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RELATIVE DEGREE OF TRACE METAL "SHIFT" IN EXPERIMENTAL CIRRHOSIS AND ALCOHOLISM

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RELATIVE DEGREE OF TRACE METAL "SHIFT" IN EXPERIMENTAL CIRRHOSIS AND ALCOHOLISM

CHAPTER I

INTRODUCTION

According to Gardner (67), Secretary of HEW:

> No other national health problem has been so seriously neglected as alcoholism. The atmosphere of moral disapproval surrounding the entire subject, and the deplorable custom of treating alcoholics as sinners or criminals have obscured the nature of the problem. But now we recognize that alcoholism is an illness - no more moral or immoral than tuberculosis or pneumonia or schizophrenia and that our ways of dealing with that illness have been shockingly inadequate.

There is a wealth of literature and voluminous books that abounds with the study of alcoholism. Many research efforts are being directed from many disciplines of science such as biochemistry, physiology, psychiatry and pharmacology hoping that some rational approach to the prophylaxis and treatment of alcoholism can be found. In spite of the mass of knowledge that have accumulated in recent years, there still exists a great deal of contradictions and inconsistencies in the understanding of the organic manifestations of the disease. Tremendous gaps are widespread regarding the metabolic pathways of ethanol in man. Speculations have ranged from inborn metabolic errors to disturbances in the metabolism of nutrients and minerals to a deficiency in a series of enzymatic reactions to a score of other postulates attempting to provide a better

understanding of the biochemical aspects of alcoholism.

Alcohol dependence is compulsive in nature and it has reached such a devastating magnitude of great complexity and scope that it has become one of the great public health problems of the world. In the United States, it ranks among the four major killers and is exceeded only by the cardiovascular and mental illness diseases (1). According to Block (11), five million people suffer from alcoholism. This means that about 6 per cent of our adult population or one out of every fifteen is afflicted with the disease.

CHAPTER II

LITERATURE REVIEW

Historical Background

Drinking alcoholic beverages has been since time immemorial a part of the ritualistic ceremonies of most civilized people. The record of mankind from one era to the next unfailingly reflects the widespread use of alcohol as a source of social gaiety, sometimes with overindulgence and the tragic consequences. It is well known that the similarity in color of wine to blood had led some people to attach symbolic value to wine and hence the pledge of loyalty and friendship was extended to include betrothals, social gatherings and baptism.

The word "alcohol" is derived from the Arabic "Al-Kouhool" which refers to a fine powder of antimony used for staining the eye lashes. According to Poznanski (147), the discovery of late Stone Age beer jugs has established the fact that some use of fermented beverages existed in the neolithic period. The Egyptians attributing the cultivation of wine to Osiris, the Greeks and the beneficient acts of Bacchus and the Hebrew tradition ascribing to Noah the art of wine manufacturing all point to the social role that alcoholic beverages played in early history. Hippocrates is said to have included wine in a list of therapeutics and used it as a wet dressing for ulcers.

According to Block (12), alcohol as a medicine was first described by Arnold of Villanova as a general panacea. He claimed that it was good for the old and for the young at every time and in every place. The Arab ian physician Rhazes is credited with the discovery of distillation in the 10^{th} century. The European alchemists believed that alcohol was an antidote to senility and hence called it the water of life.

According to Jellinek (90), the clinical manifestations resembling the condition which is generally designated today as cirrhosis have been described in the early second century before Christ. In the Greek literature there are certain indications that damage to the liver by alcohol was alluded to by the medical writings of that era. The first significant description of this condition was made by Mathew Baulie in the 18^{th} century although it did not exclude other etiological factors than alcohol in producing the disease.

In 1826, Laennec (after whom the syndrome was named) described the nodular characteristics of the atrophic liver which he specifically identified as the alcoholic form of cirrhosis, although this condition was later produced by agents other than alcohol. The term is still used today whether the condition is alcoholic or non-alcoholic in its origin.

Although alcohol has been used by man for many centuries, the development of scientific methods for determining the action of alcohol in the body is of comparatively recent undertaking. Prior to 1860, physiologists believed that alcohol was completely oxidized in the body. Following this period, experimental research has shown that alcohol could be recovered in urine as well as expired air (69).

Modern study of alcohol metabolism did not start, however, until

1870 when a group of investigators came to the conclusion that the body can utilize alcohol as a foodstuff. Widmark's micromethod for alcohol determination in 1922 was an important step toward the study of alcohol (12). In the following twenty years, the general facts about alcohol absorption, distribution and elimination in the animal and the human body were well established (89).

Definition of Terms

There are many definitions for alcoholism in the literature. Every individual seems to have his own idea of what constitutes this illness. However, whether it is the casual observation of the layman or the rejected deliberation of the scientist, one thing is agreed upon and, that is, that alcoholism is manifested by extreme ingestion of alcoholic beverages. Few definitions by highly authoritative workers in their field seems to be necessary at the outset in order to distinguish the true character of this disease from the misconception that is held by the general public.

Jellinek (90), a former consultant on alcoholism to the World Health Organization defines alcoholism as "a progressive disease characterized by an uncontrollable drinking". "Progressive" and "uncontrollable" seem to be the key words in the definition. Clinebell (33) defines an alcoholic as "anyone whose drinking interferes frequently or continuously with any other important life adjustments or interpersonal relationships". While these definitions are true statements and portray excellent characterization of the disease, they are nevertheless descriptive in nature of the manifestation of the illness and hence do not display the mechanism of the disease itself.

The official definition of alcoholism as reported by the World Health Organization's Expert Committee (214) is a technical one and is as follows:

> Any form of drinking which in its extent goes beyond the traditional and customary "dietary" use of the ordinary compliance with the social drinking customs of the whole community concerned, irrespective of the etiological factors leading to such behavior, and irrespective also of the extent to which such etiological factors are dependent upon heredity, constitution, or acquired physiopathological and metabolic influences.

This definition is quite broad in its aspect of the disease without regard for the various etiological factors that might be involved.

The American Medical Association (1) defines an alcoholic as "Those excessive drinkers whose dependence on alcohol has attained such a degree that it shows a noticeable disturbance or interference with their bodily or mental health, their interpersonal relations and their satisfactory social and economic functioning".

The definition proposed by Cain (26) for the term alcoholic is "a person whose uncontrolled drinking is causing a continuing problem" and the term alcoholism, according to him, refers to "the collective behavior of a number of such persons". The concept of "alcoholism is a disease" has been debated repeatedly although the National Council on Alcoholism as well as the American Medical Association would like to think of alcoholism as a disease. Cain (26), however, argues that alcoholism is "a disease of society", but not a physical disease caused by society.

According to Block (11), the ex-chairman of the AMA Committee on alcoholism, the term "alcoholism" is a general one describing "several species of social behavior and conduct associated with the excessive

ingestion of alcohol". He defines the term as "the use of alcohol or alcoholic beverages to the extent of causing any continuing adverse effect upon the individual or his family or community". Another term that has been adopted recently for alcoholism is problem drinking which, to some individuals, is more acceptable than the rather degrading term of "alcoholic" (26).

Jellinek (90), divides alcoholism into five different classes. The first class he calls Alpha which is typified by a psychological dependence with no apparent withdrawal symptoms or signs of progression. This type may or may not progress further into other more dangerous types. The second class is termed Beta and is characterized by polyneuropathy, gastritis and cirrhosis, which for the most part is caused by nutritional deficiency and like Alpha type it does not display any withdrawal symptoms. The third class is the Gamma type, the most prevalent in our society. This type is characterized by increased tissue tolerance, adaptive cell metabolism, physical dependence and is usually accompanied by withdrawal symptoms or craving. The fourth class is the Delta type which shares the characteristic of the Gamma type, but differs from it in the inability to abstain. It is most prevalent in the wine-drinking countries and is often unrecognized unless withdrawal symptoms ensues. The fifth class is the Epsilon type which is also called periodic alcoholism or "occasional binge" drinking.

Other classifications have also been described, but these are sociopsychological rather than biophysiological.

The term "cirrhosis" is, according to Moon (129), synonymous with "chronic diffuse hepatitis". Any agent or combination of agents

which produces chronic diffuse hepatic inflammation will result in some degree of cirrhosis. He suggests three essential features of the cirrhotic process:

(1) Degeneration and destruction of liver cells.

(2) Regeneration of liver cells from those which escaped destruction.

(3) Proliferation of connective tissue.

Probably the most concise and accurate classification of the cirrhotic process was given by Boyd (16). He states that after the original use of the term by Laennec who employed the Greek word Kirros (towny) to describe the yellowish nodules on the surface of the liver, it was realized that fibrosis was a striking feature of the lesions and hence cirrhosis came to be synonymous with fibrosis. The unfortunate thing, he indicates, is that the name suggests a disease entity, but according to him, the essence of cirrhosis is not fibrosis or scarring but destruction of liver parenchyma, followed by reconstruction of a new lobular pattern with the formation of regenerative nodules.

He contends that cirrhosis may be regarded as a chronic hepatitis with changes involving both the parenchyma and mesenchyma and hence fibrosis is secondary in nature rather than primary. He classifies cirrhosis into biliary which can be either intrahepatic or extrahepatic and portal or Laennec which can be either nutritional such as in alcoholism, post-hepatic or post-necrotic in character. Furthermore, a miscellaneous variety can include other descriptive terms such as pigmentation, cardiac, toxic and infectious.

Alcoholism as a Social and Public Health Problem

Although many people would like to blame alcoholic beverages in

its various forms for the disease, the fact is that the individual who drinks to excess is the responsible one. As Cain (26) pointed out, it is just as illogical to blame gasoline for auto accidents as it is to blame alcohol for alcoholism. It cannot be assumed, for example, that communities with a higher level of per capita alcohol consumption than others have necessarily a higher case of alcoholics. By the same reasoning, a change in the level of consumption is not necessarily accompanied by a change in a similar direction in the incidence of alcoholism (188). Therefore, one must turn to the small percentage of every population who cannot safely drink alcohol because of some unique biochemical change that is either inborn in its nature or is developed after a long period of drinking.

A regular drinker is defined as one who drinks three times a week. Anything less than that is defined as occasional drinker. Seventeen per cent of the total drinking population in the United States are regular drinkers, 45 per cent are occasional drinkers and 38 per cent are abstainers (188).

Since alcohol can act as an anesthetic agent, it is conceivable that individuals who are faced with everyday problems wish to drink excessively in order to avoid facing facts of life, hence the inability of the individual to cope realistically with the problems of living and deal with them to the best of his ability is probably the major cause of alcoholism. Block (12), refers to this state of behavior as "the preference for escape into partial or total oblivion by means of an available drug which is the most common cause of alcoholism in our society".

The real causes of alcoholism, however, are still obscure. They

have ranged from dietary deficiencies to personality traits to hormonal imbalance. Whatever the causes may be, establishing acceptable criteria is, undoubtedly, the first step to meet this illness as a public health problem. It is necessary to institute proper treatment so that the progression of the disease at the early signs can be effectively prevented. Understanding the physical and mental state of the individual may be important, but what is more important, however, are the mores and the social setting as well as the cultural and ethnic background which can give a better visibility for instituting a broad educational program. It can be hypothesized that effective measures of prevention must aim at changing philosophies and attitudes with realistic approach towards life that there are no problems without life and that there is no life without problems. This philosophy, I maintain, should be accepted particularly by young people as a part of normal life and any attempt for an escape by palliative intoxication into complete unreality must be considered an abnormal response. Many will agree that long range educational programs should be established by both private industry as well as governmental agencies to change people's attitude towards alcohol.

Within the perspective of public health many alcoholics may be regarded as "disease carriers" and their environment as "insanitary", hence the enormity and complexity of the problem. Combat team exploitation is necessary whereby the physician working with the internal environment can cooperate with those working with the external environment - in public health education, social work and guidance (188).

As Cain (26) has suggested, a truly longitudinal study of individual alcoholics should be done as opposed to statistical projects based

on cross-sectional studies. This, he argues, would represent a research instrument for a true synthesis of the multidisciplined approach favored in today's research circles. This would imply a truly long term study that embraces the function of the whole man in his total environment.

Physiological and Biochemical Changes in Alcoholism

Ethyl alcohol, the beverage kind, is only one of many alcohols. What distinguishes it from other alcohols which are non-potable is that the body oxidizes it rapidly, in contrast to methyl alcohol, (wood alcohol), for example, which would take a week for the body to destroy. Pure ethyl alcohol is a clear, colorless liquid with burning taste and little odor (69).

The distilled beverages usually have an alcohol content of 40 to 50 per cent. The term "proof" as a measure of strength comes from early distillers, who tested alcohol by wetting gunpowder with it and when the distillate was at least 50 per cent alcohol, the powder would burn. Since combustibility of the gunpowder was 100 per cent of their alcohol content, 100 per cent proof came to mean 50 per cent alcohol (69).

Forty to fifty per cent alcohol is irritating to the lining tissues of the mouth, throat, esophagus and stomach. In moderate amounts it stimulates the gastric juice and promotes stomach motility, hence the sensation of hunger after alcoholic appetizers. A large intake of alcohol shifts water from the cell into interstitial fluid, hence the thirst that usually accompanies the hangover.

Alcohol is well absorbed into the blood without digestion. A small part is absorbed from the stomach, the rest passes into the small intestine and is absorbed from there very rapidly (188). Trace amounts

of ethanol may also be synthesized endogenously (109, 134). The speed with wihich it reaches the brain depends to a great extent on the amount of the food in the stomach. Carbon dioxide in effervescent alcoholic liquors speeds up the passage of alcohol from the stomach and also minimizes the irritation of the stomach.

In a 160-pound man, the alcohol in an ounce of whisky or a bottle of beer produces an alcohol blood concentration of about 0.02 per cent, and a half pint of whisky raises the concentration to 0.15 per cent which is the level at which most states (26) consider as permissible and not as drunken driving. Six states use 0.10 per cent, which is more realistic. Only Utah uses 0.08 per cent. Many European countries use the uncompromising 0.08 cutoff point. Norway uses a stringent 0.05 per cent (7). According to Greenberg (69), intoxication is defined as "gross unmistakable disturbances of movement, coordination and intellect". A blood concentration of 0.15 per cent is probably a realistic one, but only if related to the signs and symptoms of intoxication, although with the present armamentarium of pharmaceuticals the so-called objective signs of intoxication are no longer valid and accordingly should be reevaluated due to the new advances in brain metabolism. In the words of Greenberg

> The fact that drugs and black coffee appear to have a sobering effect does not mean that they hasten the disappearance of alcohol. These stimulants merely counteract some of the depressant action of alcohol on the brain and wake the person up. Awake or asleep the man is still drunk.

The first stage in the oxidation of alcohol is its conversion to acetaldehyde which occurs in the liver with the help of alcohol dehydrogenase (139, 197). Acetaldehyde is much more toxic if it accumulates

in the body (112, 192). Fortunately, it oxides rapidly into acetic acid which is broken down further into carbon dioxide and water. Antabuse, a drug used sometimes to control alcoholism is effective because it inhibits the oxidation of acetaldehyde (46). The drug has no apparent effect as long as the patient stays away from liquor, but if a small drink is taken, the acetaldehyde formed accumulates with severe and extremely unpleasant symptoms.

An ounce of whisky liberates 75 calories of energy, about as much as four and a half teaspoons of sugar. A drinker who consumes one pint of whisky a day gets 1200 calories of his daily energy requirement, but fails to get the minerals, proteins, and vitamins that the equivalent amount of food provides. Hence, excessive drinking often produces deficiency diseases such as beriberi, pellagra and the degeneration of nerve fibers (69, 82).

The most pronounced physiological effect of alcohol is on the nervous system (1). A blood concentration of about 0.05 per cent of alcohol depresses the uppermost level of the brain, the center of inhibition, restraint and judgement. At a concentration of 0.10 per cent in the blood, from five to six ounces of whisky, alcohol depresses the lower motor area of the brain, hence depression of sensory and motor functions. A blood concentration of 0.2 per cent alcohol resulting from drinking 10 ounces of whisky disturbs the mid-brain where emotional behavior is largely controlled. At 0.3 per cent from about a pint of whisky the drinker becomes stuporous. When the alcohol reaches the level of 0.4 or 0.5 per cent, the whole perception area of the brain is suppressed and the drinker falls into a coma. However, different individuals and even

the same individual at different times vary widely in their responses to a given amount of alcohol depending on the amount of food in the stomach and the kind of liquor drunk (1, 69, 82).

Other physiological effects that have been described are on the kidney through the pituitary gland where a reduction in its activity and hence, a reduction of the antidiuretic hormone which in turn produces copious urine (112). Other glands effected are the adrenals (165) which increase their secretion in the presence of large doses of alcohol, hence the disturbance of mineral metabolism in the body (6, 87, 94, 95, 133).

The effect of alcohol on the heart and circulation has been surrounded by many misconceptions. A drink of whisky makes the heart beat faster thus giving a sensation of warmth to the skin. This perhaps is due to a transient irritation of the nerve endings in the mouth and stomach. This in turn increases blood flow, thus dilating the peripheral vessels, which in turn produces the feeling of warmth (69). Gillespie (67), on the other hand, concluded that the vasodilator effect of alcohol is not produced solely by peripheral sympathetic blockade or central inhibition, but rather as a vasodilator only when partly metabolized in the liver. Intra-arterially infused alcohol has a direct vasoconstricting effect.

Eliaser and Giensircussa (48), however, believe that the therapeutic effects of alcohol in coronary artery disease are attributable to cerebral responses rather than demonstrable increase in coronary blood flow. There is no evidence that moderate amounts of alcohol cause heart disease, high blood pressure or atherosclerosis. Getting drunk, however,

is believed to tax the heart and would be dangerous for a man with heart disease (48).

As for the effect of alcohol on longevity, it is agreed that heavy drinkers have a shorter life span than those who abstain or drink moderately. Nonetheless, there is no general agreement as to whether there is a difference between moderate drinkers and abstainers in longevity although insurance company records show no significant difference at all (1).

Alcohol as medicine has been used since time immemorial. Today rum is taken for a cold, although it does not cure a cold except for relieving pain and discomfort. Whisky is given for snake bite, although it does not counteract the venom, but does relax the victim. Brandy is given to a person who faints although it has no direct effect on the fainting process except for reviving the patient by its irritation action. However, certain beneficial medical use of alcohol is still prescribed today. For example, a drink is prescribed for the elderly with arteriosclerosis due to the dilating effect of the alcohol on the blood vessels, relief from pain, and irritability and rousing their appetite. Also, it is used to provide caloric intake in the cases of patients who cannot be fed after an operation. As a source of food, however, the amount of alcohol that the body can burn is limited to about half an ounce per hour, hence a limited part of the energy needed can be supplied by alcohol. Furthermore, it must be realized that it does not provide certain essential micronutrients which come along with the ordinary food intake (82).

As for the psychological effect of alcohol it may be said that

research is divided on this issue. Jellinek (91) summarizes the effect by saying that a small amount of alcohol increases skilled performance as the task becomes more complex. Thus small amounts which may produce relaxation usually interfere to some degree with efficiency, reduce verbal fluency and impair judgment and higher order of intellectual functioning. As for the emotional behavior, two ounces of wine hardly affected the emotional response to the sudden stress but 12 ounces diminished emotional responses very markedly (1).

It may be important to mention at this point one of the crucial differences between chronic alcohol intoxication and chronic opiate use. In opiate addiction, an individual functions well as long as he receives large enough quantities of the drug to stave off his abstinence syndrome. However, the chronically intoxicated alcoholic cannot function normally as long as he maintains an intoxicating intake of alcohol. Thus the chronic alcoholic suffers during intoxication while opiate addicts suffer primarily during abstinence. Evidences suggest, however, that withdrawal of alcohol from subjects protected from dehydration, avitaminosis and protein depletion can precipitate delirium tremens (85).

Rubin (159) presented evidence to explain the increased tolerance by alcoholics to drugs such as sedatives. He fed rats ethanol for two weeks along with diets either adequate or deficient in protein and choline. Hepatic lipids and the drug-metabolizing enzymes increased more in the deficient diet.

Ethanol and Muscular Myopathies

Perkoff (143) described a clinical syndrome associated with biochemical abnormalities in chronic alcoholism that resembles hereditary

phosphorylase deficiency. The main features of this syndrome are muscle tenderness, increased serum creatine phosphokinase, poor lactic acid response to ischemic exercise and a variable muscle phosphorylase activity. According to Wendt, et al. (207), alcoholism and cardiovascular changes were recognized as early as 1873 by Walshe. Recently Eliaser and Giansircusa (48) identified three cardiovascular syndromes and designated them as alcoholic myocardosis, nutritional heart disease and beriberi heart disease. Evans (51) suggests an early diagnosis of alcoholic cardiomyopathy with EKG which can manifest a depressed T wave, the presence of extrasystole, a bundle-branch blodk and a depression of S-T segment. Brigden (19) recognized several etiologic groups of cardiomyopathies among them is a condition he calls "alcoholic cardiomyopathies" which does not manifest itself with a development of beriberi. Gallop rhythm was common, and left ventricular failure was frequently encountered with progressively diminishing response to therapy including large doses of thiamine. Burch and Walsh (25) indicated that alcohol per se may have a direct toxic effect on the heart. They reviewed eight cases of alcoholism in which they found a classical picture of beriberi heart disease as well as a typical non-specific heart failure. Wendt, et al. (207), tend to support the concept that chronic alcoholism results in altered myocardial metabolism even in those patients who do not display any clinical evidence of heart or liver disease. This led them to classify chronic alcoholic patients into four groups on the basis of the hemodynamic as well as the metabolic changes noted. Those with clinical evidence of heart or liver disease, those with alcoholic cardiomyopathy, those with Laennec's cirrhosis and those with both Laennec's cirrhosis

and heart disease. They postulated, however, that with repeated bouts of alcoholism, the cellular and mitochondrial permeability as well as the metabolic pathways are altered. In support of this hypothesis, Ferrans and his co-workers (54) made a recent study of the cardiac muscle with alcoholic cardiomyopathy by electron microscopy and found the prescence of mitochondria swelling, a decrease in intramitochondrial enzymes and the deposition of liquid droplets in the myocardial cells.

Ethanol and the Pancreas

The so-called Zieve's syndrome has been described by Zieve (219) and subsequently by others (10, 18, 173). It is characterized by, hyperlipemia jaundice, fatty liver and hemolytic anemia. Sobel and Waye (173) found that incidence of pancreatic lesions was twice as high in patients with portal as contrasted to past necrotic cirrhosis. This suggested to them that pancreatic lesions are to be correlated with the alcoholism rather than with the liver disease.

Although the mechanism of hemolytic anemia is unknown, recent work by Beard and Knott (7) indicate that the chronic administration of ethanol resulted in the development of a normochromic normocytic anemia accompanied by a concomitant reticulocytopenia. There was also a generalized hypercellularity of the bone marrow with no change in the myeloid/erythroid ratio. These findings led them to conclude that the pathophysiologic sequelae of chronic alcohol ingestion may result from a direct depressive and toxic action of ethyl alcohol on the hematopoietic system.

Sullivan and Herbert (183) were able to correct this supression of the hematopoietic response with large doses of folic acid or by

cessation of alcohol, hence the improvement seen in anemic alcoholics after hospitalization due to both ingestion of folate containing food and to cessation of alcohol ingestion. They speculated that the mechanism involved here may be partly due to an effect on folate metabolism. However, Lindenbaum and Lieber (119) indicated that the dose related vacuole formation in RBC precursors due to alcohol could not be corrected with vitamin supplementation including large doses of folic acid.

Herbert, et al. (78), studied seventy patients with alcoholism and varying degrees of hepatic dysfunction. They found a significant correlation between serum folate deficiency and alcoholism, less significance with either macrocytosis or anemia and borderline with cirrhosis and fatty hepatitis.

Psychopharmacology of Ethanol and Its Effect on the Nervous System

The physiological effect of alcohol on the nervous system is probably the most pronounced effect to be described in detail by many workers in their field. Evidence is accumulating that alcohol or its metabolites produce at least some of its effects through the interaction of these various brain amines. Davis, et al. (40, 41), provide evidence supporting the fact that there is a "shift" in nor-epinephrine (NE) metabolism from a normal oxidative route to a reductive pathway. The mechanism involved is the increased production of NADH resulting from ethanol oxidation hence a secondary "shift" in the metabolism of 3, 4 dihydoxymandelic aldehyde and 3 methoxy-4-hydroxymandelic aldehyde to the corresponding glycols. Furthermore acetaldehyde form competes with the aromatic aldehyde for alcohol dehydrogenase; thus inhibiting their

oxidation to the acids (41).

Duritz and Truitt (44) did not find any significant increases either in nor-epinephrine or in 5-hydroxytryptamine (5HT) after ethanol in rats. However, increased acetaldehyde blood levels produced by disulfiram pretreatment before the ethanol doses or by administration of acetaldehyde itself cause statistically significant decreases in brain norepinephrine but no effect on 5HT.

It has been suggested (45) that the direct myocardial release of NE was due to acetaldehyde which has a stimulating action similar to that of tyramine. Towne (192) found amine levels of nerve cell-bodies in the catecholamine cell groups to be significantly affected by ethanol. Thus the metabolism of acetaldehyde could contribute to a number of pathologic effects through the mechanism of $NADH_2$ generation as well as through increased catecholamines release (112). Some ethanol effects on the nervous system has been attributed directly to the accumulation of acetaldehyde (44, 153, 192, 193).

Drugs are now available which in very small doses can have dramatic changes in behavioral pattern of the alcoholic. For example, chlorpromazine, a commonly used tranquilizer, delays the disappearance of alcohol from the blood in man (185). It also has the capacity to inhibit alcohol dehydrogenase although this has been challenged by Edwards and Price (46) who observed inhibition in vitro but not in vivo. Metronidazole has also been reported to inhibit ADH activity and hence decreases alcohol consumption in man (46). The drug requirement for inhibitor is much higher in vitro than in vivo. Disulfiram (antabuse), as well as metronidazole, inhibit xanthine oxidase, a molybdenum activated enzyme,

which is implicated in the metabolism of acetaldehyde.

Metabolism of Alcohol

Perhaps there is no other drug whose metabolism has been studied so intensively as alcohol. Its value as a model for understanding the mechanism involved in other drugs has been exploited by many investigators (21, 159, 176). First, for better understanding of some metabolic processes of clinical relevance, second, as a means of modifying the action of other drugs in the liver and third as an agent that is capable of altering the structure of the liver cells.

Ethanol, when taken orally, is rapidly absorbed from the stomach and the rest of the GI tract. About 2-10 per cent is eliminated via the kidney and the lungs and the rest reaches the liver via the portal vein. It is generally agreed that the site of ethanol oxidation is almost exclusively located in the liver (130, 166). Larson (106), however, found that when the concentration of ethanol in the blood reaching the liver is below 50-60 mg/l, the concentration in the liver vein, obtained by catherization is zero.

The major metabolic pathways of ethanol are diagrammed in Figure 1 (77, 112, 138).

It is generally accepted by many workers in this field (39, 130, 131, 136, 196) that the initial step in the disposal of ethanol is its catalysis by alcohol dehydrogenase, a zinc containing enzyme, which has been isolated in pure form from the soluble fraction of liver cells (136) with quantitative microtechnic. Morrison (130) found hepatic ADH to be 3/5 more abundant in centrolobular areas of the liver than in peripheral areas in both man and rat, whereas Nyberg (136) obtained the reverse with

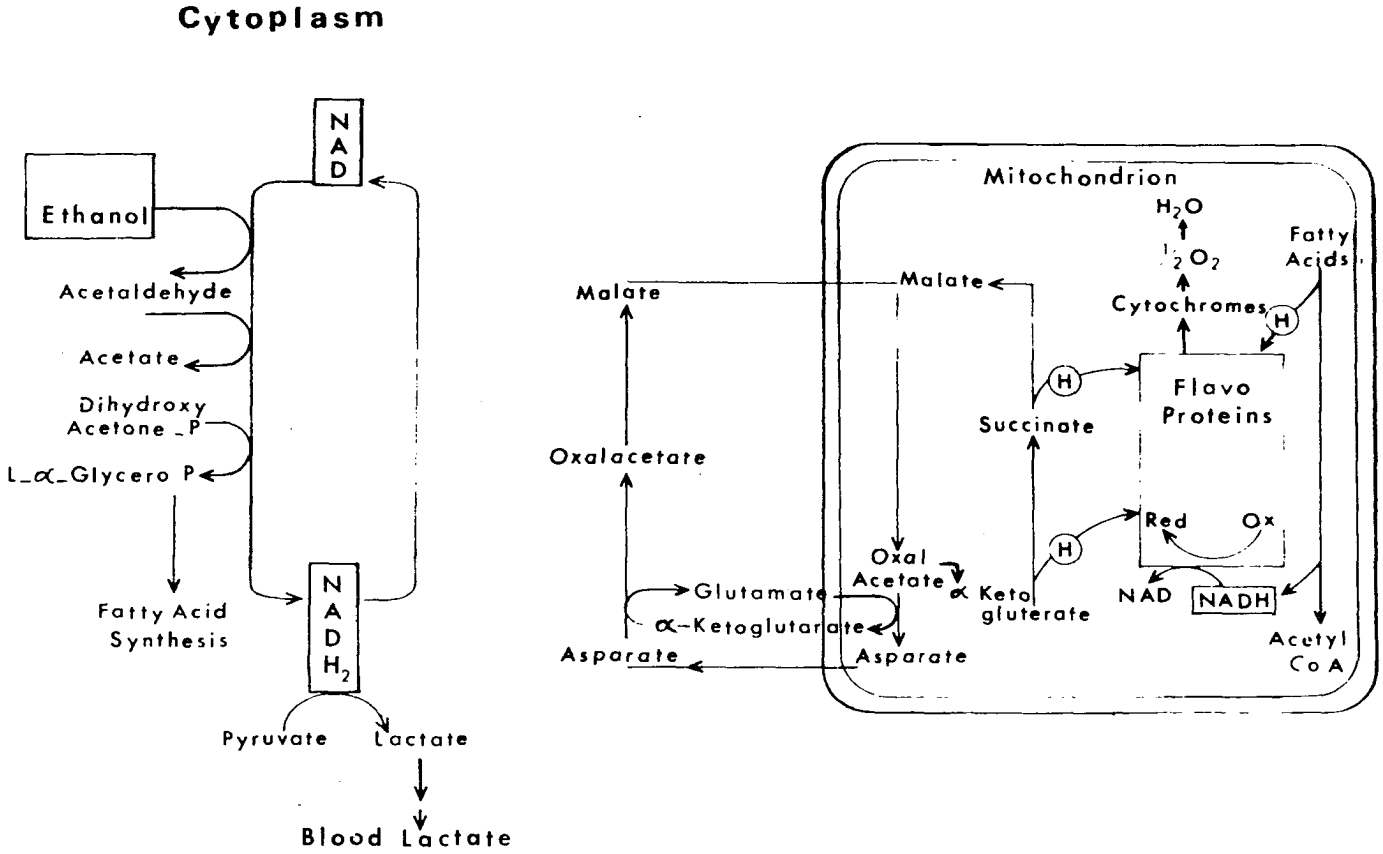

Fig. 1--A general sketch of ethanol metabolism.

histochemical procedures.

The end product of ethanol oxidation is acetate. This takes place in two steps:

$$\text{Ethanol} + NAD^{+} \rightarrow \text{Acetaldehyde} + NADH + H^{+}$$

$$\text{Acetaldehyde} + NAD^{+} \rightarrow \text{Acetate} + NADH + H^{+} \quad .$$

ADH catalyzes the first reaction and acetaldehyde dehydrogenase the second. The metabolism of acetate depends a great deal on its conversion to acetyl CoA via acetate thiokinase whose activity in the liver is very low. Hence most of the acetate formed is metabolized elsewhere in the body (77) where acetate thiokinase is relatively high. ADH, however, is mainly concentrated in the soluble fraction of the liver cell (192). Recently, however, Rubin (159) found that enzymes of the smooth endoplasmic reticulum are also capable of oxidizing alcohol to acetaldehyde, a system that requires NADPH and molecular oxygen.

Galambos, et al. (66) found an increase in glutamic pyruvic transaminase and lactic dehydrogenase in patients with liver disease given I.V. alcohol than in persons without liver injury. French (59) found a "shift" in succinic dehydrogenase histochemically localized to the centrolobular zone of rat livers fed ethanol. He suggested that this may be due to the ethanol-induced increase in permeability of the mitochondrial membrane. This led him to postulate that perhaps ethanol ingestion reduces some substances in the centrolobular zone that normally inhibits succinic dehydrogenase activity.

Mikata, et al. (127), found a progressively impaired ethanol metabolism in cirrhotic rats as well as a decreased activity of hepatic alcohol dehydrogenase.

Christophersen's (32) work on the liver mitrochondria suggests an increased stimulation of O_2 uptake with β-hydroxybutyrate as the substrate whereas a decreased uptake with pyruvate, α-ketoglutarate and succinate as the substrate. He suggests, however, that ethanol increases the oxidation of the β-hydroxybutyrate, by facilitating its transport into the mitochondria.

Kiessling and Tilander (100) studied the brain and liver content of thiamine diphosphate in rats given ethanol. This was moderately lowered in liver mitochondria and supernatant whereas its hydrolysis was partly increased in the supernatant.

Rubin and Lieber (157, 158) found mitochondrial alterations and focal cytoplasmic degradation within one day after giving ethanol in a concentration of 46 per cent of total caloric intake to humans. Fatty metamorphosis was noted within 3 to 10 days after the same dose and was associated with alterations of mitochondria and endoplasmic reticulum. This led them to conclude that alcohol exerts a direct toxic action on the liver. The same results were also obtained by other workers (64, 86, 106, 160, 189).

Towne (192) did not find any alcohol dehydrogenase in brain homogenate, whereas its activity in the liver is easily detected. His results support the conclusion that acetaldehyde formed from ethanol oxidation acts as non-specific inhibitor of liver monoamine oxidase.

According to Henley and Scholz (77), the metabolic fate of ethanol becomes of great concern as two molecules of NADH are formed from each molecule of ethanol. He suggests three possibilities which could be operative in taking care of the NADH.

1. Synthesis of triglycerides and glucose.
2. Conversion to a metabolite such as lactate.
3. NADH transport into mitochondria to provide energy by oxidative phosphorylation.

Since there are other NAD dependent reactions located in the soluble fraction of the liver cells, they will be altered by competing with ethanol for NAD. These types of reactions that need NAD are lactic dehydrogenase, glycerophosphate dehydrogenase and glyceraldehyde phosphate dehydrogenase. Reduced metabolites of these reactions (lactate, glycerphosphate and glyceraldehyde phosphate) will increase in the tissues while the oxidized form (pyruvate, dihydroxyacetone phosphate and 3-phosphoglycerate) of the metabolites will decrease.

As has been found by Salaspuro and Maenpaeae (161) and Sholz, et al. (167), the administration of ethanol increases the concentration of lactate in the blood both in vitro (167) as well as in vivo (114, 116). The increased lactate permits the release of reducing equivalents into the blood stream, while that of glycerophosphate provides the three carbon skeleton for the synthesis of triglycerides and the glyceraldehyde phosphate dehydrogenase shifts the equilibrium towards glyceraldehyde phosphate from the oxidation of glucose to gluconeogenesis.

Although this postulate seems quite plausible from the biochemical point of view it does not fit in with the clinical side of the story. Brown and Harvey (22) found a clinical picture of hypoglycemia associated with alcoholic intoxication due to some form of denatured alcohol. Cummins (38) reported two cases of hypoglycemia in children following ingestion of alcohol. He suggests that alcohol utilizes glucose as a

metabolic poison and thus interferes with gluconeogenesis or may inhibit glycogenolysis. Hypoglycemia is more pronounced when the liver is depleted of glycogen. Field, et al. (55) consistently produced hypoglycemia in humans by the ingestion of 35-50 ml of ethanol after a two day fast. Besides inhibiting glycogen synthesis, he also found that ethanol interfered with urea formation in the isolated, perfused rat liver, but not with amino acid mobilization. Freinkel, et al. (58), drew the same conclusion after an overnight fast.

Henley (77) suggests, however, that in well-fed man or animal, the blood sugar may rise due to the action of catecholamines, which will promote the formation of glucose from glycogen stores.

Madison (124) postulated as diagrammed in Figure 2 that ethanol inhibits gluconeogenesis because of the marked increase in the ratio of $NADH_2/NAD$. This, he suggests, permits a unitary explanation for several puzzling phenomenon. First, it will allow for the increased fatty acid synthesis. Second, it explains the decrease in hepatic conversion of galactose to glucose. Third, it justifies the decreased transformation of serotonin to 5-hydroxyindolacetic Acid (5 HIAC). Fourth, it gives a logical rationale for the decreased glycerol metabolism, the depression of the Krebs cycle and the decreased conversion of amino acids to glucose.

An additional factor that should be considered in the metabolism of ethanol is the fatty acids which are increased in starvation due to their increased production of acetyl CoA and possibly by inhibition of glycolysis by citrate. It is not known, however, whether fatty acids are produced de novo or are mobilized from adipose tissue into the liver.

Bouchier and Dawson (15) infused 1.0 g ethanol/kg body weight

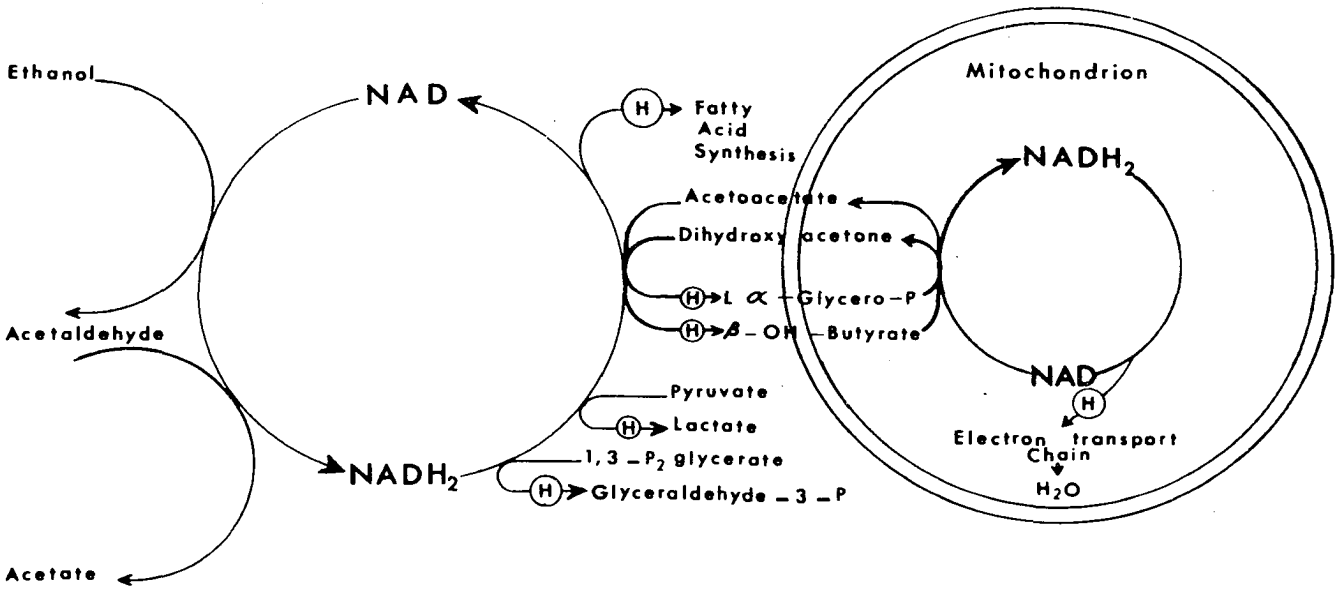

Fig. 2--Reduction of NAD to $NADH_2$ during ethanol oxidation and the pathways for disposition of the ethanol-generated reducing equivalents.

over 30 minutes and noted a decrease of free fatty acids (FFA) during infusion and an increase after the infusion had been stopped. He concluded that FFA is mobilized from adipose tissue via adrenaline. Brown (21) administered ethanol to rats orally and found a lower serum FFA and lower liver triglyceride. DiLuzio and Poggi (42) provided further support to the concept that depressed intrahepatic triglyceride is a factor in the development of steatosis following acute ethanol intoxication. Contrary to that, Elko, et al. (49) presented data indicating a significant increase in hepatic triglycerides 4-16 hours after alcohol intubation into rats. He does not subscribe to the idea that ethanol intoxication enhances lipid mobilization. Lieber, et al. (116) observed a fall in FFA levels during ethanol administration, but were unable to find any evidence that ethanol induces mobilization of FFA from the extremities. Thorpe and Shorey (189) found decreased serum FFA after 4 months of alcohol administration but no change in serum triglyceride and albumin levels.

To assess peripheral lipid mobilization, Feinman and Lieber (53) investigated the effect of ethanol on plasma glycerol and FFA in alcoholic volunteers. They found a drop in plasma FFA and glycerol 46 per cent and 36 per cent respectively from baseline values. They did not detect any change in glucose levels. Hence their data support the findings of a reduction in peripheral lipid mobilization after ethanol. French (62) found an increased phospholipid in livers of both male and female rats fed ethanol. It would seem, therefore, that the lipid that accumulates in liver has three obvious sources: dietary, whose magnitude depends on the amount of fat taken in, endogenous synthesis, and mobilization of peripheral fat depots which may be important only when large

quantities of alcohol are consumed.

As to the mechanism and cause of fatty liver development in the alcoholic, Salaspuro and Maenpaeae (161) and also Lieber and DeCarli (113) presented data to indicate that the oxidation of fatty acids and the citrate cycle are strongly inhibited during ethanol oxidation as demonstrated by lowered carbon dioxide production. Hence the "shift" toward fatty acid synthesis due to its replacement by ethanol in the energy-yielding process and the similarity of the NADH generated from the oxidation of ethanol to that generated from the oxidation of fatty acids is the main cause for the development of a fatty liver in the alcoholic (5, 37, 111).

It would seem, therefore, from the foregoing evidence that ethanol interacts with NAD which causes a fall of the $NAD/NADH_2$ ratio in the liver. Consequently a rise of lactate/pyruvate ratio follows. The decrease in the pyruvate causes an inhibition of gluconeogenesis accompanied by a rise in α-glycerophosphate concentration in the liver. All of these changes lead to the accumulation of fat in the liver, hypoglycemia, hyperlactemia and hyperuricemia (Figure 2). Krebs (105) contends that the $NAD/NADH_2$ ratio of the cytoplasm and mitochondria are maintained and regulated within relatively narrow limits and any condition that upsets their ratio leads to pathogenesis of the liver. In support to this contention, Dajani and Kouyoumjian (39) conducted in vitro studies and were able to find a correlation between the concentrations of hepatic FFA as well as triglyceride with the levels of liver ADH and $NADH_2$.

As to the effect of ethanol on ATP, French (60) presented evidence indicating an increase in acute treatment with ethanol and a

decrease after chronic ingestion. This was attributed to degenerative changes in liver mitochondria as reported by Kiessling and Tilander (100) and Kiessling and Pilstrom (101, 102).

Experimental Liver Cirrhosis and Ethanol

Alcoholic liver cirrhosis has never been reproduced in experimental animals, although fatty liver has been produced by many investigators (23, 24, 105, 146). It is well established that fatty liver may precede the development of cirrhosis, but it is not clear whether there is cause and effect relationship. Recently, however, Porta, et al. (146) described a procedure of producing fatty livers, hepatofibrosis, cirrhosis and Mallory bodies in rats that have been put on diet deficient in lipotropes (146).

Many agents have been described in the literature to produce cirrhosis. In reviewing these agents Moon (129) included various agents both inorganic elements such as phosphorus, arsenic, lead, manganese, copper and organic substances such as alcohol, chloroform, tar, carbon tetrachloride and related substances, infectious and bacterial products of protein decomposition and immune serum. He suggests that alcohol may accentuate the injurious effects of these agents. White, et al. (209) were able to produce cirrhosis in the rat by total body irradiation. Compos, et al. (27) conducted similar experiments except that the carbon tetrachloride was given by inhalation daily for 7 weeks, in conjunction with 9.5 per cent ethanol in drinking water. They suggested that cirrhotic changes in the liver increase the fraction of ethanol lost by non-oxidative means. Bunyan, et al. (24) gave carbon tetrachloride orally 2.0 ml/kg to study the ATP level in the liver. There was a slight

decline which could not be prevented by vitamin E. This suggested to him that carbon tetrachloride does not increase lipid peroxidation in vivo. Fischer (56) produced acute carbon tetrachloride poisoning by giving rabbits 2.5 ml/kg I.V. in which alcohol elimination was increased. This, he contends, supports the hypothesis that the liver NAD/NADH ratio effected alcohol elimination.

Solodkowska, et al. (174) used carbon tetrachloride induced cirrhosis to study the incorporation of carbon-1 of ethanol into lipids in both "drinker" and "non-drinker" strains. The rate of combustion of C-1 ethanol to CO_2 was significantly higher in carbon tetrachloride treated rats than untreated.

Paradoxically, Loyke (121) treated experimental hypertension in rats with carbon tetrachloride. This suggested to him that liver injury caused by carbon tetrachloride is associated with formation of a substance that inhibits circulating converting enzymes.

Cornish, et al. (34) studied serum enzyme response in rats after they were given a variety of alcohols 18 hours prior to carbon tetrachloride exposure. They found a highly significant potentiation of the enzyme. Cartier and Leroux (29) studied carbon tetrachloride poisoning in rats to determine its effect on the acid soluble free nucleotides of the liver. They found a 30 per cent decrease in pyridines, purines and ATP. These effects, he suggests, appear to be due to an alteration of the respiratory functions of liver mitochondria through alterations of oxidative phosphorylation. Lawrence and Sanat (107) used carbon tetrachloride poisoning to study the portal and hepatic beds. Shunt development correlated well with the general deterioration of liver function.

Reichard (152) investigated alterations in ornithine carbamyl transferase during severe carbon tetrachloride poisoning. Edwards and Dalton (47) gave carbon tetrachloride in olive oil in doses of 0.04cc to mice and were able to produce cirrhosis within a period of six weeks. When the compound was removed, conditions were restored to normal within one month.

Rubin, et al. (156) studied the mechanism of carbon tetrachloride induced cirrhosis in rats. They found striking proliferation of mesenchymal cells. They also noted a diminished uptake of thymidine-H^3 by hepatocytes and a progressive rise in DNA content. During non-septal fibrogenesis, collagen measured as hydroxyproline increased in like manner. During septal fibrogenesis, however, collagen increased out of proportion and the hydroxyproline/DNA ratio doubled, a fact, which they suggest might reflect irreversibility of cirrhosis.

Some workers, however, contend that there are differences in strain susceptibility to induction to cirrhosis in experimental animals. Patek, et al. (142) found this susceptibility to be greater in the Wistar, less in the Sherman and considerably less in the Long-Evans. They suggested that conditions other than food intake such as constitutionality might be responsible for the difference.

Gillespie and Lucas (68) provided further evidence that the source of supply as well as the type of strain used may be an important variable. Arvola and Forsander (2) found similar variation and inter-individual fluctuation of the voluntary intake of alcohol by laboratory rats and mice, but the average alcohol consumption within a species of animal was constant.

The Etiology of Alcoholism: Heredity vs Environmental and Nutritional Factors

There is a wide variability among individuals in the amount of alcohol required to bring about signs of intoxication or impairment of function. This fact led Williams (211) to postulate that environmental factors do not bring about alcoholism unless the individual involved possesses the type of "metabolic individuality" which predisposes toward addiction such as the pathological intoxication or drug idiosyncrasy. Hence, psychological stresses do not make a person an alcoholic unless he has inherited a metabolic pattern. Williams, et al. (212) presented evidence to show that alcoholism is a genetotropic disease which is defined by him as a disease in which genetic factors and nutritional deficiencies together are the etiological agents. The experimental work they did on rats (213) demonstrated that sugar consumption, as well as alcohol consumption are increased by deficient diets. Mardones (125) presented data to the effect that there is a genetic factor in alcohol preference in rats. Animal experiments, however, particularly those of Best (9) have established beyond any doubt the importance of dietary supplements, particularly lipotropic factors, in the prevention of nutritional liver damage. His conclusion suggests that an imbalance between caloric intake and supply of accessory food factors is the cause of liver disease. Lester, et al. (109) do not support this same view.

Although many workers in this field (13, 23, 24, 63, 108, 141, 206, 213) provided evidence along the same line as that of Best, namely, that low protein diet (6%-30% casein) developed cirrhosis in rats these conditions often aggravated but did not cause the liver damage. Experimental evidence as to whether ethanol in the presence of a normal diet

can bring about an increased triglyceride formation in the liver was provided by Lieber and DeCarli (113) and Lieber and Spritz (115) who demonstrated that partial protection from alcoholic fatty liver can be achieved with large amounts of DL-methionine or choline with an average reduction of 80 per cent of triglyceride accumulation in the liver.

Takada, et al. (186) confirmed these results by providing further proof against the hypothesis that alcohol is cirrhogenic in humans by direct hepatotoxic action. They contended that this can afford hope for achieving some recovery of liver function if high protein diet is consumed containing abundant vitamins and essential food factors simultaneously with alcohol. Porta, et al. (145) presented data to indicate that when alcohol provides less than 30 per cent of the total caloric intake of rats, harmful dietary imbalances do not result as long as the basal food mixture is reasonably adequate. However, Rubin and Lieber (157) do not agree completely with this concept. Their data do not exclude the possibility that chronic malnutrition may exacerbate alcohol-induced liver injury, but also demonstrate that alcohol itself is toxic to the liver, independent of nutritional factors. In a paired feeding experiment, Lowry, et al. (120) supported this view that alcohol increases the severity of the liver cirrhosis when rats are put on deficient diets. Furthermore, Kasliwal, et al. (96) presented data to support the same view and concluded that undernutrition may hasten cirrhogenesis by rendering the liver more vulnerable to the ill effects of hepatotoxic factors.

If alcohol is isocalorically replaced by sucrose, however, sucrose-fed animals show significantly more hepatic lipid than alcohol-fed rats (145). When sucrose-fed rats are put on a fortified diet, "Super

Diet", animals again showed a higher concentration of hepatic lipid than controls (186). In the perfused liver, the rat, unlike man, can make a histologic recovery from cirrhosis provided it receives an adequate diet (186).

As for the effect of ethanol on vitamins such as riboflavin, pyridoxine, thiamine and such lipotropic factors as choline, French (61) measured the changes in rat liver dehydrogenase and concluded ethanol ingestion is more deleterious to pyridoxine-deficient rats than controls. In a separate experiment (62), he showed that chronic ethanol feeding produced fatty changes in the liver and focal fibrosis, but not cirrhosis, particularly in vitamin B_6 deficient rats. Suomaleinen (184) gave 10 per cent ethanol to rats and found an immediate increase in the excretion of thiamine, pyridoxine and pantothenic acid. He observed no change in methionine, cobalamine and folic acid. Mardones (125) increased the alcohol intake of rats maintained on a diet deprived of thermolabile elements of the vitamin B complex, hence he advocated the use of genetotrophic etiology as a working hypothesis in the study of human alcoholism.

Wilgram (210) produced the Laennec's type of cirrhosis in monkeys and was unable to reverse the histological picture when animals were put back on a fully nutritious regimen. Auto-immunization against liver cell protein was considered by him as a possible etiology.

Dimberg, et al. (43) found increased serum enzyme values of glutamic-oxalacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT) and creatine phosphokinase in patients given a diet poor in carbohydrate which suggests liver and muscle damage.

The question that is raised by many is what would the effect of

the alcohol be on the liver that is already the site of cirrhosis. The answer was provided by Erenoglu, et al. (50) who gave alcohol daily to cirrhotic patients. One group received a nutritious diet and the other was restricted in protein. The first group of patients made steady improvement while in the second group there was definite deterioration. As indicated earlier, ethanol suppresses Krebs cycle activity in the liver. In man, unlike the rat, there is no way of reversing the lesions of cirrhosis once it sets in, although it has been shown that continued alcohol consumption with adequate diet such as in hospitalized patients is compatible with clinical improvement. According to Gyoergy and Goldblatt (72), tocopherol is an additional protective dietary factor which may compensate for the absence of sulfur-containing amino acids. In a similar experiment Gyorgy and Goldblatt (73) were able to reduce liver injury by giving from 10 to 20 mg choline daily whereas addition of 50 mg cystine daily accentuated liver cirrhosis.

Trace Metal Metabolism and Its Relation to Human Diseases

The literature on trace elements and its relationship to health and disease is immensely widespread. Trace-metal deficiencies have been known from the earliest historic times such as treatment of iron deficiency anemia with iron salts during the Roman times and the treatment of goiter with the ash of sponges which contain iodine by the Greeks. Hippocrates is known to have prescribed rusty water to restore vigor to pale persons. The physiological and biochemical role that they play in health and disease, however, is relatively of recent undertaking.

Although the term trace elements is used widely among various

disciplines of science it does connote to some, however, the idea that these elements do not play an important role. The term has been defined by many workers in this field. but it is generally agreed that any element that comprises less than 0.01 per cent of the organism is called a trace element (169). They are also known as micronutrients, oligonutrients or minor elements as opposed to macro or bulk elements. The designation "trace", however, often refers to the extent of our technical and analytical ability of detecting that particular element in biological material rather than its relative concentration. Hence, our concept of a "trace" keeps changing as we keep improving our method of detection. For example, according to a comment made by Vallee (196), zinc has lost its standing as a "trace" element because of its broad base of biological importance that has been recently discovered.

In the early twentieth century, the term "accessory food factors" was coined by Sir Frederick Gowland Hopkins. We recognize these factors today as vitamins. Although the metabolic importance of these compounds and their essentiality in the total economy of the organism at the cellular and enzymatic levels have been explored extensively, their counterpart, the trace metals, had to await the purified diet era in the early thirties in order to elucidate their role in the biological system. This was due to the fact that tools for their study were not available and even more important the advent of newer tools of biochemistry to isolate and characterize metalloenzymes were lacking. Today we recognize trace elements as an important part of the whole armamentarium of the biochemical makeup of the organism. They are associated with co-factors and enzymes for growth, repair, regeneration, homeostasis and

nutrition by acting on an electrochemical, catalytic or structural level of the cellular components.

In spite of much speculation as to how metals function in these enzymes, David Green's comment (70) that enzyme catalysis is the only rational explanation of how a trace substance can produce profound biologic effect seems to be pertinent at this point. However, one popular concept is that metals act as a bridge between substrate and the protein which activates the metal by withdrawing electrons from it, hence becoming positively charged and thus enabling it to withdraw electrons from substrate. A chemical change is precipitated and hence the free energy of activation is lowered by the electronic alteration mediated by the metal.

In other instances the metal plays an important role in the tertiary folding of the protein which may have to bring together two or three amino-acid residues that are located far apart on a polypeptide chain, but which may become active when brought into the proximity of each other. It is believed that metals are bound to proteins mainly by the histidine residues in the protein.

There are over 2000 enzymes that regulate a vast number of chemical reactions within the cell. Trace elements supply what Kench (99) calls the "Cationic Climate" and how a preferential absorption of certain trace elements can either block or promote enzyme activity and hence alter the entire biochemistry of the cell. To quote Kench (99) connection seems appropriate as to the important role of trace elements.

> The wealth of living forms is reflected at the cellular and sub-cellular level by a vast number of possible molecular inter-relationships, among which the relatively indestructible metal ions appear to have been exploited fully in a directive

> capacity, accelerating or decelerating the rate of structural change of the more evanescent carbon compounds, helping to provide the metabolic bridges and feed-back.

Enzymes activated by trace elements are divided into two classes according to Parisi, et al. (140): (1) metalloenzymes, which have a fixed amount of specific metallic ions firmly bonded to enzymes and whose metal/enzyme ratio is constant, (2) metal-activated enzymes or metal protein complexes where the metal is reversibly bonded to enzyme with a variable metal/protein ratio. Metals may substitute for each other and may also be removed by dialysis in the latter type of binding.

There are over thirty-seven elements out of the 102 in the periodic table that have been found in the human body (80). Twenty-six of these are metals and are present in only trace amounts some of which are too little to be detected by our present technique. According to Schroeder (169), there are 9 inorganic essential micronutrients, 7 metals and 2 non-metal. Four cause deficiency disease and three cause disease of accumulation. Ten elements have the capacity to be essential, four alkali or alkali earth and thirteen heavy metals to which modern man is exposed.

Schroeder (169) lists three types of trace metal deficiencies. These include simple deficiency such as iron and copper in man and Mo and Co in other mammals, and malabsorption or decreases in carriers of the blood such as zinc in Laennec's cirrhosis of the liver. Although overt toxicities of trace elements have been well established experimentally as well as clinically, small chronic exposure over long periods of time is not well defined. There are also excesses of trace elements, certain diseases such as Wilson disease and copper accumulation (8, 195),

and Parkinsonism and high manganese concentrationism (35). Other types of excesses may be due to other large dietary intakes, increased absorption or decreased excretion resulting from other disorders such as in biliary obstruction or renal insufficiency (169).

In spite of much speculation regarding essentiality of trace metals, several workers in this field (36, 144, 168, 191, 194) agree on the criteria for essentiality. First, it is present in all healthy tissues of all living things. Second, its concentration from one animal to the next is fairly constant if compared to non-essentials; even the variability of human organs as found by Perry, et al. (144) was more alike with respect to the absolute concentrations of essential than of non-essential metals. Third, its withdrawal from the body induces in a reproducible fashion the same structural and physiological abnormalities regardless of the species studied. Fourth, its addition either prevents or reverses these abnormalities. Fifth, the abnormalities manifested by the deficiency state are always accompanied by pertinent, specific biochemical changes. Sixth, these biochemical changes can be cured or prevented when the deficiency is corrected. Hence, by these criteria a beneficial effect on growth or reproduction does not qualify an element as essential.

Schroeder (169), however, has formulated a criteria of essentiality quite different. He contends that any element concentrated by the plant is essential. Such a rule is not always true, for example, lithium and rubidium are widely distributed in plants and yet they are not proven to be essential for man yet. Although elements may be present at a very low concentration it is conceivable that all of them may be present in

biological material even though we may not be able to detect them. Furthermore, lack of awareness of the importance of trace metals, in the biological system, has hampered their study. Recently, however, new tools have appeared on the horizon which have enabled us to determine qualitatively and for some elements quantitatively, concentration at the cellular level such as in various parts of the nephron (217) or the gut wall (154) by the use of such sophisticated tools as the laser and electron microprobes.

Various other analytical techniques such as emmission spectroscopy, atomic absorption, and neutron activation as well as mass spectroscopy have contributed a great deal to our understanding of trace metal distribution in the organism and particularly in man.

In spite of our great strides in trace metal biology we still lack a unified biological standard as a frame of reference and hence in reporting the amounts of trace metals in biological material, different observers used different frames of reference and different measures of central tendency. Thus, Nusbaum, et al. (135) reported values as concentrations in dry tissue. Koch, et al. (104) reported their results in modified dry ash. Thiers and Vallee (187) have used nitrogen content as the reference in calculating concentrations in cell fractions. Tipton and Cook (191) used micromoles of metal per gram tissue ash. We (215, 218) have always reported on concentrations in dry ash but also have given the ratio of dry ash to wet weight in different organs, because different organs ash differently. It is obvious, then, that there is an urgent need of securing better standard materials and a more unified reporting system in order to make inter-laboratory comparison more

meaningful.

Trace Metal Alterations and Their Relation to Health and Disease

Pathological alterations of trace metal metabolism is widespread in the literature (3, 71, 79, 92, 104, 110, 175, 190, 208). We (218) have reported trace metal alterations in autopsy material for four diseases: diastolic-hypertension, bronchogenic carcinoma, hepatic cirrhosis and myocardial infarction. Preliminary data for 5 patients from each of the 5 groups (including control group) are tabulated in Appendix I. We also sought differences between normal and arteriosclerotic aorta for trace metal content (216). There was a significant decrease in copper and zinc (Appendix II).

Magnesium

It is well established now that shifts in body magnesium have deleterious effect and can produce significant clinical manifestations (204, 205). The entire body of an adult contains about 25 g. It is the fourth most abundant cation being exceeded only by calcium, sodium, and potassium. Hence, the term "trace element" similar to that of iron can hardly be applied in this situation. Like potassium, it is mainly intracellular with a concentration of 26 mEq/l as compared to 151 mEq/l potassium and 15 mEq/l sodium. Approximately half of the magnesium resides in the skeleton and exchanges very slowly with the extracellular ions. The highest concentrations of magnesium are in the liver and muscle followed by the brain, kidney and red blood cells (203).

About one-third of the serum magnesium is bound to albumin and globulin and the remainder is in ionic form (203).

Magnesium is known to perform a large variety of functions. It activates a wide variety of enzymatic systems and frequently it acts as a necessary co-factor for enzymes concerned with hydrolysis and the transfer of large groups such as the magnesium activated adenosine-triphosphatase of mammalian muscle. It is also known to act as an irreplaceable link in keeping the ribosome components together as well as the bridging of the ribosome and m-RNA (205).

Magnesium is depressant to both the central nervous system and the peripheral nervous system and is known to maintain the normal excitability of neuromuscular junction.

The average intake of magnesium for an adult is approximately 300 mg although requirements may vary between infants and under certain conditions such as in pregnancy. Two-thirds of the magnesium intake is absorbed and that which appears in the kidneys causes diuresis, of this, normally 35 per cent is reabsorbed by the tubule (75). Diuretics which decrease the excretion of potassium increase the excretion of magnesium and vice versa. Aldosterone is known to increase the excretion of both (203). In renal failure, both magnesium and potassium are retained (205).

Diseases known to be caused by a shift in the metabolism of magnesium are widespread. Miller (128) described a normocalcemic, hypomagnesemic tetany. Martin, et al. (7) reported a magnesium depletion in heart failure and chronic nephitis. Smith and Hammarsten (170, 171) described low plasma magnesium in delirium tremens which was followed by improvement when magensium was administered.

Increased concentrations of plasma magnesium is associated with renal failure (170). This, however, is not always associated with renal

failure. Hills, et al. (81) described adrenal insufficiency in which there was high level of plasma serum. There are conflicting reports as to the levels of magnesium in portal cirrhosis and diabetes millitus (178).

Iron

Iron, like magnesium, can hardly be called a trace element since it is highly concentrated in the body. It is a vital constituent of the porphyrin enzymes such as the catalases, the peroxidases, and various members of the cytochrome system. The latter system is extremely important in the metabolism of all living cells. It is also an important constituent of the oxygen-transfer substances such as hemoglobin and myoglobin, transferrin which reduces the concentration of the ferrous ions in the blood and ferritin which transfers iron from the GI tract to other tissues. Among the non-porphyrin enzymes in which iron is an essential constituent are aconitase, aldolase and fumaric hydrogenase (194).

Concentrations of iron in the body varies with the species, age, species, nutrition, and state of health. Liver and spleen rank the highest in iron content, followed by kidney, heart, skeletal muscles, pancreas and brain (194).

Normally, the major part of iron is bound to a specific iron-binding protein, called transferrin. Other forms of iron have been described, such as the acid soluble iron and the plasma hemoglobin iron.

Excesses and deficiencies of iron have been described in the literature. Increases in the iron content of liver have been reported in human malignancies and chronic infection (190). A peculiar abnormality in iron metabolism occurs in hemochromatosis in which up to 50 g of iron can accumulate in the body. Excess dietary iron in alcoholic liquors

has led some investigators (31, 122, 123) to blame this source for a possible association between excessive consumption of alcohol and the development of iron overload. Deficiency states are well known in iron-deficiency anemia.

Zinc

Approximately 2.2 g of zinc are found in an average adult. The highest concentrations are found in the prostate and the retina: most other organs contain on the average 20-30 µg/g wet weight. Whole blood contains about 9.0 ppm (150).

One-third of the zinc is bound to globulin and the rest is loosely bound to albumin for transport purposes. Zinc forms an integral part of carbonic anhydrase and alcohol and lactic dehydrogenases (97, 196).

The average daily intake of zinc is about 12-15 µg, 4 per cent of which is excreted in the urine and the remainder is excreted in the feces. Very little of the absorbed zinc is excreted in the bile (196).

Although zinc deficiency causes paraketosis in swine the occurrence of uncomplicated primary zinc deficiency is not well established in man except for the work reported by Prasad, et al. (148). Vallee, et al. (199, 200) reported a marked decrease of zinc in the serum of patients with post-alcoholic cirrhosis as well as of patients with myocardial infarction (172). Low values of zinc were also reported in malignant tumors, hepatogenic jaundice and chronic nephritis. Vikbladh (201) observed low levels of serum zinc in leukemic patients. Vallee and Gibson (198) noted 10 per cent less zinc in the white blood cells of patients with myelogenous and lymphatic leukemia. Herring, et al. (79)

reported low levels of plasma zinc from patients with untreated pernicious anemia. Zinc levels were back to normal after patients were treated with vitamin B_{12}. Statistical analysis by Herring, et al. (80) showed no significant differences attributable to age or sex although mean plasma values for zinc were lower in the second and third decades than in the fourth decade.

Copper

It is estimated that the human body contains approximately 100-150 µg of copper, most of which is concentrated in the liver and the central nervous system. Spleen and bones have the lowest concentration. Most of the copper present in the plasma is firmly bound to alpha-2-globulin called ceruloplasmin which according to Markowitz (126) was discovered first by Holmberg and Laurell. A small fraction, about 5 per cent is bound to albumin. This copper protein complex acts as an oxidase upon many substrates such as serotonin, ascorbic acid and other polyphenols. Other enzymes activated by copper are uricase, and tyrosinase (194) and monoamine oxidase. Red blood cells also contain a copper protein that was called Markowitz "erythrocuprein" (126). Cartwright (30) believes that copper is involved in myelination of nervous tissue and the maintainance of normal skin pigmentation. Carres, et al. (28) and O'Dell (137) postulate that copper-deficient animals have defects of vascular integrity ascribable to abnormalities in the biogenesis of elastin. Its action in erythropoiesis is probably due to the increased absorption of iron and its incorporation into protoporphyrin in the formation of heme (194).

The average amount of copper ingested daily by an adult is about

2.5-5.0 mg, 99 per cent of which appears in the stool, and the majority of that portion absorbed is excreted via the bile. About 70 µg appear in the urine per day (194).

Manganese

Cotzias (35, 36) has contributed a great deal to our understanding of manganese absorpiton, excretion, turnover and excess. Ingested divalent manganese remains unchanged in the acid stomach, but as soon as it gets to the small intestine it becomes oxidized since alkaline conditions are more favorable for oxidation. Intestinal absorption, like that of iron depends a great deal on the body pool. After it is absorbed, it is transported to the liver where it is conjugated with bile and then is excreted into the intestine. Manganese absorbed is transported via B_1 globulin plasma transmanganin, the trivalent form (35).

Britton and Cotzias (20) and Borg and Cotzias (14) have demonstrated the dependence of manganese turnover upon supply. They suggested that disorders due to manganese deficiency or excess may result from slow or rapid rates of turnover. Hedge, et al. (74) have demonstrated that serum concentrations of manganese are almost universally elevated after acute coronary occlusion. Rubenstein, et al. (155) reported an unusual case of diabetes mellitus that responded to the hypoglycemic effect of the plant extract infusion of alfalfa due to the high content of manganese (45.5 µg/kg).

Recently, however, Everson and Shrader (52) reported an impairment of glucose tolerance and utilization in young adult guinea pigs deficient in manganese. Other effects were also noted in manganese deficiency such as skeletal defects including shortening of the leg bones,

enlargement and malformation of joints and abnormalities in the shape of the skull (52). Hurley (83) and Hurley, et al. (84) studied maternal dietary deficiency of manganese during pregnancy. They found irreversible congenital ataxia in the offspring of rats, mice and guinea pigs characterized by abnormal body rightly reflexes and mobility of the animal to orient itself in water. Supplementing with manganese during gestation completely prevented the development of these abnormalities.

Alterations in Trace Metal Homeostasis in Hepatic Dysfunction Due to Ethanol and Other Hepatotoxic Agents

Vallee's discovery (197) of ADH, a zinc metalloenzyme from horse liver containing 2 g atoms of zinc per mole of protein and his subsequent report of a conditional zinc deficiency in Laennec's syndrome (199) led many workers in this field to explore further the effect of alcohol and other hepatotoxic agents on the mobility of trace metals and electrolytes.

Sullivan, et al. (181) found 68 per cent of the cirrhotics and 21 per cent of other hospitalized patients with serum zinc level 3 standard deviations below the normal mean. They also found low magnesium and calcium levels in serum of cirrhotics independent of zinc concentration. Concomitant with this were a high urinary zinc and magnesium excretion and an abnormally low serum albumin and α_2-globulin. In a separate study (180), abnormal zincuria was transient, disappearing within two weeks in 80 per cent of those studied.

However, zinc excretion was not significantly changed when normal individuals ingested the alcohol. Cirrhotics, whose urinary excretion of zinc was high, were not further affected by the infusion of alcohol

(182).

Very recently, Boyett and Sullivan (17) demonstrated that low serum zinc in cirrhotics was a function of serum albumin concentration. Zinc, they found, was consistently bound to transferrin and α_2-macroglobulin. On the other hand, Prasad and Oberleas (151) presented evidence that amino acids are capable of binding zinc, hence the increased urinary excretion associated with the aminoaciduria in cirrhotic patients.

Oppenheimer, et al. (139) studied the zinc content of horse liver alcohol dehydrogenase and found that the specific activity of LADH decreases as zinc is removed from the enzyme even when the sulfhydryl groups remain intact. However, there was a linear relationship between LADH zinc content and the stoichiometry of co-enzyme binding.

Vallee (200) hypothesized that the alcohol dehydrogenase of liver is vulnerable to repeated or to continuous metabolic insults by high concentrations of ethanol, thus causing a higher dynamic tissue ethanol level and the inability of liver cells to oxidize it after a certain threshold value. This is coupled with alterations of the protein structure of this enzyme whose degradation can lead to the observed amino acid and zinc excretion reported earlier (151, 180).

Hyperzincuria and hypozincemia in cirrhotics have been repeatedly confirmed by other workers (92, 149, 181) as well as the low level of liver zinc (218).

In spite of all these reports on cirrhotic patients, there is still doubt as to the value of this hyperzincuria as a diagnostic tool in ambulatory persons with chronic alcoholism (76) since the metabolic changes manifest themselves only in advanced stages of the disease.

Doubts have also been voiced by Spry and Piper (177) relating abnormalities of zinc concentration without knowing the iron status in the body. They stipulated that iron and zinc are absorbed by different metabolic pathways and that there is an increased turnover of zinc in the iron-deficient rats possibly related to the short-life span and increased zinc concentrate of iron-deficient rat red blood cells.

As for the decreased zinc level of cirrhotic liver, doubts have also been raised as whether this is due to a change in the chemical composition of liver cells or whether it may simply reflect the replacement of normal liver cells by connective tissue which have been shown to have low content of trace metals (187).

In order to determine whether the biochemical changes are due to the pathological changes caused by alcohol per se or some other secondary effects, Moses (132) administered ethanol to rats for 28 weeks and measured urinary iron, copper, magnesium and zinc. He found negative zinc balance in the ethanol treated rats, slightly increased liver zinc, lower liver and bone iron and no change in copper and magnesium. Barak, et al. (4) investigated the zinc pattern in cirrhotic rats put on a choline deficient diet versus rats fed alcohol only. In the former group there was a deficiency of zinc while in the latter group liver manganese was low.

As mentioned under experimental liver cirrhosis, several workers used hepatotoxic agents to produce damage in the liver cells as a model for studying trace metal kinetics. Kahn and Ozeran (93) injected rats intraperitoneally with 10 per cent carbon tetrachloride to produce liver cirrhosis. They found significant decrease in the liver and serum zinc

level after five weeks of treatment. Saldeen and Brunk (162) damaged the liver of mice with 0.01 ml carbon tetrachloride to study the alkaline phosphatase distribution in the liver cells. They found a marked increase centrally in the biliary capillaries and in the cytoplasm and a moderate increase in the peripheral lobule of the liver. The increase was less pronounced when zinc was given in conjunction with carbon tetrachloride. In a similar experiment, Saldeen and Stenrum (163) gave radioactive zinc along with carbon tetrachloride and found that livers of animals that received $ZnCl_2$ and carbon tetrachloride showed higher radioactivity than controls not receiving carbon tetrachloride showing a great demand for zinc uptake in damaged cells particularly the parenchymal rather than the Kupfer cells.

In order to correct this deficiency and reverse the pathological changes caused by these hepatotoxic agents, several workers tried administration of trace metals. Vallee, et al. (199) used oral $ZnSO_4$ in doses of 19.5 µg/day in cirrhotic patients and claimed a significant improvement. Surprisingly, subsequent to these plausible findings, $ZnSO_4$ has never been used on a large scale in cirrhotic patients. Galakhova (65) gave $ZnSO_4$ to rats poisoned with carbon tetrachloride. Total lipids and phospholipids increased in the cirrhotic liver but failed to disappear with subcutaneous injection of the $ZnSO_4$. Fodor, et al. (57) pretreated cirrhotic livers with selenium and claimed a partial protection against the carbon tetrachloride-induced liver damage. Voinar, et al. (202) pretreated rats with $MnCl_2$ and were able to alter the pathological changes induced by carbon tetrachloride. Kelomiitseva (98) gave rabbits $CuSO_4$ and KI along with carbon tetrachloride. Pretreated rabbits showed more

normal liver histology.

As mentioned earlier, attempts have been made to explain the effect of zinc deficiency at the cellular level. Sandstead, et al. (164) demonstrated a decrease in thymidine incorporation in vivo into the nuclear DNA in dietary zinc deficiency. This phenomenon was reversed rapidly with a single injection of zinc. Christophersen (32) suggested that ethanol increases the oxidation of the β-hydroxybutyrate by facilitating its transport into the mitochondria.

Cation transport and electrolyte excretion have also been explored extensively by many workers during ethanol intoxication (95, 118, 179). Beard, et al. (6) presented data to indicate a retention of water, Na, K and Cl particularly in the animals receiving the higher doses of ethanol. He concluded that ethanol is capable of producing marked fluid alteration and electrolyte retention in the dog. Kalbfleisch, et al. (95) induced an acute urinary diuresis of magnesium, calcium and a decreased K excretion during ethanol administration to normal and alcoholic subjects. They stipulated that the increased magnesium excretion was not due to changes in renal blood flow or glomerular filtration. Lindeman (118) and Lindeman, et al. (117) observed magnesium deficiency in alcoholics. They reported increases in urine calcium and magnesium and a decreased urine potassium during acute ethanol ingestion. Substances such as glucose, galactose, fructose, casein, insulin and vasopressin had similar effects (118).

They postulated that the increased glucose uptake and glycolysis block divalent cation reabsorption and potassium secretion in the distal tubule of the nephron.

Kalant, et al. (94), after ethanol treatment in the rat, found a fall in the water content of the blood and a rise in K. Plasma K and Na fell down. Tissues showed a rise in intracellular water and Na and a fall in intracellular K, thus confirming the work of Beard, et al. (6). Knutsson and Katz (103) stipulated that ethanol might increase Na permeability of the membrane although at low concentration, ethanol had no effect on K membrane permeability.

Isreal-Jacard and Kallant (87) and Isreal, et al. (88) confirmed these reports by using guinea pig brain cortex slices. Initially they found no loss of K in these tissues, although it subsequently inhibited to a significant degree the reaccumulation of K.

CHAPTER III

PURPOSE AND SCOPE

There seems to be a widespread belief among many workers in this field that metabolic aberrations and impairment of the liver function may explain some of the sequelae of alcohol addiction, which in turn leads to disturbance of the endocrine system such as the hypofunction of the adrenal cortex and the pituitary gland. The dichotomy between those who hold to the belief that alcohol addiction and its sequalae of hepatic dysfunction is genetic in origin versus those who offer an explanation based on epidemiological studies of culture and social patterns as well as diet habits seems to be widening as we learn more and more about the metabolic pathways of alcohol.

The reports on dietary deficiency of vitamins and trace elements are very conflicting as indicated heretofore. Although Vallee's work (199) has been confirmed repeatedly (93), zinc as a therapeutic agent has not come into wide use in alcoholic wards among cirrhotic patients. We have used carbon tetrachloride in this investigation as a model for inducing cirrhosis of the liver in the rat in order to explore several parameters of trace metal kinetics. Since cirrhosis has not been successfully produced in the rat with the use of ethanol we used it, to compare the trace metal "shift" in non-cirrhotic rats with those that became cirrhotic due to carbon tetrachloride.

One of the major aims of this research project is to test the hypothesis proposed earlier as to whether cirrhosis is a conditioned zinc deficiency or some other trace metal deficiency. Although zinc has been explored extensively in the liver, urine and sera of the cirrhotics, little has been done to study the kinetics of other essential minerals in tissues other than liver.

We have undertaken this investigation, therefore to answer five important questions:

1. Is trace metal alteration of magnesium, iron, copper, manganese, and zinc of the same magnitude and in the same direction in the cirrhotic rat as in the alcoholic under similar experimental conditions?
2. In which of the organs, heart, kidney and liver are the changes most prominent?
3. Are there any correlations between changes in the total urine output per certain time with organ changes of magnesium, iron, copper, manganese and zinc?
4. What is the pattern of change in time as experimental conditions continue to be applied?
5. Can the administration of zinc alone ameliorate the pathological changes in the liver or affect the distribution or excretion of the other essential minerals?

Due to the controversy surrounding the value of zinc as a therapeutic agent in post-alcoholic cirrhosis, it would be of interest to test this hypothesis and to extend the study further to shed some light on changes in other vital metals which may be equally important as zinc in

the metabolism of alcohol. Furthermore, a correlation between electrolyte changes in the urine and serum with that of the essential metals in the cirrhotic and alcoholic has not been described in the literature although scanty information indicates the inhibitory effect of ethanol on the active transport of cations across cell membrane (94). The present investigation, therefore, was undertaken with the hope of resolving some of these apparent contradictions.

CHAPTER IV

EQUIPMENT, METHODS AND PROCEDURE

Forty-eight adult female Holtzman albino rats purchased from Hormone Assay Laboratories, Chicago were received at our laboratories with ample supply of water and food before they were randomly divided into groups. They were weighed at the time they were received and every week thereafter until the end of the experiment. Their body weight at this time ranged between 100-125 grams.

After each rat was weighed, it was housed in an individual plastic cage especially designed for metabolic studies. Feces and urine were separated on an inverted polyethylene funnel in which several holes were made. The round plastic cage was placed on top of a funnel of the same diameter. The bottom was made of glass rods spaced to allow feces and urine to pass through. At the lower end of the funnel neck was placed a small piece of glass wool to catch any food particles or fecal material that happened to escape. Extreme care was taken to assure proper separation with minimal contact of feces and urine.

All rats were supplied with tap water and ground Rockland rat-mouse pellets ad libitum. The tap water was replaced with deionized water at the initiation of the experiment. After the rats were acclimatized to the laboratory environment at a fairly constant temperature of 72 ±2°C, they were randomly divided into six groups of eight rats each.

The treatment each group received was as outlined below:

Group I - Intraperitoneal injections of 0.15 ml per 100 g of body weight of 40 per cent carbon tetrachloride in olive oil.

Group II - Intraperitoneal injections of 0.6 ml per 100 g body weight of 40 per cent ethanol in physiological saline.

Group III - Control which received intraperitoneal injections of saline.

Group IV - Same as group I except that in addition to the carbon tetrachloride they also received zinc solution as zinc chloride in saline intraperitoneally at the rate of 100 µg zinc per 100 g body weight.

Group V - Same as group II except that they were zinc supplemented at the same rate as group IV.

Group VI - Received saline and zinc supplemented injections.

Zinc solution was prepared from a certified atomic absorption standard of 10,000 ppm solution in dilute nitric acid as solvent. Concentration of stock solution that was injected in the rats was 400 µg per 100 ml. Injection of all groups was made on the average of three times a week, Monday, Wednesday, and Friday for ten consecutive weeks. Although initially they were started on four injections per week, the death of two rats from group I and group IV in the first two weeks made it necessary to curtail injections to three times a week. The dead ones were replaced immediately with rats from the same batch which were started on the same treatment as their respective groups.

Collection of Urine

Urine was collected daily on each rat in a 25 ml polyethylene

vial and was pooled into a 250 ml plastic bottle to which was added 0.5 ml acetic acid as preservative. All plastic bottles were kept in the refrigerator except during the time of collection. At 2, 4, 6, 8 and 10 weeks of treatment the urine volume was filtered through glass wool and measured to the nearest milliliter. Three aliquots were collected in plastic vials. One aliquot was for the analysis of magnesium, iron, copper, manganese and zinc; another, for sodium, potassium and blood urea nitrogen analyses; and a third aliquot was placed in the freezer for further study.

At the end of four weeks of treatment, and at two weeks thereafter, two rats of each group were randomly chosen to be sacrificed. Each rat was anesthetized by an intraperitoneal injection of sodium pentobarbital at the rate of 30 mg per kg of body weight. After an incision was made through the thoraco-abdominal region, a needle of gauge 20 attached to a 10 ml plastic syringe was inserted into the ventricle of the heart and blood was collected without any anticoagulant. Blood volumes obtained this way ranged between 4 ml and 7 ml on each rat. Immediately after collection, blood was squeezed out carefully into a plastic cnetrifuge tube, left to coagulate and then centrifuged once to obtain the serum which was pipetted into a polyethylene vial and then stored in the refrigerator until the time of analysis.

Organs were obtained consistently in a similar fashion in order to avoid any changes due to surgical manipulation and hence changes in wet organ weight. The heart was excised right above the auricles, blotted well of blood; placed in a plastic vial and stored in the refrigerator for analysis. The liver was carefully separated from connective

tissue, placed on a parafilm and three thin sections were sliced off with a stainless steel blade. Two of them went into a 10 per cent formalin for histopathological examination of eosin and hematoxylin stain preparation and the third one into the deep freeze for oil red fat stain. The sections were consistently obtained from the upper left lobe to avoid intrahepatic variability. The kidneys were cleaned of fat tissue and stored away in the refrigerator. All vials were weighed before and after organs were placed in them. The difference between the two weights was considered to be the wet weight. Extreme precaution was taken at every step to insure minimum contact of the sample with contaminated objects. All glassware containers were acid washed and all plastic vials were used only once.

Trace Metal Analysis

Liver, kidney, heart, urine and serum were analyzed for magnesium, iron, copper, manganese and zinc by atomic absorption spectrophotometry using the Perkin-Elmer atomic absorption spectrophotometer, Model 303 to which is attached a Digital Concentration Readout (DCR) which provides some means of averaging the signal. The DCR can display on an illuminated readout either sample concentration or absorbance readings, approximately every two seconds or maintain a reading indefinitely. We have used the scale expansion feature of the DCR which enabled us to make the readout display the concentration value of the sample instead of the absorbance. In addition to that we have utilized the correction feature of the non-linearity in the working curve. These added features of the 303 Perkin-Elmer atomic absorption did not only speed up the analysis of the large number of samples we have, but also made it possible to recheck

the readout concentration of the standards every other fifth sample to make sure that the instrument parameters were consistent throughout the daily run. Furthermore, this digital voltmeter had a significant improvement upon the ordinary limits of precision and detection.

Since the Perkin-Elmer Atomic Absorption Spectrophotometer Model 303 is a double beam system, Table 1 is presented to illustrate instrumental specifications.

TABLE 1

SPECIFICATIONS OF INSTRUMENTAL CONDITIONS FOR ATOMIC ABSORPTION

Photometer:	Double-beam system with a single detector shared in time by both beams; A-C output.
Monochromator:	Czerny-Turner grating with dispersion of 6.5A per mm in the UV region and 13.0A in the visible.
Wave Length Range:	1950 Å - 8521 Å
Resolution:	0.5 Å in the UV and 1.0 Å in the visible.
Readout:	Counter readout in per cent absorption at scale control setting of 1. Scale expansion of 1X, 2X, 5X, and 10X is available.

Optical System

As illustrated in Figure 3, the chopper which is a two segment rotating mirror with an open area between each segment creates a sample beam to M_6 and a reference beam to M_9 both modulated at 60 cycles per second. After the two beams are combined, they are focused on the entrance slit of the monochromator and then collimated by spherical mirror

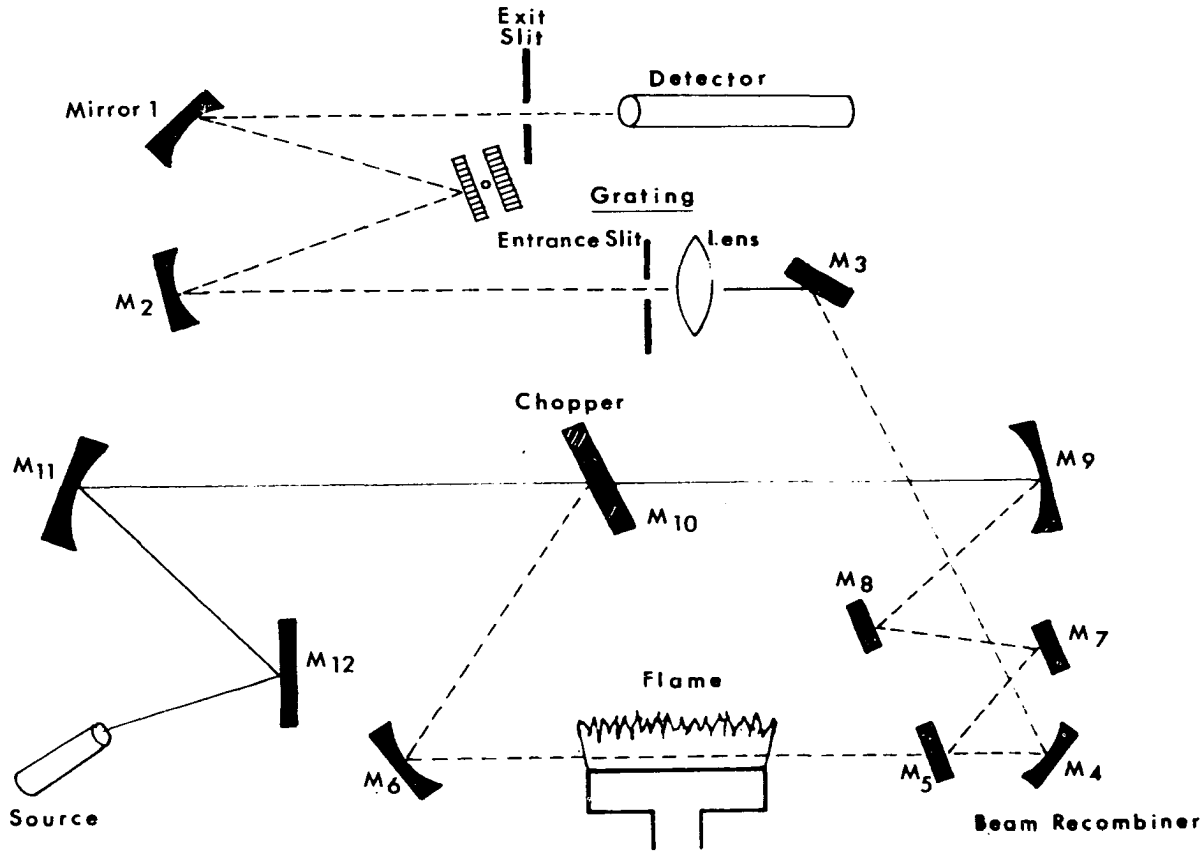

Fig. 3--Schematic diagram of the double-beam optical system of Perkin-Elmer AA Model 303.

M_2 which has a focal length of 400 mm. The beam is then directed to either of the two diffraction gratings, depending upon the setting of range control. The image formed on the exit slit is picked up by the photomultiplier detector which is located just behind exit slit.

Sample Preparations

Urine and serum were aspirated directly into the flame using a premix burner whose sample uptake was approximately 5 ml per minute. The oxidizing flame was made up of compressed air at 25 psi pressure and acetylene at a pressure of 8 psi. The tissues were dried in an oven at 110-115C in pyrex boats for 24 hours. No attempt was made to weigh the tissues after they were dried. From previous experience, it was felt that this was sufficient to get rid of most of the water and any small residue left would have been taken care of by the 24 hour period of subsequent ashing. Furthermore, tissue concentration of metals was expressed in terms of either µg per gram tissue ash or grams per 100 gram tissue ash, hence dry values were not necessary.

Ashing was done in a Tracerlab Low Temperature Asher (Model 600) for a period of 24 hours. This technique is relatively new and has the advantage of retaining most of the volatile elements in the ashed sample. Oxidation of the organic materials for each sample takes place in a closed system at low temperature which does not exceed 150°C. By virtue of the fact that the system utilizes streams of molecular oxygen which passes through an electromagnetic field of electrodeless-radio-frequency, the absorbed energy creates activated oxygen atoms which break the molecular bonds of the organic constituents of the sample and thus produce gaseous oxides such as CO_2, H_2O and SO_3 which are removed from the system

by a vacuum pump. The chambers were maintained at an oxygen flow of 70cc per minute and a vacuum of 1 mm of mercury. The radio-frequency forward power was operated at 250 watts with minimum reflected power.

It is well known that different tissues ash differently and hence require different lengths of time for complete ashing. In order to be consistent throughout the ashing procedure an equal time of 24 hours was alloted for each of the kidney, liver and heart tissues. This is three-fold the length of time recommended by the manufacturers.

Ashed tissues were collected directly from the pyrex boats into a weighed polyethylene vial. Only weighing paper was used to transfer the ash. Extreme care was taken to scrap the ashed sample well and minimize the small fraction left in the boat. The difference in weight between the empty vial and the vial with sample gave us the ash weight of the sample which was carried to one hundred thousandth of a gram.

To dissolve the ash, 0.10 ml of 6 N HCl was added to each 10 mg of ash. The vial was shaken and left overnight. Just prior to analysis the dissolved ash was then diluted 1:10 so that the final normality of the HCl was 0.60. Subsequent dilutions were made in 0.6 N HCl and this depended on metal concentration of that particular tissue. Manganese needed no further dilution in any of the organs. For copper, zinc and iron of the heart and kidney, a ten-fold dilution was necessary. In the liver a twenty-fold dilution was needed for these metals. For magnesium in all three tissues, one hundred-fold dilution was made in order to come within the range of instrumental sensitivity.

Method of Analysis

When all samples of every consecutive batch of urine, serum and

tissues were ready to be analyzed, a set of standard solutions was prepared fresh for each of the metals. For copper, manganese, iron and zinc in tissues a certified atomic absorption standard solution of 1000 ppm each were diluted with 0.6 N HCl to obtain a concentration of 40 ppm stock solution from which 0.8, 1.20, 1.60 ppm working standards were prepared for copper, manganese and zinc. Since sensitivity for iron was not as good, a higher concentration of working standards of 1.6, 3.2 and 6.4 ppm were prepared. The commercial standard solutions obtained from Fisher Scientific Company, Fairlawn, New Jersey were checked against a laboratory prepared Specpure salt solution.

For magnesium determination, a 10,000 ppm stock solution was serially diluted in a similar fashion to contain 0.04, 0.08 and 0.12 mEq/l. For sodium, potassium and blood urea nitrogen, samples were sent to the Renal Division laboratories of the Veterans Administration Hospital for analysis. Flame emmission was used for sodium and potassium and the Auto-Analyzer was utilized for the determination of blood urea nitrogen.

A working curve was prepared for each of the metals. Correction for non-linearity was switched on all the time during the analysis. Working standards were checked every fifth sample and the concentration readout on the illuminated DCR panel was recorded.

Instrumental settings for each particular metal were optimized and a warm-up period was allowed for the whole system to stabilize. The hollow cathode tube current for each metal was set according to the manufacturers recommendations as follows. Magnesium 6 ma, iron 40 ma, copper 15 ma, manganese 15 ma and zinc 15 ma. The corresponding reasonance line

for each metal was 2852 Å, 2483 Å, 3247 Å, 2794.8 Å and 2138 Å, respectively.

Data were collected and analyzed statistically at the Computer Facility to seek differences among groups for trace metal concentrations as well as the corresponding pathological changes in the liver. Analysis of variance, range, mean, standard deviation and F ratio were computed at the statistical significance of 5 per cent level. Data are tabulated in Appendix I for our published work on Tissue Trace Metal in Disease (216), in Appendix II on Decreased Zinc and Copper Content of Arteriosclerotic Aortas which was also published in Clinical Research (217) and in Appendix III which comprises all the data we have collected in this investigation.

CHAPTER V

RESULTS AND DISCUSSION

Pathological Alterations of Liver Tissue and Body Weight Changes

Success of this investigation has depended largely on the ease and speed of producing a pathological trauma in the liver or cirrhotic changes in order that the comparison of trace metal kinetics between cirrhotic and non-cirrhotic rats can be made. Fortunately our model system of carbon tetrachloride-induced cirrhosis which has been previously described was 100 per cent effective in altering liver cell architecture within four weeks of treatment.

As can be seen in Figures 4-6 a hematoxylin-eosine stain of liver sections are presented for the unsupplemented groups after eight weeks of treatment. Loss of liver cell architecture has been consistently seen in all the carbon tetrachloride treated rats except for the one that died before sacrifice (Figure 4). Furthermore, ascites was a prominent feature of most of these rats. Summary of the complete histological examination of liver sections is presented in Appendix III, Tables 7 and 8. In none of the rats that received alcohol were there any pathological changes except for occasional fatty infiltration which was confirmed with Oil Red O fat stain.

Figures 7-9 illustrate the pathological changes in the zinc

Fig. 4--Carbon tetrachloride induced cirrhosis with loss of liver architecture, pseudolobule formation by fibrous tissue, enlargement of some liver cells and hyperchromatic nuclei.

Fig. 5--Normal liver architecture of alcohol treated rats.

Fig. 6--Normal liver of control group.

HEMATOXYLIN AND EOSIN STAIN OF UNSUPPLEMENTED RAT LIVER TISSUE AFTER EIGHT WEEKS OF TREATMENT

Fig. 4

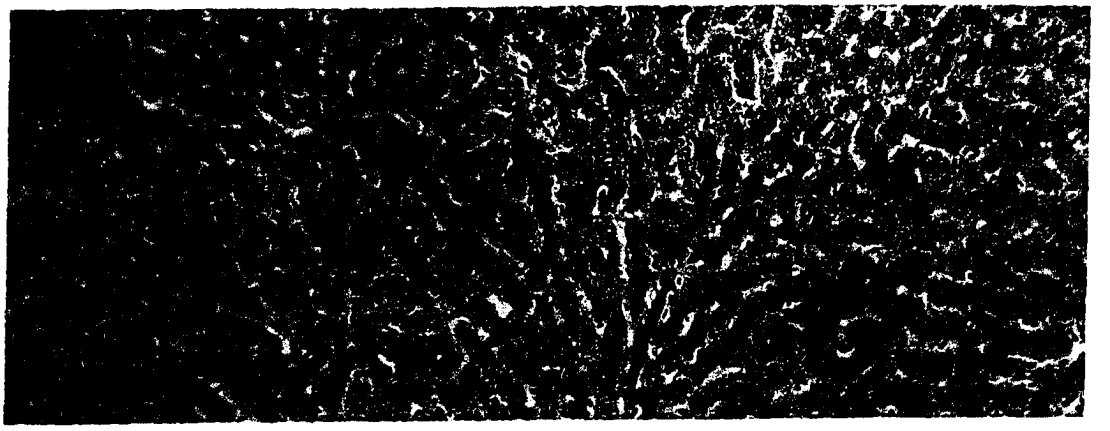

Fig. 5

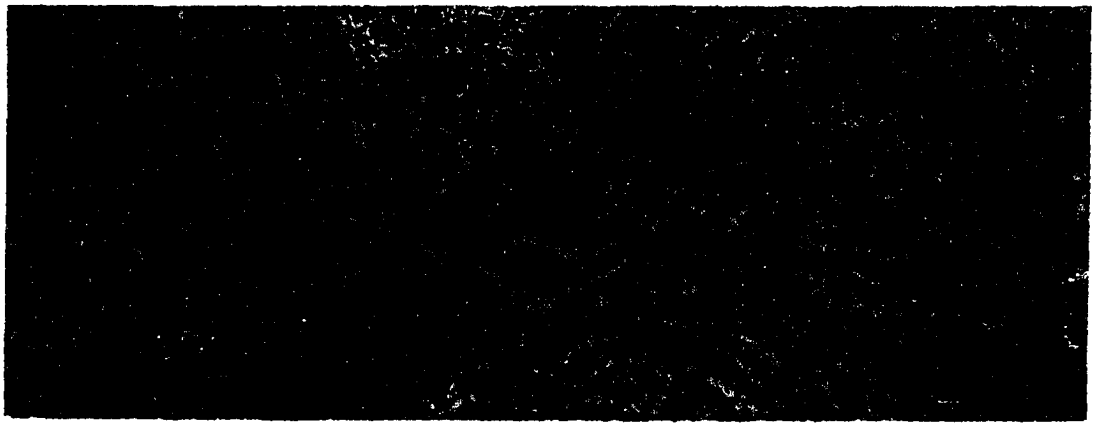

Fig. 6

Fig. 7--Carbon tetrachloride treated liver with zinc supplementation. Histological examination reveals loss of liver architecture, fibrosis around the pseudolobules and fatty infiltration.

Fig. 8--Normal liver architecture of alcohol treated and zinc supplemented rats.

Fig. 9--Normal liver of control group.

HEMATOXYLIN AND EOSIN STAIN OF ZINC SUPPLEMENTED RAT LIVER TISSUE AFTER EIGHT WEEKS OF TREATMENT

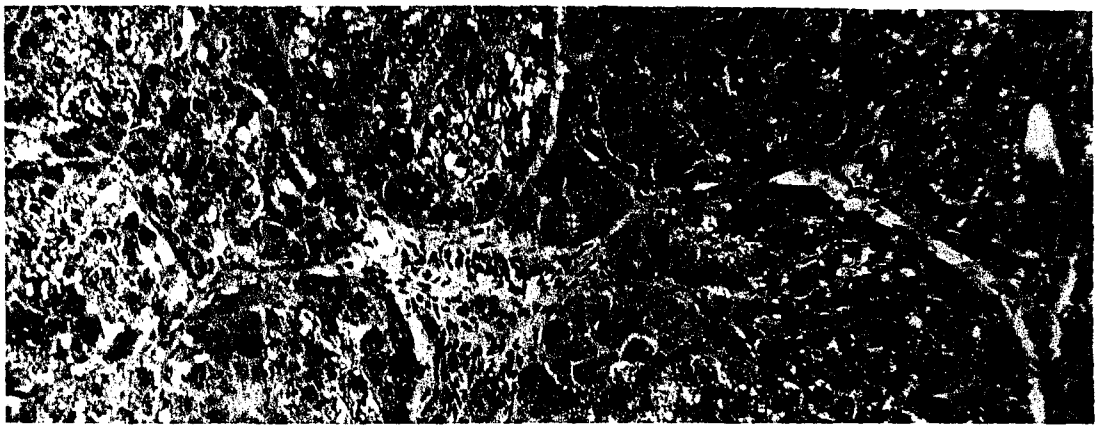

Fig. 7

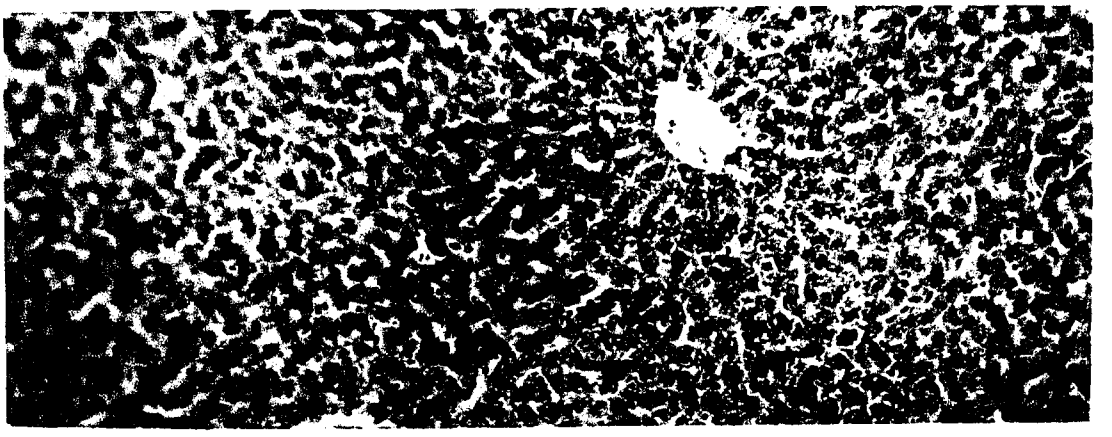

Fig. 8

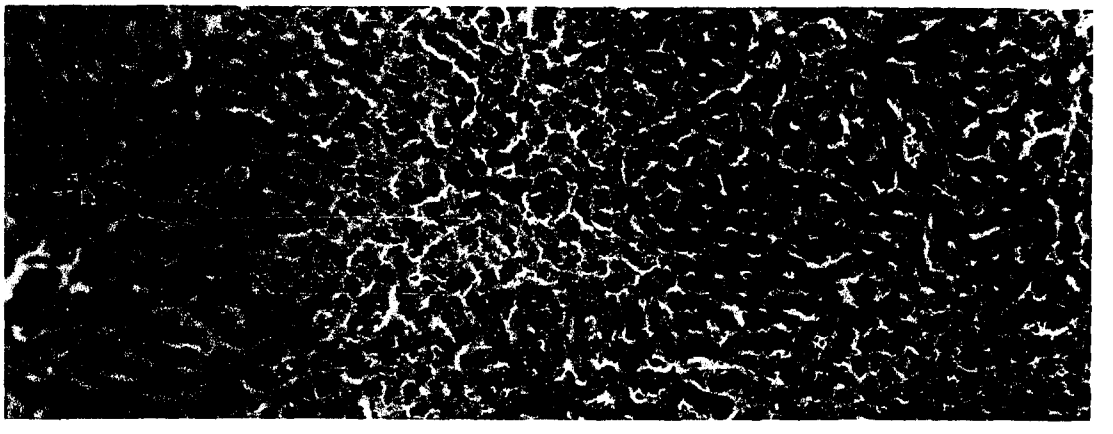

Fig. 9

supplemented group. There was no substantial improvement of the liver cell architecture as seen in Figure 7. This can lead us to the conclusion that under the present set of conditions described in this investigation optimal zinc supplementation of 1.0 mg/kg body weight does not appear to reverse the pathological trauma caused by carbon tetrachloride. It must be remembered, however, that the duration of the experiment was much too short, if an analogy was to be made between human cirrhosis which was claimed to be corrected by zinc and rat cirrhosis induced with carbon tetrachloride.

In order to assess the relative healthy conditions of the six groups, Figures 10 and 11 are presented to show the body weight gains at weekly intervals for unsupplemented and zinc supplemented groups respectively. Rats treated with carbon tetrachloride were slower in gaining weight during the course of the experiment than either alcohol treated or control groups. This was in spite of the ascitic conditions which most of these rats developed.

"Shifts" in Trace Metal Patterns

In an attempt to elucidate differences in trace metal patterns between carbon tetrachloride induced cirrhosis and chronic alcoholics without hepatic dysfunction, rat liver, heart and kidney as well as urine and sera were analyzed for five essential metals: magnesium, iron, manganese, copper and zinc. A comparison of the relative degree of metal kinetics and its interorgan mobility was made between those that were zinc-supplemented and those that were not supplemented under exactly similar experimental conditions.

In seeking statistically significant differences, data collected

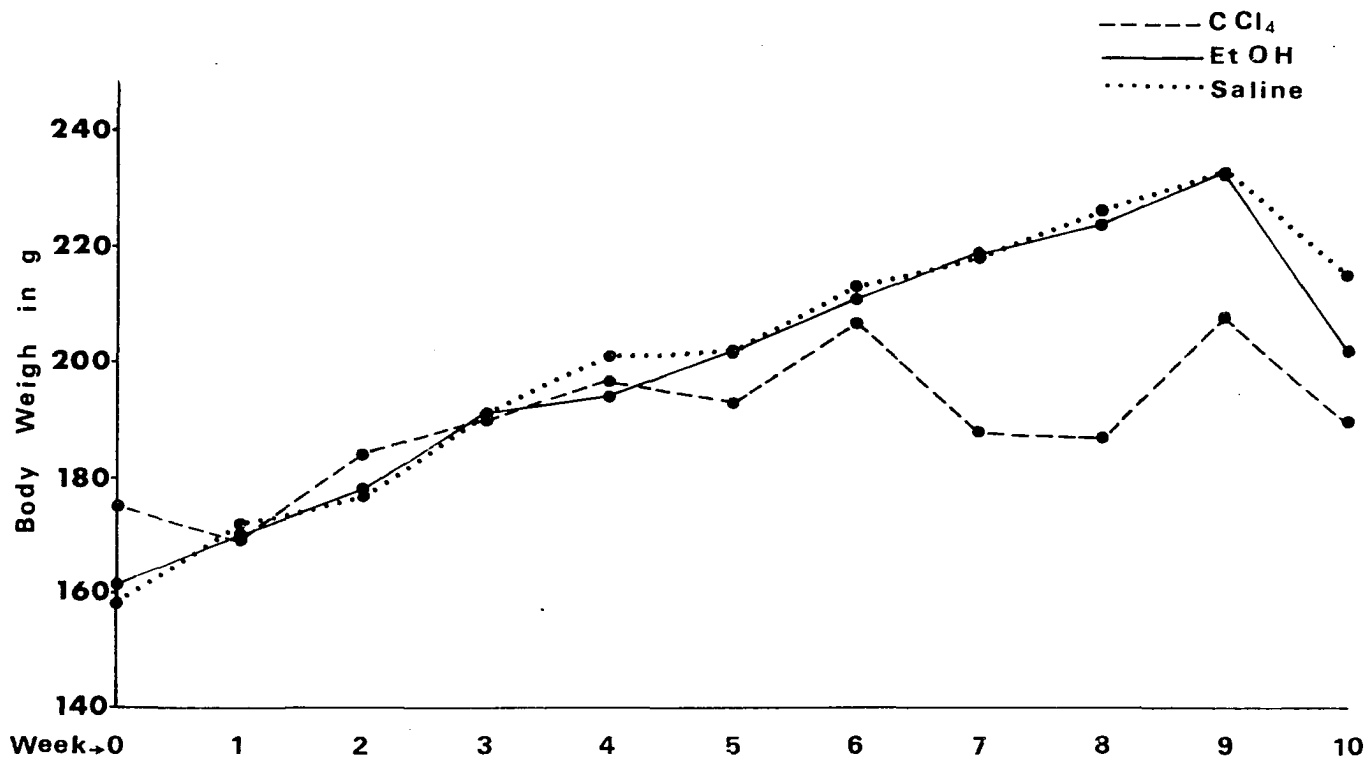

Fig. 10--Gains in body weights of unsupplemented rats.

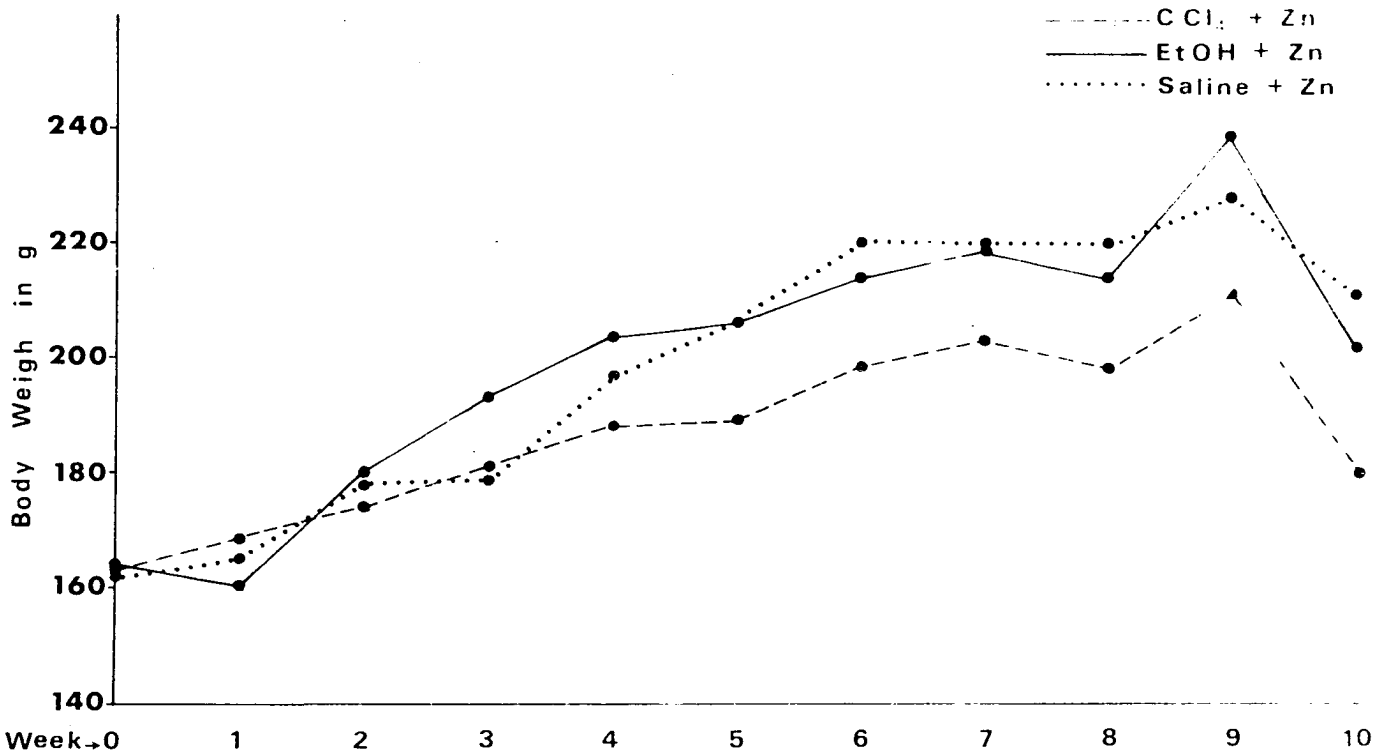

Fig. 11--Gains in body weights of Zn-supplemented rats.

were analyzed for means, standard deviation, analysis of variance and F ratios. Tabulations of the data for each metal in a particular organ or biological fluid are presented in Appendix III, Tables 9 through 36. Following each table is a corresponding bar graph (Figure 14 - Figure 34) to depict the intragroup changes as well as the variations displayed in time at 4, 6, 8 and 10 weeks of treatment. Bar graphs are not presented for serum and urine analyses. All significant differences at the 5 per cent level or less are indicated with a superscript asterisk. Summary of statistically significant increases or decreases in the concentration of five metals in three organs, urine and sera of zinc supplemented as compared with unsupplemented rats is presented in Figure 12 and Figure 13.

Heart Tissue - Zinc Supplemented vs. Unsupplemented Groups

Magnesium. As can be seen in Figure 14, concentrations of magnesium in the heart have increased uniformly in all six groups after ten weeks of treatment except in the fourth group which received zinc. Conversely, however, the first group which did not receive zinc has shown a consistently increasing concentration of magnesium in time although initially at four weeks of treatment, it was lower than the control values. In the unsupplemented groups, rats that received alcohol have maintained a higher concentration of cardiac tissue magnesium concentrations than control group in the same category. The over-all picture, then, seems to indicate that zinc supplementation helps maintain a level of magnesium in the heart similar to that of the control while in the group receiving alcohol, magnesium concentration fell down in the first eight weeks of treatment but regained its value at the end of the tenth week.

	Heart				Kidney				Liver			
	Unsupple.		Zn supple.		Unsupple.		Zn supple.		Unsupple.		Zn supple.	
	CCl_4	EtOH	CCl_4	EtOH	CCl_4	EtOH	CCl_4	EtOH	CCl_4	Et OH	CCl_4	Et OH
Mg	↑	↑	—	—	—	—	—	—	↓	—	↓	↑
Fe	↓	↓	↑	↑	↑	↓	↑	↓	↑	—	—	—
Cu	—	↑	—	↓	↑	↓	↑	—	↑	—	↓	—
Mn	↑	—	↓	—	↑	—	↑	—	—	↓	—	↑
Zn	—	—	—	—	—	—	—	—	↓	↑	↓	—

Fig. 12--Significant changes in tissue metal concentrations.

	Urine								Serum			
	Two Weeks of Treatment				Four Weeks of Treatment							
	Unsupple.		Zn supple.		Unsupple.		Zn supple.		Unsupple.		Zn supple.	
	CCl_4	EtOH	CCl_4	EtOH	CCl_4	EtOH	CCl_4	EtOH	CCl_4	EtOH	CCl_4	EtOH
Mg	↑	↑	↑	↑	↑	—	↑	↑	↑	—	↑	↑
Fe	—	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
Cu	↑	↓	↑	↓	↑	↓	↑	↓	—	↑	↑	↑
Mn	↑	↓	↑	—	↑	↓	—	↓	—	—	—	—
Zn	↑	↓	↑	↓	↑	↑	↑	↓	—	—	—	—
Na	—	↓	↓	↓	↓	—	↓	↓	—	—	—	—
K	↑	↓	↑	↑	↓	↓	↓	↓	—	—	↓	—

Fig. 13--Significant changes in urine and serum metal concentrations.

Iron. In general, levels of iron were depressed in the rats that received carbon tetrachloride during the total course of the treatmentwhile in those that were zinc supplemented, iron content of the heart tissue gradually decreased, but then increased significantly at the termination of the experiment. In the unsupplemented group that received alcohol there was an initial increase in iron and then a leveling off to normal concentrations in subsequent weeks.

Copper. There was a statistically significant difference among the groups as well as in weeks of treatment. However, in both unsupplemented as well as zinc-supplemented carbon tetrachloride groups, levels of copper were kept virtually unchanged over time. In that of the alcohol groups, changes were inconsistent, although the trend as seen in Figure 16 points towards an increase in group two and a decrease in group five.

Manganese. Several different patterns of changes seem to have occurred in the manganese concentration of the heart tissue probably more so than with any other metal. There was an intra as well as an intergroup variability which was highly significant, more markedly so in group one and four at the sixth and tenth week of treatment. Manganese concentrations appear to have increased at least 40 per cent at the end of treatment with carbon tetrachloride whereas in the corresponding group that received zinc, manganese was initially high, but dropped to almost one-third of its value at the end of treatment. Groups that received alcohol did not change significantly from those of the control groups.

Zinc. No variability was noted among groups in the concentration of this metal, although there was an inconsistent change in the

sixth week for group one and four. The change was of the same magnitude, but not unidirectional. As expected, zinc supplemented rats did not show any significant increase in cardiac zinc as compared to unsupplemented groups.

Kidney Tissue - Zinc-Supplemented vs. Unsupplemented Groups

Magnesium. At six weeks of treatment, magnesium seems to be consistently diminished in all six groups. However, if an assumption is made that this may reflect a change in instrumental parameters for that particular period of time, then excluding this group would show a decrease in the carbon tetrachloride group and in the alcohol treated group as compared to the control, and zinc supplementation would appear to have brought the concentration close to normal levels. The highly significant difference reflected in the F ratio for the weeks is related to the depressed values at the 6 week period.

Iron. Kinetics of this metal in the kidney appears to have been influenced by both carbon tetrachloride and ethanol, increasing in the former and diminishing in the latter. Zinc supplementation does not seem to have any significant effect on changing the iron pattern in the kidney. As can be seen in Table 15, intra and intergroup variability is highly significant with group one and group four having an F ratio of 6.17 and 4.85 respectively.

Copper. Alterations in the copper concentration of the kidney seem to follow the same pattern as that of iron. It was markedly and significantly increased in carbon tetrachloride treated rats in both unsupplemented and zinc-supplemented groups. Conversely, however, rats that received alcohol showed a decreased copper content in the kidney

compared to the control groups. Zinc supplementation does not seem to change the magnitude of kidney copper levels.

Manganese. As can be seen in Figure 22, kidney manganese concentrations were similar to that of copper and iron. However, zinc supplementation seems to increase its levels in the carbon tetrachloride group and to diminish it in the rats that received alcohol.

Zinc. The pattern of zinc concentration in the kidney appears to be similar to that of magnesium, namely, depressed at the sixth week of treatment in all groups but rising again on subsequent weeks. In general, zinc supplementation did not raise its concentration in the kidney as it might be expected in the carbon tetrachloride and ethanol treated rats probably due to the essentiality of this metal and the necessity of the body to conserve it, although in the zinc supplemented control group where conservation is less likely to be necessary, the zinc level has risen gradually, particularly at the end of the tenth week of treatment.

Liver Tissue

Magnesium. In all groups, magnesium levels were initially high, but after six weeks of treatment liver concentrations appear to have dropped and then gradually increased at eight and ten weeks of treatment. The average group concentration, however, remained low for group one and four compared to the control groups. Zinc supplemented groups seem to have retained more magnesium in the liver than unsupplemented ones.

Iron. Hepatic iron seemed to vary in time, increasing in all groups during the course of treatment. This is not an unexpected finding since growing rats do have a maximum sensitivity and larger requirement

for essential metals such as iron. Nevertheless, the magnitude of this increase is much less frequent in the carbon tetrachloride and ethanol treated groups. The reciprocal relationship between iron and zinc does not seem to hold true here at least in the hepatic concentration, because it appears that the zinc supplemented control group has gained more iron than any other group. Since the liver is a large pool for iron storage it would not be surprising to find a great deal of variability.

Copper. In general, hepatic copper does not seem to have varied a great deal except in the fourth group where the average level was diminished in contrast to the control group. Whether this is due to zinc-supplementation which is believed to markedly decrease levels of hepatic copper or to the combined effect of carbon tetrachloride and zinc is not clear. It seems likely that there are similar, but as yet undefined, complex relationships between other metals. In contrast, however, unsupplemented rats that received carbon tetrachloride had a highly significant increase in hepatic copper at the end of the tenth week.

Manganese. No change could be observed in the hepatic manganese concentration neither in time nor among groups, although at the sixth week of treatment it was slightly depressed in group one and two.

Zinc. In view of the great attention that this metal has received in the pathogenesis of liver disease, a more careful assessment of its role will be given here. It must be borne in mind, however, that the short course of treatment may not represent a true picture of a classical human cirrhosis that has been described elsewhere. Nonetheless, hepatic kinetics of this metal was quite variable. Intra group

differences seem to be statistically significant reflecting a decrease in group one and group four if compared with their respective control groups. However, alcohol treated groups did not vary a great deal although there was a gradual increase in the unsupplemented in time and a steady leveling off in the zinc supplemented group. Groups two, three and six gained a substantial amount of zinc after ten weeks of treatment.

Changes in Wet and Ash Organ Weights

As can be seen in Table 24 there was no apparent difference in the heart wet weights between different groups neither was there a significant difference in the ash weights. Computation of per cent ash weight as the ratio of wet weight to ash weight ranged between 1.06 and 1.34. In view of the reports regarding involvement of alcohol with cardiomyopathies it was expected that this would be reflected in cardiac wet weight. It is conceivable that changes in size of the heart would lay behind any alterations in essential metal kinetics.

In contrast, significant differences were found in kidney weights which were higher in carbon tetrachloride treated rats than control. In the zinc supplemented group, however, kidney weights were not significantly different than its corresponding control. There was no apparent change in the alcohol treated groups. Per cent ash kidney was on the average higher than that of the heart. It ranged between 1.18 - 1.58 per cent.

Grossly the liver showed great variation in weight and texture in carbon tetrachloride treated rats, sometimes being small, at other times being large. For example, the wet weight was 13.5 g and 4.2 g after four weeks and eight weeks of treatment respectively. This would

be anticipated since fibrous tissue accumulates in the early stages of the disease, but later when normal liver cells degenerate they are replaced with connective tissue. In spite of these obvious differences, the mean wet for each group as seen in Table 26 did not increase more than 15 per cent in the carbon tetrachloride and ethanol treated rats of the unsupplemented groups and 20 per cent in the zinc supplemented ones. As expected per cent ash weight of liver tissue was the highest. It ranged between 1.35 - 2.35. It is apparent that this depends a great deal on the amount of connective tissue and hence on the treatment period at which the rat was sacrificed.

Trace metal values have been expressed in terms of concentrations per ash weight; hence no attempt has been made to calculate for the total content of a metal in a particular organ. It was felt that this would be unnecessary since the study was intended to show comparative concentrations rather than absolute values.

Urine

Since urine volume was pooled for each rat over a two week period, total output of each metal was computed for each group rather than concentrations. This would represent the true value of metal mobility particularly in view of the significant differences in total urine output.

As illustrated in Table 31 total urine output was approximately twice as much in carbon tetrachloride treated rats as in the controls in unsupplemented as well as zinc supplemented groups. This difference was consistently so at four weeks of treatment as seen in Table 33 and on subsequent collections at 6, 8 and 10 weeks which is not shown in

Appendix III. These latter collections were reduced by two urines every two weeks and hence a special statistical treatment is needed to evaluate them, but a look at the raw data would seem to suggest the same trend on that of the first two collections.

Several different patterns of change in the total urinary output of zinc, copper and manganese are observed at two (Table 31) and four (Table 33) weeks of treatment. It was not unexpected to see an increased total output of zinc in the carbon tetrachloride treated rats in both unsupplemented as well as zinc supplemented groups, but it was surprising to note a decrease in the alcohol treated rats in the corresponding groups. Most surprising, however, was the unchanged pattern of urinary zinc output in the zinc supplemented groups at two weeks of treatment. Conversely, at four weeks of treatment, increased output of zinc is noted in both groups and zinc supplementation has brought the output of the three groups closer to one another.

In the carbon tetrachloride treated group after two weeks, copper output increased two-fold, manganese increased approximately 30 per cent, iron remained unchanged, magnesium increased two-fold, potassium increased slightly and sodium remained unchanged. After four weeks of treatment, the same pattern was noted except that manganese output increased further to approximately three-fold that of the normal, iron diminished 25 per cent, sodium diminished slightly and potassium remained unchanged.

In the zinc supplemented groups, magnesium, copper, manganese, zinc and potassium remained higher than normal, whereas iron and sodium were diminished.

In the alcohol treated group, magnesium remained high, iron, copper and manganese were diminished at two and four weeks of treatment in both supplemented as well as zinc supplemented groups. Zinc output was diminished at two weeks of treatment, but was raised at four weeks of treatment in the unsupplemented group. Sodium and potassium were diminished or remained unchanged.

Serum

As can be seen in Figure 13 serum metal concentrations are not as variable as that of urine or tissues. It is hard to interpret the decreased concentrations of iron in all experimental groups due to the uncertainty of obtaining serum free of hemolysis. Copper and magnesium increases are interesting since they are in reciprocal relation to the hepatic concentrations, but in the same direction as that of the urine. Because of limitation of instrumental sensitivity as well as the insufficient aliquots of serum availability for analysis, manganese determination was not performed.

An unexpected finding in this investigation was the lack of change in serum zinc concentrations in unsupplemented as well as zinc supplemented groups. In view of the hypozincemia reported by Prasad (149) and Vallee (199), it is interesting to speculate as to whether the duration of the treatment was enough to produce hyperzincuria as reported above, but not enough to deplete the body stores particularly in blood serum which acts as a shuttle mechanism for all essential metals and hence the last pool to suffer the consequences of the depletion. In support of this view is the lack of increased zinc concentration in the supplemented groups as indicated in Figure 13.

CHAPTER VI

SUMMARY

A model system of carbon tetrachloride induced cirrhosis in the rat was used in this investigation to compare the relative degree of biochemical alterations of five essential metals: magnesium, iron, copper, manganese and zinc. An analogous system of ethanol and administration which is known not to produce pathological changes in the rat liver was carried out to test the hypothesis as to whether the changes in trace metal kinetics in cirrhosis are due solely to the hepatic cellular destruction by carbon tetrachloride or increased cellular permeability caused by ethanol. Each system was run with or without zinc supplementation to test the degree of effectiveness with which zinc can alter the mobility of essential metals and/or reverse the biochemical insult in the liver of carbon tetrachloride infected rats. It must be emphasized, however, that the two systems tested were run under exactly similar experimental conditions. Each system of carbon tetrachloride and ethanol had its own control of saline and saline with zinc in the unsupplemented and zinc supplemented groups respectively.

Each of the six groups of eight rats received on the average three intraperitoneal injections per week for ten consecutive weeks. Two rats of each group were sacrificed after four weeks of treatment and every consecutive two weeks thereafter.

In the 2200 determinations made by atomic absorption spectroscopy on heart, kidney, liver, urine and serum, differences were sought between cirrhotic and non-cirrhotic on one hand and between unsupplemented and zinc supplemented on the other. Results of these findings are outlined below:

1. Histological examination of liver sections revealed loss of normal liver architecture, fibrosis and fatty infiltration in the carbon tetrachloride treated rats.
2. No pathological changes were noted in the alcohol treated rat livers supporting the thesis that rats are immune to alcoholic cirrhosis.
3. Zinc supplementation failed to reverse the pathological alterations induced by carbon tetrachloride although it did change the distribution of some trace metals.
4. Urine output of carbon tetrachloride treated groups exceeded those of alcohol and control groups by two-fold. It is not known whether this is due to the large amount of water intake observed in these rats or to some other mechanism at the cellular level.
5. Trace metal pattern in the cardiac tissue was variable, but in general, magnesium increased in the unsupplemented groups, iron diminished but increased again on zinc supplementation, and zinc remained unchanged. Manganese increased in the carbon tetrachloride group, but reversed its direction on zinc supplementation. In contrast, copper increased in ethanol treated rats but was diminished on zinc supplementation.

6. Alterations in the renal trace metal kinetics revealed significant changes in iron and copper. There was no difference between unsupplemented and zinc supplemented groups. Both iron and copper increased in carbon tetrachloride treated kidneys but diminished in ethanol treated groups.
7. In the hepatic tissue, variation was inconsistent. However, the general trend seems to indicate increased concentrations of magnesium in the carbon tetrachloride group and increased zinc concentration in the ethanol treated ones. Copper increased, but was diminished on zinc supplementation. Manganese concentrations decreased in the carbon tetrachloride treated group.
8. Excretion rates of trace metals in the urine varied widely. In brief, magnesium remained high in both groups when compared to corresponding controls. In contrast, iron and sodium remained low in all test groups. Potassium, zinc, manganese and copper were variable, but in general they increased in carbon tetrachloride treated groups and diminished in the ethanol. Zinc supplementation did not change the pattern.
9. In the serum, variation was less striking than in the urine. Iron diminished in both groups, unsupplemented and zinc supplemented, whereas magnesium and copper urinary output increased. No significant alterations were noted in the total urinary zinc output.
10. It would appear from the aforementioned results that the clinical manifestations seen in human cirrhosis could be related to shifts in the essential micronutrients of the liver other than

zinc particularly copper and manganese. Further exploration on supplementation studies of these metals singly or in combination in the rats of our model system of carbon tetrachloride induced cirrhosis could provide some answers to the pathogenesis of liver disease and hence establish a rationale for treatment and therapy in humans. Furthermore, the excretion pattern of proteinaceous macromolecules in the urine in conjunction with trace metal concentrations could shed some light on the nature of the available binding sites and hence further our understanding of the sub-cellular pathology and etiology of this disease.

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APPENDIX I

TISSUE TRACE METAL IN DISEASE*

*Yunice, A., et al: Clin. Res. 16:32, 1968.

TABLE 2

CONCENTRATIONS IN MICROGRAMS PER GRAM ASH OF INDICATED METALS OF NORMAL AND CIRRHOTIC HUMAN LIVER, KIDNEY MEDULLA, KIDNEY CORTEX AND HEART

Tissue	N	Metal	Normal	Cirrhotic
Liver	5	Zn	12,262	5,851
		Cu	1,251	639
		Mo	45	14
		Ti	9.1	7.8
Kidney Medulla	5	Cu	302	167
		Mn	100	82
		Sn	47	36
		Ti	13.2	9.8
		V	2.0	0.24
Kidney Cortex	5	Fe	4,000	4,838
		Mn	96	100
Heart	5	Zn	2,881	3,335

Only statistically significant differences are given for the underlined values at the 5 per cent level.

TABLE 3

CONCENTRATIONS IN MICROGRAMS PER GRAM ASH OF INDICATED METALS OF NORMAL AND BROCHOGENIC CARCINOMA OF HUMAN LIVER, KIDNEY MEDULLA, KIDNEY CORTEX AND HEART

Tissue	N	Metal	Normal	Bronchogenic Carcinoma
Liver	5	Zn	12,262	9,546
		Cu	1,251	704
		Mo	45	70
		Ti	9.1	33.9
Kidney Medulla	5	Cu	302	264
		Mn	100	30
		Sn	47	18
		Ti	13.2	9.2
		V	2.0	1.8
Kidney Cortex	5	Fe	4,000	4,975
		Mn	96	48
Heart	5	Zn	2,881	2,958

Only statistically significant differences are given for the underlined values at the 5 per cent level.

TABLE 4

CONCENTRATIONS IN MICROGRAMS PER GRAM ASH OF INDICATED METALS OF NORMAL AND MYOCARDIAL INFARCTION OF HUMAN LIVER, KIDNEY MEDULLA, KIDNEY CORTEX AND HEART

Tissue	N	Metal	Normal	Cardiac Infarction
Liver	5	Zn	12,262	8,325
		Cu	1,251	574
		Mo	45	44
		Ti	9.1	17
Kidney Medulla	5	Cu	302	142
		Mn	100	52
		Sn	47	18
		Ti	13.2	19.6
		V	2.0	1.3
Kidney Cortex	5	Fe	4,000	4,547
		Mn	96	64
Heart	5	Zn	2,881	1,740

Only statistically significant differences are given for the underlined values at the 5 per cent level.

TABLE 5

CONCENTRATIONS IN MICROGRAMS PER GRAM ASH OF INDICATED METALS OF NORMAL AND HYPERTENSIVE OF HUMAN LIVER, KIDNEY MEDULLA, KIDNEY CORTEX AND HEART

Tissue	N	Metal	Normal	Hypertension
Liver	5	Zn	12,262	13,100
		Cu	1,251	964
		Mo	45	40
		Ti	9.1	8.3
Kidney Medulla	5	Cu	302	187
		Mn	100	39
		Sn	47	43
		Ti	13.2	6.2
		V	2.2	3.0
Kidney Cortex	5	Fe	4,000	5,534
		Mn	96	50
Heart	5	Zn	2,881	3,508

Only statistically significant differences are given for the underlined values at the 5 per cent level.

APPENDIX II

DECREASED ZINC AND COPPER OF ARTERIOSCLEROTIC AORTAS*

*Yunice, A., et al.: Clin. Res. 17:65, 1969.

TABLE 6

CONCENTRATIONS IN MICROGRAMS PER GRAM ASH OF INDICATED METALS OF NORMAL AND ARTERIOSCLEROTIC AORTAS

	Normal Aortas	Arteriosclerotic Aortas	P
N	22	18	
$\frac{\text{Dry weight}}{\text{wet weight}} \times 100$	33.8	38.5	> 0.10
$\frac{\text{Ash weight}}{\text{wet weight}} \times 100$	2.8	6.1	< 0.001
Cu	234	64	< 0.001
Zn	1196	652	< 0.001
Fe	213	292	> 0.10
CO	1.47	2.27	> 0.10
Pb	217	150	> 0.10

APPENDIX III

TABLE 7

SUMMARY OF THE PATHOLOGICAL FINDINGS IN THE UNSUPPLEMENTED RAT LIVERS (H&E STAIN)

Rat No.	$CC\ell_4$	EtOH	Saline
1	Fatty infiltration. Young fibroblasts in between liver cells and portal area.	Normal	Normal
2	Loss of liver architecture. Pseudo-lobule formation by fibrous tissue. Some liver cells are enlarged. Contain hyperchromatic nuclei.	Normal	Normal
3	Loss of normal liver architecture. Beginning of laying down of collagen by fibroblasts.	Normal	Normal
4	Fatty infiltration	Normal	Normal
5	Fatty infiltration	Normal	Normal
6	Marked fatty infiltration. Fibroblasts in between liver cells. Loss of normal liver architecture.	Normal	Normal
7	Autolysis	Normal	Normal
8	Loss of liver architecture. Hyper-chromatic nuclei. Few giant cells. Some mitosis.	Normal	Normal

TABLE 8

SUMMARY OF THE PATHOLOGICAL FINDINGS IN THE ZN-SUPPLEMENTED RAT LIVERS (H&E STAIN)

Rat No.	$CC\ell_4$ + Zn	EtOH + Zn	Saline + Zn
1	Loss of normal liver architecture. Marked vacualiation of liver cells. Fibrosis. Pseudolobulation and bile duct proliferation.	Normal	Normal
2	Autolysis	Normal	Normal
3	Loss of normal liver architecture. Fibrosis around the pseudolobules. Fatty infiltration.	Normal	Normal
4	Marked fatty infiltration. Few fibroblasts in between liver cells.	Normal	Normal
5	Fibroblasts in between liver cells. No fat.	Normal	Normal
6	Fatty infiltration. Little fibrosis.	Normal	Normal
7	Fatty infiltration. Some fibrosis.	Normal	Normal
8	Fibrosis with pseudolobule formation. Moderate fatty infiltration.	Normal	Normal

TABLE 9

MAGNESIUM CONTENT OF HEART TISSUE
IN g/100g OF TISSUE ASH

	Group Means					
Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
Weeks						
4	1.0970	1.5015	1.3680	1.5135	1.5590	1.4960
6	1.4435	1.5925	1.5045	1.6689	1.4800	1.5440
8	1.3980	1.4919	1.2795	1.5499	1.3435	1.4985
10	1.6105	1.7084	1.5835	1.5835	1.6869	1.7324

Analysis of Variance

	SS	DF	MS	F
Weeks	0.42	3	0.14	9.509*
Groups	0.26	5	5.28	3.567*
Groups x Weeks	0.25	15	1.66	1.122
Within Cell	0.35	24	1.48	

Simple Effect

Week within Groups

Groups	SS	DF	MS	F
1	0.27	3	9.15	6.17*
2	6.08	3	2.02	1.36
3	0.11	3	3.70	2.49
4	2.65	3	88.34	0.59
5	0.12	3	4.14	2.79
6	7.53	3	2.51	1.69

Group within Weeks

Weeks	SS	DF	MS	F
4	0.29	5	5.9	3.97*
6	6.71	5	1.3	0.90
8	0.10	5	2.1	1.45
10	4.40	5	87.9	0.59

*Significant at the 5 per cent level.

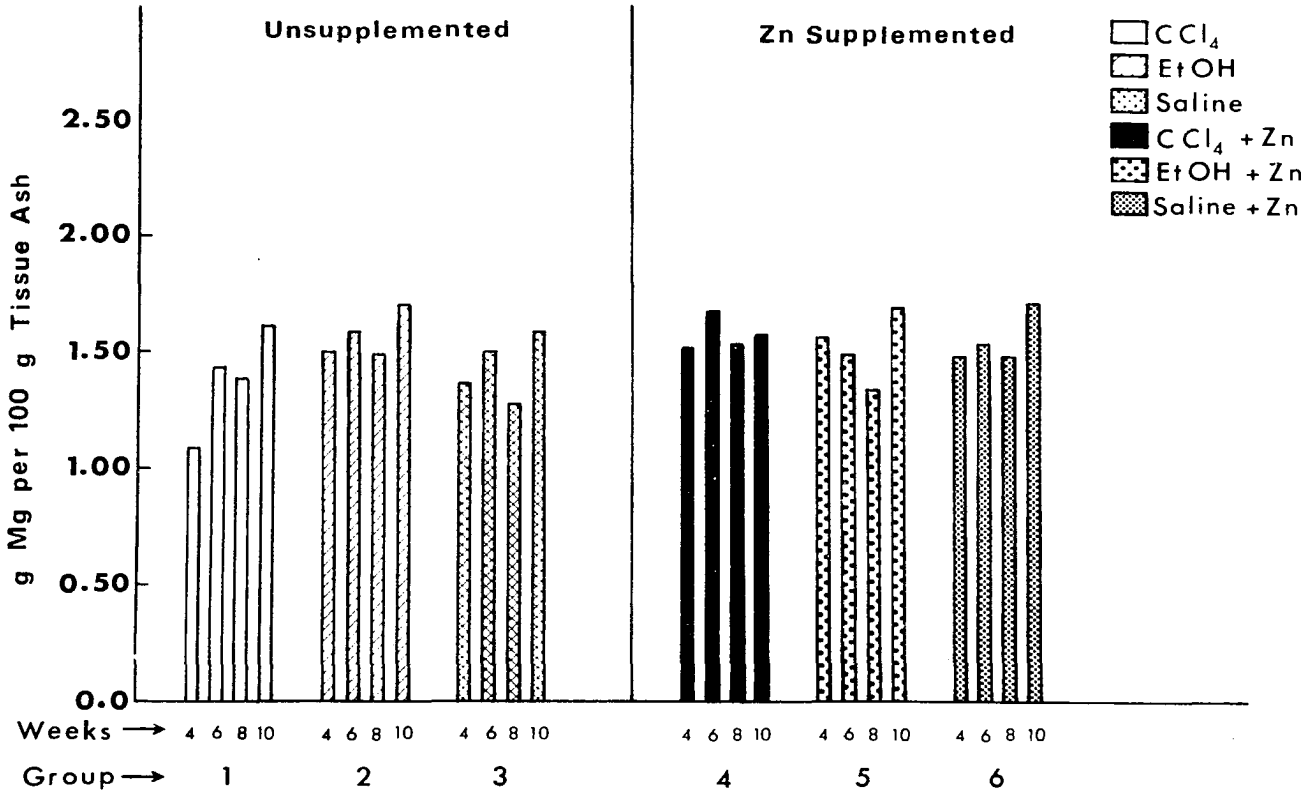

Fig. 14--Magnesium content of heart tissue.

TABLE 10

IRON CONTENT OF HEART TISSUE
IN g/100g OF TISSUE ASH

Group Means

Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
Weeks						
4	0.7200	1.1650	0.8050	0.8850	0.9900	0.8700
6	0.6550	0.9000	0.9350	0.7900	0.8549	0.9250
8	0.7400	0.7150	0.9100	0.6250	0.9599	0.7500
10	0.7050	0.8750	0.8050	1.3150	1.0250	0.9499

Analysis of Variance

	SS	DF	MS	F
Weeks	0.18	3	6.10	1.136
Groups	0.30	5	6.07	1.131
Groups x Weeks	0.66	15	4.41	0.821
Within Cell	1.29	24	5.37	

Simple Effect

Week within Groups

Group	SS	DF	MS	F
1	79	3	26	0.04
2	0.20	3	6.9	1.29
3	2.82	3	94.1	0.17
4	0.52	3	0.17	3.22*
5	0.32	3	1.07	0.20
6	4.75	3	1.58	0.29

Group within Weeks

Weeks	SS	DF	MS	F
4	0.24	5	4.82	0.89
6	0.11	5	2.26	0.42
8	0.16	5	3.19	0.59
10	0.45	5	9.01	1.67

*Significant at the 5 per cent level.

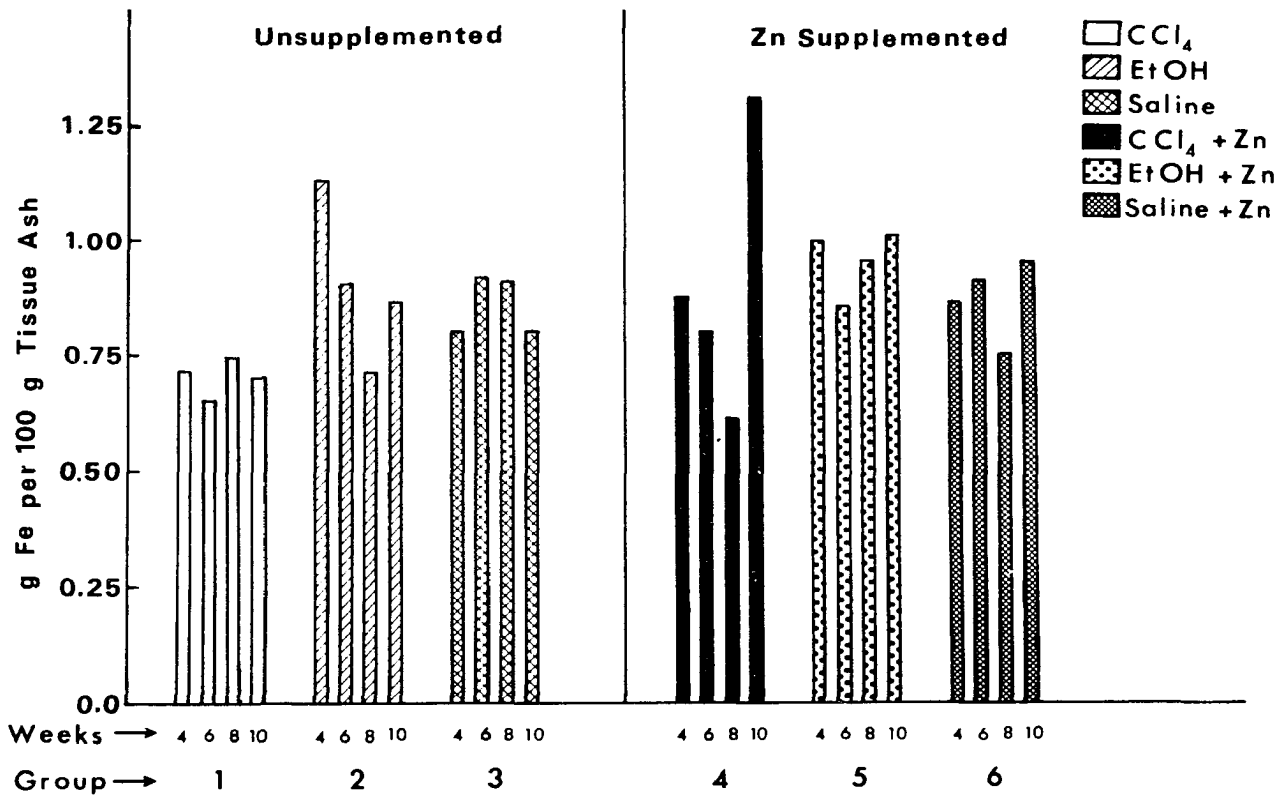

Fig. 15--Iron content of heart tissue.

TABLE 11

COPPER CONTENT OF HEART TISSUE
IN μg/g OF TISSUE ASH

Group Means

Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
Weeks						
4	365	365	355	340	420	385
6	345	405	415	355	375	385
8	325	365	300	340	280	355
10	380	445	410	335	345	430

Analysis of Variance

	SS	DF	MS	F
Weeks	27816	3	9272	6.868*
Groups	17475	5	3495	2.588*
Groups x Weeks	28808	15	1920	1.422
Within Cell	32400	24	13.50	

Simple Effect

Week within Groups

Group	SS	DF	MS	F
1	3437	3	1145	0.84
2	8800	3	2533	2.17
3	17500	3	5833	4.32*
4	450	3	150	0.11
5	20700	3	6900	5.11*
6	5737	3	1912	1.41

Group within Weeks

Weeks	SS	DF	MS	F
4	7766	5	1553	1.15
6	7500	5	1500	1.11
8	10675	5	2135	1.58
10	20341	5	4068	3.01*

*Significant at the 5 per cent level.

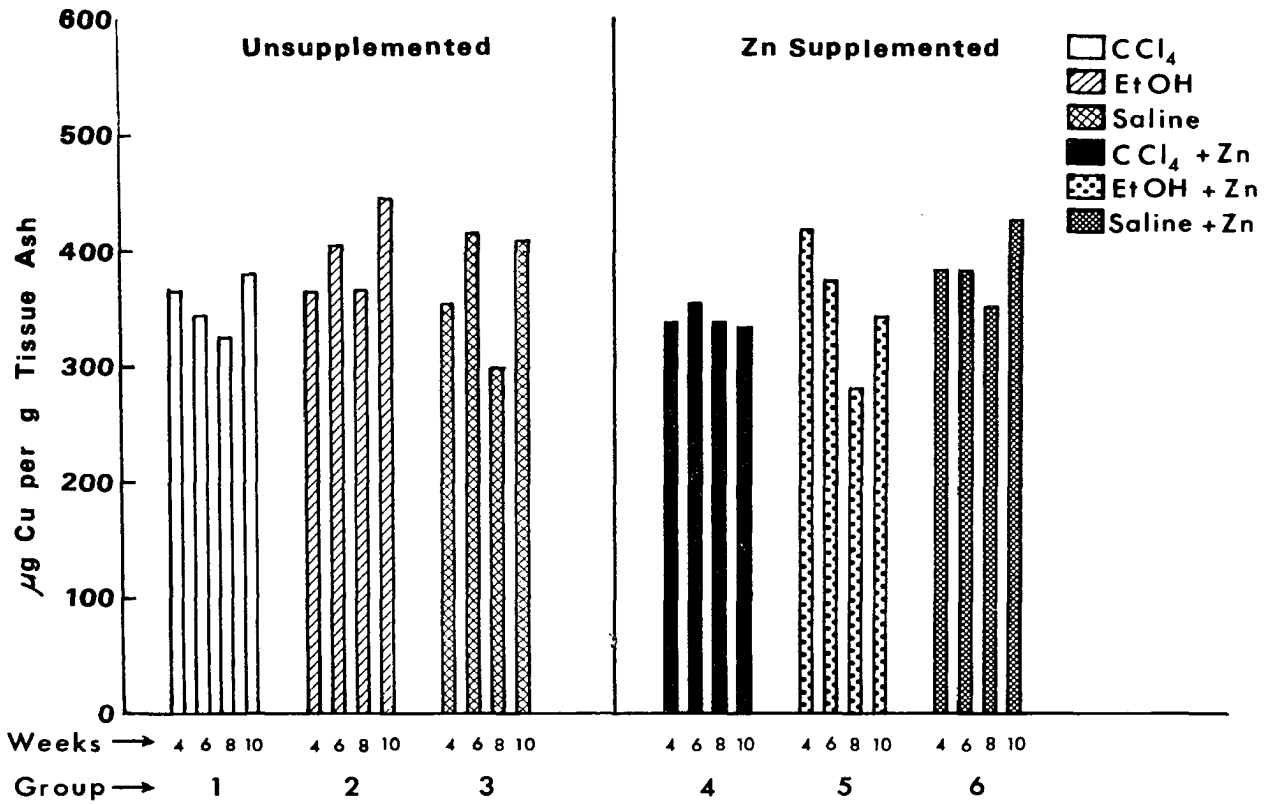

Fig. 16--Copper content of heart tissue.

TABLE 12

MANGANESE CONTENT OF HEART TISSUE
IN μg/g OF TISSUE ASH

Group Means

Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
Weeks						
4	26.0	18.5	20.0	24.5	18.5	20.5
6	29.5	23.5	26.0	41.0	21.5	25.0
8	24.0	19.0	23.5	20.0	19.5	17.5
10	43.0	24.5	20.0	15.0	20.0	27.5

Analysis of Variance

	SS	DF	MS	F
Weeks	400	3	133	9.007*
Groups	583	5	116	7.866*
Groups x Weeks	1038	15	69	4.667*
Within Cell	356	24	14.8	

Simple Effect

Weeks within Groups

Group	SS	DF	MS	F
1	439	3	146	9.87*
2	56	3	18	1.26
3	51	3	17	1.15
4	762	3	254	17.13*
5	9	3	3	0.21
6	120	3	40	2.70

Group within Weeks

Weeks	SS	DF	MS	F
4	100	5	20	1.35
6	492	5	98	6.64*
8	67	5	13	0.90
10	961	5	192	12.95*

*Significant at the 5 per cent level.

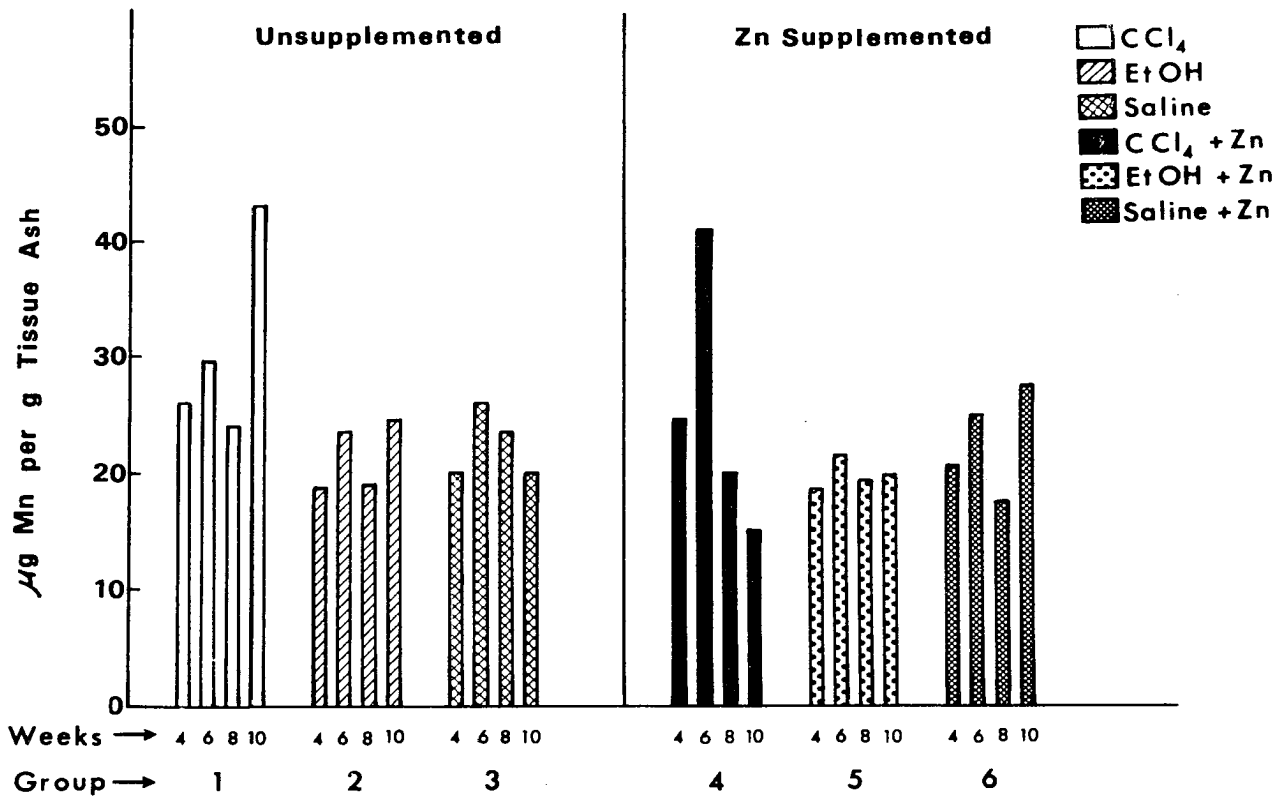

Fig. 17--Manganese content of heart tissue.

TABLE 13

ZINC CONTENT OF HEART TISSUE
IN µg/g OF TISSUE ASH

	Group Means					
Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
Weeks						
4	1300	1330	1235	1300	1450	1310
6	1400	1495	1485	1675	1460	1420
8	1165	1325	1130	1315	1260	1265
10	1465	1505	1470	1470	1485	1495

Analysis of Variance

	SS	DF	MS	F
Weeks	532508	3	177502	23.228*
Groups	84850	5	16970	2.220
Groups x Weeks	127366	15	8491	1.111
Within Cell	183400	24	7641	

Simple Effect

Week within Groups

Group	SS	DF	MS	F
1	102450	3	34150	4.46*
2	59637	3	19879	2.60
3	185300	3	61766	8.08*
4	182700	3	60900	7.96*
5	64337	3	21445	2.80
6	65450	3	21816	2.85

Group within Weeks

Weeks	SS	DF	MS	F
4	50241	5	10048	1.31
6	96341	5	19268	2.52*
8	63066	5	12613	1.65
10	2566	5	513	0.06

*Significant at the 5 per cent level.

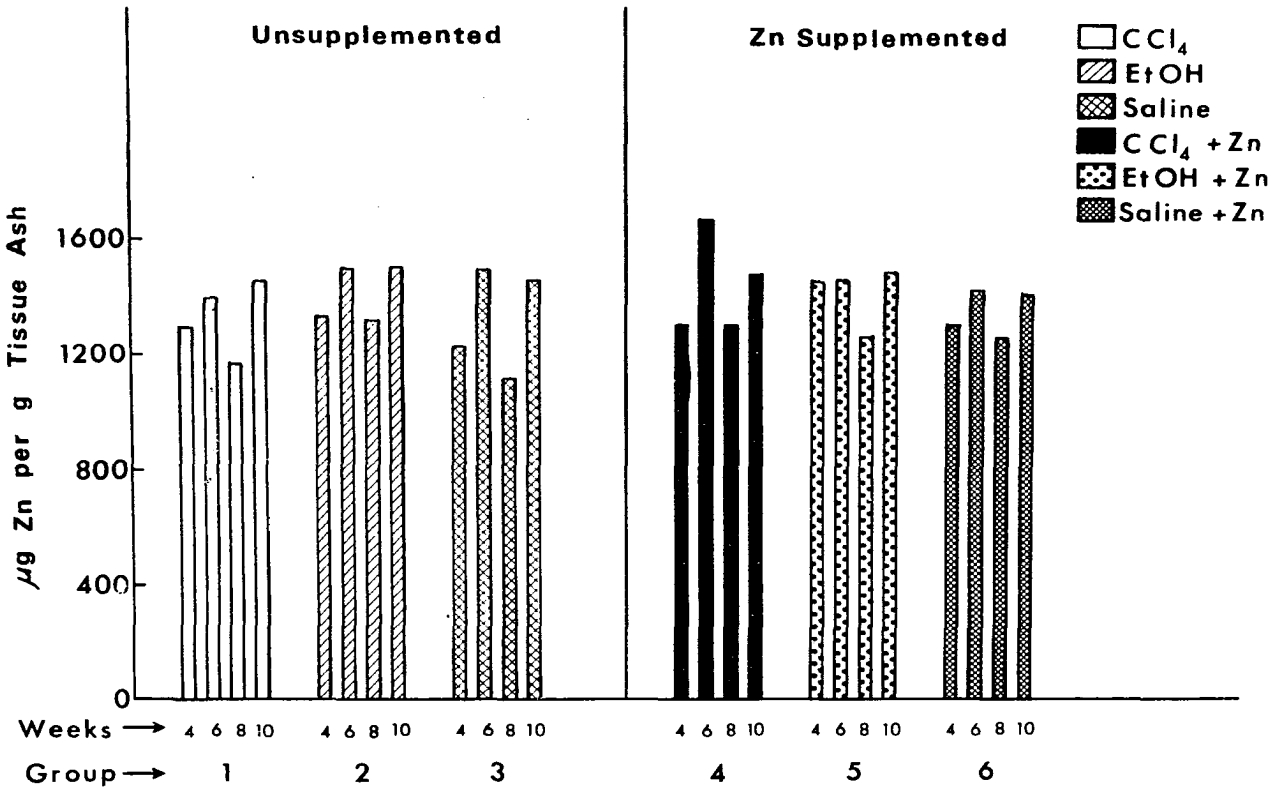

Fig. 18--Zinc content of heart tissue.

TABLE 14

MAGNESIUM CONTENT OF KIDNEY TISSUE
IN g/100g OF TISSUE ASH

Group Means

Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
Weeks						
4	1.0394	1.2669	1.3500	1.3739	1.3710	1.3009
6	0.8209	0.8270	0.8755	0.8939	0.8360	0.9390
8	1.1370	1.2285	1.2340	1.1765	1.2645	1.2220
10	1.1280	1.0035	1.2525	1.0970	1.1735	1.3100

Analysis of Variance

	SS	DF	MS	F
Weeks	1.2	3	0.40	27.491
Groups	0.15	5	3.09	2.106
Groups x Weeks	0.17	15	1.13	0.768
Within Cell	0.35	24	1.47	

Simple Effect

Week within Groups

Group	SS	DF	MS	F
1	0.129	3	4.32	2.93
2	0.253	3	8.458	5.75*
3	0.259	3	8.651	5.88*
4	0.236	3	7.891	5.36*
5	0.321	3	0.107	7.28*
6	0.181	3	6.04	4.11*

Group within Weeks

Weeks	SS	DF	MS	F
4	0.160	5	3.215	2.18
6	2.129	5	42.588	0.28
8	2.095	5	41.930	0.28
10	0.121	5	2.428	1.65

*Significant at the 5 per cent level.

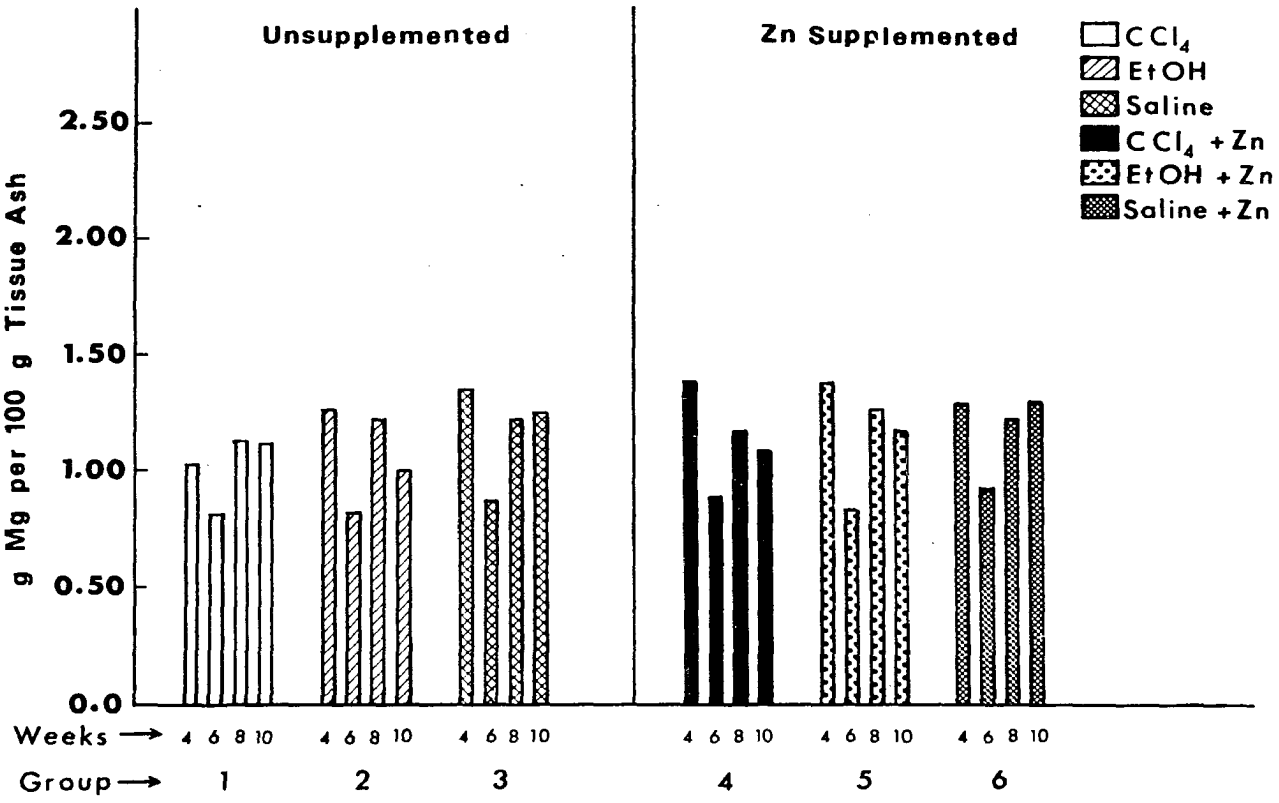

Fig. 19--Magnesium content of kidney tissue.

TABLE 15

IRON CONTENT OF KIDNEY TISSUE
IN g/100g OF TISSUE ASH

Group Means

Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
Weeks						
4	0.4849	0.3700	0.4150	0.3750	0.3200	0.4500
6	0.3700	0.2300	0.2500	0.5100	0.3550	0.3300
8	0.6100	0.3000	0.4700	0.4000	0.5700	0.4500
10	1.0700	0.3000	0.5350	0.9599	0.3549	0.6499

Analysis of Variance

	SS	DF	MS	F
Weeks	0.629	3	0.209	6.878*
Groups	0.571	5	0.114	3.745*
Groups x Weeks	0.678	15	4.520	1.481
Within Cell	0.732	24	3.050	

Simple Effect

Week within Groups

Group	SS	DF	MS	F
1	0.565	3	0.188	6.17*
2	0.196	3	65.330	0.21
3	8.925	3	2.975	0.97
4	0.444	3	0.148	4.85*
5	7.870	3	2.623	0.85
6	0.110	3	3.674	1.20

Groups within Weeks

Weeks	SS	DF	MS	F
4	3.106	5	62.133	0.20
6	0.100	5	2.012	0.65
8	0.127	5	2.549	0.83
10	0.990	5	0.198	6.49

*Significant at the 5 per cent level.

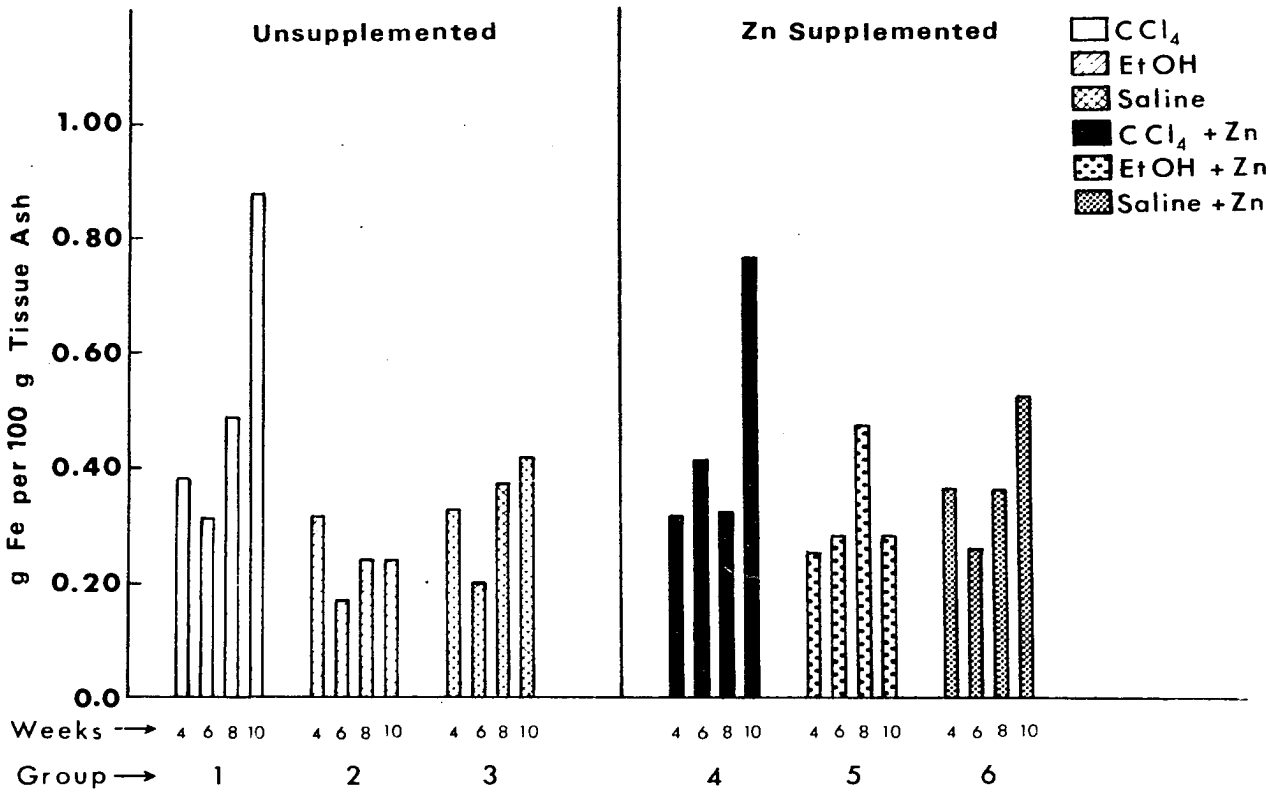

Fig. 20--Iron content of kidney tissue.

TABLE 16

COPPER CONTENT OF KIDNEY TISSUE
IN µg/g OF TISSUE ASH

Group Means

Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
Weeks						
4	375	265	340	465	455	390
6	270	150	235	350	175	280
8	610	225	345	375	270	330
10	345	210	315	415	230	355

Analysis of Variance

	SS	DF	MS	F
Weeks	134656	3	44885	5.885*
Groups	210785	5	42157	5.527*
Groups x Weeks	140256	15	9350	1.225
Within Cell	183050	24	7627	

Simple Effect

Week within Groups

Groups	SS	DF	MS	F
1	129300	3	43100	5.65*
2	13650	3	4550	0.59
3	15537	3	5179	0.67
4	15137	3	5045	0.66
5	88450	3	2948	3.86*
6	12837	3	4279	0.56

Groups within Weeks

Weeks	SS	DF	MS	F
4	55566	5	11113	1.45
6	53766	5	10753	1.40
8	180341	5	36068	4.72*
10	61366	5	12273	1.60

*Significant at the 5 per cent level.

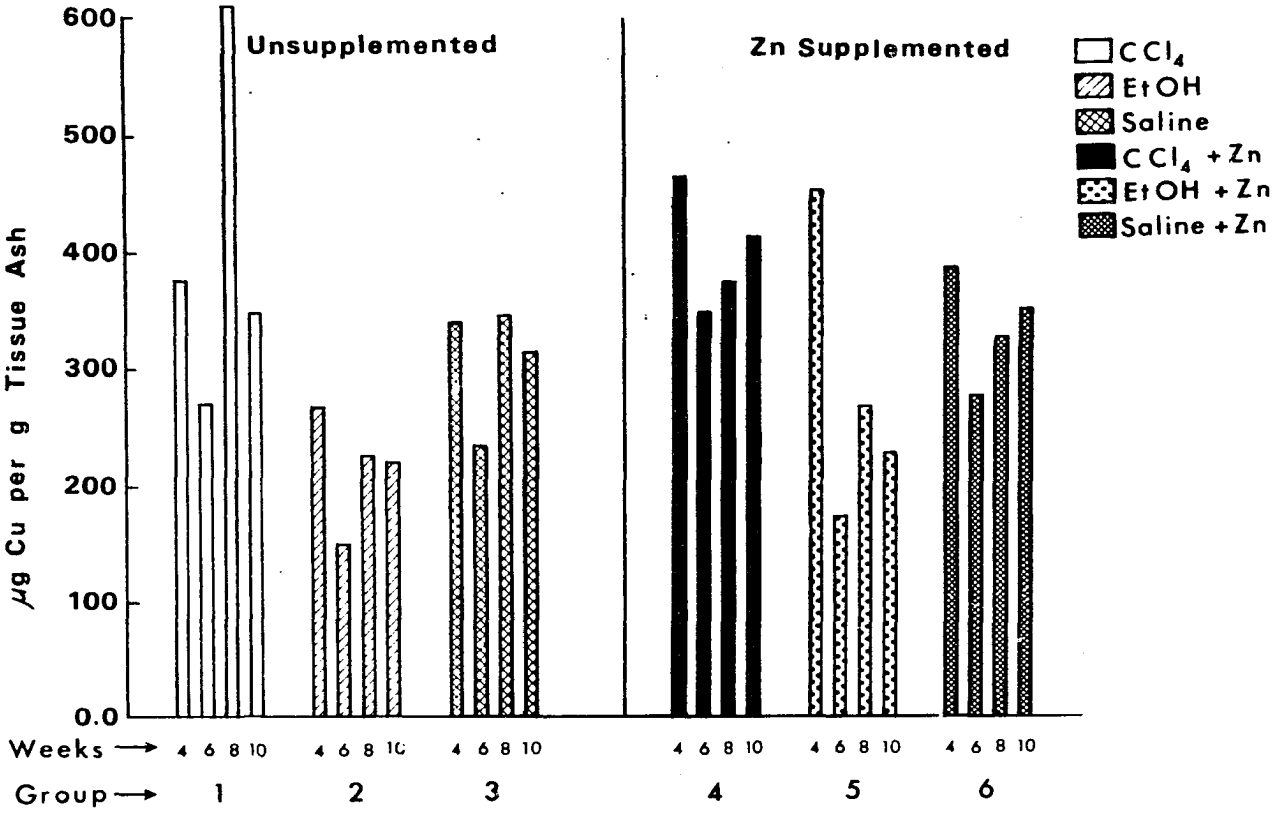

Fig. 21--Copper content of kidney tissue.

TABLE 17

MANGANESE CONTENT OF KIDNEY TISSUE
IN µg/g OF TISSUE ASH

Group Means

Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
Weeks						
4	53.0	56.5	52.5	59.0	41.5	45.0
6	31.0	24.5	30.5	46.5	27.0	32.0
8	58.0	41.0	45.5	63.0	41.5	39.0
10	68.5	32.5	50.5	69.0	46.0	56.5

Analysis of Variance

	SS	DF	MS	F
Weeks	3492	3	1164	13.627*
Groups	2685	5	537.0	6.286*
Groups x Weeks	1323	15	88	1.033
Within Cells	2050	24	85.4	

Simple Effect

Week within Groups

Group	SS	DF	MS	F
1	149	3	499	5.84*
2	1124	3	374	4.38*
3	593	3	197	2.31
4	543	3	181	2.12
5	411	3	137	1.60
6	646	3	215	2.52

Group within Weeks

Weeks	SS	DF	MS	F
4	452	5	90	1.06
6	589	5	117	1.38
8	1007	5	201	2.35
10	1959	5	391	4.58*

*Significant at the 5 per cent level.

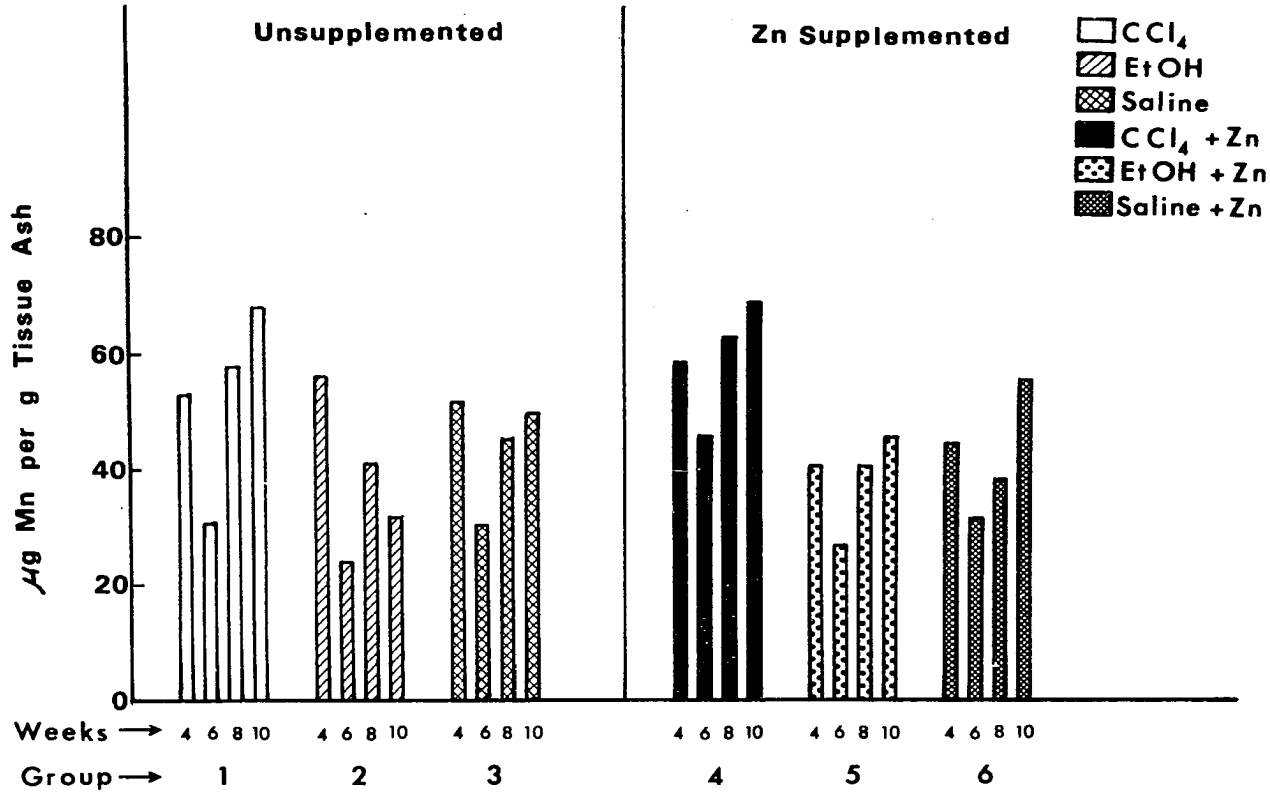

Fig. 22--Manganese content of kidney tissue.

TABLE 18

ZINC CONTENT OF KIDNEY TISSUE
IN μg/g OF TISSUE ASH

Group Means

Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
Weeks						
4	1150	1370	1375	1450	1410	1350
6	945	860	970	1055	950	1015
8	1235	1325	1350	1340	1455	1305
10	1315	1055	1455	1415	1305	1630

Analysis of Variance

	SS	DF	MS	F
Weeks	1328968	3	442996	14.860*
Groups	235710	5	47142	1.581
Groups x Weeks	335347	15	22356	0.749
Within Cell	715450	24	29810	

Simple Effect

Week within Groups

Group	SS	DF	MS	F
1	151937	3	51645	1.69
2	344250	3	114750	3.84*
3	280850	3	93616	3.14*
4	192900	3	64300	2.15
5	314100	3	104700	3.51*
6	380300	3	126766	4.25*

Group within Weeks

Weeks	SS	DF	MS	F
4	109241	5	21848	0.73
6	44541	5	8908	0.29
8	51300	5	10260	0.34
10	365975	5	73195	2.45

*Significant at the 5 per cent level.

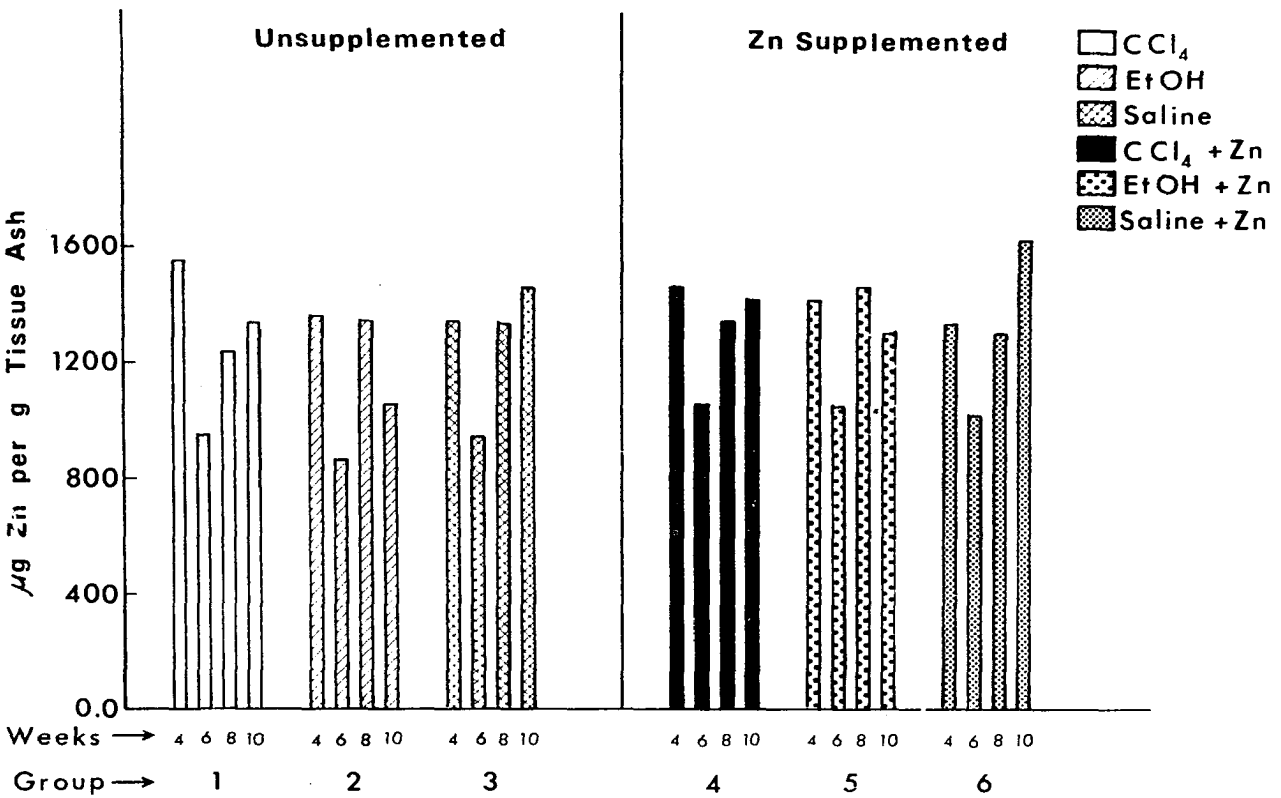

Fig. 23--Zinc content of kidney tissue.

TABLE 19

MAGNESIUM CONTENT OF LIVER TISSUE
IN g/100g OF TISSUE ASH

Group Means

Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
Weeks						
4	1.2580	1.1124	1.1885	1.1400	1.1825	1.2000
6	0.7845	0.8815	0.9939	1.0240	1.0425	1.0810
8	1.0365	1.1124	1.1585	1.0855	1.3435	1.1730
10	1.1004	1.2405	1.2094	0.9275	1.2764	1.2735

Analysis of Variance

	SS	DF	MS	F
Weeks	0.364	3	0.121	6.251*
Groups	0.199	5	3.987	2.051
Groups x Weeks	0.249	15	1.666	0.857
Within Cell	0.466	24	1.943	

Simple Effect

Week within Groups

Group	SS	DF	MS	F
1	0.232	3	7.758	3.99*
2	0.134	3	4.472	2.30
3	5.763	3	1.921	0.98
4	4.982	3	1.660	0.85
5	0.102	3	3.403	1.75
6	3.795	3	1.265	0.65

Group within Weeks

Weeks	SS	DF	MS	F
4	2.543	5	50.873	0.26
6	0.126	5	2.531	1.30
8	0.112	5	2.259	1.16
10	0.184	5	3.688	1.89

*Significant at the 5 per cent level.

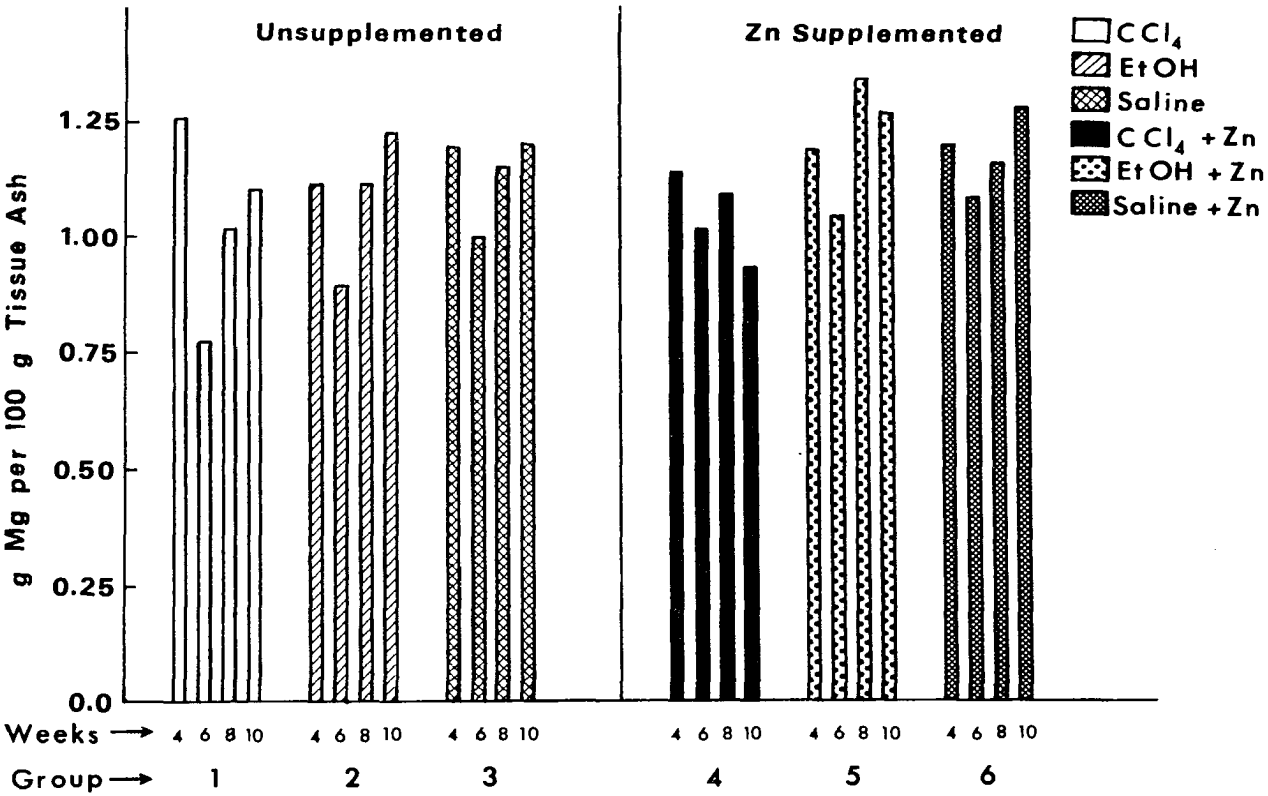

Fig. 24--Magnesium content of liver tissue.

TABLE 20

IRON CONTENT OF LIVER TISSUE
IN g/100g OF TISSUE ASH

Group Means

Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
Weeks						
4	1.2400	1.6100	1.7400	1.1400	1.4700	1.9599
6	0.9300	1.4199	1.9000	2.0800	1.2800	2.6200
8	1.6200	1.4400	2.3100	1.1699	1.9999	2.4100
10	1.9900	1.8300	2.9900	2.0800	2.5099	3.8300

Analysis of Variance

	SS	DF	MS	F
Weeks	7.085	3	2.361	9.814*
Groups	9.279	5	1.855	7.711*
Groups x Weeks	3.653	15	0.243	1.012
Within Cell	5.775	24	0.240	

Simple Effect

Week within Groups

Group	SS	DF	MS	F
1	1.269	3	0.423	1.75
2	0.217	3	7.233	0.30
3	1.865	3	0.621	2.58
4	1.171	3	0.570	2.37
5	1.844	3	0.615	2.55
6	3.829	3	1.276	5.30*

Groups within Weeks

Weeks	SS	DF	MS	F
4	0.950	5	0.190	0.78
6	3.756	5	0.751	3.12*
8	2.454	5	0.490	2.03
10	5.771	5	1.154	4.79*

*Significant at the 5 per cent level.

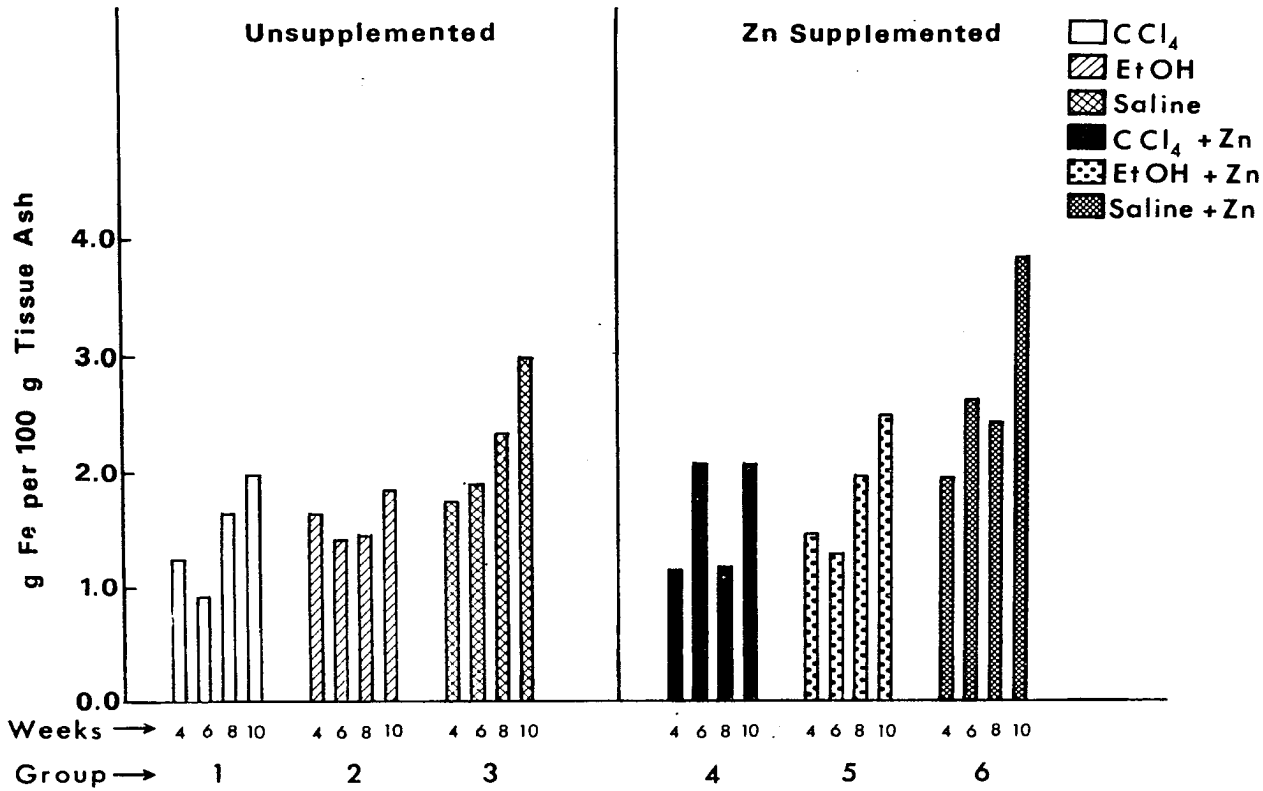

Fig. 25--Iron content of liver tissue.

TABLE 21

COPPER CONTENT OF LIVER TISSUE
IN μg/g OF TISSUE ASH

Group Means

Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
Weeks						
4	380.0	460.0	430.0	320.0	470.0	450.0
6	230.0	380.0	450.0	390.0	440.0	480.0
8	540.0	380.0	380.0	350.0	450.0	390.0
10	540.0	430.0	410.0	280.0	430.0	520.0

Analysis of Variance

	SS	DF	MS	F
Weeks	9700	3	3233	0.233
Groups	76366	5	15273	1.104
Groups x Weeks	170700	15	113800	0.822
Within Cell	332000	24	13833	

Simple Effect

Week within Groups

Group	SS	DF	MS	F
1	132950	3	44316	3.20*
2	9350	3	3116	0.22
3	5350	3	1783	0.12
4	13000	3	4333	0.31
5	1750	3	583	0.04
6	18000	3	6000	0.43

Group within Weeks

Weeks	SS	DF	MS	F
4	33366	5	6673	0.48
6	79500	5	15900	1.14
8	48300	5	9660	0.69
10	85900	5	17180	1.24

*Significant at the 5 per cent level.

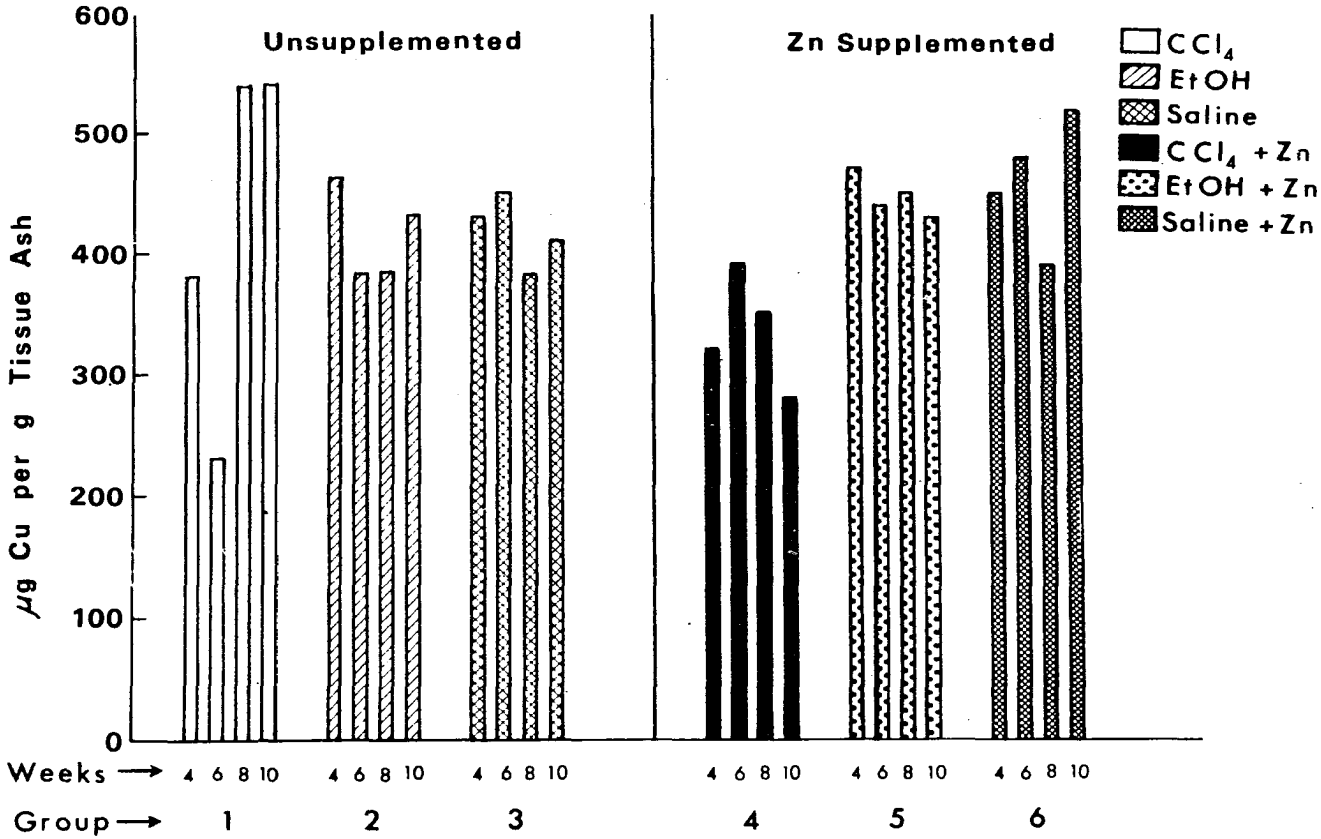

Fig. 26--Copper content of liver tissue.

TABLE 22

MANGANESE CONTENT OF LIVER TISSUE
IN µg/g OF TISSUE ASH

	Group Means					
Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
Weeks						
4	270.0	314.0	262.0	230.0	237.0	244.0
6	155.0	197.0	249.0	252.0	237.0	288.0
8	277.0	213.0	268.0	260.0	199.0	261.0
10	256.0	244.0	262.0	220.0	303.0	252.0

Analysis of Variance

	SS	DF	MS	F
Weeks	7416	3	2472	1.620
Groups	6804	5	1360	0.892
Groups x Weeks	40298	15	2686	1.761
Within Cell	36600	24	1525	

Simple Effect

Week within Groups

Group	SS	DF	MS	F
1	15748	3	5249	3.44*
2	16108	3	5369	3.52*
3	385	3	128	0.08
4	2086	3	695	0.45
5	11208	3	3736	2.44
6	2179	3	726	0.47

Group within Weeks

Weeks	SS	DF	MS	F
4	9407	5	1881	1.23
6	21942	5	4388	2.87*
8	8360	5	1672	1.09
10	7393	5	1478	0.96

*Significant at the 5 per cent level.

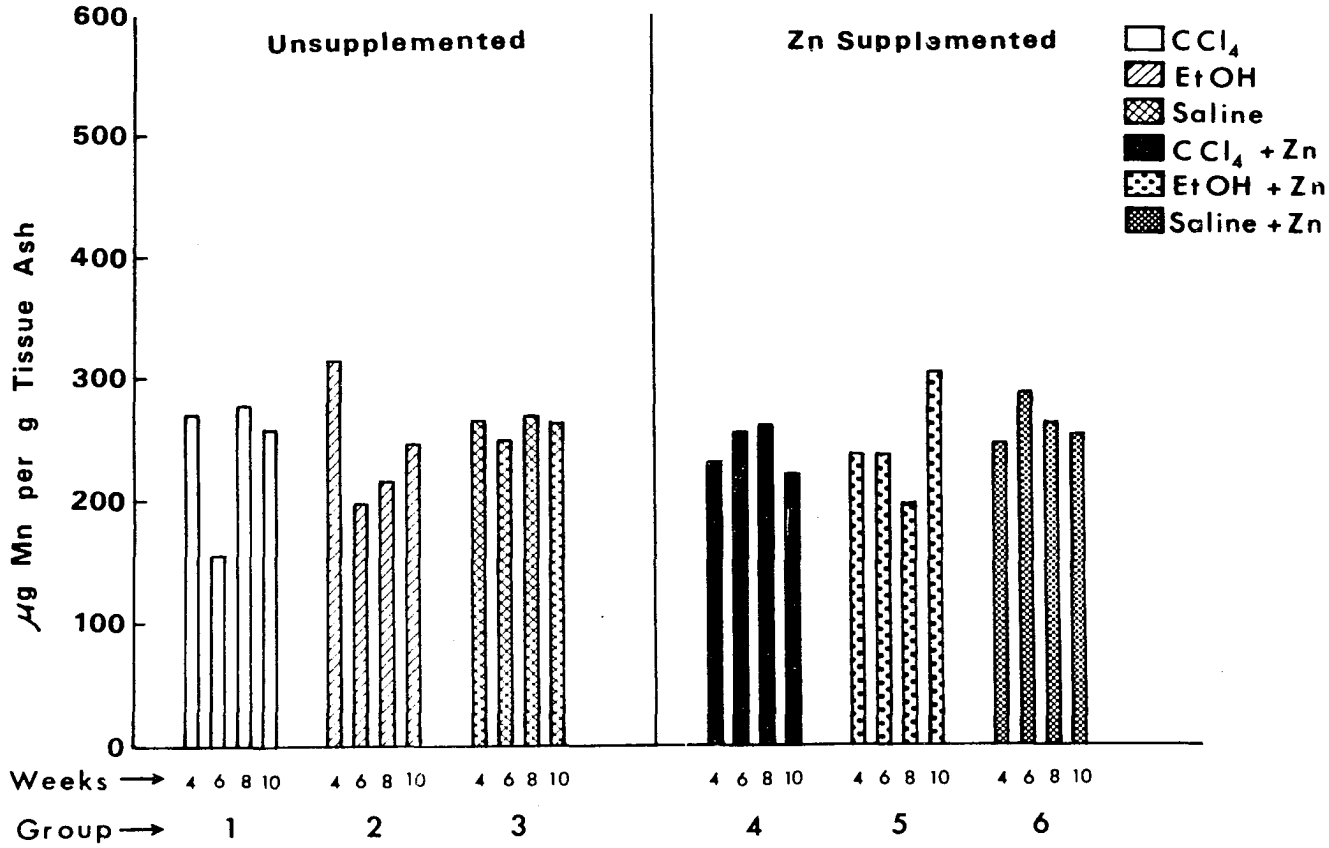

Fig. 27--Manganese content of liver tissue.

TABLE 23

ZINC CONTENT OF LIVER TISSUE
IN µg/g OF TISSUE ASH

Group Means

Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
Weeks						
4	3320	3150	2720	2700	3160	2760
6	2020	2560	2630	2650	3030	3110
8	2260	2870	2510	2410	3640	2860
10	2680	3850	3340	1940	3180	3830

Analysis of Variance

	SS	DF	MS	F
Weeks	1607633	3	535877	2.200
Groups	4540800	5	908160	3.728*
Groups x Weeks	5529266	15	368617	1.513
Within Cell	5845200	24	243550	

Simple Effect

Week within Groups

Group	SS	DF	MS	F
1	1946400	3	648800	2.66
2	1818550	3	606183	2.48
3	822000	3	274000	1.12
4	723400	3	241133	0.99
5	426950	3	142316	0.58
6	139960	3	466533	1.91

Groups within Weeks

Weeks	SS	DF	MS	F
4	74096	5	148193	0.60
6	1519460	5	303893	1.24
8	2462960	5	492593	2.02
10	5346660	5	1069330	4.39*

*Significant at the 5 per cent level.

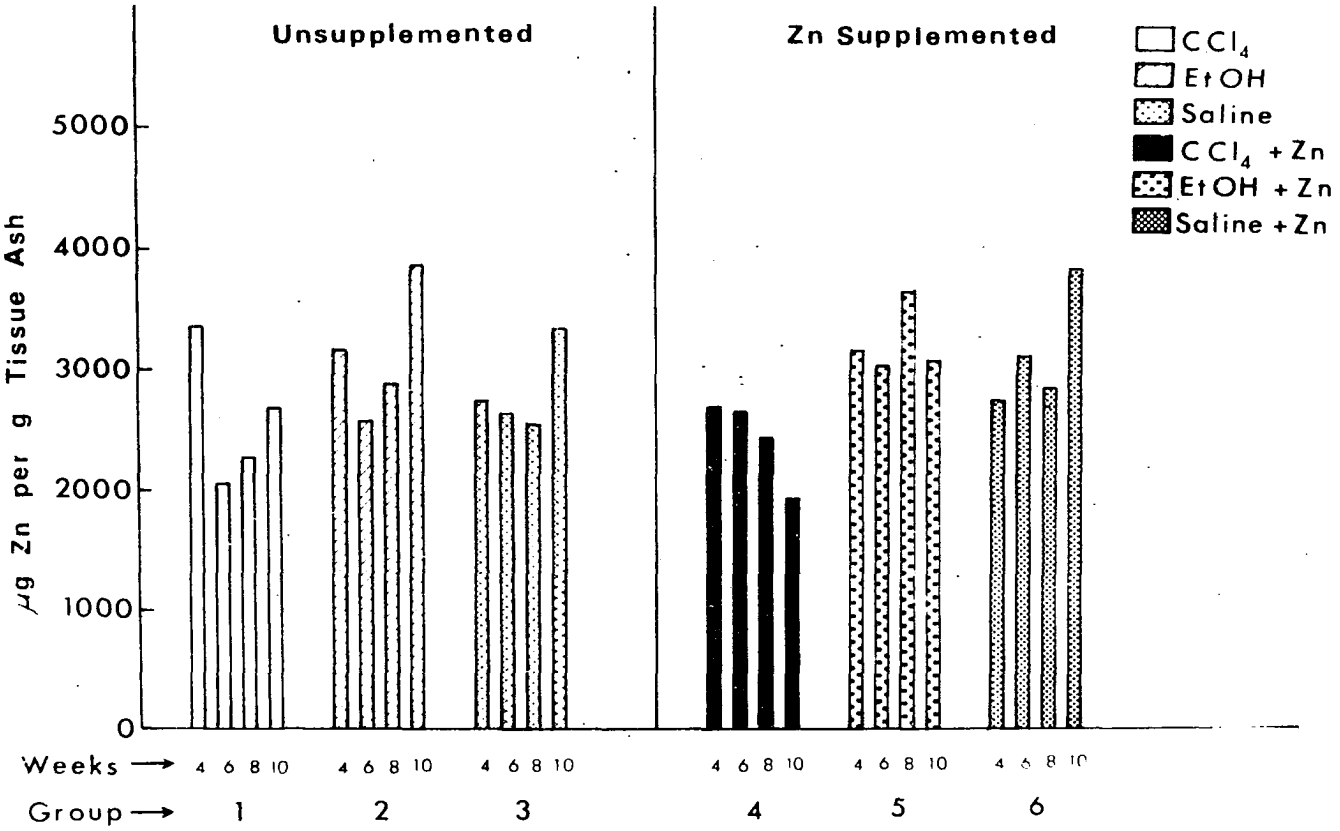

Fig. 28--Zinc content of liver tissue.

TABLE 24

WET WEIGHT OF HEART TISSUE IN GRAMS

Group Means

Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
Weeks						
4	0.7328	0.7160	0.8335	0.7445	0.7585	0.7173
6	0.8975	0.7716	0.7682	0.6741	0.7716	0.8080
8	0.6721	0.7478	0.8631	0.8110	0.8508	0.7141
10	0.8143	0.7733	0.7710	0.7168	0.7990	0.7727

Analysis of Variance

	SS	DF	MS	F
Weeks	70	3	23	0.258
Groups	3	5	63	0.700
Groups x Weeks	0.11	15	73	0.818
Within Cell	0.21	24	90	

Simple Effect

Weeks within Groups

Group	SS	DF	MS	F
1	6	3	1.9	2.13
2	43	3	14	0.15
3	1.3	3	44	0.49
4	2	3	66	0.73
5	1	3	33	0.37
6	1.2	3	41	0.45

Groups within Weeks

Weeks	SS	DF	MS	F
4	2	5	38	0.42
6	5	5	1.0	1.15
8	6	5	1.2	1.32
10	1	5	22	0.24

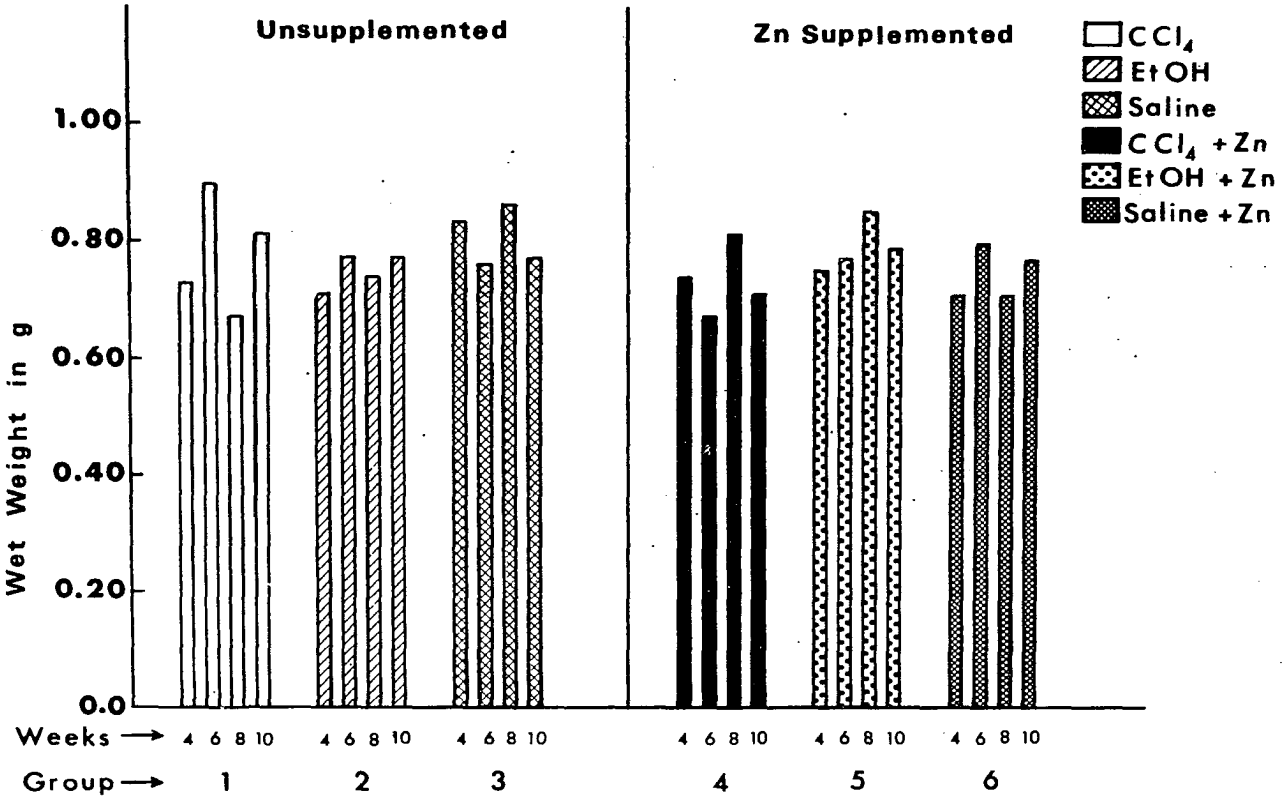

Fig. 29--Wet weight of heart tissue.

TABLE 25

WET WEIGHT OF KIDNEY TISSUE IN GRAMS

Group Means

Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
Weeks						
4	1.7706	1.3302	1.7829	1.5671	1.7818	1.6018
6	2.1021	1.6563	1.7020	1.8888	1.7566	1.6795
8	1.8322	1.6889	1.7279	1.9715	1.8513	1.6955
10	2.6133	1.6808	1.6060	1.7686	1.6521	1.5360

Analysis of Variance

	SS	DF	MS	F
Weeks	0.23	3	8	3.611*
Groups	1.20	5	0.2	11.252*
Groups x Weeks	1.1	15	7	3.423*
Within Cell	0.52	24	2.2	

Simple Effect

Weeks within Groups

Group	SS	DF	MS	F
1	0.9	3	0.3	13.51*
2	0.2	3	6	2.75
3	3	3	1	0.50
4	0.2	3	6	2.83
5	4	3	1	0.62
6	3	3	1	0.50

Group within Weeks

Weeks	SS	DF	MS	F
4	0.3	5	6	2.94*
6	0.3	5	6	2.67*
8	0.1	5	2	1.12
10	2	5	0.3	14.77*

*Significant at the 5 per cent level.

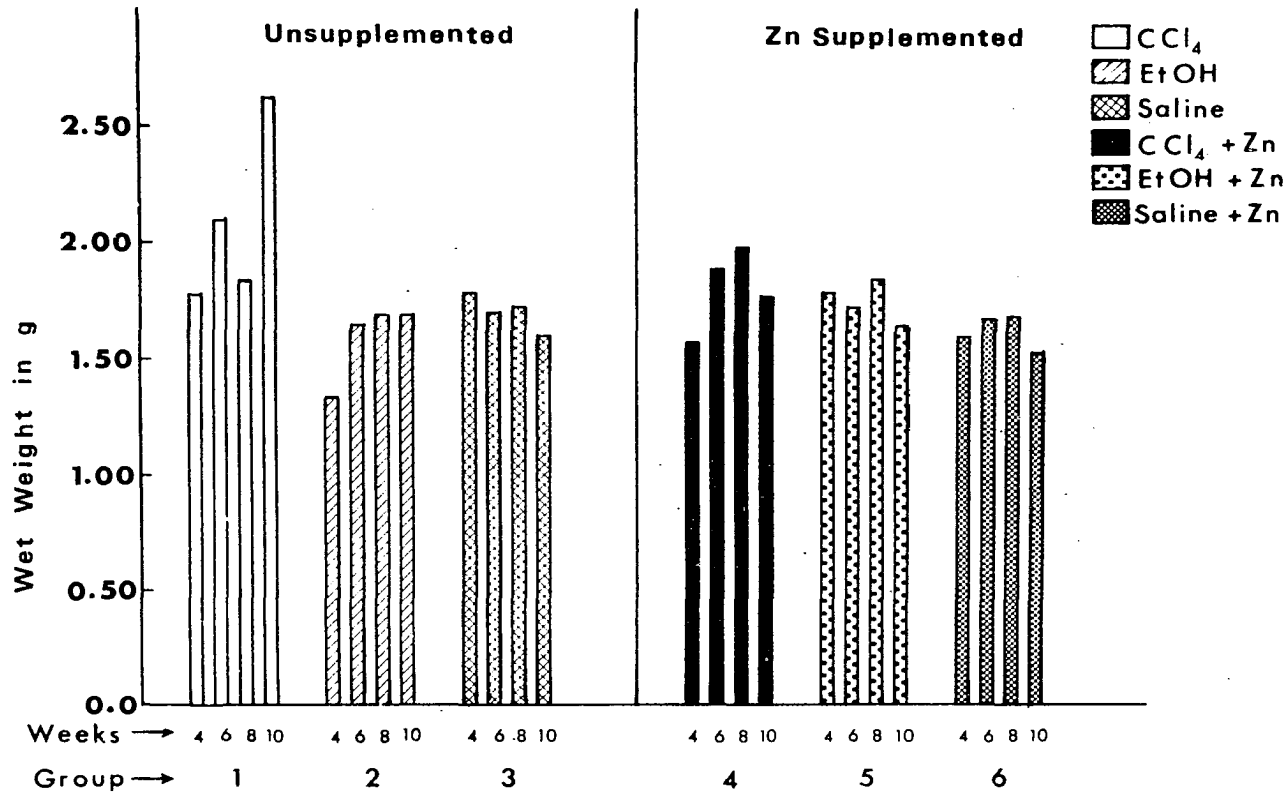

Fig. 30--Wet weight of kidney tissue.

TABLE 26

WET WEIGHT OF LIVER TISSUE IN GRAMS

Group Means

Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
Week						
4	8.7620	6.5319	7.6137	9.8187	8.2252	7.0306
6	10.6301	8.7929	7.9902	8.5866	9.0551	8.0204
8	7.7278	10.4637	8.0124	8.8985	6.6688	7.3138
10	7.4968	7.7815	6.4283	8.3914	7.7157	6.1180

Analysis of Variance

	SS	DF	MS	F
Weeks	14	3	5	1.310
Groups	19	5	4	1.072
Groups x Weeks	30	15	2	0.557
Within Cell	86	24	3.6	

Simple Effect

Week within Groups

Group	SS	DF	MS	F
1	12	3	4	1.13
2	17	3	6	1.53
3	3	3	1	0.30
4	2	3	0.79	0.22
5	6	3	2	0.55
6	4	3	1	0.34

Group within Weeks

Weeks	SS	DF	MS	F
4	14	5	3	0.79
6	10	5	2	0.52
8	18	5	4	1.00
10	8	5	1.5	0.42

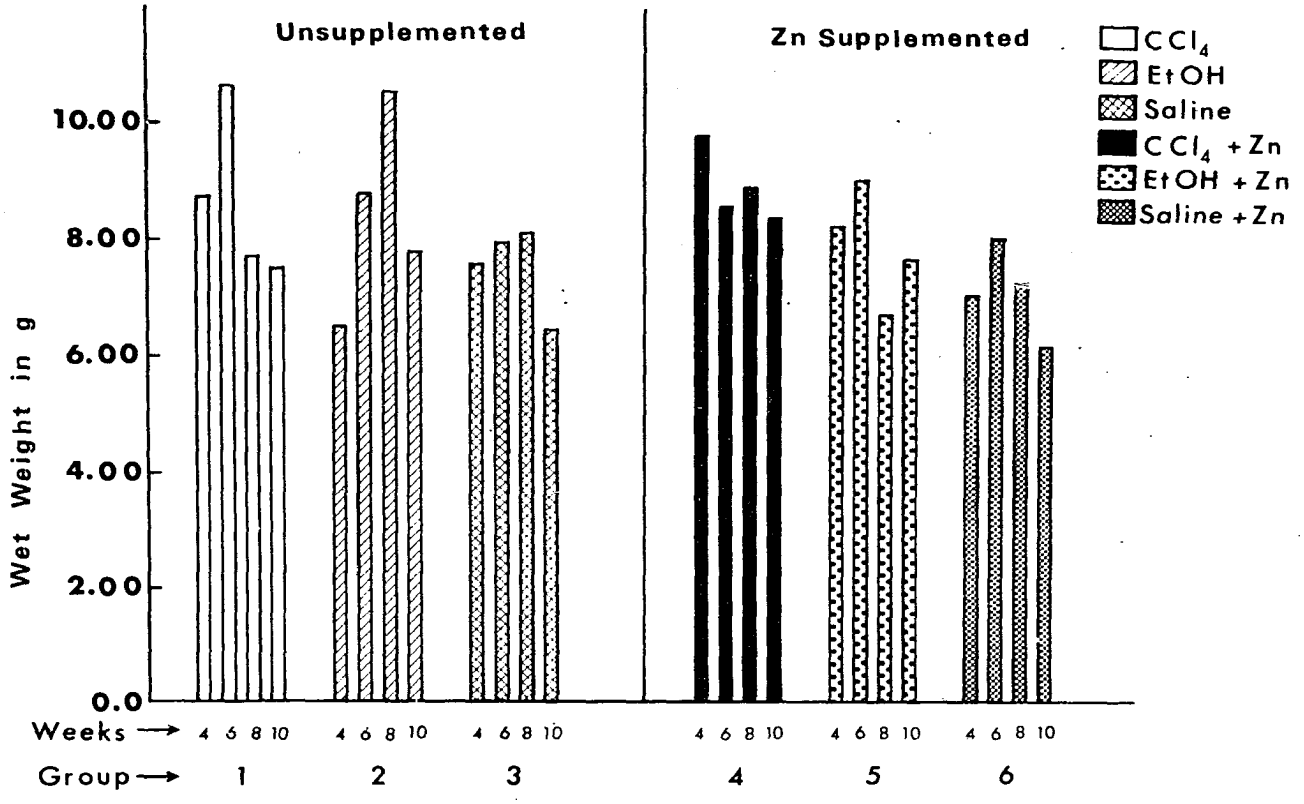

Fig. 31--Wet weight of liver tissue.

TABLE 27

ASH WEIGHT OF HEART TISSUE IN GRAMS

Group Means

Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
Weeks						
4	0.0097	0.0090	0.0104	0.0096	0.0100	0.0096
6	0.0114	0.0094	0.0095	0.0086	0.0100	0.0105
8	0.0082	0.0087	0.0108	0.0096	0.0105	0.0088
10	0.0086	0.0082	0.0090	0.0071	0.0090	0.0097

Analysis of Variance

	SS	DF	MS	F
Weeks	1158	3	38624	1.996
Groups	1081	5	21635	1.118
Groups x Weeks	1942	15	12949	0.669
Within Cell	4643	24	19348	

Simple Effect

Week within Groups

Group	SS	DF	MS	F
1	1208	3	40270	2.08
2	15350	3	511666	0.26
3	37962	3	12654	0.65
4	84062	3	28020	1.44
5	22909	3	763645	0.39
6	29012	3	967083	0.49

Group within Weeks

Weeks	SS	DF	MS	F
4	21493	5	429875	0.22
6	94485	5	18897	0.97
8	1083	5	21663	1.11
10	78116	5	15623	0.80

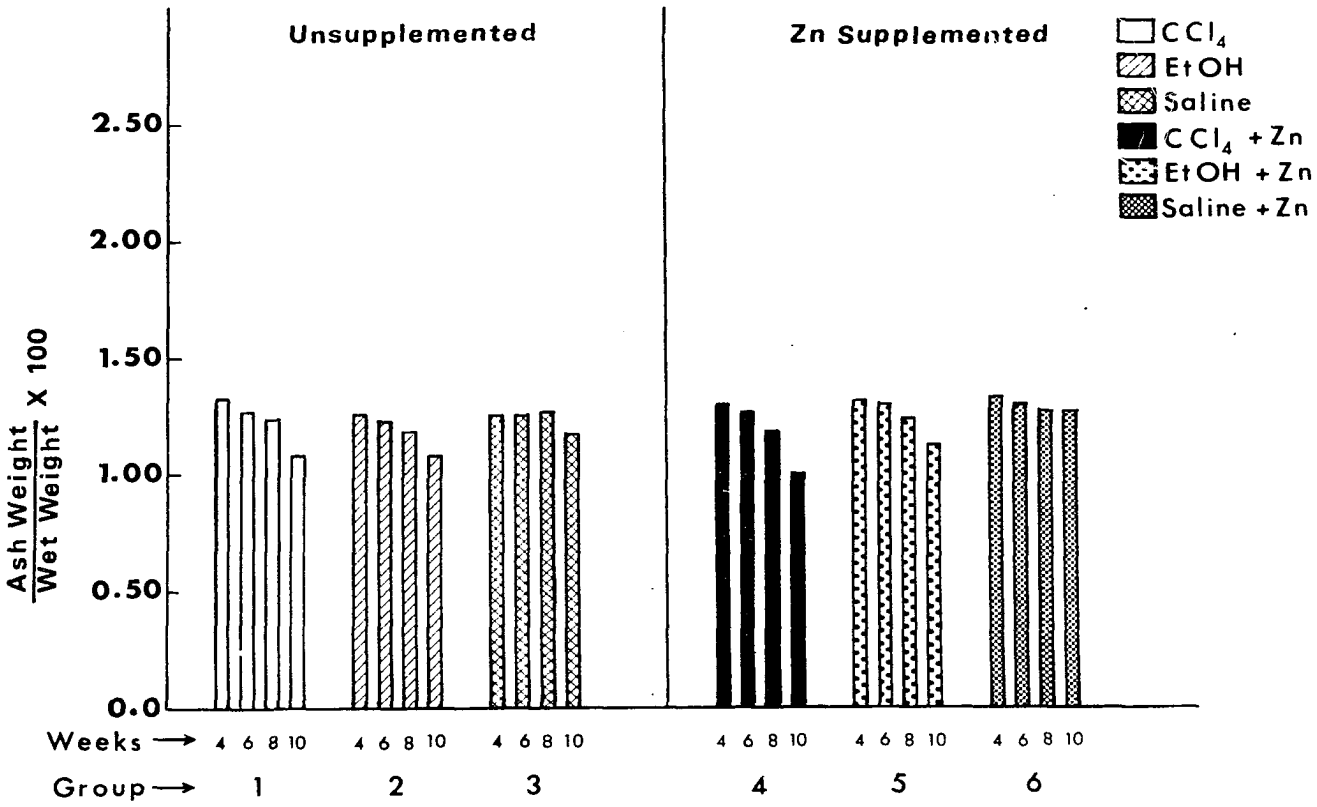

Fig. 32--Per cent ash weight of heart tissue.

TABLE 28

ASH WEIGHT OF KIDNEY TISSUE IN GRAMS

Group Means

Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
Weeks						
4	0.0265	0.0194	0.0244	0.0229	0.0261	0.0230
6	0.0360	0.0361	0.0358	0.0386	0.0369	0.0339
8	0.0260	0.0233	0.0227	0.0269	0.0241	0.0228
10	0.0308	0.0221	0.0218	0.0234	0.0244	0.0190

Analysis of Variance

	SS	DF	MS	F
Weeks	13.79	3	459.90	47.874*
Groups	151.54	5	3030.90	3.154*
Groups x Weeks	129.20	15	86152.2	0.896
Within Cell	230.58	24	96075	

Simple Effect

Week within Groups

Group	SS	DF	MS	F
1	130	3	4357	4.33*
2	332	3	110	11.53*
3	256	3	8560	8.91*
4	321	3	107	11.14*
5	221	3	7387	7.68*
6	246	3	8204	8.53*

Group within Weeks

Weeks	SS	DF	MS	F
4	6724.6	5	1334.9	1.39
6	2336.3	5	46727.1	0.48
8	3146.6	5	62933.7	0.65
10	158.7	5		3.30*

*Significant at the 5 per cent level.

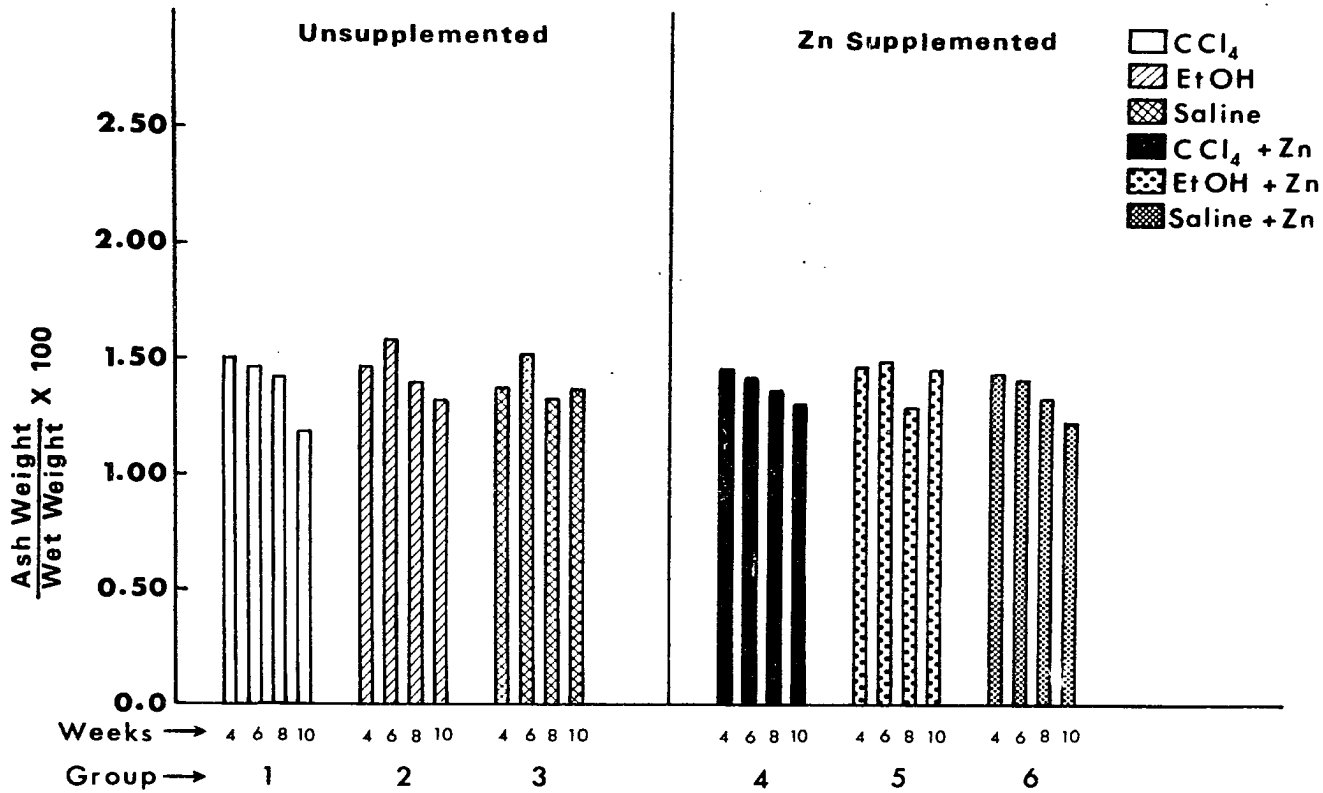

Fig. 33--Per cent ash weight of kidney tissue.

TABLE 29

ASH WEIGHT OF LIVER TISSUE IN GRAMS

Group Means

Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
Weeks						
4	0.1279	0.0995	0.1100	0.1429	0.1268	0.1043
6	0.2568	0.1666	0.1293	0.1354	0.1493	0.1324
8	0.1289	0.1605	0.1138	0.1297	0.0956	0.1042
10	0.0866	0.1116	0.0871	0.1348	0.1125	0.0801

Analysis of Variance

	SS	DF	MS	F
Weeks	2.3	3	76	2.940
Groups	1.1	5	23	0.894
Groups x Weeks	2.4	15	16	0.628
Within Cell	6.2	24	26	

Simple Effect

Week within Groups

Group	SS	DF	MS	F
1	3.3	3	1.01	4.19*
2	69	3	23	0.88
3	18	3	609	0.23
4	176	3	5871	0.02
5	31	3	10	0.39
6	27	3	917	0.35

Group within Weeks

Weeks	SS	DF	MS	F
4	28	5	555	0.21
6	2.4	5	47	1.81
8	5.3	5	11	0.41
10	44	5	887	0.34

*Significant at the 5 per cent level.

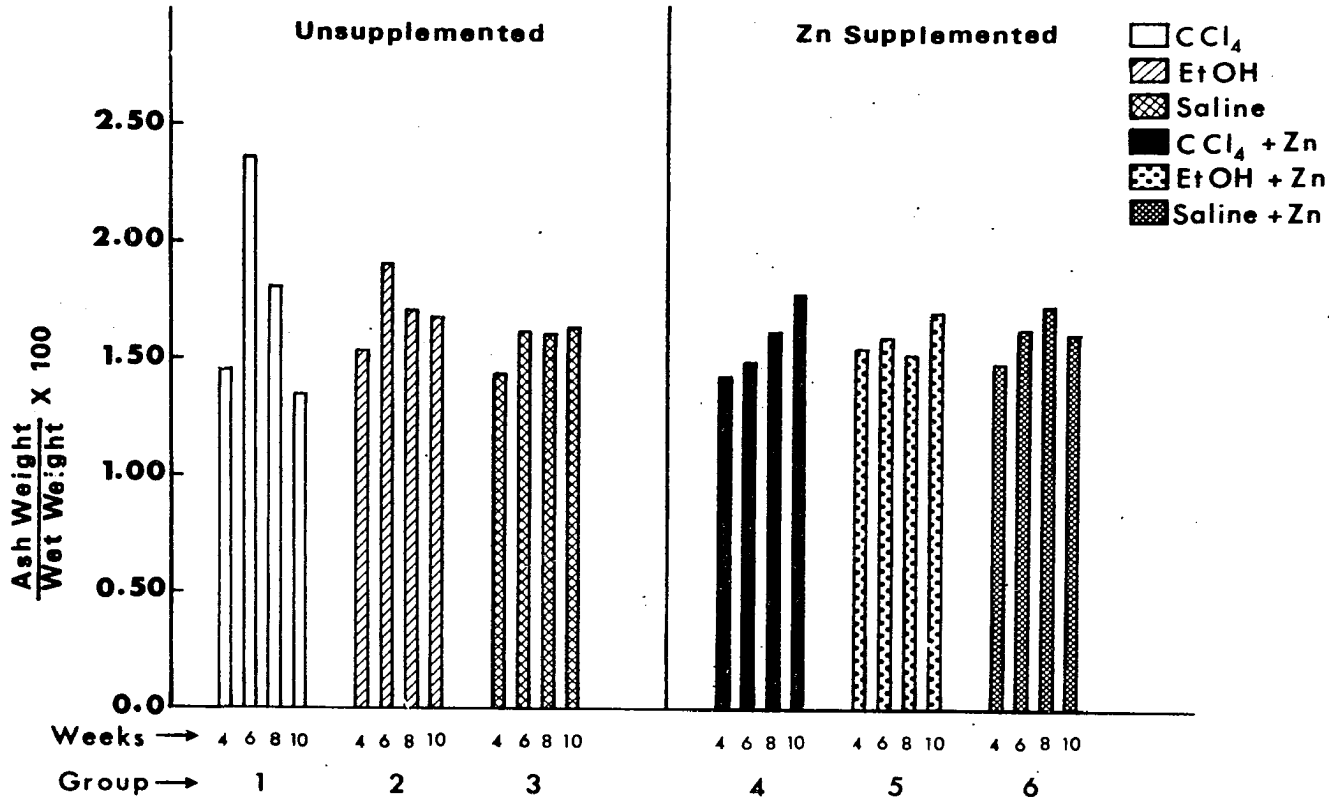

Fig. 34--Per cent ash weight of liver tissue.

TABLE 30

ZINC, COPPER AND MANGANESE CONTENT OF TOTAL URINE VOLUME AT TWO WEEKS OF TREATMENT

Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
N	8	8	8	8	8	8
Total Volume of urine in ml						
Mean ± S.D.	224.4± 56.2	145.8± 57.0	125.3± 23.8	233.0± 61.6	144.8± 47.8	134.4± 27.3
Spread	168.0-345.0	65.0-226.0	88.0-150.0	171.0-325.0	80.0-215.0	100.0-175.0
Total Zinc Output in µg						
Mean ± S.D.	145.85± 31.16	87.46± 39.76	119.24± 56.54	142.63± 37.75	87.22± 19.97	116.94± 42.96
Spread	128.18-217.35	39.65-160.55	70.06-220.0	100.00-221.88	66.0 -116.1	78.20-210.60
Total Copper Output in µg						
Mean ± S.D.	44.0± 11.1	19.1± 8.5	24.6± 9.4	34.4± 9.8	17.2± 3.6	29.8 - 23.6
Spread	26.9- 65.4	11.7- 37.2	13.5- 43.5	23.4- 51.6	12.1- 21.5	14.5 - 83.0
Total Manganese Output in µg						
Mean ± S.D.	13.91±10.83	8.3 ± 5.08	10.73± 6.64	13.75±10.33	7.51± 3.35	7.66± 5.53
Spread	4.42-37.84	3.25-18.07	1.76-20.24	2.50-32.5	2.96-13.2	2.20±20.30

TABLE 31

IRON, MAGNESIUM, SODIUM AND POTASSIUM CONTENT OF TOTAL URINE VOLUME AT TWO WEEKS OF TREATMENT

Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
N	8	8	8	8	8	8
Total Volume of urine in ml						
Mean ± S.D.	224.4± 56.2	145.8± 57.0	125.3± 23.8	233.0± 61.6	144.8± 47.8	134.4± 27.3
Spread	168.0-345.0	65.0-226.0	88.0-150.0	171.0-325.0	80.0-215.0	100.0-175.0
Total Iron Output in µg						
Mean ± S.D.	209.2± 98.9	126.6±101.7	200.4±146.0	160.4± 82.6	111.3± 42.3	220.0±199.6
Spread	120.0-393.3	27.0-324.0	70.2-406.0	48.6-299.3	49.6-183.5	72.5-688.5
Total Magnesium Output in mEq						
Mean ± S.D.	4.00±1.44	3.17±2.29	1.83±1.19	3.84±1.02	3.84±1.38	2.84±1.67
Spread	1.93-6.38	0.58-6.65	0.18-3.94	2.32-5.80	2.60-5.93	0.84-6.52
Total Sodium Output in mEq						
Mean ± S.D.	21.33± 3.02	18.14± 5.97	21.57± 3.73	19.64± 1.12	20.11± 2.72	23.07± 3.87
Spread	16.24-24.62	8.50-25.31	17.75-27.82	18.45-21.93	17.38-25.91	18.09-29.40
Total Potassium Output in mEq						
Mean ± S.D.	30.57± 2.71	24.11± 6.90	27.41± 4.61	27.01± 1.87	27.01± 1.87	25.72± 4.17
Spread	25.53-33.50	13.71-32.20	22.08-35.25	24.66-30.70	24.66-30.70	20.07-34.72

TABLE 32

ZINC, COPPER AND MANGANESE CONTENT OF TOTAL URINE VOLUME AT FOUR WEEKS OF TREATMENT

Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
N	8	8	8	8	8	8
Total Volume of urine in ml						
Mean ± S.D.	250.8± 77.4	175.3± 53.4	145.5± 25.5	279.9± 88.7	177.9± 66.5	152.0± 24.1
Spread	140.0-337.0	103.0-293.0	109.0-177.0	182.0-445.0	87.0-291.0	122.0-189.0
Total Zinc Output in µg						
Mean ± S.D.	187.72± 53.21	146.54± 26.88	116.60± 14.91	222.85± 29.98	164.84± 78.77	188.17± 42.97
Spread	116.20-283.65	115.36-189.00	91.65-130.90	187.45-275.90	76.56-338.80	122.00-245.34
Total Copper Output in µg						
Mean ± S.D.	44.50± 16.81	19.68± 4.69	26.17± 9.48	75.85± 17.96	26.81± 13.68	34.92± 10.47
Spread	23.60- 71.04	11.34- 24.00	11.48- 37.40	51.20-105.00	15.12- 58.08	20.79- 52.14
Total Manganese Output in µg						
Mean ± S.D.	22.0± 8.00	7.86± 3.31	8.81± 2.11	10.87± 4.12	7.70± 4.25	9.86± 2.46
Spread	10.71- 32.80	4.86- 14.65	6.40- 13.12	4.94- 17.46	3.48- 15.54	6.2 - 13.77

TABLE 33

IRON, MAGNESIUM, SODIUM AND POTASSIUM CONTENT OF TOTAL URINE VOLUME AT FOUR WEEKS OF TREATMENT

Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
N	8	8	8	8	8	8
Total Volume of Urine in ml						
Mean ± S.D.	250.8± 77.4	175.3± 53.4	145.5± 25.5	279.9± 88.7	177.9± 66.5	152.0± 24.1
Spread	140.0-337.0	103.0-293.0	109.0-177.0	182.0-445.0	87.0-291.0	122.0-189.0
Total Iron Output in µg						
Mean ± S.D.	149.8± 98.7	168.8± 83.5	217.5±101.7	189.3± 96.0	247.1±157.4	355.0±127.7
Spread	42.8-346.3	60.8-263.7	81.8-336.	92.8-390.0	68.7-549.3	165.9-509.0
Total Magnesium Output in mEq						
Mean ± S.D.	3.17± 1.95	1.20± 0.86	1.16± 1.15	3.01± 1.64	2.95± 2.05	0.54± 0.14
Spread	1.18- 6.52	0.41- 2.28	1.09- 3.74	0.63- 5.08	0.65- 6.95	0.34- 0.76
Total Sodium Output in mEq						
Mean ± S.D.	19.52± 4.66	21.38± 3.27	22.53± 2.77	18.63± 3.45	20.73± 4.29	23.14± 2.93
Spread	13.31-27.97	15.86-24.25	19.24-26.80	13.37-23.13	15.68-26.77	19.22-27.31
Total Potassium Output in mEq						
Mean ± S.D.	29.39± 5.62	28.63± 4.42	30.09± 3.91	28.19± 3.82	28.92± 6.05	31.00± 4.30
Spread	21.42-39.09	20.75-32.45	25.94-35.12	21.66-32.30	20.57-38.84	25.01-37.98

TABLE 34

MEAN SERUM CONCENTRATIONS OF Mg, Na AND K IN mEq/l

Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
			mEq/l Mg			
Weeks						
6	2.32	2.32	2.33	2.98	3.18	2.62
8	2.41	1.68	2.03	1.91	2.75	2.50
10	SNS	2.63	2.22	SNS	2.50	2.10
			mEq/l Na			
Weeks						
6	130	133	135	141	141	135
8	131	129	135	125	135	131
10	SNS	137	133	130	130	136
			mEq/l K			
Weeks						
6	4.15	4.65	4.20	4.75	5.12	6.72
8	4.35	4.95	4.45	3.85	4.35	4.85
10	SNS	11.95	12.50	1.70	16.50	12.50
mg% BUN Pooled Sera	24.9	24.0	26.0	29.0	38.9	27.9

SNS = Sample not sufficient.

TABLE 35

MEAN SERUM CONCENTRATIONS OF Fe, Cu AND An
IN µg PER 100 ml

Group	1	2	3	4	5	6
Treatment	$CC\ell_4$	Ethanol	Saline	$CC\ell_4$ + Zn	Ethanol + Zn	Saline + Zn
			µg Fe per 100 ml			
Weeks						
6	295	300	330	240	387	390
8	164	200	365	120	80	340
10	SNS	455	550	340	445	555
			µg Cu per 100 ml			
Weeks						
6	104	136	91	105	138	102
8	80	172	94	83	107	90
10	SNS	136	99	204	129	109
			µg Zn per 100 ml			
Weeks						
6	110	104	113	79	107	94
8	76	84	95	82	89	101
10	86	91	101	116	95	92

SNS = Sample not sufficient.