical level of significance was chosen for this study. All statistical analyses were carried out under the direction of Dr. R. C. Duncan at the Computer Facility, the University of Oklahoma Medical Center, Oklahoma City.

# CHAPTER IV

## OBSERVATIONS AND DISCUSSION

Observations on control and exposed experimental rat groups were made over an extended period of 12 weeks. Both groups of rats were treated identically except the chronic group received a CO gas exposure of approximately 50 ppm, 5 days per week, 5 hrs per day. During the first week of exposure the exposed group appeared excitable and nervous, but returned to normal within a few days. There was no other noticeable behavioral manifestation. Both experimental groups appeared to be in good health throughout the experiment.

Approximately 9 chronic and 6 control rats were sacrificed every 3 weeks until the 12 week exposure period was terminated. The analyses were carried out on blood serum and RBC, pooled urine, organs, and liver nuclear, mitochondrial, and supernatant fractions.

This experiment was designed to establish baseline data and to examine the trace metal technique as a unique experimental test parameter of a potentially fruitful area for more detailed and specific future investigations at the molecular level. The following observations and discussion include interpretations of possible or probable metal translocation patterns, but verification of these

interpretations require a more detailed investigation.

This statistical study used analysis of variance for completely randomized design followed by Duncan's New Multiple Range Test (DNMRT). The analyses were done both within the treatment groups across the weeks of study and between the treatment groups for specific weeks. Time effects will not be reported in this dissertation although the data representing time changes are presented in the tables. Consideration of time changes were used in interpretation of the comparisons between control and exposed groups.

The specific data is located in the appendix. Tables 1-5 are whole organ trace metal concentration data, Tables 6-8 are liver fraction trace metal concentration data, Tables 9 and 10 are blood fraction trace metal concentration data, Table 11 is hematocrit data, Table 12 is pooled urine trace metal concentration data, Table 13 is the pooled urine volume data, Tables 14-16 are the whole organ weight data, Tables 17-19 are liver fraction weight data, and Table 20 is the rat body weight data.

## Brain

Exposure to 50 ppm of CO had no appreciable effects on rat brain weight parameters during the first 9 weeks. Sacrifice after 12 weeks demonstrated a nonsignificant decrease in brain wet weight and significant decreases in dry and ash weights. Only two isolated significant alterations in metal concentration occurred; copper was decreased at the end of 6 weeks and zinc was elevated at the end of 12 weeks. By the parameters examined, the brain was the least

sensitive to change of the organs studied.

## Heart

The heart size was found to be decreased only at the 6-week period. Zinc and copper appeared to be mobilized out of the heart throughout the first 6 weeks but the effect was not maintained during the last 6 weeks. Copper demonstrated no further changes and at the end of 12 weeks zinc showed an elevated concentration. Cobalt and iron were depressed only at the termination of 12 weeks. Magnesium demonstrated tendency toward a more consistent pattern of mobilization into the heart during the entire examination period.

## Kidneys

The kidney weight parameters were not appreciably changed as a response to 12 weeks of low-level CO exposure. Several trace metal concentration alterations were evidenced at each 3-week interval. Zinc concentration progressed from one change by week 3, to a depressed concentration by week 6. By the end of week 9 a resilience of zinc concentration had occurred to a higher than normal level. By the end of week 12, the kidney zinc concentration was compatible with control values. A nonsignificant rebound phenomenon in copper concentration appeared to be exhibited in that copper levels were elevated by week 3, no change at week 6, and depressed by the end of week 9 and week 12. The kidney was the only organ which showed any indication of a possible accumulation in copper and even this indication was transient at the week 3 period and was a nonsignificant

value at the 0.05 level. Cobalt accumulation by the kidney was apparent only during the initial 6 weeks with no further significant trace metal alterations. This increased migration of trace metals to the kidney was reflected in an increased urinary output during the 6 to 7 week period. Iron values showed no changes prior to week 12, at which time a diminished concentration was observed. At weeks 3, 6, and 9 kidney magnesium concentration values indicated a progressive accumulation becoming a highly significant value by the end of week 12.

## Lungs

The various weight measurements of the lungs during the course of the investigation indicated an initial small increase in size by week 3, and thereafter it appeared to be depressed in size. Zinc concentration was significantly decreased at 3 weeks but not altered from control values thereafter. During the first 9 weeks copper appeared to migrate out of the lungs, and by the end of 12 weeks, the copper concentration had returned to a normal level. Prolonged low-level CO inhalation appeared to have little if any effect on lung iron concentration. Magnesium translocation into the lungs appeared to be similar in magnitude but opposite in direction to that observed in the heart during weeks 3 and 6.

## Spleen

During the course of the 12-week low-level CO exposure, the spleen demonstrated no evidence of significant alterations in wet, dry, and ash weights. Although the results were not significant at the 0.05 level, zinc concentration values were increased at each of the 4

exposure periods (weeks 3, 6, 9, 12), and one could speculate that higher CO exposure concentrations or longer exposure periods at 50 ppm CO might result in a significant increase in the spleen zinc concentration. Copper, cobalt, and iron remained relatively unaltered in the spleen under the experimental conditions. As in the heart, kidney, and lungs, the spleen magnesium concentration values demonstrated evidence of a migration into the spleen.

## Liver Fractions

The weight parameters recorded in the appendix for the liver nuclear, mitochondrial, and supernatant fractions little apparent value outside ash weight considerations deriving metal concentrations other than demonstrating that a consistency in experimental technique resulted. Nuclear zinc was altered appreciably only during week 6 and this alteration was an increase. Mitochondrial zinc was nonsignificantly decreased by week 3 and remained at this lowered level for each of the other 3-week intervals. Supernatant zinc was depressed at 3 and 6 weeks but was significantly elevated by week 9 with no appreciable change from control values by week 12.

Nuclear copper appeared to migrate out of this fraction, but was not appreciably altered in the mitochondrial fraction, and was lost from the supernatant only during the first 6 weeks. In general, copper appeared to be removed from the liver nuclear and supernatant fractions, while the mitochondrial content remained unchanged.

Cobalt was significantly lowered in concentration in all three liver fractions, indicating a rather sharp depression in total liver

cobalt stores. Significantly increased cobalt concentration was observed in urine at week 5 and week 7.

Iron followed its own unique pattern. During weeks 3 through 6 there was no appreciable alteration in nuclear iron concentration, while the mitochondrial and supernatant concentrations were concomitantly decreased. This early decrease was completely reversed by the end of week 9 and week 12, since iron concentrations were increased above control values in all three liver fractions.

Magnesium was elevated in the nuclear fraction, apparently unchanged in the mitochondrial fraction. The supernatant fraction lost magnesium during the first 6 weeks.

Generally, the nuclear fraction demonstrated losses of copper and cobalt and a postponed accumulation of iron. The mitochondrial fraction yielded no indication of metal accumulation, no apparent change in copper and magnesium, but losses of zinc, cobalt and iron were observed. An examination of supernatant fraction data strongly suggests an initial loss of all 5 metals persisting throughout the first 6 weeks). By week 9, with the exception of cobalt, compensation for the initial loss had occurred so much so that all 4 metals (Zinc, copper, iron,and magnesium) are significantly elevated above control concentrations. By week 12 all supernatant fraction metal concentrations had returned to control values.

## Blood Fractions

The hematocrit was significantly altered only at the week 9 observation. This alteration was shown as an increase. In contrast

to the obvious metallic translocations occurring in the various organs, none of these translocations were evidenced in the plasma. The packed RBC yielded a significantly depressed zinc level only at the 6-week period, and no significant change in copper was noted. Cobalt levels were not sufficiently sampled in the blood serum and RBC due to instrument limitations. Red blood cell iron levels remained appreciably unchanged for the first 6 weeks after which they were significantly depressed. The observed depression in RBC magnesium concentrations indicated a possible source required to meet the demands of elevated magnesium levels in the heart, kidneys, lungs, spleen, and liver nuclear fraction.

## Urine

An effect of CO exposure was manifested as a decreased urinary volume. Since the concentrations of copper, iron and magnesium remained unaltered, the total daily excretion of these three metals decreased. However, the concentrations of zinc and cobalt were increased for as long as 8 weeks, and in spite of the decreased urinary volume, the total daily excretion of zinc and cobalt was generally increased during the first half of the exposure period. This would suggest the probability of a net loss of these two metals to the rat as the result of translocations occurring from a source(s) other than the blood (which did not change). Since zinc is primarily excreted in the feces, any increase in urinary zinc is usually considered to be a direct result of kidney zinc loss. The time period of the increased urinary zinc is consistent with the observed loss of kidney zinc.

## Zinc

In general zinc appeared to migrate out of the liver (mitochondrial and supernatant fractions), and to a lesser degree, out of the kidney. These decreases in zinc levels appeared to result from a translocation of zinc into the spleen and urine. Probably all the urinary zinc had the kidney as its source, and under the parameters examined this would indicate the liver as being the major source of the splenic zinc increases resulting from the effects of chronic CO exposure.

## Copper

Copper appeared to migrate out of the liver (nuclear and supernatant fractions), heart, and lungs, while the kidneys and spleen concentrations were not appreciably affected. The potential receptor of this mobilized copper was not suggested since the serum, RBC, and urine concentrations remained relatively unchanged.

## Cobalt

The spleen, brain, and heart demonstrated no appreciable accumulation or loss of cobalt. Although the spleen showed a statistically significant change during week 12 the biological meaningfulness is not clear. It would appear that relatively massive quantities of cobalt were lost from the liver, some of which may have been utilized in the small accumulation of this metal by the lungs. A reasonable speculation would be that this mobilization of liver cobalt is apparently collected and concentrated (at least in part and limited to the

first 7 weeks) by the kidneys and subsequently excreted in the urine.

Analysis of cobalt in bone marrow was not carried out, therefore it is not known if the cobalt migration from the liver had an effect upon the erythropoietic RBC stimulating system in the bone marrow, but since there was a statistically significant increase in the cobalt level in the spleen by week 12, there exists the possibility that cobalt is required in the spleen after a prolonged chronic CO exposure. The lungs also showed a statistically significant cobalt increase by 12 weeks of exposure.

## Iron

Other than in the liver and RBC, no iron translocation pattern seems to be suggested. It would appear that during the first 6 weeks iron is lost from the liver (mitochondrial and supernatant fractions). During the latter 6 weeks iron migrated into all liver fractions in concentrations greater than control quantities with significant increases in nuclear and supernatant fraction iron during week 9. The data suggests the RBC as a major source of iron during the latter 6 weeks in which a high demand for iron occurred.

## Magnesium

Prolonged and extensive translocations appeared in the magnesium data. Depletion of RBC and of the liver supernatant fraction could be conceived as sources of the increased magnesium concentrations observed in the liver nuclear fraction and the heart, kidneys, lungs, and spleen. Since an elevated quantity of magnesium is apparently

required, and since urinary magnesium concentration was not appreciably changed while urinary volume decreased, it would seem as a reasonable speculation that the total urinary magnesium excretion was decreased in order to partly supply this apparently elevated requirement.

# CHAPTER V

## SUMMARY

This investigation was concerned with the effects of chronic carbon monoxide (CO) exposure to rats as reflected by trace metal alterations. The concentrations of zinc, copper, cobalt, iron, and magnesium were determined in the brain, heart, kidneys, lungs, spleen, liver nuclear fraction, liver mitochondrial fraction, liver supernatant fraction, blood serum, packed RBC, and urine. Approximately 9 chronic and 6 control rats were sacrificed every 3 weeks until the 12-week exposure period was terminated. All groups of rats were treated identical except the chronic group received a CO gas exposure of approximately 50 ppm, 5 hrs per day, 5 days per week.

Based on statistical analyses of over 5,200 determinations made throughout this investigation, the following generalized conclusions have been drawn for the effects of chronic CO exposure as compared to their respective controls:

1. In general, CO exposure had small effects on organ weight parameters which were reflected as a diminution of size, being primarily noted in the lungs.
2. Zinc appeared to migrate out of the liver (mitochondrial and supernatant fractions), and to a lesser degree out

of the kidney, heart and lungs. These decreases in zinc levels appeared to result from a translocation of zinc into the spleen and urine.

3. Copper appeared to be mobilized out of the liver (nuclear and supernatant fractions), heart and lungs while the kidneys, spleen and brain concentrations were not appreciably affected. The potential receptor of the mobilized copper was not suggested sinch the plasma, RBC, and urine concentrations remained relatively unchanged.
4. The spleen, brain, and heart demonstrated no appreciable accumulation or loss of cobalt. It would appear that relatively massive quantities of cobalt were lost from the liver, some of which may have been utilized in the small accumulation of this metal by the lungs. During the first 6 weeks, the kidney increased its cobalt concentration and urinary cobalt concentration also increased.
5. No pattern of iron translocation seemed to be apparent other than in the liver and RBC. It would appear that during the first 6 weeks iron is lost from the liver (mitochondrial and supernatant fractions), and during the last 6 weeks iron migrates into all liver fractions in concentrations greater than control quantities. During this latter time period, the RBC iron concentration decreases.
6. Prolonged and extensive translocations appear in the

magnesium data. Depletion of RBC and of the liver supernatant fraction could be conceived as sources of the increased magnesium concentration observed in the liver nuclear fraction, heart, kidneys, lungs, and spleen.

7. As might be expected, each organ presented an individual pattern of trace metal content and changes due to chronic CO exposure. The various liver fractions had appeared to be the most responsive to metal translocations while the brain and blood serum were the least responsive.
8. The level of CO exposure (50 ppm) used in this experiment is the currently accepted "no effect" Threshold Limit Value; however it would appear that certain effects do, in fact occur, and that these effects may be precursors to more clinically manifested effects. Further investigation is strongly indicated.

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# APPENDIX

TABLE 1

COMPARISON OF TRACE METAL CONCENTRATION (ug/ml) IN BRAIN BY WEEK AND TREATMENT GROUP

| Metal | Week | Control | | | Exposed | | | Significance Level of Difference |
|---|---|---|---|---|---|---|---|---|
| | | Mean | Variance | N | Mean | Variance | N | |
| Zinc | 3 | 1.173 | 0.0706 | 5 | 1.408 | 0.0629 | 9 | n.s. |
| | 6 | 1.566 | 0.0484 | 6 | 1.468 | 0.0129 | 9 | n.s. |
| | 9 | 1.544 | 0.0120 | 6 | 1.519 | 0.0043 | 9 | n.s. |
| | 12 | 1.559 | 0.0316 | 6 | 1.710 | 0.0203 | 8 | $p<.005$ |
| Copper | 3 | 0.175 | 0.0053 | 5 | 0.180 | 0.0018 | 9 | n.s. |
| | 6 | 0.158 | 0.0005 | 6 | 0.134 | 0.0003 | 9 | $p<.050$ |
| | 9 | 0.122 | 0.0004 | 6 | 0.128 | 0.0002 | 9 | n.s. |
| | 12 | 0.179 | 0.0009 | 6 | 0.199 | 0.0006 | 8 | n.s. |
| Cobalt | 3 | - | - | - | 0.055 | 0.0047 | 9 | - |
| | 6 | 0.182 | 0.0040 | 6 | 0.180 | 0.0028 | 9 | n.s. |
| | 9 | 0.106 | 0.0013 | 6 | 0.103 | 0.0020 | 9 | n.s. |
| | 12 | 0.133 | 0.0045 | 6 | 0.142 | 0.0026 | 8 | n.s. |
| Iron | 3 | 0.585 | 0.0054 | 5 | 0.649 | 0.0267 | 9 | n.s. |
| | 6 | 0.751 | 0.0674 | 6 | 0.953 | 0.0720 | 9 | n.s. |
| | 9 | 0.983 | 0.0192 | 6 | 1.015 | 0.0267 | 9 | n.s. |
| | 12 | 0.785 | 0.0111 | 6 | 0.751 | 0.0065 | 8 | n.s. |
| Magnesium | 3 | 0.371 | 0.0139 | 5 | 0.292 | 0.0010 | 9 | n.s. |
| | 6 | 0.223 | 0.0065 | 6 | 0.283 | 0.0073 | 9 | n.s. |
| | 9 | 0.291 | 0.0036 | 6 | 0.248 | 0.0025 | 9 | n.s. |
| | 12 | 0.239 | 0.0091 | 6 | 0.240 | 0.0013 | 8 | n.s. |

TABLE 2

COMPARISON OF TRACE METAL CONCENTRATION (ug/ml) IN HEART BY WEEK AND TREATMENT GROUP

| Metal | Week | Control | | | Exposed | | | Significance Level of Difference |
|---|---|---|---|---|---|---|---|---|
| | | Mean | Variance | N | Mean | Variance | N | |
| Zinc | 3 | 1.167 | 0.0051 | 5 | 1.019 | 0.0072 | 9 | $p<.010$ |
| | 6 | 1.368 | 0.0027 | 6 | 1.363 | 0.0096 | 9 | n.s. |
| | 9 | 1.254 | 0.0012 | 6 | 1.266 | 0.0016 | 9 | n.s. |
| | 12 | 1.304 | 0.0021 | 6 | 1.356 | 0.0016 | 8 | $p<.050$ |
| Copper | 3 | 0.343 | 0.0005 | 5 | 0.255 | 0.0007 | 9 | $p<.005$ |
| | 6 | 0.316 | 0.0004 | 6 | 0.256 | 0.0095 | 9 | n.s. |
| | 9 | 0.303 | 0.0003 | 6 | 0.303 | 0.0008 | 9 | n.s. |
| | 12 | 0.288 | 0.0006 | 6 | 0.309 | 0.0010 | 8 | n.s. |
| Cobalt | 3 | - | - | - | 0.026 | 0.0029 | 9 | - |
| | 6 | 0.193 | 0.0061 | 6 | 0.172 | 0.0024 | 9 | n.s. |
| | 9 | 0.068 | 0.0083 | 6 | 0.098 | 0.0010 | 9 | n.s. |
| | 12 | 0.102 | 0.0014 | 6 | 0.012 | 0.0006 | 8 | $p<.005$ |
| Iron | 3 | 1.787 | 0.0744 | 5 | 1.706 | 0.0423 | 9 | n.s. |
| | 6 | 2.111 | 0.0635 | 6 | 2.832 | 2.6022 | 9 | n.s. |
| | 9 | 2.556 | 0.0063 | 6 | 2.411 | 0.0416 | 9 | n.s. |
| | 12 | 1.809 | 0.0120 | 6 | 1.583 | 0.0173 | 8 | $p<.010$ |
| Magnesium | 3 | 0.514 | 0.0015 | 5 | 0.371 | 0.0084 | 9 | $p<.010$ |
| | 6 | 0.307 | 0.0003 | 6 | 0.456 | 0.0145 | 9 | $p<.025$ |
| | 9 | 0.359 | 0.0005 | 6 | 0.391 | 0.0019 | 9 | n.s. |
| | 12 | 0.310 | 0.0027 | 6 | 0.431 | 0.0061 | 8 | $p<.010$ |

TABLE 3

COMPARISON OF TRACE METAL CONCENTRATION (μg/ml) IN KIDNEY BY WEEK AND TREATMENT GROUP

| Metal | Week | Control | | | Exposed | | | Significance Level of Difference |
|---|---|---|---|---|---|---|---|---|
| | | Mean | Variance | N | Mean | Variance | N | |
| Zinc | 3 | 2.987 | 0.0033 | 5 | 3.019 | 0.0160 | 9 | n.s. |
| | 6 | 3.519 | 0.0078 | 6 | 3.326 | 0.0038 | 9 | $p<.005$ |
| | 9 | 3.054 | 0.0049 | 6 | 3.191 | 0.0127 | 9 | $p<.025$ |
| | 12 | 3.429 | 0.0079 | 6 | 3.209 | 0.0894 | 8 | n.s. |
| Copper | 3 | 0.767 | 0.0371 | 5 | 0.965 | 0.0244 | 9 | n.s. |
| | 6 | 0.996 | 0.0298 | 6 | 0.974 | 0.0469 | 9 | n.s. |
| | 9 | 1.199 | 0.6255 | 6 | 0.943 | 0.0673 | 9 | n.s. |
| | 12 | 3.974 | 0.0638 | 6 | 1.171 | 0.0433 | 8 | n.s. |
| Cobalt | 3 | 0.013 | 0.0009 | 5 | 0.105 | 0.0029 | 9 | $p<.010$ |
| | 6 | 0.112 | 0.0036 | 6 | 0.207 | 0.0075 | 9 | $p<.050$ |
| | 9 | 0.177 | 0.0031 | 6 | 0.139 | 0.0007 | 9 | n.s. |
| | 12 | 0.110 | 0.0037 | 6 | 0.106 | 0.0041 | 8 | n.s. |
| Iron | 3 | 3.287 | 0.0717 | 5 | 3.648 | 0.4658 | 9 | n.s. |
| | 6 | 4.179 | 0.5776 | 6 | 4.481 | 0.6359 | 9 | n.s. |
| | 9 | 5.333 | 1.6066 | 6 | 5.341 | 0.5490 | 9 | n.s. |
| | 12 | 4.606 | 0.4330 | 6 | 3.493 | 0.2631 | 8 | $p<.005$ |
| Magnesium | 3 | 0.571 | 0.0005 | 5 | 0.606 | 0.0031 | 9 | n.s. |
| | 6 | 0.400 | 0.0070 | 6 | 0.474 | 0.0035 | 9 | n.s. |
| | 9 | 0.445 | 0.0031 | 6 | 0.468 | 0.0034 | 9 | n.s. |
| | 12 | 0.406 | 0.0017 | 6 | 0.519 | 0.0080 | 8 | $p<.025$ |

TABLE 4

COMPARISON OF TRACE METAL CONCENTRATION (µg/ml) IN LUNGS BY WEEK AND TREATMENT GROUP

| Metal | Week | Control | | | Exposed | | | Significance Level of Difference |
|---|---|---|---|---|---|---|---|---|
| | | Mean | Variance | N | Mean | Variance | N | |
| Zinc | 3 | 2.601 | 0.0612 | 5 | 2.235 | 0.0106 | 9 | p<.005 |
| | 6 | 2.734 | 0.0030 | 6 | 2.804 | 0.0784 | 9 | n.s. |
| | 9 | 2.583 | 0.0307 | 6 | 2.563 | 0.0944 | 9 | n.s. |
| | 12 | 2.780 | 0.0145 | 6 | 2.823 | 1.2263 | 8 | n.s. |
| Copper | 3 | 0.159 | 0.0030 | 5 | 0.120 | 0.0026 | 9 | n.s. |
| | 6 | 0.180 | 0.0045 | 6 | 0.124 | 0.0022 | 9 | n.s. |
| | 9 | 0.174 | 0.0012 | 6 | 0.125 | 0.0006 | 9 | p<.010 |
| | 12 | 0.167 | 0.0021 | 6 | 0.191 | 0.0011 | 8 | n.s. |
| Cobalt | 3 | 0.067 | 0.0056 | 5 | 0.102 | 0.0029 | 9 | n.s. |
| | 6 | 0.150 | 0.0004 | 6 | 0.139 | 0.0060 | 9 | n.s. |
| | 9 | 0.107 | 0.0007 | 6 | 0.141 | 0.0011 | 9 | n.s. |
| | 12 | 0.038 | 0.0003 | 6 | 0.111 | 0.0007 | 8 | p<.005 |
| Iron | 3 | 3.377 | 0.1733 | 5 | 2.801 | 0.0789 | 9 | p<.010 |
| | 6 | 4.694 | 0.1955 | 6 | 4.591 | 0.6933 | 9 | n.s. |
| | 9 | 4.389 | 0.2808 | 6 | 4.697 | 0.3374 | 9 | n.s. |
| | 12 | 4.936 | 0.4680 | 6 | 4.743 | 0.1367 | 8 | n.s. |
| Magnesium | 3 | 0.510 | 0.0011 | 5 | 0.574 | 0.0019 | 9 | p<.025 |
| | 6 | 0.503 | 0.0033 | 6 | 0.368 | 0.0082 | 9 | p<.010 |
| | 9 | 0.380 | 0.0021 | 6 | 0.427 | 0.0042 | 9 | n.s. |
| | 12 | 0.379 | 0.0035 | 6 | 0.531 | 0.0032 | 8 | p<.005 |

TABLE 5

COMPARISON OF TRACE METAL CONCENTRATION (µg/ml) IN SPLEEN BY WEEK AND TREATMENT GROUP

| Metal | Week | Control | | | Exposed | | | Significance Level of Difference |
|---|---|---|---|---|---|---|---|---|
| | | Mean | Variance | N | Mean | Variance | N | |
| Zinc | 3 | 1.007 | 0.0085 | 5 | 1.083 | 0.0742 | 9 | n.s. |
| | 6 | 1.189 | 0.0056 | 6 | 1.569 | 0.1896 | 9 | n.s. |
| | 9 | 1.101 | 0.0006 | 6 | 1.126 | 0.0020 | 9 | n.s. |
| | 12 | 1.088 | 0.0034 | 6 | 1.144 | 0.0033 | 8 | n.s. |
| Copper | 3 | 0.034 | 0.0001 | 5 | 0.043 | 0.0003 | 9 | n.s. |
| | 6 | 0.050 | 0.0005 | 6 | 0.086 | 0.0138 | 9 | n.s. |
| | 9 | 0.047 | 0.0004 | 6 | 0.060 | 0.0003 | 9 | n.s. |
| | 12 | 0.055 | 0.0002 | 6 | 0.052 | 0.0003 | 8 | n.s. |
| Cobalt | 3 | 0.225 | 0.2133 | 5 | 0.083 | 0.0016 | 9 | n.s. |
| | 6 | 0.107 | 0.0031 | 6 | 0.081 | 0.0066 | 9 | n.s. |
| | 9 | 0.086 | 0.0007 | 6 | 0.073 | 0.0041 | 9 | n.s. |
| | 12 | 0.038 | 0.0028 | 6 | 0.109 | 0.0031 | 8 | $p<.050$ |
| Iron | 3 | 4.839 | 0.1280 | 5 | 4.716 | 1.3719 | 9 | n.s. |
| | 6 | 6.296 | 0.8072 | 6 | 4.909 | 4.5271 | 9 | n.s. |
| | 9 | 4.926 | 1.1135 | 6 | 7.172 | 5.8694 | 9 | n.s. |
| | 12 | 7.033 | 2.8066 | 6 | 7.737 | 27.7548 | 8 | n.s. |
| Magnesium | 3 | 0.755 | 0.0023 | 5 | 0.893 | 0.0249 | 9 | n.s. |
| | 6 | 0.846 | 0.0535 | 6 | 0.652 | 0.0110 | 9 | $p<.050$ |
| | 9 | 0.546 | 0.0044 | 6 | 0.704 | 0.0286 | 9 | $p<.050$ |
| | 12 | 0.816 | 0.1047 | 6 | 0.964 | 0.1870 | 8 | n.s. |

TABLE 6

COMPARISON OF TRACE METAL CONCENTRATION (μg/ml) IN LIVER NUCLEAR FRACTION BY WEEK AND TREATMENT GROUP

| Metal | Week | Control | | | Exposed | | | Significance Level of Difference |
|---|---|---|---|---|---|---|---|---|
| | | Mean | Variance | N | Mean | Variance | N | |
| Zinc | 3 | 3.021 | 0.0059 | 5 | 2.847 | 0.3612 | 9 | n.s. |
| | 6 | 3.414 | 0.0367 | 6 | 4.108 | 0.0794 | 9 | p<.005 |
| | 9 | 2.768 | 0.0680 | 6 | 2.466 | 0.1483 | 9 | n.s. |
| | 12 | 1.996 | 0.0720 | 6 | 2.287 | 0.0984 | 8 | n.s. |
| Copper | 3 | 0.380 | 0.0295 | 5 | 0.267 | 0.0196 | 9 | n.s. |
| | 6 | 0.420 | 0.0187 | 6 | 0.454 | 0.0413 | 9 | n.s. |
| | 9 | 0.146 | 0.0039 | 6 | 0.101 | 0.0027 | 9 | n.s. |
| | 12 | 0.321 | 0.0140 | 6 | 0.169 | 0.0040 | 8 | p<.010 |
| Cobalt | 3 | 0.115 | 0.0012 | 5 | 0.034 | 0.0020 | 9 | p<.005 |
| | 6 | 0.128 | 0.0040 | 6 | 0.052 | 0.0012 | 9 | p<.010 |
| | 9 | 0.120 | 0.0013 | 6 | 0.062 | 0.0020 | 9 | p<.025 |
| | 12 | 0.116 | 0.0024 | 6 | 0.058 | 0.0009 | 8 | p<.025 |
| Iron | 3 | 2.865 | 0.0351 | 5 | 2.842 | 0.3397 | 9 | n.s. |
| | 6 | 4.989 | 2.1776 | 6 | 4.524 | 0.3204 | 9 | n.s. |
| | 9 | 3.283 | 0.3801 | 6 | 4.328 | 0.4400 | 9 | p<.010 |
| | 12 | 3.798 | 0.5459 | 6 | 4.103 | 0.5465 | 8 | n.s. |
| Magnesium | 3 | 0.466 | 0.0218 | 5 | 0.707 | 0.0116 | 9 | p<.005 |
| | 6 | 0.570 | 0.0217 | 6 | 0.638 | 0.0099 | 9 | n.s. |
| | 9 | 0.423 | 0.0079 | 6 | 0.495 | 0.0047 | 9 | n.s. |
| | 12 | 0.364 | 0.0039 | 6 | 0.483 | 0.0026 | 8 | p<.005 |

TABLE 7

COMPARISON OF TRACE METAL CONCENTRATION (ug/ml) LIVER MITOCHONDRIAL FRACTION BY WEEK AND TREATMENT GROUP

| Metal | Week | Control | | | Exposed | | | Significance Level of Difference |
|---|---|---|---|---|---|---|---|---|
| | | Mean | Variance | N | Mean | Variance | N | |
| Zinc | 3 | 0.713 | 0.0566 | 5 | 0.526 | 0.1422 | 9 | n.s. |
| | 6 | 0.988 | 0.0695 | 6 | 0.729 | 0.0836 | 9 | n.s. |
| | 9 | 1.211 | 1.4773 | 6 | 0.868 | 0.0776 | 9 | n.s. |
| | 12 | 0.707 | 0.0127 | 6 | 0.737 | 0.0672 | 8 | n.s. |
| Copper | 3 | 0.007 | 0.0002 | 5 | 0.011 | 0.0002 | 9 | n.s. |
| | 6 | 0.031 | 0.0001 | 6 | 0.021 | 0.0001 | 9 | n.s. |
| | 9 | 0.031 | 0.0001 | 6 | 0.025 | 0.0007 | 9 | n.s. |
| | 12 | 0.094 | 0.0023 | 6 | 0.025 | 0.0001 | 8 | $p<.005$ |
| Cobalt | 3 | 0.058 | 0.0028 | 5 | 0.057 | 0.0054 | 9 | n.s. |
| | 6 | 0.134 | 0.0005 | 6 | 0.019 | 0.0007 | 9 | $p<.005$ |
| | 9 | 0.081 | 0.0010 | 6 | 0.024 | 0.0006 | 9 | $p<.005$ |
| | 12 | 0.086 | 0.0009 | 6 | 0.054 | 0.0019 | 8 | n.s. |
| Iron | 3 | 3.079 | 2.1207 | 5 | 1.050 | 0.5718 | 9 | $p<.005$ |
| | 6 | 3.561 | 0.3701 | 6 | 2.142 | 2.1899 | 9 | $p<.050$ |
| | 9 | 1.703 | 0.5139 | 6 | 2.199 | 0.5632 | 9 | n.s. |
| | 12 | 3.273 | 0.2218 | 6 | 2.661 | 1.0405 | 8 | n.s. |
| Magnesium | 3 | 0.708 | 0.0218 | 5 | 0.395 | 0.0410 | 9 | $p<.010$ |
| | 6 | 0.467 | 0.0140 | 6 | 0.416 | 0.0089 | 9 | n.s. |
| | 9 | 0.347 | 0.0029 | 6 | 0.361 | 0.0033 | 9 | n.s. |
| | 12 | 0.393 | 0.0067 | 6 | 0.421 | 0.0062 | 8 | n.s. |

TABLE 8

COMPARISON OF TRACE METAL CONCENTRATION (µg/ml) LIVER SUPERNATANT FRACTION BY WEEK AND TREATMENT GROUP

| Metal | Week | Control | | | Exposed | | | Significance Level of Difference |
|---|---|---|---|---|---|---|---|---|
| | | Mean | Variance | N | Mean | Variance | N | |
| Zinc | 3 | 5.499 | 0.0084 | 5 | 4.804 | 0.5686 | 9 | n.s. |
| | 6 | 5.670 | 0.0043 | 6 | 4.465 | 1.7286 | 9 | $p<.050$ |
| | 9 | 5.794 | 0.0084 | 6 | 6.118 | 0.0102 | 9 | $p<.005$ |
| | 12 | 5.674 | 0.0117 | 6 | 5.678 | 0.0111 | 8 | n.s. |
| Copper | 3 | 1.075 | 0.0366 | 5 | 0.481 | 0.1341 | 9 | $p<.010$ |
| | 6 | 1.139 | 0.2678 | 6 | 0.644 | 0.3821 | 9 | n.s. |
| | 9 | 0.444 | 0.0161 | 6 | 1.033 | 0.0890 | 9 | $p<.005$ |
| | 12 | 1.111 | 0.0748 | 6 | 1.026 | 0.0344 | 8 | n.s. |
| Cobalt | 3 | 0.071 | 0.0019 | 5 | 0.031 | 0.0033 | 9 | n.s. |
| | 6 | 0.103 | 0.0008 | 6 | 0.038 | 0.0006 | 9 | $p<.005$ |
| | 9 | 0.109 | 0.0046 | 6 | 0.050 | 0.0005 | 9 | $p<.050$ |
| | 12 | 0.091 | 0.0005 | 6 | 0.094 | 0.0032 | 8 | n.s. |
| Iron | 3 | 7.080 | 13.6970 | 5 | 5.711 | 2.2911 | 9 | n.s. |
| | 6 | 13.116 | 3.5830 | 6 | 7.811 | 15.5911 | 9 | $p<.010$ |
| | 9 | 8.487 | 3.3115 | 6 | 15.135 | 3.2790 | 9 | $p<.005$ |
| | 12 | 11.813 | 2.4183 | 6 | 12.306 | 2.8519 | 8 | n.s. |
| Magnesium | 3 | 1.423 | 0.0255 | 5 | 0.929 | 0.0212 | 9 | $p<.005$ |
| | 6 | 1.411 | 0.0284 | 6 | 0.423 | 0.0905 | 9 | $p<.005$ |
| | 9 | 0.847 | 0.0223 | 6 | 1.188 | 0.0226 | 9 | $p<.005$ |
| | 12 | 1.139 | 0.0256 | 6 | 1.259 | 0.0237 | 8 | n.s. |

TABLE 9

COMPARISON OF TRACE METAL CONCENTRATION (μg/ml) IN BLOOD SERUM BY WEEK AND TREATMENT GROUP

| Metal | Week | Control | | | Exposed | | | Significance Level of Difference |
|---|---|---|---|---|---|---|---|---|
| | | Mean | Variance | N | Mean | Variance | N | |
| Zinc | 3 | 0.219 | 0.0015 | 5 | 0.224 | 0.0043 | 9 | n.s. |
| | 6 | 0.216 | 0.0009 | 6 | 0.206 | 0.0010 | 9 | n.s. |
| | 9 | 0.191 | 0.0007 | 6 | 0.219 | 0.0005 | 9 | n.s. |
| | 12 | 0.174 | 0.0006 | 6 | 0.177 | 0.0002 | 8 | n.s. |
| Copper | 3 | 0.151 | 0.0018 | 5 | 0.199 | 0.0017 | 9 | n.s. |
| | 6 | 0.119 | 0.0006 | 6 | 0.126 | 0.0006 | 9 | n.s. |
| | 9 | 0.133 | 0.0004 | 6 | 0.155 | 0.0005 | 9 | n.s. |
| | 12 | 0.184 | 0.0016 | 6 | 0.161 | 0.0008 | 8 | n.s. |
| Cobalt | 3 | - | - | - | - | - | - | - |
| | 6 | - | - | - | - | - | - | - |
| | 9 | - | - | - | - | - | - | - |
| | 12 | - | - | - | - | - | - | - |
| Iron | 3 | 0.576 | 0.0233 | 5 | 0.657 | 0.0195 | 9 | n.s. |
| | 6 | 0.422 | 0.0224 | 6 | 0.521 | 0.0607 | 9 | n.s. |
| | 9 | 0.574 | 0.0122 | 6 | 0.484 | 0.0291 | 9 | n.s. |
| | 12 | 0.377 | 0.0301 | 6 | 0.259 | 0.0074 | 8 | n.s. |
| Magnesium | 3 | 1.192 | 0.0076 | 5 | 1.044 | 0.0855 | 9 | n.s. |
| | 6 | 2.236 | 0.0954 | 6 | 2.149 | 0.0188 | 9 | n.s. |
| | 9 | 1.826 | 0.0515 | 6 | 1.614 | 0.0462 | 9 | n.s. |
| | 12 | 1.944 | 0.0205 | 6 | 2.122 | 0.0443 | 8 | n.s. |

TABLE 10

COMPARISON OF TRACE METAL CONCENTRATION (µg/ml) IN BLOOD RBC BY WEEK AND TREATMENT GROUP

| Metal | Week | Control | | | Exposed | | | Significance Level of Difference |
|---|---|---|---|---|---|---|---|---|
| | | Mean | Variance | N | Mean | Variance | N | |
| Zinc | 3 | 0.296 | 0.0005 | 5 | 0.444 | 0.1078 | 9 | n.s. |
| | 6 | 0.250 | 0.0001 | 6 | 0.219 | 0.0004 | 9 | $p<.025$ |
| | 9 | 0.267 | 0.0009 | 6 | 0.283 | 0.0011 | 9 | n.s. |
| | 12 | 0.275 | 0.0001 | 6 | 0.290 | 0.0003 | 8 | n.s. |
| Copper | 3 | 0.047 | 0.0003 | 5 | 0.046 | 0.0001 | 9 | n.s. |
| | 6 | 0.046 | 0.0002 | 6 | 0.023 | 0.0005 | 9 | n.s. |
| | 9 | 0.024 | 0.0002 | 6 | 0.040 | 0.0004 | 9 | n.s. |
| | 12 | 0.012 | 0.0002 | 6 | 0.149 | 0.0553 | 8 | n.s. |
| Cobalt | 3 | - | - | - | - | - | - | - |
| | 6 | - | - | - | - | - | - | - |
| | 9 | - | - | - | - | - | - | - |
| | 12 | - | - | - | - | - | - | - |
| Iron | 3 | 10.120 | 2.7520 | 5 | 8.238 | 9.1423 | 9 | n.s. |
| | 6 | 1.553 | 0.0922 | 6 | 2.205 | 0.8876 | 9 | n.s. |
| | 9 | 5.183 | 4.7816 | 6 | 3.005 | 0.5477 | 9 | $p<.025$ |
| | 12 | 7.849 | 5.5340 | 6 | 5.168 | 0.4925 | 8 | $p<.010$ |
| Magnesium | 3 | 0.316 | 0.0002 | 5 | 0.241 | 0.0019 | 9 | $p<.005$ |
| | 6 | 1.231 | 0.0829 | 6 | 1.047 | 0.0789 | 9 | n.s. |
| | 9 | 0.708 | 0.0215 | 6 | 0.673 | 0.0414 | 9 | n.s. |
| | 12 | 0.804 | 0.0067 | 6 | 0.581 | 0.0118 | 8 | $p<.005$ |

TABLE 11

COMPARISON OF HEMATOCRIT BY WEEK AND TREATMENT GROUP

| Week | Control | | | Exposed | | | Significance Level of Difference |
|---|---|---|---|---|---|---|---|
| | Mean | Variance | N | Mean | Variance | N | |
| 3 | 44.300 | 5.7000 | 5 | 44.555 | 11.9027 | 9 | n.s. |
| 6 | 42.083 | 1.1416 | 6 | 43.388 | 2.1111 | 9 | n.s. |
| 9 | 42.166 | 6.266 | 6 | 44.611 | 0.9236 | 9 | $p<.025$ |
| 12 | 44.000 | 0.700 | 6 | 44.312 | 1.0669 | 8 | n.s. |

TABLE 12

COMPARISON OF TRACE METAL CONCENTRATION (µg/ml) IN POOLED URINE BY WEEK AND TREATMENT GROUP

| Metal | Week | Control | | | Exposed | | | Significance Level of Difference |
|---|---|---|---|---|---|---|---|---|
| | | Mean | Variance | N | Mean | Variance | N | |
| Zinc | 4 | 0.884 | 0.0361 | 19 | 0.820 | 0.0422 | 26 | n.s. |
| | 5 | 0.781 | 0.0223 | 18 | 0.955 | 0.0215 | 26 | p<.005 |
| | 6 | 0.653 | 0.0109 | 19 | 0.824 | 0.0275 | 26 | p<.005 |
| | 7 | 0.756 | 0.0114 | 12 | 0.873 | 0.0233 | 17 | p<.050 |
| | 8 | 0.627 | 0.0118 | 12 | 0.837 | 0.0230 | 17 | p<.005 |
| | 9 | 0.626 | 0.0114 | 12 | 0.743 | 0.0234 | 8 | n.s. |
| | 10 | 0.721 | 0.0150 | 6 | 0.771 | 0.0233 | 8 | n.s. |
| | 11 | 0.758 | 0.0162 | 6 | 0.784 | 0.0286 | 8 | n.s. |
| | 12 | 0.660 | 0.0208 | 6 | - | - | - | - |
| Copper | 4 | 0.406 | 0.0075 | 19 | 0.413 | 0.0076 | 26 | n.s. |
| | 5 | 0.357 | 0.0043 | 18 | 0.370 | 0.0030 | 26 | n.s. |
| | 6 | 0.340 | 0.0040 | 19 | 0.336 | 0.0035 | 26 | n.s. |
| | 7 | 0.353 | 0.0019 | 12 | 0.390 | 0.0036 | 17 | n.s. |
| | 8 | 0.315 | 0.0013 | 12 | 0.330 | 0.0023 | 17 | n.s. |
| | 9 | 0.320 | 0.0026 | 12 | 0.315 | 0.0011 | 8 | n.s. |
| | 10 | 0.394 | 0.0040 | 6 | 0.373 | 0.0012 | 8 | n.s. |
| | 11 | 0.343 | 0.0011 | 6 | 0.327 | 0.0011 | 8 | n.s. |
| | 12 | 0.350 | 0.0012 | 6 | - | - | - | - |
| Cobalt | 4 | 1.117 | 0.1038 | 19 | 1.215 | 0.1243 | 26 | n.s. |
| | 5 | 1.200 | 0.0791 | 18 | 1.435 | 0.1113 | 26 | p<.025 |
| | 6 | 0.848 | 0.0484 | 19 | 0.931 | 0.0525 | 26 | n.s. |
| | 7 | 0.671 | 0.0351 | 12 | 0.813 | 0.0281 | 17 | p<.050 |
| | 8 | 0.943 | 0.0334 | 12 | 1.062 | 0.0691 | 17 | n.s. |
| | 9 | 0.749 | 0.0230 | 12 | 0.881 | 0.0333 | 8 | n.s. |
| | 10 | 0.830 | 0.0361 | 6 | 0.853 | 0.0393 | 8 | n.s. |
| | 11 | 0.891 | 0.0334 | 6 | 0.793 | 0.0323 | 8 | n.s. |
| | 12 | 0.640 | 0.0414 | 6 | - | - | - | - |

TABLE 12 (continued)

COMPARISON OF TRACE METAL CONCENTRATION (μg/ml) IN POOLED URINE BY WEEK AND TREATMENT GROUP

| Metal | Week | Control | | | Exposed | | | Significance Level of Difference |
|---|---|---|---|---|---|---|---|---|
| | | Mean | Variance | N | Mean | Variance | N | |
| Iron | 4 | 1.479 | 0.2728 | 19 | 1.519 | 0.2953 | 26 | n.s. |
| | 5 | 1.268 | 0.1864 | 18 | 1.448 | 0.3019 | 26 | n.s. |
| | 6 | 0.985 | 0.0960 | 19 | 1.097 | 0.5591 | 26 | n.s. |
| | 7 | 1.006 | 0.0381 | 12 | 1.069 | 0.0184 | 17 | n.s. |
| | 8 | 1.133 | 0.1328 | 12 | 1.138 | 0.0253 | 17 | n.s. |
| | 9 | 1.200 | 0.8320 | 12 | 1.031 | 0.0110 | 8 | n.s. |
| | 10 | 1.004 | 0.0561 | 6 | 0.941 | 0.0203 | 8 | n.s. |
| | 11 | 0.985 | 0.0166 | 6 | 0.954 | 0.0284 | 8 | n.s. |
| | 12 | 0.867 | 0.0289 | 6 | - | - | - | - |
| Magnesium | 4 | 9.722 | 49.4874 | 19 | 6.856 | 11.5779 | 26 | n.s. |
| | 5 | 2.701 | 1.2690 | 18 | 2.776 | 1.4133 | 26 | n.s. |
| | 6 | 4.141 | 3.2808 | 19 | 4.528 | 2.8010 | 26 | n.s. |
| | 7 | 4.239 | 1.1272 | 12 | 4.118 | 1.9118 | 17 | n.s. |
| | 8 | 3.082 | 0.7775 | 12 | 2.782 | 0.6816 | 17 | n.s. |
| | 9 | 2.571 | 1.3367 | 12 | 2.961 | 0.2578 | 8 | n.s. |
| | 10 | 3.733 | 1.1146 | 6 | 3.359 | 0.6613 | 8 | n.s. |
| | 11 | 3.404 | 1.4335 | 6 | 2.881 | 0.4988 | 8 | n.s. |
| | 12 | 2.633 | 2.8276 | 6 | - | - | - | - |

TABLE 13

COMPARISON OF POOLED URINE VOLUME (MILLITERS) BY WEEK AND TREATMENT GROUP

| Week | Control | | | Exposed | | | Significance Level of Difference |
|---|---|---|---|---|---|---|---|
| | Mean | Variance | N | Mean | Variance | N | |
| 4 | 46.94 | 468.60 | 19 | 41.30 | 275.60 | 26 | n.s. |
| 5 | 57.27 | 358.33 | 18 | 42.38 | 331.85 | 26 | $p<.025$ |
| 6 | 54.31 | 316.45 | 19 | 33.96 | 233.23 | 26 | $p<.005$ |
| 7 | 62.41 | 223.35 | 12 | 50.11 | 283.48 | 17 | n.s. |
| 8 | 60.41 | 304.81 | 12 | 44.41 | 285.13 | 17 | $p<.025$ |
| 9 | 68.58 | 389.71 | 12 | 55.49 | 143.14 | 8 | n.s. |
| 10 | 65.33 | 515.06 | 6 | 61.24 | 579.35 | 8 | n.s. |
| 11 | 61.66 | 271.06 | 6 | 61.37 | 940.26 | 8 | n.s. |
| 12 | 64.16 | 470.56 | 6 | - | - | - | - |

TABLE 14

COMPARISON OF WET WEIGHT (GRAMS) BY WEEK AND TREATMENT GROUP FOR VARIOUS ORGANS

| Organ | Week | Control | | | Exposed | | | Significance Level of Difference |
|---|---|---|---|---|---|---|---|---|
| | | Mean | Variance | N | Mean | Variance | N | |
| Brain | 3 | 1.703 | 0.0253 | 5 | 1.808 | 0.0140 | 9 | n.s. |
| | 6 | 1.817 | 0.0069 | 6 | 1.782 | 0.0068 | 9 | n.s. |
| | 9 | 1.801 | 0.0104 | 6 | 1.889 | 0.0102 | 9 | n.s. |
| | 12 | 2.054 | 0.0088 | 6 | 1.957 | 0.0065 | 8 | n.s. |
| Heart | 3 | 1.103 | 0.0021 | 5 | 0.973 | 0.0282 | 9 | n.s. |
| | 6 | 1.227 | 0.0142 | 6 | 1.019 | 0.0211 | 9 | $p<.025$ |
| | 9 | 1.223 | 0.0101 | 6 | 1.234 | 0.0135 | 9 | n.s. |
| | 12 | 1.322 | 0.0077 | 6 | 1.301 | 0.0214 | 8 | n.s. |
| Kidneys | 3 | 2.438 | 0.0387 | 5 | 2.389 | 0.0942 | 9 | n.s. |
| | 6 | 2.711 | 0.0200 | 6 | 2.508 | 0.0278 | 9 | $p<.050$ |
| | 9 | 2.650 | 0.0530 | 6 | 2.688 | 0.1448 | 9 | n.s. |
| | 12 | 2.782 | 0.0140 | 6 | 2.900 | 0.0663 | 8 | n.s. |
| Lungs | 3 | 2.517 | 0.0337 | 5 | 2.458 | 0.0638 | 9 | n.s. |
| | 6 | 2.602 | 0.0855 | 6 | 2.208 | 0.0946 | 9 | $p<.050$ |
| | 9 | 3.087 | 0.3256 | 6 | 2.786 | 0.1875 | 9 | n.s. |
| | 12 | 3.600 | 0.0401 | 6 | 3.060 | 0.2440 | 8 | $p<.050$ |
| Spleen | 3 | 0.796 | 0.0045 | 5 | 1.273 | 2.5379 | 9 | n.s. |
| | 6 | 0.746 | 0.0091 | 6 | 0.669 | 0.0148 | 9 | n.s. |
| | 9 | 0.694 | 0.0132 | 6 | 0.688 | 0.0059 | 9 | n.s. |
| | 12 | 0.868 | 0.0226 | 6 | 0.766 | 0.0169 | 8 | n.s. |

TABLE 15

COMPARISON OF DRY WEIGHT (GRAMS) BY WEEK AND TREATMENT GROUP FOR VARIOUS ORGANS

| Organ | Week | Control | | | Exposed | | | Significance Level of Difference |
|---|---|---|---|---|---|---|---|---|
| | | Mean | Variance | N | Mean | Variance | N | |
| Brain | 3 | 0.369 | 0.0010 | 5 | 0.397 | 0.0002 | 9 | $p<.050$ |
| | 6 | 0.406 | 0.0001 | 6 | 0.407 | 0.0003 | 9 | n.s. |
| | 9 | 0.414 | 0.0003 | 6 | 0.404 | 0.0012 | 9 | n.s. |
| | 12 | 0.462 | 0.0014 | 6 | 0.416 | 0.0003 | 8 | $p<.025$ |
| Heart | 3 | 0.254 | 0.0001 | 5 | 0.239 | 0.0003 | 9 | n.s. |
| | 6 | 0.288 | 0.0005 | 6 | 0.245 | 0.0013 | 9 | $p<.025$ |
| | 9 | 0.288 | 0.0004 | 6 | 0.272 | 0.0002 | 9 | n.s. |
| | 12 | 0.283 | 0.0003 | 6 | 0.274 | 0.0008 | 8 | n.s. |
| Kidneys | 3 | 0.595 | 0.0021 | 5 | 0.601 | 0.0022 | 9 | n.s. |
| | 6 | 0.648 | 0.0007 | 6 | 0.615 | 0.0019 | 9 | n.s. |
| | 9 | 0.656 | 0.0021 | 6 | 0.659 | 0.0013 | 9 | n.s. |
| | 12 | 0.633 | 0.0007 | 6 | 0.638 | 0.0024 | 8 | n.s. |
| Lungs | 3 | 0.441 | 0.0005 | 5 | 0.411 | 0.0010 | 9 | n.s. |
| | 6 | 0.415 | 0.0016 | 6 | 0.370 | 0.0008 | 9 | $p<.025$ |
| | 9 | 0.464 | 0.0032 | 6 | 0.431 | 0.0016 | 9 | n.s. |
| | 12 | 0.476 | 0.0041 | 6 | 0.404 | 0.0046 | 8 | n.s. |
| Spleen | 3 | 0.172 | 0.0002 | 5 | 0.157 | 0.0016 | 9 | n.s. |
| | 6 | 0.168 | 0.0004 | 6 | 0.171 | 0.0011 | 9 | n.s. |
| | 9 | 0.164 | 0.0003 | 6 | 0.151 | 0.0001 | 9 | n.s. |
| | 12 | 0.182 | 0.0008 | 6 | 0.160 | 0.0006 | 8 | n.s. |

TABLE 16

COMPARISON OF ASH WEIGHT (MILLIGRAMS) BY WEEK AND TREATMENT GROUP FOR VARIOUS ORGANS

| Organ | Week | Control | | | Exposed | | | Significance Level of Difference |
|---|---|---|---|---|---|---|---|---|
| | | Mean | Variance | N | Mean | Variance | N | |
| Brain | 3 | 25.719 | 161.4520 | 5 | 29.333 | 26.6050 | 9 | n.s. |
| | 6 | 36.866 | 2.4986 | 6 | 38.255 | 57.9727 | 9 | n.s. |
| | 9 | 32.999 | 12.7240 | 6 | 34.099 | 8.9450 | 9 | n.s. |
| | 12 | 34.183 | 4.5016 | 6 | 30.312 | 3.5069 | 8 | $p<.005$ |
| Heart | 3 | 12.139 | 0.4530 | 5 | 11.977 | 0.9369 | 9 | n.s. |
| | 6 | 14.866 | 1.686 | 6 | 13.199 | 0.8625 | 9 | $p<.025$ |
| | 9 | 13.866 | 1.1066 | 6 | 13.455 | 0.9502 | 9 | n.s. |
| | 12 | 13.749 | 1.1710 | 6 | 13.262 | 2.2883 | 8 | n.s. |
| Kidneys | 3 | 33.040 | 5.8180 | 5 | 32.766 | 9.7600 | 9 | n.s. |
| | 6 | 36.450 | 3.7150 | 6 | 34.566 | 5.2800 | 9 | n.s. |
| | 9 | 36.000 | 4.2520 | 6 | 36.077 | 3.2694 | 9 | n.s. |
| | 12 | 33.533 | 1.8946 | 6 | 33.874 | 7.5678 | 8 | n.s. |
| Lungs | 3 | 24.139 | 3.8780 | 5 | 27.033 | 4.5025 | 9 | $p<.050$ |
| | 6 | 26.983 | 9.1016 | 6 | 23.666 | 16.1175 | 9 | n.s. |
| | 9 | 24.350 | 7.5670 | 6 | 25.099 | 6.1175 | 9 | n.s. |
| | 12 | 26.049 | 2.0030 | 6 | 21.874 | 18.3478 | 8 | $p<.050$ |
| Spleen | 3 | 12.399 | 2.2450 | 5 | 12.788 | 1.0186 | 9 | n.s. |
| | 6 | 13.166 | 1.8986 | 6 | 12.477 | 2.4494 | 9 | n.s. |
| | 9 | 11.633 | 2.3826 | 6 | 11.344 | 0.7677 | 9 | n.s. |
| | 12 | 13.300 | 5.2040 | 6 | 11.974 | 4.5707 | 8 | n.s. |

TABLE 17

COMPARISON OF LIVER TRIM WEIGHT (GRAMS) BY WEEK AND TREATMENT GROUP

| Week | Control | | | Exposed | | | Significance Level of Difference |
|---|---|---|---|---|---|---|---|
| | Mean | Variance | N | Mean | Variance | N | |
| 3 | 11.701 | 0.4747 | 5 | 12.628 | 1.4085 | 9 | n.s. |
| 6 | 13.316 | 3.3609 | 6 | 13.474 | 2.2631 | 9 | n.s. |
| 9 | 14.821 | 0.5288 | 6 | 14.232 | 2.2046 | 9 | n.s. |
| 12 | 13.660 | 1.1154 | 6 | 14.303 | 0.9796 | 8 | n.s. |

TABLE 18

COMPARISON OF DRY WEIGHT (GRAMS) BY WEEK AND TREATMENT GROUP FOR LIVER FRACTIONS

| Liver Fraction | Week | Control | | | Exposed | | | Significance Level of Difference |
|---|---|---|---|---|---|---|---|---|
| | | Mean | Variance | N | Mean | Variance | N | |
| Nuclear | 3 | 2.426 | 0.0114 | 5 | 2.290 | 0.5530 | 9 | n.s. |
| | 6 | 3.382 | 0.1097 | 6 | 2.517 | 0.0740 | 9 | $p<.005$ |
| | 9 | 3.003 | 0.0351 | 6 | 2.748 | 0.0693 | 9 | n.s. |
| | 12 | 2.941 | 0.0942 | 6 | 2.311 | 0.0359 | 8 | $p<.005$ |
| Mito-chondrial | 3 | 1.120 | 0.0630 | 5 | 1.067 | 0.0350 | 9 | n.s. |
| | 6 | 1.353 | 0.0545 | 6 | 1.220 | 0.1034 | 9 | n.s. |
| | 9 | 1.500 | 0.0192 | 6 | 1.223 | 0.0586 | 9 | $p<.050$ |
| | 12 | 1.721 | 0.0274 | 6 | 1.739 | 0.1045 | 8 | n.s. |
| Super-natant | 3 | 5.029 | 0.2175 | 5 | 5.664 | 1.4978 | 9 | n.s. |
| | 6 | 5.775 | 0.1502 | 6 | 5.816 | 0.4848 | 9 | n.s. |
| | 9 | 6.763 | 7.5188 | 6 | 6.576 | 1.0457 | 9 | n.s. |
| | 12 | 7.423 | 0.3877 | 6 | 7.020 | 0.3303 | 8 | n.s. |

TABLE 19

COMPARISON OF ASH WEIGHT (MILLIGRAMS) BY WEEK AND TREATMENT GROUP FOR LIVER FRACTIONS

| Liver Fraction | Week | Control | | | Exposed | | | Significance Level of Difference |
|---|---|---|---|---|---|---|---|---|
| | | Mean | Variance | N | Mean | Variance | N | |
| Nuclear | 3 | 61.22 | 14.78 | 5 | 54.96 | 155.12 | 9 | n.s. |
| | 6 | 85.95 | 77.19 | 6 | 87.78 | 294.58 | 9 | n.s. |
| | 9 | 89.02 | 258.28 | 6 | 88.92 | 167.22 | 9 | n.s. |
| | 12 | 76.80 | 115.70 | 6 | 77.05 | 150.97 | 8 | n.s. |
| Mito-chondrial | 3 | 5.60 | 1.32 | 5 | 13.38 | 55.96 | 9 | $p<.050$ |
| | 6 | 7.18 | 4.44 | 6 | 13.28 | 84.93 | 9 | n.s. |
| | 9 | 13.58 | 21.89 | 6 | 8.91 | 13.85 | 9 | $p<.050$ |
| | 12 | 7.33 | 2.81 | 6 | 8.96 | 11.66 | 8 | n.s. |
| Super-natant | 3 | 67.64 | 57.59 | 5 | 119.63 | 4937.64 | 9 | n.s. |
| | 6 | 82.50 | 181.34 | 6 | 203.87 | 20794.02 | 9 | n.s. |
| | 9 | 100.50 | 150.05 | 6 | 79.40 | 150.80 | 9 | $p<.010$ |
| | 12 | 78.52 | 74.77 | 6 | 67.34 | 608.41 | 8 | n.s. |

TABLE 20

COMPARISON OF RAT WEIGHT (GRAMS) BY WEEK AND TREATMENT GROUP

| Week | Control | | | Exposed | | | Significance Level of Difference |
|---|---|---|---|---|---|---|---|
| | Mean | Variance | N | Mean | Variance | N | |
| 2 | 358.77 | 160.75 | 22 | 348.23 | 718.30 | 35 | n.s. |
| 4 | 395.89 | 293.40 | 18 | 369.88 | 823.55 | 26 | $p<.005$ |
| 6 | 437.11 | 209.63 | 18 | 409.04 | 822.36 | 26 | $p<.005$ |
| 8 | 449.33 | 171.33 | 12 | 425.41 | 746.63 | 17 | $p<.025$ |
| 10 | 468.50 | 474.70 | 6 | 438.62 | 1093.41 | 8 | n.s. |
| 12 | 489.16 | 877.37 | 6 | 454.75 | 1107.07 | 8 | n.s. |