ROLE OF ZINC DEFICIENCY AND OTHER FACTORS IN ESOPHAGEAL CARCINOGENESIS

by

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Submitted to the Department of Nutrition and Food Science in partial fulfillment of the requirements for the degree of

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ROLE OF ZINC DEFICIENCY IN ESOPHAGEAL CARCINOGENESIS

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ABSTRACT

Male weanling Sprague-Dawley rats, fed either 7 ppm or 3 ppm zinc deficient diet and exposed by gastric intubation to the esophagus-specific carcinogen, methylbenzylnitrosamine, experienced significantly enhanced tumor incidence compared to control diet ad libitum rats. This effect was verified in three separate studies. Control diet rats pair fed to the food intake of zinc deficient rats had significantly reduced tumor incidences, to a level half that of control diet ad libitum rats. This indicated that reduced food intake characteristic of zinc deficient rats was not responsible for the tumor enhancing effects of this diet.

Zinc deficiency applied only up to and through the dosing phase, or only after the dosing phase caused a tumor incidence in between that of control diet throughout and zinc deficiency throughout. This suggested that both phases contributed to the tumor enhancing effects of zinc deficiency. The length of time on post dosing control diet in rats switched from zinc deficient diet after dosing to control diet altered the tumor enhancing effects of the deficiency.

Chronic alcohol consumption enhanced tumor incidence in both the control and zinc deficient dietary groups but this effect was not significant. The decreased nutrient:calorie ratio may have accounted for this lack of effect.

Zinc excess did not inhibit the carcinogenic process so that the dramatic enhancement of tumor incidence in a zinc deficient state cannot be explained as the absence of an anti-carcinogenic effect of zinc.

Vitamin A did not appear to mediate the effects of zinc deficiency since there was no vitamin A deficiency and the analogue of vitamin A, 13 cis retinoic acid, did not inhibit tumor incidence.

The enhancement of MBN esophageal carcinogenesis by zinc deficiency can be explained in two ways: (1) zinc deficiency enhanced DNA synthesis prior to carcinogen exposure which can increase vulnerability of cells to MBN. Zinc deficiency also enhanced DNA synthesis during tumor growth; and (2) zinc deficiency enhanced the level and persistence of adduct formation, particularly the promutagenic base, O6-methylguanine.

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I. INTRODUCTION

That environmental factors contribute to the majority of cases with which we have to contend is now obvious, and there is no need to review the evidence in detail. It rests on the great variation in the incidence of most cancers, both from place to place and almost certainly also from time to time; on the experience of migrants, among whom the risk of cancer changes to that of their adopted country in the course of one or two generations...

(Berg, 1977)

Judging from the epidemiology of cancer in man, it seems clear that almost all forms of cancer are caused, largely or entirely, by factors in our environment that vary from one place to another and from one generation to the next. Even without knowing what these factors are, we can deduce -- in principle at least -- that cancer should be a preventable disease.

(Doll, 1969)

Epidemiologic evaluation of world wide cancer rates indicates that environmental factors contribution to the etiology of 80 - 90% of all cancers (Doll, 1977, Cairns, 1978).

Recognition of these circumstances greatly enhances understanding of the cancer problem; it suggests that identification and manipulation of certain environmental factors might significantly alter cancer incidence. This understanding and approach is in sharp contrast to the perception that cancer is the inevitable result of a breakdown in certain intrinsic cellular processes and that, therefore, a curative approach is the only appropriate response to the disease once it has manifested itself.

Unfortunately there is confusion as to the meaning of environmental factors. In the context of cancer causation
environmental factors are usually equated with pollution -- of the water (industrial waste, heavy metal contamination, etc.); of the air (car and industrial exhaust); of the soil (pesticides, insecticides, commercial waste dumps); and of food (additives, contaminants, etc.). Although many of these factors are carcinogenic and important to the cancer process, risk of the major cancers (excluding lung) does not correlate well with the distribution and increased use over time of these factors.

Other apparently benign factors have shown strong correlation to the risk of many cancers and so must be considered as environmental factors. These include diet, such as amount and type of protein, fat and fiber, as well as deficiencies or imbalances of nutrients; gut flora; status of the immune system; stress; and, in the case of cervical cancer, sexual activity. The data suggest that although these factors are not in themselves carcinogenic they may predispose a host in such a way as to provide the critical difference between carcinogenic potential of a chemical and actual tumor production.

The purpose of this thesis was to examine the effects of a noncarcinogenic factor, in this case a nutrient deficit, on a known carcinogen and to better understand how this factor exerts such an effect. Esophageal cancer was chosen as an ideal model for the study of the effects of environmental factors on carcinogenesis because its extraordinary geographic and temporal variation strongly implicates environmental factors in its etiology. Zinc deficiency was investigated
because it is prominent among a number of nutrient deficits arising from a diet that has been consistently correlated as a major risk factor to esophageal cancer in some of the highest risk areas of the world. By examining the effects of a deficit of this trace metal on methylbenzylnitrosamine-induced esophageal cancer in a well controlled and well defined animal model, we hoped to better understand how diet interacts with cancer causing agents in a way that significantly alters their effects.
II. LITERATURE REVIEW

A. Epidemiology of Esophageal Cancer

Esophageal cancer exhibits an exceptional world wide variation in incidence. Muir (1978) suggests this cancer varies more than any other cancer. Doll (1977) indicates that esophageal cancer varies more than 300 fold, greater than that of any other common cancer. Other investigators suggest a 500-fold range of variation (Cook-Mazaffari, 1979).

This neoplasm is relatively rare in the U.S. and much of Western Europe, but is common or very common in many other parts of the world. Areas of high frequency include a band across Central Asia -- Turkmenistan and the Guriev district of Kazakhstan in Russia and the southern Littoral of the Caspian sea in Iran; areas within East Africa and the Bantus and Transkei of Southern Africa; and sections of China in which esophageal cancer accounts for 50% of all GI cancers, being the second most common cancer after stomach cancer. There is also a high incidence in Puerto Rico, Chile and selected areas of Japan. A moderate frequency of the disease is found in India and parts of France, as well as areas in Central and South America. Besides the U.S. and most of Western Europe, the lowest incidences are exhibited in Manitoba (Canada), Bulgaria, Greece and the Netherlands. (Levin, et al., 1979; Bailey, 1965; Wydner and Bross, 1961; Jussawalla, 1977;

The remarkable variation in incidence of the disease is seen more vividly by looking at fluctuations of rates within small countries or regions. In Japan incidence varies as much as 100 fold (Okada, et al., 1978). Within Eastern and Southern Africa high incidence rates contrast to the virtual absence of the disease in neighboring West Africa (Cook, 1971), and along the Southern littoral of the Caspian sea, within a narrow strip of land no more than 600 km in length, there is a 30 fold gradient of variation of incidence in men and a 10 fold gradient of variation in women (Hormozdiari, 1975). Such well-defined distinctions in incidence rates between areas of such close proximity offer the opportunity to identify factors associated with the fluctuations and to test these factors experimentally to further delineate their role. Following is a review of a few factors associated with variation in incidence of esophageal cancer.

1. Carcinogens

Nitrosamines and mycotoxins are frequently mentioned agents responsible for esophageal cancer risk (Mahboub, et al., 1977; Warwick and Harrington, 1973; Lijinsky et al.,
1970; Druckrey et al., 1967). In areas of China nitrosamine content of pickled foods consumed by humans and scraps consumed by chickens correlate to human esophageal cancer and gullet cancer in chickens (Kaplan and Tsuchitani, 1978). Fong et al., (1977) and Huang et al. (1978) also confirmed the presence of nitrosamines in preserved foods consumed by Chinese in Hong Kong who have a high incidence of esophageal cancer; whether these nitrosamine contents are not seen in comparable low incidence areas is unclear.

A report by McGlasen et al. (1968) implicates nitrosamines in the spirits distilled from sugar and maize husks for the high incidence of esophageal cancer in Zambia. However, the methods used for nitrosamine determination have been questioned. More recent determination of nitrosamine content in food and beverages in the high incidence areas of the Transkei show uniformly low levels (< 0.1 ppm) and no difference with lower incidence areas.

Second to nitrosamines, mycotoxins are the other major group of chemical agents suspected in the etiology of human esophageal carcinogenesis. The mycotoxin producing species F. verticillioides has been associated with foodstuffs in many high incidence areas including Linxian county in the Hunan Province of China and parts of Africa (Liu and Qu, 1976). Corn consumption is associated with esophageal risk in Africa and fungal contamination of the Fulsarium species is common. A study (Marasas et al., 1979) of Fulsarium fungi did find higher levels in high risk compared to low risk areas but the authors concluded the evidence was not strong enough to indi-
cate a causal relationship. Furthermore the carcinogenicity of the isolates from these fungi is not established.

Taken together there is no strong evidence to consistently and clearly implicate nitrosamines, mycotoxins or other carcinogens as the major cause of esophageal cancer in these higher incidence areas. This is somewhat surprising in light of the dramatic variation of the disease within short distances which might be expected to pinpoint such potent carcinogens. Somewhat paradoxically, the dramatic world-wide variation in esophageal cancer, a model of the epidemiologic approach, highlights the apparent complexity of interactions, rather than clarifying a simple relationship. This data suggests there is a synergism among carcinogens thus complicating a simple correlation of a single environmental factor to the disease. The data also suggest that additional, non-carcinogenic factors which might predispose an individual to the disease, are likely involved.

2. Alcohol.

In areas of low or moderate incidence the correlation of esophageal cancer to consumption levels of alcohol is rather strong. In the United States increases in the disease are associated with increased per capita sales of alcoholic beverages (Rothman, 1975; Wydner & Bross, 1961; Schoenberg et al., 1971).

A confounding factor in this association is the effect of
cigarette smoke since most heavy drinkers smoke (and vice versa) and the carcinogenic properties of cigarette smoke are well established. These factors alone show little increased risk for esophageal cancer. It is only in combination that a strong association of these factors to risk of this neoplasm exists. Because to date no evidence has implicated alcohol as a carcinogen, it is assumed that cigarette smoke provides the necessary carcinogenic modifying effect. What is noteworthy is that when one of these factors is held constant and the other varied, alcohol consumption, rather than smoking levels, correlates more strongly to increased risk. One study (Wydner & Bross, 1961) showed risk of heavy drinkers to be 25 times that of non-drinkers. What this suggests is that given the presence of a carcinogen, it is the level of a non carcinogen modifying factor that may determine level of susceptibility and risk.

In other areas of the world, especially those exhibiting higher incidences of esophageal cancer, association to alcohol is based on type of drink rather than amount. In France, fluctuations in incidence from low to moderate follow a regional pattern; each region usually produces its own alcoholic beverages (Warwick and Harrington, 1973; Toyno, 1970). Epidemiological studies in Africa by Cook (1971) have shown that the geographic and temporal pattern of esophageal cancer risk corresponds strongly in most instances to maize as the primary crop and its use in the production of alcoholic beverages. Malawi gin among South African Bantu males (Cook, 1971) and Pai Kin among Chinese (Wydner and Bross, 1961) have
been associated with increased risk of the disease.

Despite the extensive association of alcohol to esophageal cancer, there are many places where no such association exists (Wydner and Bross, 1961; Kmet and Mahboubi, 1972). In Finland there are three times as many male heavy drinkers as female heavy drinkers; nevertheless risk of the disease is shared equally between the sexes (Kiviranta, 1952). In Hawaii, the alcohol consumption pattern among men, even smokers, correlates poorly to the disease, although there is a high correlation for women (Kolonel, 1979). In India and Iran where incidence can be very high numerous investigators have noted a lack of association of alcohol to the disease (Warwick and Harrington, 1973; Mahboubi, 1976; Cook-Mozaffari, 1979). In these areas the most commonly associated risk factors are related to diet, as discussed in the following section.

3. Dietary Factors

Esophageal cancer is much more prevalent among lower socioeconomic groups and poorer countries (Warwick and Harrington, 1973). Common to these groups are multiple nutrient deficiencies resulting from limited food intake (both calories and variety of foods) and poor food and soil quality. In high incidence areas across Asia, in parts of China and Africa, in which no association to alcohol exists, it is an unvaried limited diet that is most consistently
associated to increased risk. Because nutrient deficiencies can exert profound effects on the normal growth, development and integrity of tissues and organs they might enhance the vulnerability of the esophagus to carcinogens or in some way compromise the response of the host to carcinogenic insult.

A number of dietary factors have been associated to esophageal cancer: low intake of vitamins A, C and riboflavin, reduced amounts of fresh fruits and vegetables and animal proteins (Cook, 1971; Jussawalla, 1972; Day, 1975). In Japan consumption of hot rice is associated with a 2-3 fold increase in risk of the disease (Okado, 1970), in many areas of the world, such as China and Puerto Rico, increased risk is associated with consumption of very hot beverages.

Prominent in the areas of greatest dietary restriction and highest esophageal cancer incidence is a deficit of the trace metal zinc.

Northeastern Iran exhibit. one of the highest incidences of esophageal cancer in the world with one of the sharpest variations in such incidence from one area to another (Pfeiffer, 1982). Alcohol and tobacco are ruled out as risk factors. A poor, restricted diet is most strongly associated to high risk.

It is this same area in which the existence and seriousness of zinc deficiency in humans was first observed (Prasad, 1961; Halstead, 1972). Patterns of severe zinc deficiency coincide with patterns of highest risk of esophageal cancer. Prasad concluded that the profound tissue and organ
disturbances resulting from this deficiency can cause severe growth retardation. As Crespi et al. (1977) states, "The diet in the high incidence area is markedly restricted and even within this area high-risk individuals have the most restricted diet." The authors go on to note that no single food is associated to risk or suspected of being carcinogenic. Rather the increasingly restricted diet is characterized by greater reliance on a single source of food - bread - and large amounts of tea for sustenance. Not only does such a narrow diet lack many nutrients, but high levels of phytates in bread and tannins in tea prevent the absorption of trace metals, such as zinc. Zinc deficiency is one of the most common and prominent nutrient deficits in these high risk areas -- causing (nutritional dwarfism), sexual immaturity (hypogonadism) and anemia - could lead to a variety of problems and complications. Sadeghi et al. (1971) listed zinc deficiency as the possible etiological factor of significance in esophageal cancer.

In Transkei of Africa, another very high incidence area, dietary restriction figures prominently. States Rose (1982), "Unexpectedly, the strongest associations have been with geological strata and poor farming conditions. In comparing the habits and customs of people, the most convincing associations have been with an unvaried diet." A strong correlation of low trace element profiles to the disease was seen in the Beaufort Strata (Marais and Drewes, 1962). Deficiencies of Zn, Cu, Fe and Mg were marked in garden plants of esophageal cancer sufferers vs. non cancer sufferers (Burrell et
al., 1966). Food from an experimental garden in the high risk area fed to rats caused degenerative changes in the esophageal basal-cell nuclei (Van Rensberg et al., 1979). Warwick and Harrington (1973) noted the lack of zinc in the diet of high risk Transkei and that these individuals suffer from a number of diseases known to lead to excessive excretion of zinc.

High incidence areas of china are characterized by restricted, unvaried diet consisting primarily of low zinc cereals. Many are known to suffer from diseases such as tuberculosis and liver disease (Halstead et al., 1968) conditions that lead to excessive excretion of zinc. Patients with esophageal cancer had reduced levels of zinc in hair, serum and esophageal tissue as compared to normals or other types of cancer patients (Fong et al., 1977; Lin et al., 1977).

These data suggest that although carcinogens may be necessary for the development of cancer they are likely not in themselves sufficient or even the critical determinant for tumor development. Other non carcinogenic factors may provide a powerful modifying effect. In esophageal cancer epidemiological data implicate a severely restricted diet as a major modifying factor in high incidence areas. Prominent within this restricted diet is a deficit of the trace mineral zinc.
B. **Mechanisms**

1. **Esophagus**

   a. **Histology**

   The digestive tract consists of four layers or coats: mucosa, sub-mucosa, muscularis and adventitia or serose (Bevelander, 1961). A lining of stratified squamous epithelium distinguishes the mucosa of the esophagus from the rest of the digestive tract. In humans and rats the epithelium is cornified or keratinized at the surface. The epithelial layer, from which most esophageal tumors arise, consists of four layers: (1) the innermost basal layer, in which mitosis occurs; (2) the stratum spinosum, consisting of prickly cells; (3) stratum granulosum, the layer in which cells flatten and accumulate granules of keratohyalin as they are pushed toward the surfaces and (4) the outermost, surface stratum corneum in which the nuclei and all cytoplasmic organelles disappear and keratin forms, conferring a tough barrier.

   As mitosis occurs in the basal layers, daughter cells may either remain at that level to divide again or may migrate towards the surface and differentiate, becoming prickly, then more star shaped, flattening out and finally losing all cellular components and filling with keratin. Normal stratified squamous epithelium, therefore, is the result of a well regulated process of differentiation that confers a distinct organization on a number of different cell forms.
b. **Pathology of Esophageal Cancer – Clinical and Experimental**

Esophageal cancer is a painful and very uncomfortable disease which, by the time of onset of symptoms is so advanced that it is quite resistant to either surgical or radiation treatment, with a poor prognosis. In one study (O'Brien et al., 1982), of 199 cases 137 were inoperable. There was one survivor after 5 years. Of the 62 operable cases 50 also received radiation. There were seven survivors after five years – an 11% survival rate among operable cases and a 4% survival rate among all cases. These statistics underline the importance of an approach that might aid in the prevention of the disease.

Carcinoma of the esophagus is generally of the squamous cell type, with a rare adenocarcinoma. In one study of 150 esophageal carcinomas, one was adenocarcinoma, the other 149 being squamous cell carcinoma in situ or early invasive (Liu and Qu, 1982). Most carcinomas are accompanied by severe, chronic inflammatory cell infiltration in the area adjacent to the tumor. Of 418 cases in Iran, only 18 (4.3%) had normal mucosa adjacent to tumors (Liu and Qu, 1982). Most carcinomas are ulcerated and fungating with varying degrees of infection and necrosis.

A study by Napalkov and Pozharisski (1969) followed the histopathological changes of esophageal tumor development in 42 rats given N-methyl-N-nitrosoaniline. Early changes
included vacuolization of epithelial cells, acantholysis, spongiolysis and granulosis. Lymphoid-polyblastic elements infiltrated the deep layers of the epithelium to some degree.

Leukoplakia was the first gross change observed in the esophageal mucosa. This patch was located at either end of the esophagus and in the mid portion. Some authors consider this to be a pre-malignant condition. There was also an increase in cell number and size leading to a general thickening of the epithelium lining; there was also marked hyperkeratosis and parakeratosis.

Papillomas resulted from the proliferative activity of the epithelium and there was progressing hyperplasia of the connective tissue papillae. After 13-14 months the epithelial structures of the papillomas showed a syrphasia, and foci of intro epithelial cancer appeared. These were characterized by complete disorganization of the stratified structure and increased mitosis in the superficial layers of the epithelium.

A study of the histopathology of MBN-induced esophageal tumors (Stinson et al., 1978) indicated that 100% of rats developed papillomas (89% pedunculated, 13% sessile), 67% developed pedunculated papillary carcinomas, and 63% developed invasive carcinomas. Half of the carcinomas invaded to the depth of the muscularis and the other half to deeper layers; 20% penetrated the muscle wall. The neoplasms were well differentiated with various degrees of keratinization. Less differentiated tumors tended to be more cellular, showed less keratinization and a higher degree of disorganization.

As the investigators point out, fungating and
infiltrating carcinomas, the most common tumors found in this study, parallel what is found in human esophageal cancer. Although the less common ulcerative form in humans was not seen in the animal studies, areas of ulceration were present in other cancers. Also as in humans, the cancers were well differentiated and developed in similar parts of the esophagus. Together these results suggest that chemically induced esophageal cancer in the rat appears to be a good model for the study of human esophageal carcinogenesis.

2. Nitrosamines

   a. Synthesis.

Nitrosamines are formed by the reaction of nitrous acid with a secondary amine (Figure 1). Because nitrous acid is not stable, the reaction usually involves nitrite and a secondary amine in an acid medium (pH 3.0). The acid medium converts nitrite to nitrous acid which then immediately nitrosates the secondary amine to form a nitrosamine.

Nitrites are formed by the reduction of nitrates; a wide range of bacteria, found in plants and human saliva, contain nitro reductases (Nason, 1962; Lam and Nicolas, 1969) capable of this transformation (Zobell, 1932; Iida, K. and Taniguchi, 1959). Nitrates occur widely in the water supply and in root and green leafy vegetables (Sinois et al., 1965). As a result of nitro reduction, large concentrations of nitrites are found in stored green vegetables, especially spinach, celery and
Figure 1. Formation of nitrosamine from nitrite and secondary amine. Metabolism of methylbenzyl nitrosamine.
\[
\text{HONO} \quad \xrightarrow{\text{R-NH}} \quad \text{R-N-N=O}
\]

Nitrous Acid \hspace{1cm} \text{Secondary Amine} \hspace{1cm} \text{Nitrosamine}

\[
\begin{align*}
\text{O}=\text{N}-\text{N} & \quad \text{CH}_3 \\
\downarrow & \quad \text{methylbenzyl nitrosamine} \\
\text{O}=\text{N}-\text{N} & \quad \text{CH}_3 \\
\text{CH} & \quad \text{OH} \\
\text{HO-N=N=CH}_3 + \text{HC}_{\text{CH}} & \quad \text{methyldiazenium benzaldehyde} \\
\downarrow & \quad \text{hydroxide} \\
\text{N}_2 + \text{CH}_3 & \quad \text{benzyldiazenium hydroxide} \\
\end{align*}
\]

α - carbon hydroxylation on methylene bridge of benzyl moiety

α - carbon hydroxylation on methyl group

\[
\begin{align*}
\text{N}_2 + \text{CH}_3 & \quad \xrightarrow{\text{Nu}} \quad \text{Nu CH}_3 \\
\text{H}_2\text{O} & \quad \xrightarrow{\text{Nu}} \quad \text{Nu CH}_3 \\
\end{align*}
\]

Formaldehyde
salad (Lijinsky and Epstein, 1970).

Secondary amines occur in fish meal and fish products (Lijinsky and Epstein, 1970), cereals and tea, and in tobacco and tobacco products (Neurath et al., 1966).

Because the gastric juice offers an acid medium optimal to nitrosation, it has been generally accepted the nitrosamine formation can occur in the stomach if both nitrites and secondary amines are present. Some nitrosamines, however, are basic enough - such as dimethyl- and diethyl- nitrosamines - that nitrosation is not favored in the mildly acidic medium (Sander et al., 1968).

In a study by Druckerey (1967) rats given nitrite and methyl-benzyl amine developed esophageal tumors to the same degree as did rats receiving methyl benzyl nitrosamine. This was confirmed by Sander et al. (1968) who fed rats for eight weeks with diets containing equal concentrations of the amine and 0.5% sodium nitrite followed by a normal diet. Animals began to die with multiple tumors of the esophagus after six weeks on the diet while those fed either the nitrite or the amine alone developed no tumors.

b. **Affects.**

As a group nitrosamines are multipotent carcinogens. (Magee and Barnes, 1967). They are carcinogenic in a number of different organs over a wide variety of species tested (Magee and Barnes, 1967). One of the most interesting features of these carcinogens is their organ specificity.
Nitrosopiperidine produces tumors of the esophagus and other sites irrespective of route of administration - intravenously, orally or subcutaneously. Diethyl- and dibutyl- but not dimethyl- nitrosamines cause tumors of the esophagus and of other sites (Magee and Barnes, 1967; Mohr et al., 1967; Takayama and Imaizuma, 1969). Otherwise, esophagus-specific carcinogens are asymmetrical - methyl butyl-, methylphenyl-, butylethyl- and methylbenzyl- nitrosamines. Of 65 nitrosamines studied by Druckerey et al. (1967) methyl benzyl-nitroamine (MBN) was found to be the most carcinogenic for the esophagus.

c. Mechanics.

It is generally accepted that nitrosamines, including MBN, must be activated to a reactive electrophilic species to exert a carcinogenic effect and that this activation occurs by the P-450 enzyme system (Hodgson et al., 1980). It is this ultimate carcinogen, the product of this enzyme system, that is capable of reacting with nucleophillic sites on cellular macromolecules (DNA, RNA, proteins). The P-450 system is responsible for hydroxylating compounds (Miller, 1978). This hydroxylation increases the polarity of the compound or allows for additional chemical reactions that further increase the polarity of the compound thereby facilitating removal from the blood by the kidneys and excretion from the body via the urine.

In nitrosamine metabolism hydroxylation occurs as α carbon hydroxylation (hydroxylation of the carbon that is alpha...
to the nitrogen). Because the P-450 system exists in highest concentrations in the liver, it might be assumed that the greatest metabolism of carcinogens and production of the ultimate reactive species would occur in this organ. However, it has been postulated (Hodgson et al., 1980) that subclasses of this enzyme may exist with a higher level of activity in extraheptic tissues. The higher activity may be limited to a group of carcinogens with a particular structure that have a high degree of stereochemical affinity to this subclass of enzyme. This may also account in part for the tissue specificity of certain carcinogens.

Evidence that a subclass of P-450 enzyme exists in the esophagus with an affinity for the esophagus-specific asymmetrical nitrosamines in general and MBN in particular is provided by Hodgson et al. (1980). 10 minutes after I.V. injection of \(^{14}\text{C}-\text{methyl}\) benzyl nitrosamine both the total amount of radioactivity and of MBN were higher in the liver, kidney and serum than in the esophagus. However, through h.p.l.c. separation, 50% of the radioactivity in the esophagus was in the form of metabolites whereas only 10-22% of the radioactivity in the other tissues represented metabolites; this indicated that although uptake of MBN was actually higher in tissues other than the esophagus, metabolic conversion to the carcinogenic species was greatest in the esophagus.

Because of the asymmetrical nature of MBN, a carbon hydroxylation can produce two different reactive species: (1) a methylating species resulting from a carbon hydroxylation of the methylene bridge of the benzyl moiety; and (2) a
benzylating species resulting from α carbon hydroxylation of the methyl group (see figure 1). The esophageal carcinogeneity of MBN can therefore be assessed in two ways: the importance of ultimate carcinogen and type and level of adducts formed.

(1) **Methylation vs. benzylation.**

Druckerey et al. (1967) found the benzyl residue sufficient for induction of esophageal tumors. Tannenbaum et al. (1977), however, noted that MBN, hydroxylated on the benzyl moiety (leading to a methylating species) was more toxic than the hydroxylation of the methyl component (leading to a benzylating species). Hodgson et al. (1980) state that there is no evidence to indicate that metabolism to benzylating species or binding of benzylating species occurs to any significant extent in the esophagus. Ivankovic (1978) found that N-benzyl-N-nitrosourea, capable of producing a benzylating species, induced carcinomas of the forestomach in rats. This suggested this residue might be implicated in the carcinogenic process.

*In vitro* microsomal incubation with MBN by Chin et al. (unpublished) showed both methylation and benzylation of esophageal DNA, methylation being 5-10 times greater than benzyl at all time points. Methylation increased linearly with time of incubation, benzyl increased linearly up to 30 minutes and then plateaued.

The literature suggests that the methylating species is
likely the more important one to MBN carcinogenesis although the issue is not resolved.

(2) Adducts.

Electrophilic alkylating species, the product of metabolism of many carcinogens, including nitrosamines, by the monooxygenase system, are capable of reacting with nucleophilic sites on DNA -- oxygens, nitrogens and the phosphate backbone (Sun and Singer, 1975; Singer, 1976). These reactions can produce at least 10 different alkyl-nucleotide products (Lawley, 1974; Magee et al., 1975) including 1-, 3-, and 7-alkyladenine, 3- and 7- alkyl guanine, 3-alkylcytosine, 3-alkylthymine, O^4 alkyl thymine, O^6-alkylguanine and phosphotriesters (Lawley, 1972; Lawley, 1973; Singer, 1975; O'Conner et al., 1975; Lawley et al., 1971; Lawley et al., 1973; Lawley 1974; Pegg and Nicoll 1976; Loveless, 1969; Goth and Rajewsky, 1974; Kleihues and Margison, 1974).

The importance of these various adducts in carcinogenesis can be evaluated in two ways: (1) whether the amount of adduct formed can be correlated to the carcinogenicity of the compound. This can be assessed by a dose response relationship of a single carcinogen, an appropriate distinction between a weak and strong carcinogen, or between adduct formation in target and non-target tissue; and (2) whether the adduct formed can lead to a permanent change in the genetic material.

Based upon the first criteria alkylations in the 1 and 3
positions of guanine, in the 1, 2 and 7 positions of adenine, in the 3 positions of cytosine and thymine and the O^4 of thymine do not correlate well with carcinogenesis (Magee et al., 1975; Swann and Magee, 1968; Schoental, 1969; Swann and Magee, 1971; Pegg and Nicoll, 1976; Nicoll et al., 1975; O'Conner et al., 1973). Questions have been raised on the importance of the phosphotriesters (Kirtikar and Goldtwain, 1974) although there is strong evidence against this involvement. Frei et al. (1978) showed no difference in the level of these between weak and strong carcinogens; thus methanesulphonate, a weak carcinogen, produced more phosphotriesters than did MNU, a very strong carcinogen. The triester products also persisted in vivo in the liver, an organ resistant to this tumor production. Many of the more minor products were extremely labile, making measurement by standard laboratory procedures very difficult, if not impossible (O'Conner et al., 1973). Furthermore, the speed with which these products rapidly drop off raises questions about their ability to effect permanent, heritable damage to the DNA.

The strongest correlation to carcinogenicity by all the different measures of dose-dose response, weak vs. strong carcinogen and target vs. non target tissue has been shown for the O^6 alkylguanine (Pegg, 1977; Loveless and Hampton, 1969; Loveless, 1969; Lawley and Martin, 1975; Nicoll et al., 1975; Margison & Kleihuis, 1975; Craddock, 1975a,b; Lawley, 1974).

O^6-methylguanine has also been shown to be a promutagenic base, capable of causing base mispairing (GC —> AT) (Pegg, 1977). This is not the case with 7-methyl guanine or
most of the other minor adducts (Pegg, 1977). 3-methyl cytosine is capable of base mispairing (Ludlum and Wilhem, 1968; Ludlum, 1970, 1971; Singe and Frankel-Conrat, 1970), but similar levels of this adduct have been found in liver, for instance, when exposed to either dimethylnitrosamine (DMN), a liver carcinogen, or methylmethanesulfate, a non liver carcinogen (O'Conner et al., 1973).

O$_6$-methylguanine has been shown to pair with thymine rather than cytosine, leading to the GC $\rightarrow$ AT base shift (Abbott and Saffhill, 1979; Gerchman and Ludlum, 1973). Gerchman and Ludlum (1973) showed that CTP in vitro did not block incorporation of TMP, indicating cytosine was no longer an appropriate base for O$_6$-methylguanine. Abbott and Saffhill (1979) provide evidence that there is competition between cytosine and thymine, with a thymine incorporated for every two or three O$_6$-alkylguanines.

Measurements of O$_6$-methylguanine are usually made with 7-methylguanine since the latter occurs in the greatest quantity and this provides a baseline of approximately how much alkylating material is available and a background rate of removal. Evidence indicates that 7-methylguanine is removed by spontaneous depurination or an enzyme system not as specific as that for O$_6$-methylguanine (Nemoto and Takayama, 1974; Margism et al., 1976). By using ratios of O$_6$:N-7-MG between target and non target tissues, for instance, background tissue variation will be eliminated and real differences in rate of removal will be observed (Swann and Magee, 1968; Schoental, 1969; Swann and Magee, 1971; Pegg and Nicoll, 1976). Of the
remaining adducts, none have shown as strong a correlation either to carcinogenic potential or to genetic transformation as has O\textsuperscript{6} methylguanine (Pegg, 1977; Loveless and Hampton, 1969; Loveless, 1969; Lawley and Martin, 1975).

A number of studies have shown that either the initial formation or the persistence of O\textsuperscript{6}methylguanine is greater in target vs. nontarget tissues or in strong vs. weak carcinogens. Goth and Rajewsky (1974) found O\textsuperscript{6}methylguanine produced by ethynitrosourea was removed much more slowly in the brain - the target organ - than in the liver. This effect was substantiated by Kleihues and Marginson (1974) using methylnitrosourea, also a brain carcinogen. In both studies N-7 methylguanine was removed at similar rates in both brain (target tissue) and liver (non target tissue). In the MNU study O\textsuperscript{6}-methylguanine was lost at an intermediate rate (between liver and brain) in the kidney which shows some susceptibility to MNU. A single dose of DMN to normal adult rats produces kidney tumors but not liver tumors (Pegg, 1977; Magee and Barnes, 1967; Swann and Magee, 1968). In the kidney after a single dose O\textsuperscript{6}methylguanine was removed much more slowly as compared to the liver (Magee et al, 1975; Pegg and Nicol, 1976; Nicoll et al, 1975). When multiple doses of DMN were administered, producing liver tumors, the rates of removal of O\textsuperscript{6}methylguanine in the liver decreased appreciably (Pegg, 1977). O'Conner et al. (1973) showed no difference in the liver of 7-methylguanine or 3-methylcytosine between DMN, a liver carcinogen and MMS a non liver carcinogen, and Frei et al. (1978) showed no difference for 7 methylguaine, the minor
adducts or the triesters between a week and strong carcinogen. In both studies differences appropriate to carcinogenic potential were found for O^6^-methylguanine. Frei et al (1978) found dose response rela! rships of 4.5 between methyl- and ethyl-nitrosourea for production of thymomas. The ratio of O^6^-methylguanine in the DNA for these two carcinogens was 5.3, compared to ratios for the other adducts showing 10 fold greater variations from 4.5.

The evidence suggests a specific enzyme repair system for the O^6^-alkylguanine compared to the N-7 alkylguanine. Evidence showing a differential rate of O^6^-methylguanine removal between target and non target tissue, compared to a similar rate for N-7 methylguanine also eliminates the possibility that the differential rate in O^6^- removal is simply due to different rates of cell necrosis or synthesis (which would dilute out the adduct) since if this were the case N-7 methylguanine rates would vary to a similar degree (Nicoll et al., 1975).

The exact enzymes responsible for the removal of alkylated bases, in particular O^6^-alkylguanine, is unclear and controversial (Pegg and Hui, 1978). Kurtikar and Goldthwait (1974) showed an endonuclease in E. coli that releases O^6^-methylguanine and 3-methyl adenine but not 7-methylguanine. Whether this also occurs in humans is unclear. It has not been established whether the same enzyme responsible for the glycosidase activity releasing the purines and the strand breakage (Kirtikar et al., 1976). Pegg and Hui (1978) report that O^6^-methylguanine removed from liver and kidney in their experiment is carried out a similar glycosidase and the
apurine site remaining is repaired by a separate endonuclease. Another experiment by Pegg (1978) indicates N-glycosidase not involved in the excision of O6-methylguanine. Other work suggests an enzyme capable of dealkylating O6 methyl- or O6 ethyl guanine leaving the nucleoside intact (no depurination) (Miller et al., 1973). There may be multiple enzymes involved since some removal appears to act quickly whereas others are initiated at later times (Goth-Goldstein, 1977) or different dose levels affect removal rates in a way suggestive of multiple enzymes (Pegg and Hui, 1978).

Taken together, the initial levels and rate of removal of O6-methylguanine, relative to 7-methylguaine, provide a strong basis for assessing the carcinogenicity of many compounds. Hodgson (1981), utilizing the esophagus specific carcinogen, methylbenzylnitrosamine showed that methylation was highest in the esophagus, followed by liver, lung and forestomach. Esophageal O6-methylguanine was six times higher than in the lung and nine times higher than in liver 4 hours after dosing. Also the O6-methyl:7-methylguanine ratio was highest in esophageal DNA; this indicates that enzymatic removal of O6-methylguanine was much lower in the esophagus than liver or lung. Fong et al. (1979) also found a higher O6:N-7-methylguanine ratio in esophagus compared to lung but found lower a ratio in the more tumorigenic zinc deficient diet than the control diet which was inconsistent with the relationship of O6-methylguanine levels to carcinogenicity. This study as well as one by Kraft et al. (1980) showed an increase of overall methylation over time.
In determining the level of methylation by measurement of $^{14}$C radioactivity it is important to distinguish actual methylation -- attachment of the methyl group to intact molecules -- from biosynthetic incorporation of the labeled methyl group into macromolecules, specifically DNA. Methyldiazonium hydroxide, produced by $\alpha$-carbon hydroxylation of the methylene bridge of the benzyl moiety, in turn produces the ultimate carcinogen, the methyl cation. This highly electrophilic species, in addition to reacting with nucleophilic sites on DNA, such as oxygens, nitrogens and phosphorus -- and so forming adducts -- can also react with water to form methanol (Figure 1). This can be rapidly oxidized to formaldehyde which then enters the C$_1$-pool, a source of carbon for purine biosynthesis, as well as other macromolecular biosynthesis. Formaldehyde can attach to tetrahydrofolinic acid (THF) and be oxidized to 5,10 methenyl-THF and 10-formyl-THF or reduced to 5-methyl-THF. The methyl component of the two oxidized forms can be incorporated at two different stages of purine biosynthesis (Metzler, 1979). HPLC technology is necessary to separate purines from adducted purine so as to distinguish the sources of radioactivity.

(3) Adduct formation and DNA synthesis

Since the persistence of damage to DNA is most likely critical to the carcinogenicity of compounds, the time and rate of cell proliferation and DNA synthesis is of great significance. This can affect how much of the damage is repaired and
how accurate that repair is (Lewis and Swenberg). Therefore in addition to cell proliferation altering the initial susceptibility of cells to carcinogens, proliferation after exposure might determine to a large degree how much of the damage is eventually "fixed" and expressed.

Most carcinogens inhibit DNA synthesis upon exposure (Bates et al., 1968; Hennings and Boutwell, 1969; McCarter and Quastler, 1962; Paul, 1969). In most cases stronger carcinogens inhibit more effectively than weaker carcinogens or non-target tissue carcinogens (Slaga et al., 1974). A second feature is that target tissue carcinogens usually cause an enhancement of DNA synthesis above normal levels following the inhibition whereas a non-target tissue carcinogen, although initially inhibiting DNA synthesis somewhat does not cause a significant enhancement (Slaga et al., 1983). This distinction might be part of the reason for tissue specificity of a carcinogen. Carcinogens may act as promoters with enhanced synthesis serving to "fix" damage.

In support of this it was shown that reducing the levels of 7,12-dimethylbenz[a]anthracene from a carcinogenic dose which caused an enhancement of DNA synthesis following inhibition, to an initiating dose (in mouse skin) the stimulation of DNA synthesis following inhibition was removed, leaving only the inhibition (Slaga et al., 1974).

Also, urethan, a pure initiator, only causes inhibition with no enhancement. This is not always the case since acetic acid, neither an initiator, promoter or carcinogen, can cause some inhibition and enhancement of DNA synthesis (Slaga
et al., 1974).

Comparing an esophagus-specific carcinogen - methyl-n-
amyl nitrosamine (MAN) with dimethylnitrosourea (DMN), which
does not produce esophageal tissues, Mirvish et al. (1978)
found that 10 times more DNA was required to produce the same
degree of inhibition of DNA synthesis as did MAN. The inhibi-
tion from DMN was very brief, lasting a sixth to an eighth of
the time as that caused by MAN. There was an enhancement with
DMN, as well as MAN; however, the dose required for the ini-
tial inhibition was so large (20 mg/kg) that it was postulated
that the enhancement reflected a response to massive cell
necrosis rather than some mechanisms of damaged DNA. Mirvish
showed a strong dose response correlation between esophageal
specific carcinogens vs. non target carcinogen in both the
depth and length of inhibition. He concluded that the incorpo-
ration of $^3$H-thymidine might be useful as a short term
bioassay for "discovering cocarcinogens or other factors that
may affect esophageal carcinogenesis...".

d. Summary

The esophageal specificity of asymmetrical nitrosamines
appears to be likely due to either a subclass of P450 enzyme
that exists in the esophagus and preferentially metabolizes
this class of nitrosamine, or to the persistence of promutage-
nic adducts, rather than due to route of administration or
tissue uptake. The level and persistence of $O^6$methylguanine,
an adduct strongly linked to the carcinogenicity of compounds,
is greater in the target tissue esophagus than non target tissues such as lung and liver. Therefore measurements of this adduct appear useful in determining and understanding the effectiveness of compounds on esophageal carcinogenesis.

The carcinogenicity of compounds correlates to the inhibition of esophageal DNA synthesis. This, in connection with the time and strength of synthesis enhancement as it relates to the persistence of adducts, may further clarify the mode of action of other factors that enhance the carcinogenic process in the esophagus.

3. **Zinc and Carcinogenesis**

   Indeed, it is possible that the zinc status of a cell may control certain key cellular functions by regulating the activities of key cellular enzymes. This is of obvious importance in understanding the role of zinc in both initiation and suppression of tumorigenesis (Phillips and Kindred, 1980).

   Epidemiologic studies indicate that zinc may play a role in the etiology of esophageal carcinogenesis. Clinical and experimental evidence supports a role for zinc in the carcinogenic process in general and provides a starting point from which to approach the question of zinc's role in esophageal carcinogenesis.

   a. **Clinical Zinc Levels and Cancer**

      In malignant disease, tissues and fluid levels of many
nutrients, including zinc, change. Serum and esophageal tissue levels of zinc become reduced in esophageal cancer patients (Fong, et al., 1977; Lin et al., 1977). The significance of such a change remains unclear; whether the change reflects an involvement of the nutrient in the etiology of the disease or a response of the organism to the disease has not been established.

Wright and Dormandy (1972) showed low zinc content in tumors and a higher than normal zinc content both in organs containing metastases and in apparently uninvaded livers of cancer patients. They showed a zinc concentration gradient from the necrotic core (lowest) to the surrounding healthy tissue (highest). The authors interpreted this as some type of tissue defense reaction to malignancy analogous to what is seen in wound healing. This protective effect of zinc is what was suggested by the experiment of Poswillo and Cohen (1970) in which a cheek would prior to DMBA application led to increased zinc levels and decreased tumor incidence.

Kew and Mallet (1974) found normal liver tissue to contain 78 ± 41 µg zinc/g wet liver compared to 18 ± 7 µg zinc/g in primary liver cancer. Kaltenback & Egan (1978) showed increased zinc concentration in liver metastases. Other studies showed increased liver concentrations of zinc in bronchiogenic, gastric and nasopharyngial cancer.

Mulay et al. (1971) reported that apparently healthy tissue of invaded mammary glands had higher zinc concentrations than normal mammary tissue. Schwartz et al. (1974) showed cancerous mammary tissue to contain 5.7 times normal
levels. This was consistent with the results of another study (Tupper et al., 1955) showing the uptake of $^{65}$Zn was increased in cancerous mammary tissue compared to normal. Serum zinc concentrations were reduced in bronchial and colon cancer, primary osteosarcoma, lymphoma, leukemia, Hodgkins disease and multiple Melanoma (Davies, et al., 1968; Beeley, et al., 1974; Davies et al., 1968b; Fisher et al., 1976). Some investigators have interpreted these results as a response of the body to the malignancy -- not a simple drop in zinc levels but a shift in zinc from certain parts of the body to other parts; other investigators have suggested that rapidly growing tumors have an increased need for zinc and are able to mobilize and concentrate it. Lower levels of zinc in tumors may simply reflect the rapid dilution of the zinc present by continually dividing cells. Andronikashvi et al. (1974) reported that DNA and RNA fractions isolated from tumor tissues contained two to four times more zinc than similar fractions from non cancerous tissue such as liver.

b. Experimental Evidence

In examining experiments employing zinc deficient or zinc excess diets it is important to understand what these terms mean. Use of these terms without a clear definition implies a well accepted standard. A review of the literature suggests otherwise. In many studies "zinc deficient" diet simply refers to any diet containing less zinc than the designated control diet. Depending upon the study cited this can refer
to anywhere from 0 ppm zinc to 40 ppm zinc.

One study, (Swenerton and Hurley, 1968) utilizing two control levels of diet - 60 ppm and 100 ppm zinc - found that although both diets led to similar growth rates a closer examination found coarseness of hair, dermatitis and esophageal lesions in the 60 ppm group, symptoms of zinc deficiency. A later study (Diamond et al., 1971) found no difference in the long term effects of these two dietary zinc levels.

Barney et al. (1968) referred to 2.5 ppm zinc in diet as a "low zinc diet" whereas 0.5 ppm was considered a truly zinc deficient diet. Beach et al. (1980), on the other hand, in studying the effects of four levels of zinc referred to 100 ppm as control, 9 ppm as marginal zinc, 5 ppm as moderate and 2.5 ppm as severe. Therefore, depending upon the investigator, a specific level of zinc can be referred to as a "severe deficiency" or as a "low zinc diet." In terms of quantifying effects to level of zinc deficient, the arbitrary use of these descriptive terms can create confusion.

Another complicating factor is the protein source used in the diet. Plant protein sources, such as soy meal, contain phytate and fiber, both of which bind to zinc, as well as other minerals, and make it less available for absorption. This raises the minimum requirement for zinc. In this type of diet, calcium levels interact with phytate and zinc to further block zinc absorption; thus minimum sufficient zinc levels in a phytate containing diet also depend on calcium levels. The water used (deionized vs. non deionized), access to feces, and removal of zinc contamination from cages -- all of these fac-
tors can significantly affect the animals response to a certain dietary level of zinc.

At present the agreed upon minimum levels of zinc to support normal growth and tissue function are 18 ppm in phytate containing diet (Forbes et al., 1960; National Research Council, 1978) and 12 ppm in an animal protein (non-phytate) diet. Palluf and Kirchgessner (1971) found 8 mg/kg diet sufficient for growth, but 12 mg/kg necessary for adequate serum and liver zinc levels (Forbes et al., 1960; Luecke, et al., 1970.

Based upon a review of the literature and studies in this laboratory, a moderate zinc deficient diet - one that produces significantly reduced serum and hair zinc levels, approaching that found in terminal zinc deficiency, but which allows survival of the animal - ranges from between 3 and 5 ppm for a nonphytate containing diet to 7 ppm for a phytate containing diet. 20-60 ppm is considered a normal range and greater than 100 ppm considered an excess.

Zinc excess to levels of 1000 ppm has shown no toxic effects. At levels of 1% (10,000 ppm) death usually ensues within 3-5 weeks (Smith and Larson, 1946). At .5-.7% in diet, animals usually do not die but show distrubance of a number of copper and iron containing enzymes (later section) (Smith and Larson, 1946; Duncan et al., 1953; Van Reen, 1953).

An early study in this laboratory (Fong et al., 1978) demonstrated that a moderate zinc deficiency (7 ppm in the diet) significantly enhanced chemically induced cancer of the esophagus at four different levels of the carcinogen. This
study also demonstrated that zinc deficiency decreased the lag time for tumor induction. The use of control diet groups pair fed to zinc deficient groups indicated that the enhancing effect of zinc deficiency was not due to inanition or growth retardation but to the deficiency per se.

Other studies have shown different effects of zinc deficiency and zinc excess on the carcinogenic process of other tissues. McQuitty et al. (1970) showed zinc deficiency (zero ppm zinc) decreased the growth of Walker 256 carcinosarcoma implanted into male weanling Sprague Dawley rats and increased the survival time of the recipients, compared to controls. Zinc excess only slightly inhibited tumor growth and did not affect survival time. Because this tumor is fast growing and involves neovascularization, another study (Dewys and Pories, 1972) looked at the effects of zinc deficiency on a slow growth lung tumor (Lewis tumor) and on an ascites tumor (does not require vascularization) to determine if the earlier results were simply due to zinc's generally inhibiting effects on growth or were specific for the tumor. The results were similar to those of the Walker tumor indicating a specific inhibiting effect of zinc deficiency on the growth of tumors. One conclusion from these studies was that zinc deficiency may exert its effect primarily on tumor growth whereas zinc excess may exert its effect on tumor induction.

Poswillo and Cohen (1970) demonstrated that zinc excess inhibited dimethylbenzantracene (DMBA) induced cancer of the hamster cheek pouch (11% in zinc supplemented vs 85% in control). They also showed a lower tumor incidence in a group
of hamsters that just received a wound in the cheek pouch that eventually received DMBA application. Since zinc is mobilized to the site of a wound, the authors reasoned that reduced tumor incidence in this case was due to increased zinc levels at the site of DMBA application.

A study by Duncan et al. (1974) showed that the growth of a transplanted tumor induced by 3-methyl-7-dimethylaminoazo-benzene was significantly reduced in both zinc deficient and zinc supplemented groups. A later study indicated that either treatment significantly reduced DNA synthesis and also inhibited the induction of 3-methylcholanthracene induced carcinogenesis. (Duncan and Dreositi, 1975).

The enhancement of carcinogenesis in the esophagus of zinc deficient animals was consistent with epidemiological data showing enhanced incidences in nutritionally deficient groups. This enhancement was also consistent with the protective effects of zinc excess, i.e., enhancement due to a deficiency might reflect the absence of a protective effect of zinc. This protective effect was suggested in the clinical experiments cited, which showed a mobilization of zinc to tissue adjacent to tumors.

The zinc deficient enhancement of esophageal tumors was not consistent, however, with the effects of zinc deficiency on other tissues, in which tumor incidence was inhibited. There may be two reasons for this effect: (1) a moderate zinc
deficiency was used in the esophageal cancer studies whereas a severe, terminal zinc deficiency was used in the other studies. The inhibition of tumor growth in the severe zinc deficient state may have been part of a generalized, overall, complete cessation of growth, leading to early death, rather than a specific tumor inhibiting effect. Any subtle enhancing effects of the deficiency would have been overshadowed by this condition; and (2) in addition to the absence of a protective effect of zinc, zinc deficiency may produce distinctive effects in the esophagus capable of enhancing carcinogenesis.

c. Zinc and Esophageal Cancer

(1) Esophageal Lesions

Despite a 1934 report by Todd et al. (1934) that zinc was necessary for life in animals the ubiquity of zinc in the environment led investigators to believe that a zinc deficient state and problems resulting from it were subjects for esoteric discussion rather than matters of practical concern. As a result, almost thirty years passed from the time of the Todd report until the role of zinc in the health of animals and humans were seriously considered. In the mid 50's Tucker and Salmon (1955) showed that zinc deficiency was responsible for the severe parakeratotic condition of pigs which caused great economic loss. In the early 1960's the importance of zinc in humans was demonstrated by Prasad (1961) and Halstead (1972) in Egypt and Iran, respectively, as the cause of severe growth
retardation (nutritional dwarfism) and sexual immaturity (hypogonadism). Since that time a flourishing of research on zinc metabolism has revealed its wide ranging involvement in a variety of physiological and biochemical processes and the profound derangement in these processes than can ensue from a deficiency of this trace metal.

Zinc deficiency has been described in rats, mice, swine, chickens, turkeys, cattle, goats, lambs, rabbits, hamsters, guinea pigs and others (Halstead, 1974). Despite the variety of species affected by zinc deficiency the symptoms resulting are almost uniformly the same: retarded growth, anorexia and emaciation, inanition, alopecia, ocular lesions, testicular atrophy and characteristic lesions of the esophagus and skin.

Most relevant to this study on esophageal carcinca are the characteristic lesions of the esophagus and skin that develop in a zinc deficient state. An early study by Follis et al. (1941) demonstrated the effects of an extremely low zinc diet on the tissues of rats. In the zinc deficient animals the cells of the basal layer were more numerous and closely packed than in the controls, although there was no increase in the mitotic figures. The overlying layers increased from 2 - 3 cells thick to 6 - 8 cells thick. The nuclei became smaller but did not disappear, as normally occurs; instead there was 4 or 5 layers of small dark nuclei. There was only slight keratinization. Follis identified this condition as extreme parakeratosis, the thickened epithelial wall resulting from either incomplete keratinization or
increased proliferation of cells.

More recent studies (Swenerton and Hurley, 1968; Hurley, 1969) confirmed these earlier findings. Swenerton and Hurley, (1968), exposing both male and female weanling rats to a diet almost devoid of zinc (1 ppm in diet) produced esophageal lesions of parakeratosis, hyperkeratosis and hyperplasia. In rats fed the deficient diet for 4 - 7 weeks and returned to the control diet, gross dermal lesions began to improve within 2 - 3 days, with all signs of zinc deficiency absent within 6 - 8 weeks. A group fed the control diet but pair fed to the zinc deficient group showed no abnormalities, indicating the effect was specific to the zinc deficiency rather than the anorexia and growth retardation resulting from it.

In her study, Hurley (1969) found zinc deficiency produced the same characteristic esophageal lesions of extensive parakeratosis and hyperplasia in the developing rat embryo.

An additional study (Diamond et al., 1971) followed more closely the histological changes in zinc deficient - zinc repletion animals by inducing a zinc deficiency in male weanling rats for 28 days and then repleting animals with zinc. Animals were then killed for examination at intervals of 3, 6, 9, 12, 15, 21, 24, and 27 days following initiation of zinc repletion. Again after only seven days on deficient diet the characteristic lesions of the esophagus - epithelial hyperplasia, hyperkeratosis and parakeratosis - were evident. Upon repletion, partial reversal of the lesion was evident at day 6, complete disappearance of symptoms occurred by day 15.

Barney et al. (1968) carried out a similar study on male
weanling rats to look at the more microscopic changes occurring during zinc deficiency and repletion. Parakeratosis was first evident on day 16 of the zinc deficient diet (0.5 ppm zinc in diet). In rats displaying this imperfect keratinization the mucosa was 5-6 cell layers thick, compared to 4-5 layers of thickness in the controls (which never developed any lesion) pair led to the zinc deficient group. Whereas in the pair fed controls the layers immediately subadjacent to the stratum corneum consisted of stellate shaped cells containing keratonylin granules (which are considered one type of precursor to keratin) the granular layer of the zinc deficient group contained polyhedral cells with ovoid nuclei. By day 22 - 23 of the zinc deficiency these animals had a mucosal thickness of 11-12 layers under the parakeratotic zone, consisting almost entirely of polyhedral cells. Keratohylin granules were in the cells of the mid layers. This increased thickness was greater than that reported by Follis (1941) but may simply reflect a more severe zinc deficiency.

At three hours post repletion, the first morphological changes were noted in the deepest layers - a dense zone of stellate shaped cells immediately above the basal layer with a less dense zone 8-9 layers in thickness of polyhedral cells. By 12 hours of repletion there was a total thickness of 9-10 cells, with an increase in keratohylin granules in the mid layers. By 18 hours of repletion the esophageal mucosa consisted of 7-8 cell layers in thickness below the parakeratotic lesion with stellate shaped cells, containing keratohylin granules approximate to the stratum corneum. By 24 hours the
morphology of the repleted animals was virtually indistinguishable from that of the pair fed controls, except for the gross lesion.

Studies showing that parentally injected 65 zinc concentrates in the esophageal mucosa (Miller et al., 1961) and that, in vitro, the granular layer of the esophageal mucosa has a marked affinity for zinc (Reaven and Cox, 1962; Reaven and Cox, 1963; Cox and Reaven, 1967) suggest that the locus of the effect of zinc deficiency might be at the layer at which cells differentiated into fully keratinized cells - the point at which keratoeylin granules and other cellular constituents change to keratin. Although this may be true the study by Barney (above) indicated that zinc was exerting an effect at the deepest layers of mucosa, the first effects of repletion being the transition of the deep cells to stellate shaped cells, followed by a similar change in the more superficial layers, presumably due to the sloughing off of the polyhedral cells and replacement by stellate cells that moved up.

This effect of zinc was substantiated in the study by Follis (1941) which showed an increased proliferation of basal cells, as well as other layers. A study of the effects of zinc deficiency on fetal rats (Diamond and Hurley, 1970) also showed an increase in the number of basal cells. Furthermore, the cells immediately above the basal cell layer had plumper cell nuclei than that found in controls, and the nuclei were no longer at 90° angle to the basal layer. There were also irregularities in the nuclear size and chromatin pattern.

The hyperplastic effects of zinc deficiency in the
esophageal mucosa may be key to its enhancing effects on carcinogenesis of this tissue. In support of the enhancing effect of hyperplasia on carcinogenesis are the classic studies of Bereblum (1959) and Frei et al., (1964) using the irritant croton oil on the skin following application of a chemical carcinogen such as benzo(a)pyrene or urethane; the production of hepatomas or kidney tumors by x-ray or neutrons when following mitotic stimulation or during regeneration of tissues (Cole and Nowell, 1965; Curtis et al, 1968; Rosen and Cole, 1962); and the production of tumors from DMBA exposure in zymbol's gland (Huggins, et al., 1961), the ovary (Uematsu and Huggins, 1968), the mammary gland (Huggins et al, 1961) and skin during wound healing (Hennings et al, 1968). Bates et al. (1968) and Shinozuka and Ritchie (1967) showed that actinomycin D inhibited DMBA tumor production of skin presumably due to its inhibiting effect on DNA synthesis and hence cell reproduction. In an organ with a low level of cell proliferation, such as the liver, a 2/3 partial hepetectomy (and the resultant high level cell proliferation characteristic of the regenerative process), a hepatocarcinogen such as acetylaminofluorine (Laws, 1959) induces tumors more quickly and nonhepatocarcinogens such as 12-methyl-4-dimethylaminoazo-benzene (Warwick, 1967) and urethan, (Chernozemski and Warwick, 1970; Hollander and Bentvelzen, 1968; Lane et al., 1970; Pound, 1968) cause hepatomas. Urethan, as well as other chemicals (Terracini, 1969, Toth, 1968) are hepatocarcinogenic for newborn animals in which proliferate activity is great. These studies indicate that cell proliferation can have pro-
found enhancing effects on both the induction phase and the growth phase of carcinogenesis.

The hyperplastic effect of zinc deficiency on the esophageal epithelium is in distinct contrast to its effects on all other tissues (except skin) examined. In these tissues zinc caused hypoplasia, the condition opposite of hyperplasia. This distinction was most clearly noted in the study of Diamond and Hurley (1970) on fetal rats. As the authors point out, the rats and all organs showed growth retardation and hypoplasia, including the esophagus, which attained only one-half to two-thirds its normal size, whereas the esophageal mucosa displayed pronounced hyperplasia -- "a hyperplasia in a generalized milieu of hypoplasia."

This disparity of the carcinogenic effects of zinc deficiency on the esophagus compared to a number of other organs is actually consistent with its effects on the morphology of these various organs and adds to the importance of hyperplasia in its esophageal tumor enhancing effects.

Why this hyperplasia is limited to the esophageal epithelium is not established. Measurement of thymidine kinase (TK) activity by the incorporation of $^{14}C$-thymidine, as reflective of DNA synthesis, indicates an inhibition in most tissues tested in a zinc deficient state, consistent with observed hypoplasias. But this has not been measured in esophageal epithelium so its importance in the distinction cannot be assessed. Prasad and Oberleas (1974) demonstrated that TK activity was significantly lower in zinc deficient rats, as compared to controls, as early as six days after start of the diet; by day
17 no TK activity was detected. A comparable drop was not found in control diet animals pair fed to zinc deficient animals, indicating that inanition and reduced growth were not the cause. To be sure, this drop in TK activity appears to precede reduced food intake, indicating that it causes reduced food intake and not the reverse. This conclusion was also reached by other investigators (Weigand and Kirchgressner, 1977); in response to zinc deficiency, TK activity dropped off in a matter of hours whereas food intake decreased over days or weeks depending on the degree of the deficiency.

Whether the drop in TK activity is directly due to the dependence of this enzyme on zinc which is not unavailable or is due to decreased levels of RNA which are necessary for the synthesis of TK is unclear. However, Prasad and Oberleas (1974) indicated that the drop in TK activity occurs earlier than a drop in RNA polymerase activity or change in ribonuclease activity; this is confirmed by other studies (Prasad and Oberleas, 1973; Terhune and Sandstead, 1972). A study by Duncan and Dreosti (1976) on heactectomized rats that were zinc depleted postoperationally showed a drop in TK activity after 10 hours on the regimen, compared to a drop in DNA synthesis after 18 hours and a drop in protein synthesis after 48 hours. In the esophageal epithelium either TK activity is increased or is bypassed to account for the hyperplasia.

A second explanation for the distinctive hyperplasia in the esophagus in a zinc deficient state is provided by the nature of the epithelium and that of the skin, the only other
tissue exhibiting hyperplastic effects. Both are stratified squamous epithelium. As already mentioned, each involves a high degree of organization and a well regulated differentiation process. This process involves the programmed death of cells as they move from the underlying basal layer to the outer stratified squamous layer. A loss of this well regulated process could lead to excess of viable basal cells, a hyperplasia.

A number of investigators have suggested that zinc may play a key regulatory or homeostatic role in cellular function (Phillips and Kindred, 1980). Just as zinc deficiency can inhibit certain enzymes, so too can the presence of zinc inhibit others. Slight fluctuations would lead to increased activity of some enzymes and decreased activity of other enzymes. Because loss of regulation of cellular growth and function is central to the carcinogenic process this role of zinc may be of great importance.

Mustaga et al. (1971) and Donaldson et al. (1971) have shown both Mg$^{2+}$ dependent and Na$^+$- and K-stimulated ATPases are inhibited by zinc. Zinc also effectively inhibits phospholipase A$_2$ (Stossel et al., 1970) and guanine cyclase (Hardman and Siotherland, 1969). Zinc is capable of inhibiting electron transport in rat mitochondria (Kleiner, 1974) acting at a specific site between ubiquinone and cytochrome.

A structural-regulator function of zinc is also apparent in nucleic acids. Shin and Eichhorn (1968a, b) indicate an association of zinc to DNA, probably bound to the phosphate
groups of the molecule; they show that heated uncoiled DNA will, upon cooling, rewind in the presence of zinc. Wacker and Vallee (1959) and Prask and Piocke (1971) have shown an association of zinc to RNA, at different levels of its structure (tertiary and quartenary). Fernandez-Madrid et al. (1973) observed a decrease in polysomes and an increase in inactive monosomes in connective tissue of zinc deficient animals. The administration of zinc stimulated polysome formation (Sandstead et al. 1971). Zinc has also been associated with the histone fraction of nuclei; these proteins are involved in regulation of gene expression. Vokaer et al (1974) showed that zinc inhibits phosphoprotein phosphatase activity in rat uterus; it also produces increased phosphorylation of nucleolar proteins in Novikoff hepatoms ascites cells (Kang et al., 1974) and of F₁ histones from HTC cells (Tanphaichiti and Chalkley, 1976).

These studies indicate zinc can exert a regulatory function on key cellular enzymes. Such an effect might be most dramatically expressed in a tissue that involves a well regulated differentiation process as does the stratified squamous epithelium.

The parakeratotic lesion of the esophagus resulting from zinc deficiency represents a breach of the protective barrier provided by the keratinized squamous epithelium. Berg (1977) has stated that in order for a tumor to be produced in the esophagus an extrinsic carcinogen must come in contact with the premitotic basal cells of the esophageal epithelium. Either a breach of this barrier or a dysplasia would allow
this to occur. However, since route of administration is not relevant to nitrosamine induced carcinogenesis the importance of this effect on tumor enhancement is unclear.

(2) DNA and RNA polymerases.

DNA and RNA polymerases are involved in the removal and repair of DNA/RNA damaged by chemical carcinogens (Roberts, 1980). Since many of these enzymes are zinc dependent and their activity has been shown to be reduced in a zinc deficient state (Kirchgeessner and Roth, 1980) promutagenic damage may remain, thereby enhancing the carcinogenic process. This possibility becomes especially critical if there is also increasing DNA synthesis which reduces the time for adduct removal before the damage becomes fixed.

Removal of zinc from a growth medium of kidney cells led to decreased DNA polymerase activity, (Lieberman and Cve, 1962). A DNA-dependent RNA polymerase from *Escherica coli*, a DNA polymerase from *E. coli* and sea urchin (Slater, et al., 1971; Springate et al., 1973 a & b) were found to contain zinc. Terhune and Sandstead (1972) found that zinc deficiency caused a steady decrease in DNA-dependent RNA polymerase in the nuclei of liver cells of suckling rats. In another study Sandstead et al., (1972) found reduced RNA polymerase in brain of zinc deficient prenatal rats, as well as reduced DNA synthesis.
(3) **Interrelationship with nutrients.**

The interaction between nutrients is well recognized (Hoekstra, 1964) and might be quite important to the effects of zinc deficiency and excess; it is rare when the change in one nutrient does not affect another. Following is a review of a few such interrelationships that are most relevant to the effects of zinc.

(a) **Zinc-mineral interactions.**

Zinc has been shown to interact with copper, iron, magnesium and other minerals (Hoekstra, 1964; Halstead et al., 1974; Davis, 1980). Burch et al. (1975) has demonstrated that many of these nutrients change during zinc deficiency in the pig. Copper decreases in kidney and increases in liver; manganese and selenium decrease in heart and liver. Calcium levels do not appear to change. Burch has suggested that these various changes in tissue levels may partially account for the physiological and enzymatic derangements in zinc deficiency, without an apparent zinc deficiency in a particular affected tissue. However, mineral levels were not measured in the esophagus, which displays such characteristic effects of zinc deficiency.

Prasad et al. (1967) found that copper, magnesium and calcium contents of testes, bones, esophagus, kidneys and muscle were not changed in zinc deficient rats (although zinc levels were decreased).
Murthy (1974) noted an inverse relationship between serum zinc and serum, hair, heart and liver copper levels, indicating an antagonistic relationship between these two minerals.

Hoeffer et al. (1960) and Wallace et al. (1960) indicated that in swine parakeratosis could be alleviated by copper. However, in a paper on mineral interrelationships, Hoekstra (1964) notes that studies have repeatedly shown no beneficial effect of copper in curing or preventing zinc deficiency in swine. O'Hara et al. (1960) reported that zinc deficiency occurred in pigs fed a high level of copper but not in those receiving no copper.

Decreases of iron in the liver and serum (Reinhold, 1967) have been noted in zinc deficient animals. The effects of decreased iron on certain enzymes, such as mixed function oxidases, could be important to the carcinogenic process. Moses and Parker (1962) observed that iron and copper accumulated in tissues of rats on low zinc diets.

Fisher et al. (1976) noted that in osteosarcomas increased serum copper: zinc ratios can distinguish between primary and metastatic disease as well as the most advanced cases with the poorest prognosis. Pfeilsticker (1965) noted a three fold increase in urinary zinc excretion, accompanied by a decline in molybdenum excretion in cancer patients such that a Zn:Mo ratio of greater than 300 was indicative of advanced cancer.

Taken together these studies indicate that changes in zinc status - and the effects exerted by such changes - do not
occur in isolation but rather are part of a matrix of changes and effects that must be considered together in assessing the significance of changed zinc levels on esophageal (as well as other) cancers. Changes in these minerals can effect certain biochemical or physiological processes that might be quite relevant to the carcinogenic process.

(b) **Zinc and Vitamin A.**

Zinc may play a critical role in the transport of Vitamin A from the liver to peripheral tissues. Vitamin A is dependent on a protein, Retinol Binding Protein (RBP) for transport. Because of zinc's effect on protein synthesis due to its involvement in nucleic acid metabolism, zinc deficiency may cause a decrease in this protein as it appears to do with other proteins (Halstead, 1974). An inadequate supply of RBP would lead to a Vitamin A deficiency in tissues and blood.

One study (Smith et al., 1974) did show that zinc deficiency lowered RBP levels in liver and serum compared to controls; Vitamin A levels were highest in controls, lowest in zinc deficient and intermediate in controls pair fed to zinc deficient. In a further study (Smith et al., 1976), however, involving 3 experiments, there was no difference in plasma Vitamin A levels between the control animals -- food or weight restricted -- and zinc deficient animals in two of the experiments, suggesting that the effects of zinc deficiency were mediated by growth reduction rather than zinc deficiency per se. A study by Carney et al. (1976) did show lower levels of
plasma Vitamin A in zinc deficient animals compared to controls pair fed to zinc deficient animals, but the appearance in the plasma of intubated $^3$H-retinyl acetate was just as great and just as rapid in zinc deficient as in controls, indicating no difference in plasma transport of Vitamin A. Nevertheless, the exact effect of zinc deficiency on Vitamin A has not been resolved and will be considered in this study of esophageal cancer.

Vitamin A is important in maintaining the normal integrity of various types of epithelial tissue (Moore, 1957; Anderson, 1949). Vitamin A deficiency causes squamous metaplasia of the epithelial tissue (Wolbach and Howe, 1925). Changes in the mucosa of trachea and bronchial tubes were noted in animals on a Vitamin A deficient diet. Chu and Malgren (1965) showed that rats kept on a Vitamin A deficient diet showed a thickening of the squamous epithelium with a marked degree of keratinization. This suggested Vitamin A has a role in maintaining the integrity of esophageal epithelium as well as other epithelia.

A number of studies have shown an inhibiting effect of Vitamin A on chemically caused cancers. Saffoti et al. (1967) showed that twice weekly stomach feedings of Vitamin A palmitate following benzo(a)pyrene treatment prevented all tumors and squamous metaplasia normally seen in the bronchus and trachea. Hitchcock (1954) showed an enhancing effect of methylcholanthrene on forestomach squamous tumors in animals on a Vitamin A deficient diet. Studies have shown that Vitamin A deficiency enhanced tumor induction in the colon by
aflatoxin B₁ or 1, 2 dimethylhydrazine (Newberne and Rogers, 1973; Newberne, 1976).

(c) **Alcohol consumption and zinc deficiency**

Alcohol's carcinogen enhancing effect may be mediated through induction of nutrient deficiencies which in turn can predispose the host to greater risk (Wydner and Bross, 1961; Wydner and Mabuchi, 1973; Warwick and Harrington, 1973). Alcohol consumption can affect a nutrient deficiency in two ways: (1) provide a certain (significant) fraction of total daily caloric intake that is nutrient-free, thereby increasing the likelihood that nutrient need will not be met within remaining daily caloric intake; and (2) induce urinary loss of nutrients. Studies have shown that chronic alcohol consumption induces zincuria (Gudbjarnascon and Prasad, 1969) and significantly reduces serum zinc levels (Weisman et al., 1976). Since zinc deficiency may play a role in esophageal carcinogenesis (suggested most directly in those geographic areas in which alcohol is not involved) a possible zinc-mediated effect of chronic alcohol consumption in the etiology of esophageal cancer must be considered.

4. **Conclusion**

Epidemiological data does not clearly implicate any single carcinogen or group of carcinogens in the etiology of
esophageal cancer. This finding is all the more striking in the context of such dramatic changes in incidence and underlines the importance of modifiers in this carcinogenic process. A major modifier in high incidence areas is apparently dietary restriction, zinc deficiency prominent among the nutrient deficits. Even in the lower incidence areas the importance of high alcohol consumption may be mediated by its ability to create a malnourished condition. Zinc may exert a protective effect on the carcinogenic process demonstrated by greater inhibition of carcinogenesis with excess zinc. An enhancement due to a deficiency of zinc may partially reflect the removal of this protective effect. But zinc deficiency in itself may exert an enhancing effect by creating one or more of three conditions: (1) a hyperplasia that can enhance cell susceptibility to carcinogens and can later enhance the "fixing" of DNA damage; (2) a reduction in the removal and repair of damaged DNA, particularly the promutagenic adduct O6-methylguanine; and (3) a change in the status other nutrients particularly vitamin A, that may in turn modify the carcinogenic process. Zinc may also affect the immunocompetence of the animal which could enhance carcinogenesis but this possibility will not be explored in the present set of experiments.
III. MATERIALS AND METHODS

Two sets of experiments were designed to accomplish the specific aims of this thesis. Carcinogenesis experiments were performed to examine the effects of zinc deficiency and related factors on methylbenzyl nitrosamine-induced esophageal cancer. Biochemical experiments were performed to elucidate mechanisms to explain the observed effects of the carcinogenesis experiments.

A. CARCINOGENESIS EXPERIMENTS

1. General Design

Three carcinogenesis experiments were conducted to determine the effects of zinc deficiency and related factors on methylbenzyl nitrosamine (MBN)-induced esophageal carcinogenesis. Zinc excess was examined to assess anticarcinogenic effect of zinc, a possible reason for tumor enhancement in zinc deficient animals. Chronic alcohol consumption was examined based upon its epidemiological significance for esophageal cancer. The nutritional deficits that ethanol consumption can induce provide possible common biochemical and physiological correlates with zinc deficiency in the etiology of this cancer. 13-cis retinoic acid (13-cis RA), a synthetic
analogue of vitamin A, was examined because of the protective effects it has exerted on tumor growth in other models; Vitamin A deficiency, associated with a zinc deficiency, might be important to the tumor enhancing effects of zinc deficiency. In addition to examining the effect of these factors over the course of the experiment, these dietary treatments were applied during either the dosing or post dosing phase to better determine whether an effect occurred primarily during one stage.

2. Experimental Design

Male weanling Sprague Dawley rats were placed on diet for 28 days. Rats were then dosed twice weekly for four weeks in the first study (8 doses), 3 weeks in the second study and 3 weeks in the third study (6 doses). Rats were maintained on diets after dosing for 21 weeks in first study, 8 weeks in second study and 12 weeks in third study (Figures 2, 3 and 4).

The first study served to substantiate the effects of zinc deficiency on methylbenzylnitrosamine-induced esophageal cancer that were shown in earlier studies. It also examined the effects of zinc deficiency when applied only up to and through the dosing period; of zinc deficiency in combination with alcohol; and of zinc deficiency and alcohol with 13-cis R.A. added to the diet after the end of dosing.

The second study retested the dietary treatments of the first study and examined tumor development at three time
Figure 2. Protocol for Study 1.
Figure 3. Protocol for Study 2.
65 CD AD LIB -> -15 -> -15

25 CD PF

70 ZD -> -16 -> -15

70 ZD -> CD AD LIB -> -15

70 CD + ETOH -> -17 -> -15

35 ZD + ETOH

70 ZD + ETOH -> -17 -> -17
+ 13 CIS RA
Figure 4. Protocol for Study 3.
points. The third study examined the effects of zinc deficiency, zinc excess and chronic alcohol consumption applied during either the dosing or postdosing stages of carcinogenesis.

Certain biochemical analyses were performed on animals in these carcinogenesis experiments. These included mineral and vitamin analyses, as appropriate in hair, serum, tissue and tumor. P-nitroanisol activity in liver was measured and light microscopic evaluation of a number of tissues was carried out (Table I).

a. Animal Care and Treatment

(1) Animals.

Male weanling outbred Sprague Dawley rats, weighing 45 ± 5 grams were used in all the experiments. Upon arrival all rats were weighed, identified by number and distributed in cages according to standard randomization procedures. In the first study rats were housed two each in plastic cages with a plastic grid floor. In the second and third studies rats were housed individually in stainless steel cages with mesh floors, on stainless steel racks. Clean stainless steel cages were soaked in a nitric acid or Radic solution, either of which removed zinc contamination, and rinsed in deionized water. Cages were changed weekly.

(2) Diet. The diet was semipurified, the com-
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tumor zinc</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>p NitroAnisol Demethylase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
position of which is listed in Tables II and III. In the first study soy protein was used as the protein source; in the second and third studies egg white was used as the protein source. Because soy protein contained zinc it was necessary to first remove this mineral and then add it back in to the desired level. The zinc content in soy was reduced by multiple washings with a solution of the tetrassium salt of EDTA, followed by rinsings with deionized water. This reduced zinc content to 2 ppm. Zinc carbonate was added to provide 60 ppm zinc in control diet and 7 ppm in zinc deficient diet.

In the latter two studies it was not necessary to wash the protein source since egg white was zinc free (less than 0.3 ppm). Zinc was added to this diet at 250 ppm excess diet, 60 ppm control diet and 3 ppm deficient diet. The low zinc level used in the egg white diet, compared to the soy protein diet, was because egg white contains no phytates, as does soy protein. Phytates in soy protein chelate (bind) zinc making it unavailable for absorption: this necessitated higher zinc levles in the diets using soy proteins. Studies determined that 3 ppm zinc in egg based diets caused the same course and level of zinc deficiency previously determined in washed soy protein. This was determined by serum zinc levels, rate of growth, and development of zinc related deficiency symptoms, i.e., alopecia, course hair, and hyperkeratosis of skin (see results, pilot study).

13-cis RA was added to diet of rats receiving alcohol and zinc deficiency at a level of 67 ppm. All dietary changes that took place following dosing were started 48 hours after
<table>
<thead>
<tr>
<th>Component</th>
<th>Amount/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean protein</td>
<td>300 g</td>
</tr>
<tr>
<td>Methionine</td>
<td>7 g</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>265 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>270 g</td>
</tr>
<tr>
<td>Cellulose fiber</td>
<td>50 g</td>
</tr>
<tr>
<td>Mazola corn oil</td>
<td>50 g</td>
</tr>
<tr>
<td>Mineral mix&lt;sup&gt;1&lt;/sup&gt;</td>
<td>35 g</td>
</tr>
<tr>
<td>Vitamin mix&lt;sup&gt;2&lt;/sup&gt;</td>
<td>20 g</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>3 g</td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>50 µg</td>
</tr>
</tbody>
</table>

<sup>1</sup> Composition of mineral mix (grams): CaCO<sub>3</sub>, 190.5; CaHPO<sub>4</sub>, 144.1; K<sub>2</sub>HPO<sub>4</sub>, 403.8; MgCO<sub>3</sub>, 73.2; MgSO<sub>4</sub>-7H<sub>2</sub>O, 191.7; FeSO<sub>4</sub>-7H<sub>2</sub>O, 17.4; MnSO<sub>4</sub>-H<sub>2</sub>O, 15.4; KI, 0.02; CuSO<sub>4</sub>-5H<sub>2</sub>O, 1.96; NaF, 0.22; NaCl, 82.4; Na<sub>2</sub>CO<sub>3</sub>, 40.6; Cr<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>-H<sub>2</sub>O, 0.009; Na<sub>2</sub>SeO<sub>3</sub>, 0.02; Glucose, 1,041.

<sup>2</sup> Composition of vitamin mix (grams): menadione, 0.1; riboflavin, 0.4; thiamine HCl, 0.8; pyridoxine, 0.8; calcium pantothenate, 2; folic acid, 1; nicotinic acid, 5; inositol, 25; Roche A & D mix (500/50), 6; dl-a-tocopherol acetate (500 U/g), 22.5; sucrose, 1,396.
# TABLE III

## COMPOSITION OF BASAL ZINC-FREE DIET

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg Protein</td>
<td>200 gms</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>322 gms</td>
</tr>
<tr>
<td>Sucrose</td>
<td>320 gms</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50 gms</td>
</tr>
<tr>
<td>Mazola corn oil</td>
<td>50 gms</td>
</tr>
<tr>
<td>Mineral Mix$^1$</td>
<td>35 gms</td>
</tr>
<tr>
<td>Vitamin Mix$^2$</td>
<td>20 gms</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>3 gms</td>
</tr>
<tr>
<td>Vitamin B$_{12}$</td>
<td>50 μgms</td>
</tr>
</tbody>
</table>

---

1 Mineral mix (grams): CaCO$_3$, 190.5; CaHPO$_4$, 144.1; K$_2$KPO$_4$, 403.8; MgCO$_3$, 73.2; MgSO$_4$-7H$_2$O, 191.7; FeSO$_4$-7H$_2$O, 17.4; MnSO$_4$-H$_2$O, 15.4; KI, 0.02; CuSO$_4$-5H$_2$O, 1.96; NaF, 82.4; NaCl, 40.6; Na$_2$CO$_3$, 0.009; Cr$_2$(SO$_4$)$_3$H$_2$O, 0.02; Na$_2$SeO$_3$, 0.02; Glucose, 1,041.0; ZnCO$_3$, 250 ppm excess, 60.0 ppm control, 3.0 ppm def.

2 Composition of vitamin mix (grams): menadione, 0.10; riboflavin, 0.4; thiamine HCl, 0.8; pyridoxine, 0.8; calcium pantothenate, 2.0; folic acid, 1.0; nicotinic acid, 1.0; inositol, 25.0; Roche A & D mix (500/50), 6.0; dl-a-tocopherol acetate (500 μg/g), 22.5; sucrose, 1,941.3
administration of last dose.

All utensils used for diet preparation were either glass or stainless steel and were soaked for at least three hours in 10% nitric acid solution and then rinsed with deionized water to remove all zinc.

Diet was prepared every other week and kept frozen except for a three day supply which was refrigerated. Ad libitum animals were fed every other day.

(3) **Pair-feeding.**

Each rat in the group was fed the control diet in an amount limited to the food intake of a paired zinc deficient rat. This was done in the second and third study by weighing the amount of food consumed by the individual zinc deficient rats. This controlled for any effect that a reduced food intake of the zinc deficient rats might have on tumor incidence. The food intake of each zinc deficient rat was measured every morning and an equal amount of control diet was fed to the corresponding pair-fed rat. This amount was divided into two equal parts, one given in late afternoon, the other early evening, so as to mimic normal eating patterns.

Zinc deficient rats were fed each morning after food intake had been measured. Food was provided in sufficient amounts so that animals could feed ad libitum, without so much food that much spillage could take place. New paper was placed under cages at times of feeding so that any spillage could be recorded. The diet was served in glass or ceramic
jars, cleaned with nitric acid to remove zinc. Deionized water was supplied in nitric acid cleaned water bottles with stainless steel water spouts and zinc-free neoprene stoppers that were also nitric acid cleaned. Samples of deionized water provided to the rats were taken at regular intervals and measured for zinc content.

(4) Alcohol Intake.

Ethyl alcohol (ethanol) was provided in the drinking water. In the first study ethanol content was 4%, in the last two studies ethanol content was 10% and 5%, respectively. Total caloric intake from ethanol was determined by measuring daily intake of water containing alcohol, determining total ethanol consumed from this amount, and calculating calories derived from alcohol.

(5) Weighing.

All animals were weighed weekly.

(6) Carcinogen dosing.

Methylbenzylxnitrosamine (MBN), slightly soluble in water (0.45g/100ml) was prepared in a 2% ethanol-saline solution. The small amount of ethanol assured solubility and the saline maintained physiological conditions since the solution was administered by intragastric intubation. Methylbenzylxnitro-
samine was diluted to a concentration of 50 mg per 100 ml (500 µg per ml), well below the limit of solubility of 450 mg/100 ml.

In the first study rats were dosed 2 mg/kg body weight (or 400 µg for average 200 gm rat) twice a calendar week for 4 weeks, a total dose of 16 mg/kg body weight. Rats would be dosed, for instance, on Tuesday and Friday of each of the four weeks, meaning there were 3 days between one dose and 4 days between the other. Twenty five days elapsed from the first day of dosing to the last. In the second study animals were dosed twice weekly for three calendar weeks (6 doses), a total dosing time of 18 days. In the third study, rats were dosed six times every three days, a total dosing period of 16 days. The dosage was increased from 2.0 mg/kg body weight to 2.5 mg/kg body weight, in the last two studies a total dosage of 15 mg/kg body weight (vs. 16 mg/kg in the first study). Control rats received vehicle without carcinogen.

(7) Sample Collection.

Hair and serum samples were collected at the beginning of the experiment, at the end of the one month feeding period, at the end of dosing and at the end of the experiment. As noted in Table 1, additional select samples were taken at other times. Tissue samples were taken at the end of each experiment. In experiment 2 tissue samples were also taken at the end of dosing and one month after the end of dosing and at time of sacrifice.
All animals were sacrificed by carbon dioxide unless enzyme measurements were involved, in which case decapitation was used.

Hair was removed from the dorsal side of animals with electric clippers, placed in zinc free polyethylene bags, tightly sealed and stored until analyses were done. Hair removal at beginning of experiment provided baseline mineral concentration and also provided an area for new hair growth under changed nutrient status.

Blood samples were taken in microcapillary tubes by puncture of the retro orbital plexus, covered with parafilm, let stand for 30 minutes and then centrifuged for 20 minutes at 2000 RPMS with serum separators. Serum was then removed, placed in plastic snap vials and stored in -20° freezer for later analysis. For vitamin A analysis blood was handled in a darkened room. All containers were kept wrapped.

Routine autopsies were performed on all animals. The head was preserved in Bouin's solution for histologic evaluation of the nasal sinuses and other tissues of interest. After gross examination the esophagus, liver, kidneys, spleen and testes were placed in 10% buffered formalin solution for routine histological processing and analysis. Any other organs with gross abnormalities including tumors were saved for histologic evaluation.

b. Analysis

(1) About 30 mg of hair was washed with a
solvent comprised of hexane, alcohol and deionized water (1:1:1), rinsed twice with deionized water and transferred by teflon-coated spatula to a teflon beaker. 5 ml of ion-free water was added and beaker was placed on a hot plate until the water evaporated. An additional 5 ml of ion-free water was added to further cleanse the hair. The remaining sample was digested in mixture of HClO₄ and HNO₃. When only liquid remained, sample was diluted to 5 ml with deionized water and analyzed by direct aspiration by atomic absorption spectrophotometry (AAS).

A different lamp was used for analysis of each mineral. Standards were made fresh daily. All equipment and containers used in the preparation of standards and samples were acid washed, rinsed with deionized water and kept separate from all other equipment. Hair mineral content was determined by extrapolating from standard curve.

(2) **Tissue Mineral Analysis.**

Tissues taken for mineral analysis were removed with stainless steel scissors and forceps and pre-washed with nitric acid and deionized water. Samples were rinsed in deionized water, blotted, placed in nitric acid-cleaned polyethylene containers, and frozen until analysis.

At time of analysis tissue samples were thawed, placed in dried, pre-weighed, acid cleaned test tubes, and weight recorded. The tubes were then transferred to a beaker on a hot plate; the beaker was then covered with a larger inverted
beaker. Air temperature inside was maintained at 80° and tissue dried for 2 hours to constant weight, cooled in a dessicater and weighed. From the original sample about 5 mg of dry tissue remained. One ml of 1.0 mol/liter HNO₃ was added, and the sample acid-digested on hot plate for another 24 hours at 80°. The 1.0 mol/liter HNO₃ was then slowly evaporated on a hot plate followed by the addition of 2.0 ml of 10 mmol/liter HNO₃ and vigorous mixing.

The solution was analyzed directly by atomic absorption spectrophotometer (AAS).

(3) **Serum Mineral.**

Serum was diluted 1:8 to 1:4 with deionized water, depending on initial concentration. This was then analyzed by direct aspiration into AAS using appropriate lamp.

(4) **Serum Vitamin A.** - Procedure in Appendix A.

c. **Statistical Analyses**

All analyses were made using the IBM-370 computer, the Apple computer or a hand held calculator. Statistical packages used on the IBM computer were Statistical Package for Social Sciences, BMDP or Statistical Analysis System. Tests applied included Chi squared, Student T-test and Analysis of variance (ANOVA).
B. BIOCHEMICAL EXPERIMENTS

Two major biochemical experiments were performed: DNA synthesis and formation of carcinogen-DNA adducts. Each of these experiments provided information that complemented the other. This was valuable in the interpretation of the results of the prior studies and suggested possible mechanisms in MBN-induced carcinogenesis. The pattern, timing and intensity of DNA synthesis could clarify the importance of level, type and persistance of adduct formation.

1. DNA Synthesis

   a. Scintillation counting of incorporated $^3$H-thymidine.

   (1) Experimental Design.

   DNA synthesis was measured by the incorporation of $^3$H-thymidine in the esophagus (target organ) and, to a lesser extent, liver (non target organ) of rats in the three dietary groups, control ad libitum, control pair fed and zinc deficient. Rats in these three dietary groups received either no carcinogen, 1 dose of carcinogen or 6 doses of carcinogen, a total of nine carcinogen-dietary groups.

   DNA synthesis was measured at time intervals (Tables IV and V) over the course of the experiment for the nine carcin-
# TABLE IV

**ESOPHAGI AND LIVER SYNTHESIS EXAMINED AT MULTIPLE TIME POINTS**

<table>
<thead>
<tr>
<th>Feeding Period</th>
<th>Time after dosing (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>MBN</td>
<td>8/6</td>
</tr>
<tr>
<td>CD Ad lib</td>
<td>6/1</td>
</tr>
<tr>
<td>CD Pair fed</td>
<td>6/1</td>
</tr>
<tr>
<td>Zinc def.</td>
<td>6/1</td>
</tr>
</tbody>
</table>

^1 Dose MBN

| CD Ad lib       | 4/4 | 6/4 | 4/- | 6/4 | 6/- | 6/4 | 4/- | 6/4 | 6/- |
| CD Pair fed     | 4/4 | 6/4 | 4/- | 6/4 | 6/- | 6/4 | 4/- | 6/4 | 6/1 |
| Zinc def.       | 4/4 | 6/4 | 4/- | 6/4 | 6/- | 6/4 | 4/- | 6/4 | 6/- |

^1 No. Esophagi/No. Liver
ogen-diet groups. 444 rats were involved in this experiment which was divided into three smaller experiments. The first two experiments compared the dietary groups receiving no carcinogen with those receiving one dose of carcinogen. The third experiment compared the dietary groups receiving no carcinogen with those receiving six doses of carcinogen.

The protocol for these experiments was identical to that for the tumor experiments. Rats were placed on diet for 1 month, then some were given either one or six doses of MBN, the latter at at three day intervals. The sacrifice schedule and number of esophageal or liver samples is shown in tables IV and V.

In the untreated group, rats were sacrificed at time 0, 2 and 4 weeks of the four week feeding period, 3 and 7 days after the time at which the first dose would have been administered, and at weekly intervals thereafter up to 8 weeks (56 days) post the initial dose. Esophageal DNA synthesis was examined at all time points. Liver DNA synthesis was examined at times 0, 2, and 4 weeks of the feeding period, and 1, 2, 4 and 7 weeks after the time of when initial dose would have been administered.

Rats in the single MBN dose group were sacrificed at four hours, 1, 2, 3, 5, 7, 10, 14, 21 and 28 days after the single dose. Esophageal DNA synthesis was examined at all time points; hepatic DNA synthesis was examined at 4 hours, 1, 3, 7, 14 and 21 days after the single dose.

In the six dose MBN group, rats were sacrificed 1, 3, 5, 7, 10, 14, 21, 28 and 35 days after the last of six doses.
Esophageal DNA synthesis was examined at all time points; hepatic DNA synthesis was examined at 1, 3, 7, 14, and 28 days after the last dose.

(2) Procedures.

Rats were injected intraperitoneally with 50 μci $^3$H-thymidine (Amersham)/100 gm body weight 90 minutes prior to sacrifice (by decapitation). This 90 minute period was determined to be optimal by a pilot study. Animals scheduled to be sacrificed 4 hours or 24 hours post carcinogen dosing, for example, were injected with $^3$H-thymidine 2.5 hours and 22.5 hours, respectively, after dosing for sacrifice 90 minutes later at 4 hours and 24 hours. Except for the 4 hour post dosing group, all animals were dosed with carcinogen at 10 a.m., and on the subsequent day of sacrifice were injected with $^3$H-thymidine at 8:30 a.m. and sacrificed at 10 a.m. In the 4 hour post dosing group rats were carcinogen dosed at 7 a.m., injected with $^3$H-thymidine at 9:30 a.m. and sacrificed at 11 a.m. All animals were dosed in specific order at 5 minute intervals and killed in same order at 5 minute intervals to maintain an equal amount of time from dosing to sacrifice for all animals. The five minute interval provided sufficient time to remove organs and begin processing them.

(a) Isolation of esophageal DNA.

After decapitation the entire esophagus was excised and
loose connective tissue was removed. After splitting longitudinally, each esophagus was incubated in 5 ml Krebs-Ringer bicarbonate buffer (Appendix B) containing hyaluronidase (1 mg/ml), bovine serum albumin (1 mg/ml) and hydroxyurea (0.5 mg/ml) for 30 minutes at 37°C in shaking water bath. Elastase (1 mg/ml) was then added and incubation continued for another 30 minutes. The esophagi were then placed on a parafilm covered glass plate resting on ice and the epithelial layers were gently teased away from stroma with forceps.

The epithelial layer was cut into small pieces and placed in 10 ml of Hepes-potassium sucrose buffer (0.05 m Hepes, pH 7.0, 0.25 M Sucrose; 25 mM KCl; 5 mM MgCl₂, Appendix C) and gently homogenized with 3 five second bursts of polytron (speed 2).

The preparation was centrifuged for 10 minutes at 800 x g and the supernatant and floating residue was discarded. The remaining pellet was resuspended in the above HKM sucrose buffer which also contained 0.5% Triton X-100 detergent. A pastuer pipette was used to agitate solution for three minutes to wash nuclear preparation. This was spun down 800 x g for 10 minutes and this procedure was repeated once more.

After the third spin down, the sample was transfered to glass homogenizing vessel containing 1.03 ml buffered 3M CsSO₄, 1.67 Sarkosyl lysing buffer and 2 ml of buffered 6M CsCl. This solution was homogenized by hand for a few minutes and then left to stand for 15 minutes. It was then transferred to centrifuge tubes. The remaining 7.3 ml of buffered CsCl was used to rinse homogenizer and pestle before addition to
centrifuge tube to complete 12 ml CsCl gradient (Appendix D).

Centrifuge tubes were placed in Beckman Vti 50 vertical rotor and spun for a minimum of 8 hours in Beckman ultracentrifuge at 49,500 RPM. Density of the gradient, 1.72 g/ml, led to a sharp banding of the DNA in the middle of the gradient. Protein collected at the top of the gradient and RNA at the bottom. The sample was harvested from the top by pumping Florinert through the bottom of the tube. As the sample was pumped out through the top absorbance at 254 nm was recorded graphically and the sample was then fractionated into 0.375 ml aliquots.

The aliquots were brought up to 1 ml with deionized water and absorbance at 260 nm and 280 nm was measured on a spectrophotometer to determine amount and purity of DNA. A 260/280 ratio of 1.72 was considered the minimum acceptable level of purity (protein contamination) to continue with the experiment. DNA, RNA and protein content was also determined on a number of samples by the more definitive assays, diphenylamine, orcinol and Biorad, respectively (Appendix E).

Twelve mls of Aquasol scintillation cocktail were added to each fraction and all fraction were counted on a standard scintillation counter. Standards of $^{3}$H-thymidine were counted to determine efficiency of machine and blanks were counted to determine background levels. From these two measurements it was possible to calculate from CPMs the DPMs/µg DNA. By measuring and graphing the absorbance and counts of all fractions the patterns of the two could be compared to determine
that the radioactivity peak coincided with DNA peak, determined by optical density. Once the consistency of counts was established the four or five fractions in the DNA peak were pooled and the total absorption and counts for a single animal were determined from the single pooled sample.

(b) Isolation of liver DNA.

Upon removal of the esophagus from animal, liver was perfused with ice cold saline and excised. Liver was placed in tissue press and one gram of pressed liver was removed and added to glass homogenizing vessel containing 10 ml of ice cold HKM-sucrose buffer. Sample was homogenized and filtered once through course 160 mesh nylon forbric (Nytex) and once through cheesecloth. Preparation was then centrifuged 800 x g and supernatant poured off. Remaining pellet was then washed twice with HKM-0.5% Triton X-100 detergent and processed as described for esophagus.

b. \(^{3}\text{H}-\text{thymidine incorporation - autoradiography.}\)

The protocol and animal treatment for this study were indentical to that in the previous experiment but of a much more limited scope. Rats of the control ad lib and zinc deficient diets were examined after 4 weeks on diet and 4 hours and 7 days after a single carcinogen dose. There were five rats in each group; they were injected with 50 μci/100 gram
body weight $^3$H-thymidine 90 minutes prior to sacrifice. At sacrifice esophageal and liver tissue was placed in 10% formalin, then processed for routine histological analysis. Just prior to deparafinizing, some slides containing mounted sections were processed in a dark room for autoradiography. The procedure is described in Appendix F. Measurement was number of labeled cells per 100 cells counted. A labeled cell was one whose nucleus contained 6 or more silver grains. There were 10 readings per animal (total 1000 cells).

c. Mitotic Counts

1000 cells were counted per animals, five animals per group for control ad lib and zinc deficient diets. Measurements were made after four weeks on diet and 4 hours and 7 days after single dose.

2. Carcinogen Binding Studies

The binding of the carcinogen, methylbenzylnitrosamine (MBN), to esophageal (target) and liver (nontarget) DNA was determined using liquid scintillation counting of $^{14}$C-methylbenzylnitrosamine. This compound was labeled on either the methyl or benzyl moieties to measure the binding capacities of each to DNA. Location of the label is shown in Figure 5.
Figure 5. Two positions of $^{14}$C label in MBN.
$^{14}\text{CH}_3$

$\text{N} \quad \text{N} \quad \equiv \equiv \text{O}$

$\text{CH}_2$

$\text{苯基}$

$^{14}$C-METHYL BENZYL NITROSAMINE

$\text{H}_3\text{C}$

$\text{N} \quad \text{N} \quad \equiv \equiv \text{O}$

$\text{14CH}_2$

$\text{苯基}$

METHYL $^{14}$C-BENZYL NITROSAMINE
Pilot studies were first performed to ascertain whether the methodologies and amount of radioactivity used was sufficient to detect binding levels. Positive results then led to complete experiments.

a. Benzylating Species.

(1) Pilot Study.

Male weanling rats were placed on control diet for four weeks and then dosed with methyl-[\(^{14}\)C-benzyl]-nitrosamine (specific activity 20 mci/mm) intraperitoneally in doses ranging from 10 \(\mu\)ci to 100 \(\mu\)ci. Animals received either a single dose of MBN or three doses. They were sacrificed by decapitation 1, 4, 8, 12 or 24 hours after the dose. DNA was obtained and processed in the same manner as described in section on \(^3\)H-thymidine incorporation. Fractionated gradients were counted and DNA peaks were pooled and counted. Peaks were also dialysed before counting. DNA peaks from gradients of a number of rats were pooled, dialyzed and counted. Because no radioactivity was detected in any of the DNA fractions the experiment on benzylating species was carried no further.

b. Methylating Species.
(1) **Pilot Study.**

Male rats, 7 weeks of age (3 week weanling placed on diet for 4 weeks) were injected intraperitonealy with [\(^{14}\)C-methyl]benzyl nitrosamine (specific activity 20 mci/mm) at a level equivalent to 2.5 mg/kg body weight (same as used in last two tumor studies) or 333 μci/kg body weight. Animals were decapitated at 2, 4, 8 or 12 hours to determine time of optimal binding.

The esophageal epithelium was isolated in a manner different from that described in the previous studies; the method of Läbuc and Archer (1982) was used in this study. After removal, instead of opening the esophagus longitudinally, two forceps were applied to the middle of the excised esophagus, and with one forcep holding the esophagus firmly, the other forcep, with pressure applied, was pulled away from the center toward the end of esophagus, stripping off the outer muscular and connective tissue layers, leaving only the epithelium. The same procedure was applied to the other half of esophagus. Residual non-epithelial tissue remaining was gently scraped away. This method yielded as clean an epithelium as the enzyme method and also eliminated an additional hour of processing at physiological temperature in which degradative enzymes might affect bound carcinogen. The epithelium was then processed as in the \(^{3}\)H-thymidine experiments.

Fractions (0.375 ml) of the 12 ml gradient were brought up to 1 ml with deionized water and the 260/280 absorbances were measured to determine amount and purity of DNA peak.
The samples were then handled in one of two ways: liquid scintillation cocktail was added to each fraction and the entire gradient was counted for $^{14}\text{C}$ radioactivity; or, the 32 fractions were combined into five pools: 6-10 (pre DNA), fractions 11-16 (DNA peak) and fractions 17-32 (post DNA). Fractions 1-5 contained cellular debris and proteins and were counted separately.

1 ml of pooled sample was removed and counted. The remainder of the pooled samples were transferred to dialysis tubing and placed in beaker containing HKM buffer. At end of each hour buffer was replaced with fresh buffer. After the third replacement the dialysis tubing was left in buffer overnight in refrigerator (5°C). 1 ml was removed from each of the three pools of each gradient and with addition of scintillation fluid was counted. Based on presence of radioactivity further experiments were pursued.

(2) HPLC Techniques.

HPLC separation techniques were necessary with the methyl labeled group to distinguish radioactivity due to adduct formation from radioactivity due to biosynthetic incorporation. HPLC was also necessary to determine level of N-7 methylguanine, O$_6$-methylguanine and 3-methyladenine. To accomplish this a cation exchange column (Biorad) was used with 500 mM ammonium formate, pH 3.9, 57°C at a flow rate of 0.7 ml/min. This provided a minimum separation of 2 minutes (1.4 ml) between guanine, adenine, N-7 methyl guanine, O$_6$-methylguanine
and 3-methyladenine. The time and location of these standards was determined by addition of authentic standards.

Once optimal separation conditions were obtained with standards, samples were prepared by acid hydrolysis. To pooled DNA peak fractions that had been dialyzed, concentrated HCl was added to a pH of 1 (0.1 M HCl). Samples were heated to 70°C in shaking water bath for 40 minutes and then cooled on ice. Supernatant was transferred to rotoevaporator and reduced to 250 µl and then injected into column.

Optical density (254 nm) of elutant was continuously monitored. The entire run which took approximately 120 minutes was fractionated into one minute samples (0.7 ml, flow rate 0.7 ml/min), a total of 120 aliquots. Scintillation fluid was added to all fractions and CPMs were determined from scintillation counting. Background counts were determined from blanks and efficiency of counting from standard 14C-toluene. From this DPMs could be calculated.

Adenine and guanine were present in sufficient amounts to be calculated from optical density, based on extinction coefficients. The adducts were calculated from radioactivity present in appropriate peaks. Based upon specific activity of compounds it was possible to calculate amount of adduct from DPMs.

Although location for elution of adducts was already determined by authentic standards, these were also added in large enough amounts to test samples to provide an optical peak that coeluted with the radioactive peak to further substantiate the location of the adduct. These unlabeled standards were added to
dialysate prior to acid hydrolysis. By adding known amount of these standards and ultimately measuring optical density it was possible to assess whether acid hydrolysis, sample transfers, or rotoevaporation caused significant loss of adduct. Addition of unlabeled standard did not interfere with sample amounts of adducts since these were calculated from radioactivity rather than optical density.

(3) **Definitive Studies**

It was necessary to pool animals for these experiments to obtain a sufficient amount of esophageal DNA to determine adduct levels, particularly O\textsuperscript{6}methylguanine. Approximately 4-8 rats were required, based on age and dietary status.

(a) **Dose Response Study.**

18 rats were placed on control diet for one month. At end of month 6 rats each received one of three dose levels of \textsuperscript{14}C-methyl]benzyl nitrosamine (S.A. 20 mci/mm): 1.5 mg/kg body weight, 2.5 mg/kg body weight or 3.5 mg/kg body weight. Animals were sacrificed 4 hours post dosing and esophageal and liver tissue were processed as already described. Samples were pooled prior to dialysis.

(b) **Single Dose Study.**

38 male weanling rats were placed on one of three diets
for four weeks: control (6), pair fed (16) or zinc deficient (16). At end of four week period each animal received single dose of [¹⁴C-methyl]benzyl nitrosamine (S.A. 20 mci/mm) at level of 2.5 mg/kg body weight i.p. at 8 a.m. Four hours later rats were decapitated and esopagi and livers were processed as already described. DNA from six control diet rats was combined into single pool. DNA from 16 pair fed and 16 zinc deficient was combined into two pools for each respective diet.

(c) **Multiple Dose Study.**

To study persistence of adducts after complete carcinogenic dosing regimen 48 male weanling rats were placed on one of three diets for four weeks: control ad lib (12), control pair fed (18) or zinc deficient (18). At end of one month all rats received 3 unlabeled doses of methylbenzylnitrosamine (2.5 mg/kg body weight) by gavage at three day intervals, as was the dosing schedule in the tumor study. Rats then received three more doses of labeled methylbenzylnitrosamine (20 mci/mm; 2.5 mg/kg body weight) at three day intervals i.p. Rats were sacrificed by decapitation at 4 hours, 24 hours and 48 hours after the final dose. At each sacrifice 4 rats were sacrificed in control ad lib diet group and 6 rats each were sacrificed in control pair fed and zinc deficient dietary groups. DNA from esophagus and liver was combined from the number of rats used for each group, so that there was one measurement for each dietary group at each time point.
IV. RESULTS

A. CARCINOGENESIS STUDIES

1. Tumor Yield

   a. Study 1

   This experiment consisted of 212 male weanling Sprague Dawley rats divided into six groups (Table VI), the first group of 12 rats on control diet receiving no MBN, the other five groups of 40 rats each receiving MBN treatment: control diet ad lib; zinc deficient diet; zinc deficient diet repleted with control diet after MBN treatment; zinc deficient diet plus 4% ethanol in drinking water; and zinc deficient diet plus 4% ethanol in drinking water plus addition of 13 cis retinoic acid (13 cis R.A.) to diet after dosing. Rats received diets for 4 weeks, were dosed twice weekly 2 mg/kg body weight for four weeks and then maintained another 21 weeks.

   There were no tumors in the untreated control diet groups (Table VI). In groups that received MBN, tumor incidence ranged from 40% (control diet) to 94% (zinc deficient plus 4% ethanol plus 13-cis R.A.). In MBN treated rats zinc deficient diet throughout significantly enhanced tumor incidence above that of control diet rats (75% vs. 40%, p < 0.005). Zinc deficiency throughout also significantly enhanced tumor incidence compared to the group that was switched from zinc deficiency to
<table>
<thead>
<tr>
<th>Dietary Treatment</th>
<th>Tumors No.</th>
<th>%</th>
<th>Total No. Tumors</th>
<th>Avg. No. Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. CD, AD LIB(^2),-MBN</td>
<td>0/12</td>
<td>--</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>2. CD, AD LIB</td>
<td>14/35</td>
<td>40.0</td>
<td>63</td>
<td>4.5</td>
</tr>
<tr>
<td>3. ZD(^3)</td>
<td>25/35</td>
<td>75.7</td>
<td>76</td>
<td>3.04</td>
</tr>
<tr>
<td>4. ZD, ZR(^4)</td>
<td>18/35</td>
<td>51.4</td>
<td>87</td>
<td>4.83</td>
</tr>
<tr>
<td>5. ZD, ETOH(^5)</td>
<td>29/30</td>
<td>85.3</td>
<td>99</td>
<td>3.41</td>
</tr>
<tr>
<td>6. ZD, ETOH, 13-CIS RA(^6)</td>
<td>33/35</td>
<td>94.3</td>
<td>76</td>
<td>2.30</td>
</tr>
</tbody>
</table>

1 60 ppm zinc control diet; 7 ppm zinc deficient diet; with MBN unless noted.

2 CD AD LIB is control diet Ad libitum, without MBN.

3 ZD is Zinc Deficient Diet.

4 ZD, ZR is Zinc Deficient, Zinc Repleted after end dosing.

5 ETOH is 4% Ethanol in drinking water.

6 13-CIS RA is 13-CIS Retinoic Acid added at 67 μg/gram diet to diet after dosing.
control diet after MBN dosing (76\% vs. 51\%, p < 0.05). This zinc deficient, zinc repleted group had a tumor incidence that was greater than control diet (51\% vs. 40\%) but not to a significant degree. Addition of ethanol to the zinc deficient diet group enhanced tumor incidence from 75\% to 85\% although this increase was not significant. Addition of 13-cis R.A. to the zinc deficient-ethanol group further enhanced tumor incidence to 94\%, which was also not significant. Although this group had the highest tumor incidence (33 of 35 rats) it had the lowest average number of tumors per tumor bearing rat, 2.3. This appeared to be due primarily to the coalescing of tumors that made counting of individual tumors difficult. The alcohol treated group, having twice the incidence of the control diet group, had fewer tumors per tumor bearing rat, 3.4 vs. 4.5.

Total tumor incidence consisted of both papillomas and carcinomas. Because of the length of time following dosing most tumors were papillomas. Table VII lists numbers of papillomas and carcinomas of total tumors for each group. The control diet group and the zinc deficient group that was returned to control diet for 21 weeks after dosing had 4.8\% and 5.7\% incidence of carcinomas, respectively. The zinc deficient, zinc deficient plus ethanol and zinc deficient plus ethanol plus 13-cis R.A. groups had 10.5\%, 9.0\% and 9.2\% carcinomas respectively, twice the incidence found in the control diet-based groups. The zinc deficient diet throughout produced the highest incidences of carcinomas.
<table>
<thead>
<tr>
<th>Condition</th>
<th>Tumor Incidence</th>
<th>Total Tumors</th>
<th>Papillomas</th>
<th>Carcinomas Num.</th>
<th>Carcinomas %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control ad lib</td>
<td>40</td>
<td>63</td>
<td>60</td>
<td>3</td>
<td>4.8</td>
</tr>
<tr>
<td>Zinc deficient</td>
<td>76</td>
<td>76</td>
<td>68</td>
<td>8</td>
<td>10.5</td>
</tr>
<tr>
<td>Zinc deficient - Control</td>
<td>51</td>
<td>87</td>
<td>82</td>
<td>5</td>
<td>5.7</td>
</tr>
<tr>
<td>Zinc deficient, Ethanol</td>
<td>85</td>
<td>99</td>
<td>90</td>
<td>9</td>
<td>9.0</td>
</tr>
<tr>
<td>Zinc deficient, Ethanol, 13 CIS R.A.</td>
<td>94</td>
<td>76</td>
<td>69</td>
<td>7</td>
<td>9.2</td>
</tr>
</tbody>
</table>
b. Study 2

This experiment (Table VIII) included a control diet group pair fed to the food intake of the zinc deficient group and a control diet group that consumed ethanol, in addition to the other five MBN treated groups of Study 1. Rats consumed 20% egg protein diet with 3 ppm zinc in the zinc deficient group. The final sacrifice was eight weeks after the end of dosing. The pair fed and zinc deficient plus ethanol groups were sacrificed only at this final two month time. The other five groups were also sacrificed immediately after dosing and one month after dosing.

As depicted in Table VIII and Figure 6, the tumor incidences of the zinc deficient, zinc deficient returned to control diet after dosing and zinc deficient plus ethanol plus 13-cis R.A. groups increased with each successive sacrifice. Tumor incidence was identical (27%) for the first two sacrifice periods in control diet rats but increased to 39% at 2 months post dosing. The control diet plus ethanol group had a tumor incidence that increased on month after dosing from the level immediately after dosing, but then decreased two months post dosing (47% to 60% to 53%). As Fig. 6 illustrates the sharpest rise in tumor incidence over the sacrificing periods was in the zinc deficient, ethanol, 13 cis R.A. group. The relative position of the groups in terms of tumor incidence remained the same over the course of the three sacrifices except for the control diet plus ethanol, which had a tumor incidence greater than the zinc deficient and zinc deficient
<table>
<thead>
<tr>
<th>Dietary Treatment</th>
<th>No. with Tumors/No. Rats</th>
<th>(% with Tumors)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1 CD AD LIB</td>
<td>4/15</td>
<td>4/15</td>
</tr>
<tr>
<td></td>
<td>(27)</td>
<td>(27)</td>
</tr>
<tr>
<td>2 CD, PF</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>3 ZD</td>
<td>7/16</td>
<td>11/16</td>
</tr>
<tr>
<td></td>
<td>(44)</td>
<td>(69)</td>
</tr>
<tr>
<td>4 ZD, ZR</td>
<td>6/16</td>
<td>9/15</td>
</tr>
<tr>
<td></td>
<td>(37)</td>
<td>(60)</td>
</tr>
<tr>
<td>5 CD, ETOH</td>
<td>8/17</td>
<td>9/15</td>
</tr>
<tr>
<td></td>
<td>(47)</td>
<td>(60)</td>
</tr>
<tr>
<td>6 ZD, ETOH</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 ZD, ETOH</td>
<td>8/17</td>
<td>14/17</td>
</tr>
<tr>
<td></td>
<td>(47)</td>
<td>(82)</td>
</tr>
</tbody>
</table>

1 Time Points are:
1 End of Dosing
2 One Month Post Dosing
3 Two Months Post Dosing

2 Control diet 60 ppm zinc; zinc deficient 3 ppm zinc.
3 CD AD LIB is control diet ad libitum.
4 CD, PF is control diet pair fed to group 3.
5 ZD is zinc deficient.
6 ZD, ZR is zinc deficient, zinc repleted after dosing.
7 ETOH is 10% ethanol in drinking water.
8 13 CIS is 13-CIS retinoic acid.
Figure 6. Tumor Incidence Levels at Three Time Points.
returned to control at the initial sacrifice but significantly lower than both at the final sacrifice.

Zinc deficiency throughout the experiment significantly enhanced tumor incidence over the control diet ad lib (88% vs. 39%, p < 0.005). Pair feeding led to a significant decrease in tumor incidence (16%, p < 0.01), indicating that decreased food intake characteristic of zinc deficient animals was not the cause of enhanced tumor incidence in that group. Zinc deficiency applied only before and during dosing caused a significant enhancement in tumor incidence over control diet (71% vs. 39%, p < 0.01). This level was lower than that attained by zinc deficiency throughout the experiment, although not significantly less. Consumption of ethanol increased tumor incidence in control diet rats, although not significantly whereas no change was seen in zinc deficient rats consuming ethanol. Addition of 13 cis R.A. to the zinc deficient plus ethanol group enhanced tumor incidence to 100%.

Table IX shows distribution by size of total tumors per group. In Groups 1, 2, and 5, control diet based groups, more than 70% of tumors were 2 mm or less in size. In Groups 3, 4 and 6, zinc deficient diet based groups, less than 50% of tumors were 2 mm or less in size.

The average number of tumors per tumor bearing rat correlated well with tumor incidence levels (Table IX). The pair fed group, which had the lowest tumor incidence, fed, had 1.75 tumors per tumor bearing rat, whereas, excluding the 13-cis group, the zinc deficient plus ethanol group had the highest tumor incidence and greatest number of tumors per tumor
Tumors counted, counted as single clumps.

6 T3-CIS RA is T3-CIS retinoic acid added to diet after dosing.

5 ETHOH is 10% ethanol in drinking water.

4 ZD, ZR is zinc dectetion, switched to control diet after dosing.

3 ZD is zinc dectetion.

2 CD, PR is control diet, pair fed to group 3.

1 CD AD IIB is control diet ad libitum.

<table>
<thead>
<tr>
<th>Tumor Rate</th>
<th>Tumors</th>
<th>Tumor Size (mm)</th>
<th>Incidence (%)</th>
<th>Dietary Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVG. #</td>
<td>TOTAL</td>
<td>18.8</td>
<td>39</td>
<td>CD AD IIB</td>
</tr>
</tbody>
</table>

| 3.0          | 60     | 13.9          | 10.0          | ETHOH             |
| 4.1          | 122    | 22.9          | 9.1           | ETHOH             |
| 6.9          | 50     | 6.3           | 6.3           | ETHOH             |
| 2.6          | 61     | 2.1           | 2.1           | ETHOH             |
| 3.46         | 97     | 25.9          | 25.9          | ETHOH             |
| 1.75         | 7      | 3             | 3             | CD PR             |
| 2.76         | 36     | 18.8          | 39            | CD AD IIB         |

TABLE IX

% TUMOR BY SIZE

(1)
bearing rats, 4.1. The 13-cis group was excluded from this calculation since tumors coalesced making counting of individual tumors difficult.

During the first two sacrifice periods there were no carcinomas in any dietary groups. All papillomas were non invasive. The number and percent of carcinomas based on total tumors from final sacrifice are shown in Table X. There were six in the zinc deficient group compared to 1 in the control diet group which, based on total tumors of each group, was twice the incidence (6% vs. 3%).

c. Study 3.

Table XI depicts the groups and tumor incidences in study 3. In this study all rats were sacrificed 12 weeks after end of dosing. Zinc deficient diet significantly enhanced tumor incidences above control diet ad lib (88% vs. 31.4%). Pair feeding significantly reduced tumor incidence in control diet rats by half (15% vs. 31%, p < 0.005). Both the zinc deficient group switched to control diet after dosing and the control diet group switched to zinc deficient diet after dosing produced tumor incidences (68% and 62%, respectively) that were significantly greater than control diet throughout (p < 0.01; p < 0.05, respectively) and significantly less than zinc deficient diet throughout (p <0.05; p <0.01, respectively). The zinc excess groups that were switched in the same way produced slight increases in tumor incidence (41% and 46%) which were not significant. Ethanol during the pre-
### Table X

**MORPHOLOGY OF TUMORS STUDY 2**

<table>
<thead>
<tr>
<th></th>
<th>Tumor Incidence %</th>
<th>Total Tumors</th>
<th>Papillomas</th>
<th>Carcinomas Number %</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD AD LIB(^1)</td>
<td>39</td>
<td>32</td>
<td>31</td>
<td>1</td>
</tr>
<tr>
<td>CD PF(^2)</td>
<td>16</td>
<td>7</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>ZD(^3)</td>
<td>88</td>
<td>97</td>
<td>91</td>
<td>6</td>
</tr>
<tr>
<td>ZD, ZR(^4)</td>
<td>72</td>
<td>61</td>
<td>69</td>
<td>3</td>
</tr>
<tr>
<td>CD, ETOH(^5)</td>
<td>53</td>
<td>50</td>
<td>49</td>
<td>1</td>
</tr>
<tr>
<td>ZD, ETOH</td>
<td>91</td>
<td>122</td>
<td>114</td>
<td>8</td>
</tr>
<tr>
<td>ZD, ETOH, 13 CIS RA(^6)</td>
<td>100</td>
<td>60</td>
<td>58</td>
<td>2</td>
</tr>
</tbody>
</table>

\(^1\) CD AD LIB is control diet ad libitum.

\(^2\) CD, PF is control diet, pair fed to group 3.

\(^3\) ZD is zinc deficient.

\(^4\) ZD, ZR is zinc deficient, switched to control diet after dosing.

\(^5\) ETOH is 10% ethanol in drinking water.

\(^6\) 13-CIS RA is 13-cis retinoic acid added to diet after dosing.
TABLE XI
DIETARY TREATMENT AND TUMOR INCIDENCE - STUDY 3

<table>
<thead>
<tr>
<th>Dietary Treatment</th>
<th>Number with/Number Tumors/ rats</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 CD AD LIB²</td>
<td>11/35</td>
<td>(31.4)</td>
</tr>
<tr>
<td>2 CD, PF³ to ZD</td>
<td>5/35</td>
<td>(14.7)</td>
</tr>
<tr>
<td>3 ZD⁴</td>
<td>30/34</td>
<td>(88.2)</td>
</tr>
<tr>
<td>4 ZD, ZR⁵</td>
<td>23/34</td>
<td>(67.2)</td>
</tr>
<tr>
<td>5 CD, ZD⁶</td>
<td>21/34</td>
<td>(61.7)</td>
</tr>
<tr>
<td>6 CD + ETOH, CD⁷</td>
<td>11/35</td>
<td>(31.4)</td>
</tr>
<tr>
<td>7 CD, EX⁸</td>
<td>14/34</td>
<td>(41.2)</td>
</tr>
<tr>
<td>8 EX, CD⁹</td>
<td>15/32</td>
<td>(46.8)</td>
</tr>
</tbody>
</table>

1 Control Diet 60 ppm zinc; Zinc Deficient Diet 3 ppm zinc, excess 250 ppm.
2 Control Ad libitum.
3 Control pair fed to group 3.
4 Zinc Deficient.
5 Zinc Repleted following dosing.
6 Control switched to Zinc Deficient Diet After Dosing
7 5% Ethanol up to and through dosing.
8 Control diet switched to excess after dosing.
9 Excess diet switched to control diet after dosing.
and dosing period produced a tumor incidence identical to that of control diet.

Although tumor incidences of the control diet and the control diet with ethanol included through dosing were identical, the ethanol group had approximately half as many tumors and half the average number of tumors per tumor bearing rat (Table XII). This was the lowest average for all groups. The pair fed group, having the lowest tumor incidence had the second lowest average number of tumors per tumor bearing rat (1.82). The zinc deficient group, having the highest tumor incidence, had the highest average number of tumors per tumor bearing rat (3.82). The zinc excess groups which had tumor incidences higher than control diet rats, although not significantly so, had a smaller average number of tumors per tumor bearing rats than did control diet. The two zinc deficient groups switched with control diet, had significantly greater tumor incidences than control diet rats but a smaller average number of tumors per tumor bearing rat than control.

80% of tumor bearing animals in the control diet pair fed group had only 1 or 2 tumors per esophagus (Table XII). In the control diet group receiving ethanol before and during dosing 100% had 1 or 2 tumors. In the zinc deficient group a third of rats had one or two tumors and half had four or more compared to half in the control diet group that had one or two and a third had four or more. Also, in the zinc deficient group, rats with more than four tumors had from 5 to 17, compared to the control diet which were 5 or 6. In the two groups switched from either zinc deficient diet to control
### TABLE XII

**NUMBER OF TUMORS PER ESOPHAGUS**

<table>
<thead>
<tr>
<th>Dietary Treatment</th>
<th>Total Tumors</th>
<th>Number per Esophagus (%)</th>
<th>TTA¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>1 CD AD LIB¹</td>
<td>30</td>
<td>5(45)</td>
<td>1(9)</td>
</tr>
<tr>
<td>2 CD, PF³</td>
<td>9</td>
<td>3(60)</td>
<td>1(20)</td>
</tr>
<tr>
<td>3 ZD⁴</td>
<td>113</td>
<td>4(13)</td>
<td>7(23)</td>
</tr>
<tr>
<td>4 ZD, ZR⁵</td>
<td>57</td>
<td>6(26)</td>
<td>6(26)</td>
</tr>
<tr>
<td>5 CD, ZD⁶</td>
<td>46</td>
<td>9(43)</td>
<td>3(14)</td>
</tr>
<tr>
<td>6 CD + ETOH, CD⁷</td>
<td>16</td>
<td>6(54)</td>
<td>5(46)</td>
</tr>
<tr>
<td>7 CD, EX⁸</td>
<td>25</td>
<td>5(36)</td>
<td>6(43)</td>
</tr>
<tr>
<td>8 EX, CD⁹</td>
<td>35</td>
<td>6(40)</td>
<td>3(20)</td>
</tr>
</tbody>
</table>

---

1 Average Number Tumors/Tumor Bearing Animal

2 Control diet ad libitum.

3' Control diet pair fed to group 3.

4 Zinc deficient diet.

5 Zinc deficient diet switched to control diet after dosing.

6 Control diet switched to zinc deficient after dosing.

7 Control diet plus 5% ethanol switched to control diet only after dosing.

8 Control diet switched to zinc excess after dosing.

9 Excess diet switched to control diet after dosing.
diet or control diet to zinc deficient diet the percent of
animals with 1 or 2 tumors was similar whereas there were
twice as many animals that had 4 or more tumors in the group
switched to control diet after dosing compared to the group
switched to zinc deficient diet after dosing (31% vs. 14%).

The two zinc excess groups switched with control diet had
the highest percentage (67% and 60%) of tumors 2mm or less
(Table XIII). The control diet group switched to zinc deficiency
after dosing had 59% of tumors 2 mm in size or less compared
to the zinc deficient group that was switched to control
following dosing which had 40% of tumors 2mm in size or less.
This was the same level found for control diet ad lib and
similar to that found for zinc deficiency throughout the
experiment (42%). The two groups that had significant numbers
of tumors 4 mm in size and larger were the zinc deficient
group throughout and to a lesser extent the zinc deficient
group switched to control after dosing.

2. **Body Weight**

   a. **Pilot Study**

   Table XIV shows body weights for rats receiving 60, 7, 5,
or 3 ppm zinc in a 30% or 20% soy or egg protein based diet
for one month from weanling age. Rats weighed more in both of
the soy based diets than the egg based diets at 60 ppms. At 7
ppm weight declined more in the soy based diet groups than in
### TABLE XIII

**TUMORS BY SIZE - STUDY 3**

<table>
<thead>
<tr>
<th>Dietary Treatment</th>
<th>Tumor Incidence</th>
<th>Total Tumors</th>
<th>Tumor Size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1 CD AD LIB(^1)</td>
<td>31.4</td>
<td>30</td>
<td>9</td>
</tr>
<tr>
<td>2 CD, PF(^2)</td>
<td>14.7</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>3 ZD(^3)</td>
<td>88.2</td>
<td>113</td>
<td>16</td>
</tr>
<tr>
<td>4 ZD, ZR(^4)</td>
<td>67.6</td>
<td>57</td>
<td>4</td>
</tr>
<tr>
<td>5 CD, ZD(^5)</td>
<td>61.7</td>
<td>46</td>
<td>20</td>
</tr>
<tr>
<td>6 CD + ETOH, CD(^6)</td>
<td>31.4</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>7 CD, EX(^7)</td>
<td>41.2</td>
<td>26</td>
<td>10</td>
</tr>
<tr>
<td>8 Excess, CD(^8)</td>
<td>46.8</td>
<td>35</td>
<td>11</td>
</tr>
</tbody>
</table>

---

1 Control diet ad libitum.
2 Control diet pair fed to group 3.
3 Zinc deficient diet.
4 Zinc deficient diet switched to control diet after dosing.
5 Control diet switched to zinc deficient after dosing.
6 Control diet plus 5% ethanol switched to control diet only after dosing.
7 Control diet switched to zinc excess after dosing.
8 Excess diet switched to control diet after dosing.
## TABLE XIV

**BODY WEIGHT AFTER ONE MONTH ON DIET**

<table>
<thead>
<tr>
<th>Protein Source</th>
<th>60 ppm</th>
<th>7 ppm</th>
<th>5 ppm</th>
<th>3 ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Soy</td>
<td>193 ± 18</td>
<td>148 ± 13</td>
<td>136 ± 10</td>
<td>106 ± 7</td>
</tr>
<tr>
<td>20% Soy</td>
<td>184 ± 15</td>
<td>131 ± 16</td>
<td>124 ± 8</td>
<td>118 ± 9</td>
</tr>
<tr>
<td>30% Egg</td>
<td>160 ± 21</td>
<td>150 ± 11</td>
<td>134 ± 14</td>
<td>96 ± 8</td>
</tr>
<tr>
<td>20% Egg</td>
<td>178 ± 16</td>
<td>161 ± 13</td>
<td>153 ± 6</td>
<td>124 ± 3</td>
</tr>
</tbody>
</table>
the egg based diet groups so that the latter two groups weighed slightly more. The 30% soy weighed more than the 20% soy whereas the 20% egg weighed more than the 30% egg diet. There was a slight body weight decline at 5 ppm in all groups. At 3 ppm, the 30% soy group exhibited the lowest body weight but this did not vary greatly from that of 30% egg diet (106 ± 7 vs. 110 ± 9). Weights were higher in the 20% soy and 20% egg based groups, 118 ± 9 vs. 124 ± 13. The body weight for the 3 ppm 20% egg protein diet, was comparable to that of the 7 ppm 20% soy protein diet 124 vs. 131, respectively.

b. Tumor Studies

Figures 7, 8 and 9 show weekly body weights for dietary groups of the three tumor studies, respectively. There was no significant body weight differences between control diet groups with or without MBN. The zinc deficient groups had body weights significantly less than controls after three weeks on 7 ppm zinc (Fig. 7) and two weeks on 3 ppm zinc (Fig. 8 and 9). Animals on a zinc deficient diet and repleted with control diet following the end of carcinogen dosing experienced sharp weight gains during the 2-3 weeks after start of repletion at which time weights were no longer significantly less than weight of rats that had only received control diet.

Rats switched from control diet to zinc deficient diet showed a slowing rate of weight gain after two weeks with a significantly lowered weight by six weeks. Rats receiving excess zinc showed no difference in weight gain from control
Figure 7. Weekly Body Weight for Study 1.
Body Weight (grams)

WEEKS POST WEANLING

△ CONTROL, -MBN
○ CONTROL, +MBN
▲ ZINC Def. +MBN
▪ ZINC Def./ZINC Repletion +MBN
○ ZINC Def., Alcohol + MBN
● ZINC Def., Alcohol + 13 CIS R.A. + MBN
Figure 8. Weekly Body Weight for Study 2.
BODY WEIGHT (grams)

TIME (WEEKS)

CONTROL AD LIB
CONTROL + ETOL
ZINC DEF
ZINC DEF + ETOL
ZINC DEF, REPLETION
PAIR FED
ZINC DEF + ETOL + 13C1S R.A.
Figure 9. Body Weight for Study 3.
diet animals. Those animals receiving ethanol with either control diet or zinc deficient diet at the 4% or 5% level (Figure 7 and 9, respectively) showed lower weights than the comparable non alcohol diet groups although these weights were not significantly less. 10% ethanol (Study 2, Fig. 8) caused a greater weight reduction that was not significantly less. Addition of 13-cis retinoic acid did not alter weight gain.

Pair feeding (Figures 8 and 9) caused weight gain similar to zinc deficient rats although the mean weight of this group was usually slightly less.

3. Food, Alcohol and Caloric Intake

As table XV shows, caloric intake for the 30% soy diet and the 20% egg diet were very similar, 4.34 and 4.22 cal/gm diet. Control ad lib, after six weeks on diet, consumed an average 23 gm/day or 97 cal/day compared to the zinc deficient group which consumed about 13 gm/day or 55 cal/day. Pair fed rats had similar intake levels.

Alcohol consumption decreased food intake in the control ad lib group from 23 gm/day to 21 and 19/day for the 4% and 10% alcohol levels, respectively. Intake was reduced in the zinc deficient group from 13 gm/day to 11.5 and 10.5 due to the 4% and 10% alcohol concentrations, respectively. Caloric intake from food was reduced by 10% and 9% in the control ad lib group and aby about 10% and 10% in the zinc deficient group. These drops were more than compensated for by added
## TABLE XV

**FOOD, ALCOHOL AND CALORIC INTAKE**

<table>
<thead>
<tr>
<th></th>
<th>Food Intake(g)</th>
<th>Cal/day</th>
<th>Water Intake(ml)</th>
<th>Cal/day</th>
<th>Total Cal</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD AD LIB</td>
<td>23</td>
<td>97</td>
<td>21</td>
<td>-</td>
<td>97</td>
</tr>
<tr>
<td>CD PF</td>
<td>13</td>
<td>55</td>
<td>15</td>
<td>-</td>
<td>55</td>
</tr>
<tr>
<td>ZD</td>
<td>13</td>
<td>55</td>
<td>13</td>
<td>-</td>
<td>55</td>
</tr>
<tr>
<td>CD + ETOH 4% (10%)</td>
<td>21(19)</td>
<td>89(80)</td>
<td>18(16)</td>
<td>5.0(11)</td>
<td>94(91)</td>
</tr>
<tr>
<td>ZD + ETOH 4% (10%)</td>
<td>11.5(10.5)</td>
<td>49(44)</td>
<td>12(10)</td>
<td>3.4(7)</td>
<td>52.4(51)</td>
</tr>
<tr>
<td>EX</td>
<td>21</td>
<td>89</td>
<td>20</td>
<td>-</td>
<td>89</td>
</tr>
<tr>
<td>ZD + ETOH, 13 CIS RA</td>
<td>11</td>
<td>46</td>
<td>11</td>
<td>-</td>
<td>46</td>
</tr>
</tbody>
</table>

---

**cal/gm diet**

- 30% Soy: 4.34
- 20% Egg White: 4.22

**cal/gm diet**

<table>
<thead>
<tr>
<th>Minerals(g)/100 calories</th>
</tr>
</thead>
<tbody>
<tr>
<td>non ethanol</td>
</tr>
<tr>
<td>4% ethanol</td>
</tr>
<tr>
<td>CD</td>
</tr>
<tr>
<td>ZD</td>
</tr>
<tr>
<td>10% ethanol</td>
</tr>
<tr>
<td>ZD</td>
</tr>
</tbody>
</table>
calories in the drinking water, thereby diluting out the effects of the mineral: calorie ratio, from 1.19 in the nonalcohol groups to 1.12 in the control and zinc deficient groups consuming 4% ethanol and to 1.03 and 1.00 in the control and zinc deficient groups, respectively, for the 10% alcohol level.

4. **Mineral Levels**

   a. **Serum**

   (1) **Pilot Study**

   Serum zinc levels for 30% and 20% soy and egg based diets were similar in the diet containing 60 ppm zinc (Table XVI). The range was from 112-117 μg/100 ml serum. Zinc levels dropped significantly in the 7 ppm soy based diets to 68 and 64 μg/100 ml (p < 0.01). Levels also dropped in the egg based diets to 92 and 85 μg/100 ml but this was not significant. At 5 ppm there was not a significant drop in the zinc levels of the soy based diets although the levels were approximately 10 μg/100 ml lower. There was a greater drop in the 30% and 25% egg based groups to 70 and 74 μg/100 ml, respectively, which was significantly lower than the 60 ppm level (p < 0.05).

   In the 3 ppm group serum zinc levels dropped to 39 and 44 μg/100 ml in the 30% and 20% soy based diet, a level con-
<table>
<thead>
<tr>
<th>Protein Source</th>
<th>60 ppm</th>
<th>7 ppm</th>
<th>5 ppm</th>
<th>3 ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Soy</td>
<td>117.1</td>
<td>68.2</td>
<td>55.3</td>
<td>39</td>
</tr>
<tr>
<td>20% Soy</td>
<td>115.3</td>
<td>64.3</td>
<td>52.1</td>
<td>44</td>
</tr>
<tr>
<td>30% Egg</td>
<td>112.8</td>
<td>92.4</td>
<td>70.1</td>
<td>59</td>
</tr>
<tr>
<td>20% Egg</td>
<td>114.3</td>
<td>85.0</td>
<td>74.2</td>
<td>66.2</td>
</tr>
</tbody>
</table>

1 After one month on diet containing various concentrations of zinc.
sidered a severe zinc deficiency. In the egg based groups, there was a slight decline from the 5 ppm level to 59 µg/100 ml in the 30% egg group and 66 µg/100 ml in the 20% egg group. This slight decline increased the significant difference of this level from the 60 ppm level from p < 0.05 to p < 0.01. The 3 ppm level in the 20% egg diet was comparable to the 7 ppm level in the soy based diet groups.

(2) Study 1

Serum was taken from rats and measured for zinc and copper levels at the start of the experiment, immediately before carcinogen dosing, at end of carcinogen dosing and one month after dosing (Table XVII). Zinc ranged from 115 to 120 µg/100 ml serum in the different dietary groups. After one month on diets, zinc serum values were significantly depressed in zinc deficient groups (p < 0.05). Ethanol did not depress levels further. Treatment with MBN increased serum zinc levels slightly (not significantly) in all groups. One month after returning zinc deficient rats to control diets serum zinc levels approximated that of control diet rats. Addition of 13-cis retinoic acid had no affect on serum zinc levels.

Serum copper levels were unchanged in the control diet group with or without MBN. Copper levels approached a significant increase (P=0.05) in the zinc deficient group after 1 month. Copper levels increased significantly after 3 months in the zinc deficient plus alcohol group and after 2 months in the zinc deficient plus alcohol plus 13-cis retinoic acid group.
<table>
<thead>
<tr>
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<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD AD LIB, (zinc)</td>
<td>116 ± 74</td>
<td>-</td>
<td>-</td>
<td>119 ± 7</td>
</tr>
<tr>
<td>-MBN (copper)</td>
<td>109 ± 6</td>
<td>-</td>
<td>-</td>
<td>112 ± 5</td>
</tr>
<tr>
<td>CD AD LIB</td>
<td>115 ± 9</td>
<td>119 ± 8</td>
<td>130 ± 8</td>
<td>128 ± 11</td>
</tr>
<tr>
<td></td>
<td>109 ± 13</td>
<td>111 ± 12</td>
<td>121 ± 11</td>
<td>121 ± 7</td>
</tr>
<tr>
<td>ZD</td>
<td>115 ± 7</td>
<td>63 ± 102</td>
<td>74 ± 62</td>
<td>80 ± C2</td>
</tr>
<tr>
<td></td>
<td>108 ± 11</td>
<td>131 ± 8</td>
<td>125 ± 13</td>
<td>129 ± 12</td>
</tr>
<tr>
<td>ZD, CD</td>
<td>115 ± 7</td>
<td>60 ± 72</td>
<td>75 ± 62</td>
<td>110 ± 10</td>
</tr>
<tr>
<td></td>
<td>107 ± 11</td>
<td>118 ± 13</td>
<td>127 ± 13</td>
<td>121 ± 10</td>
</tr>
<tr>
<td>ZD, ETOH</td>
<td>113 ± 6</td>
<td>-</td>
<td>65 ± 92</td>
<td>77 ± 122</td>
</tr>
<tr>
<td></td>
<td>107 ± 11</td>
<td>-</td>
<td>114 ± 12</td>
<td>146 ± 183</td>
</tr>
<tr>
<td>ZD, ETOH, 13-CIS</td>
<td>114 ± 6</td>
<td>-</td>
<td>65 ± 92</td>
<td>77 ± 122</td>
</tr>
<tr>
<td></td>
<td>108 ± 10</td>
<td>-</td>
<td>156 ± 213</td>
<td>157 ± 93</td>
</tr>
</tbody>
</table>

1 Time of Analysis

1. Initial
2. One month feeding
3. After dosing
4. One month after dosing

2 Zinc levels significantly different from initial baseline levels, p < 0.005.

3 Copper levels significantly different from initial baseline levels, p < 0.005.

4 µg/100 ml ± S.E.
(3) **Study 2**

Serum zinc and copper levels followed similar patterns (Table XVIII) to those seen in Study 1 for comparable groups. The pair feeding group had mineral levels comparable to that seen in the control diet ad lib groups. Serum zinc was reduced in the control diet plus ethanol but not in the zinc deficient diet plus ethanol. Serum copper was unchanged in these two groups.

(4) **Study 3**

Serum zinc and copper levels (Table XIX) were comparable to those seen in the same groups of studies 1 and 2. Zinc excess caused significantly increased zinc levels and reduced levels of copper but not significantly.

b. **Hair Mineral Content**

(1) **Study 1**

Hair zinc and copper displayed a pattern similar to that found in the serum mineral levels, substantiating the accuracy of serum measurements to determine mineral status of the animal (Table XX). Zinc levels decreased significantly from baseline levels after one month of zinc deficient diet. Addition of ethanol or 13-cis R.A. to zinc deficient diet did not alter levels. Zinc repletion did not increase zinc levels after one month as
<table>
<thead>
<tr>
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<th>4</th>
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<tbody>
<tr>
<td><strong>CD AD LIB (zinc)</strong></td>
<td>118.1 ± 7^4</td>
<td>120 ± 3</td>
<td>122 ± 8</td>
<td>128 ± 9</td>
</tr>
<tr>
<td></td>
<td>(copper)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>108 ± 2</td>
<td>111 ± 8</td>
<td>109 ± 12</td>
<td>104 ± 11</td>
</tr>
<tr>
<td><strong>CD, PF</strong></td>
<td>116 ± 6</td>
<td>114 ± 6</td>
<td>116 ± 4</td>
<td>119 ± 13</td>
</tr>
<tr>
<td></td>
<td>107 ± 5</td>
<td>104 ± 2</td>
<td>103 ± 2</td>
<td>101 ± 8</td>
</tr>
<tr>
<td><strong>ZD</strong></td>
<td>119 ± 5</td>
<td>68 ± 6^2</td>
<td>66 ± 10^2</td>
<td>64 ± 14^2</td>
</tr>
<tr>
<td></td>
<td>104 ± 10</td>
<td>129 ± 8^3</td>
<td>121 ± 9</td>
<td></td>
</tr>
<tr>
<td><strong>ZD, CL</strong></td>
<td>112 ± 8</td>
<td>63 ± 7^2</td>
<td>68 ± 10^2</td>
<td>108 ± 16</td>
</tr>
<tr>
<td></td>
<td>108 ± 7</td>
<td>130 ± 11</td>
<td>134 ± 3^3</td>
<td>117 ± 13</td>
</tr>
<tr>
<td><strong>CD, ETOH</strong></td>
<td>113 ± 7</td>
<td>98 ± 6</td>
<td>94 ± 9</td>
<td>91 ± 12</td>
</tr>
<tr>
<td></td>
<td>105 ± 4</td>
<td>107 ± 8</td>
<td>101 ± 10</td>
<td>101 ± 6</td>
</tr>
<tr>
<td><strong>ZD, ETOH</strong></td>
<td>120 ± 9</td>
<td>60 ± 4^2</td>
<td>64 ± 7^2</td>
<td>68 ± 9^2</td>
</tr>
<tr>
<td></td>
<td>107 ± 9</td>
<td>120 ± 3</td>
<td>119 ± 10</td>
<td>104 ± 8</td>
</tr>
<tr>
<td><strong>ZD, ETOH, 13-CIS</strong></td>
<td>117 ± 6</td>
<td>59 ± 8^2</td>
<td>68 ± 10^2</td>
<td>66 ± 16^2</td>
</tr>
<tr>
<td></td>
<td>109 ± 2</td>
<td>122 ± 6</td>
<td>118 ± 6</td>
<td>104 ± 11</td>
</tr>
</tbody>
</table>

1 Time  
1. Initial  
2. One month feeding  
3. After dosing  
4. One month after dosing

2 Serum zinc significantly different from initial baseline levels, p < 0.001.

3 Serum copper significantly different from initial baseline levels, p < 0.005.

4 μg/100 ml ± S.E.
<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
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<th>5</th>
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<tbody>
<tr>
<td>CD AD (zinc)</td>
<td>120 ± 76</td>
<td>119 ± 10</td>
<td>124 ± 11</td>
<td>128 ± 16</td>
<td></td>
</tr>
<tr>
<td>LIB (copper)</td>
<td>107 ± 3</td>
<td>104 ± 8</td>
<td>107 ± 10</td>
<td>101 ± 13</td>
<td></td>
</tr>
<tr>
<td>Cd, PF</td>
<td>118 ± 6</td>
<td>114 ± 6</td>
<td>109 ± 6</td>
<td>116 ± 9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>104 ± 10</td>
<td>109 ± 10</td>
<td>107 ± 11</td>
<td>105 ± 8</td>
<td></td>
</tr>
<tr>
<td>ZD</td>
<td>120 ± 6</td>
<td>69 ± 11&lt;sup&gt;2&lt;/sup&gt;</td>
<td>64 ± 14&lt;sup&gt;2&lt;/sup&gt;</td>
<td>62 ± 13&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>109 ± 4</td>
<td>121 ± 12&lt;sup&gt;5&lt;/sup&gt;</td>
<td>128 ± 75</td>
<td>123 ± 12</td>
<td></td>
</tr>
<tr>
<td>ZD, CD</td>
<td>114 ± 8</td>
<td>61 ± 8&lt;sup&gt;2&lt;/sup&gt;</td>
<td>64 ± 6&lt;sup&gt;2&lt;/sup&gt;</td>
<td>108 ± 6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>101 ± 7</td>
<td>130 ± 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>132 ± 14&lt;sup&gt;5&lt;/sup&gt;</td>
<td>119 ± 12</td>
<td></td>
</tr>
<tr>
<td>CD, ZD</td>
<td>122 ± 6</td>
<td>114 ± 12</td>
<td>120 ± 6</td>
<td>91 ± 16</td>
<td>77 ± 17&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>107 ± 4</td>
<td>101 ± 14</td>
<td>109 ± 7</td>
<td>111 ± 12</td>
<td>119 ± 10</td>
</tr>
<tr>
<td>CD, EX</td>
<td>114 ± 2</td>
<td>121 ± 8</td>
<td>117 ± 11</td>
<td>148 ± 17&lt;sup&gt;4&lt;/sup&gt;</td>
<td>168 ± 14&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>197 ± 10</td>
<td>107 ± 8</td>
<td>101 ± 14</td>
<td>104 ± 16</td>
<td>99 ± 10</td>
</tr>
<tr>
<td>EX, CD</td>
<td>108 ± 6</td>
<td>171 ± 13&lt;sup&gt;3&lt;/sup&gt;</td>
<td>161 ± 14&lt;sup&gt;3&lt;/sup&gt;</td>
<td>142 ± 18</td>
<td>131 ± 17</td>
</tr>
<tr>
<td></td>
<td>99 ± 14</td>
<td>94 ± 8</td>
<td>97 ± 11</td>
<td>100 ± 12</td>
<td>104 ± 13</td>
</tr>
<tr>
<td>CD + ETOH, CD</td>
<td>121 ± 7</td>
<td>103 ± 8</td>
<td>107 ± 11</td>
<td>112 ± 11</td>
<td>112 ± 10</td>
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<td></td>
<td>104 ± 6</td>
<td>107 ± 9</td>
<td>103 ± 10</td>
<td>101 ± 14</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> Times
1. Initial
2. One month feeding
3. After dosing
4. One month after dosing
5. End of experiment

<sup>2</sup> Serum zinc significantly different from baseline levels, p < 0.005.
<sup>3</sup> Serum zinc significantly different from baseline levels, p < 0.01.
<sup>4</sup> Serum zinc significantly different from baseline levels, p < 0.05.
Serum copper significantly different from baseline levels, $p < 0.005$

$\mu g/100 \text{ ml} \pm \text{S.E.}$
### TABLE XX

**HAIR ZINC AND COPPER**

**CONCENTRATIONS DURING CARCINOGENESIS**

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD AD LIB, - MBN</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(zinc)</td>
<td>228 ± 21</td>
<td>-</td>
<td>215 ± 18</td>
</tr>
<tr>
<td>(copper)</td>
<td>20 ± 4</td>
<td>-</td>
<td>20 ± 4</td>
</tr>
<tr>
<td><strong>CD AD LIB</strong></td>
<td>238 ± 12</td>
<td>210 ± 39</td>
<td>201 ± 19</td>
</tr>
<tr>
<td></td>
<td>21 ± 3</td>
<td>20 ± 4</td>
<td>22 ± 5</td>
</tr>
<tr>
<td><strong>ZD</strong></td>
<td>231 ± 12</td>
<td>175 ± 27&lt;sup&gt;3&lt;/sup&gt;</td>
<td>182 ± 18&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>20 ± 3</td>
<td>-</td>
<td>21 ± 4</td>
</tr>
<tr>
<td><strong>ZD, CD</strong></td>
<td>228 ± 20</td>
<td>181 ± 24&lt;sup&gt;3&lt;/sup&gt;</td>
<td>187 ± 13&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>20 ± 2.4</td>
<td>20 ± 4</td>
<td>22 ± 6</td>
</tr>
<tr>
<td><strong>ZD + ETOH</strong></td>
<td>230 ± 10</td>
<td>-</td>
<td>177 ± 12&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>20 ± 3</td>
<td>-</td>
<td>22 ± 7</td>
</tr>
<tr>
<td><strong>ZD + ETOH, 13-CIS</strong></td>
<td>229 ± 19</td>
<td>-</td>
<td>180 ± 14&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>19 ± 5</td>
<td>-</td>
<td>18 ± 2</td>
</tr>
</tbody>
</table>

<sup>1</sup> ppm ± S.E.

<sup>2</sup> Times

1. Start of experiment
2. End of 1 month feeding
3. One month after dosing

<sup>3</sup> Significantly different from control levels, p < 0.01.
was seen in serum zinc levels. There was no significant change in copper hair levels.

(2) Studies 2 and 3

Hair zinc and copper levels were similar to those of Study 1 for same dietary groups. Ethanol in control diet reduced zinc levels but not significantly. In zinc diet ethanol had no effect. Zinc repletion led to increased zinc levels 2 months after repletion in study 2 (Table XXI). In the third study (Table XXII) a measurement at six weeks post repletion revealed an increase towards control diet levels of hair zinc. Zinc excess caused significantly increased zinc levels 2 months after start of diet. In the group switched from control diet to zinc deficient diet after dosing hair zinc was not significantly reduced until 2 months on diet.

c. Tissue Mineral Content

Esophageal and liver zinc were measured in experiments 2 and 3. In experiment 2, with multiple sacrifice periods for all groups except the pair fed and zinc deficient plus ethanol groups, measurements were made before dosing with MBN, immediately after dosing, one month after dosing and two months after dosing. In experiment 3, measurements were made immediately before MBN dosing, one month after dosing, and at the end of the experiment, three months after dosing. In this experiment select measurements were made on tumors
### TABLE XXI
HAIR ZINC AND COPPER CONCENTRATIONS DURING CARCINOGENESIS $^1$ - STUDY 2

<table>
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<th>3 $^3$</th>
<th>4 $^3$</th>
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<tbody>
<tr>
<td>CD AD LIB</td>
<td>233 ± 17</td>
<td>217 ± 20</td>
<td>211 ± 11</td>
<td>218 ± 20</td>
</tr>
<tr>
<td></td>
<td>23 ± 7</td>
<td>26 ± 8</td>
<td>21 ± 6</td>
<td>23 ± 10</td>
</tr>
<tr>
<td>CD, PF</td>
<td>224 ± 12</td>
<td>229 ± 17</td>
<td>231 ± 20</td>
<td>222 ± 4</td>
</tr>
<tr>
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<td>20 ± 4</td>
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<td>20 ± 8</td>
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<tr>
<td>ZD, ETOH</td>
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<td>184 ± 17 $^3$</td>
<td>190 ± 12</td>
<td>196 ± 17 $^3$</td>
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<td>20 ± 6</td>
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<td>181 ± 12 $^3$</td>
<td>176 ± 20 $^3$</td>
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<td>22 ± 8</td>
<td>23 ± 6</td>
<td>21 ± 4</td>
<td>28 ± 11</td>
</tr>
</tbody>
</table>

1 ppm ± S.E.

2 Time
   1. Initial
   2. One month feeding
   3. After dosing
   4. End of experiment (two months after dosing)

3 Significantly different from initial values, $p < 0.05$. 
<table>
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<td>229 ± 13</td>
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<td>21 ± 9</td>
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<td>24 ± 12</td>
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<td>CD, PF</td>
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<td>238 ± 21</td>
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<td>201 ± 20</td>
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<td>CD, EX</td>
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<td>17 ± 8</td>
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<tr>
<td>EX, CD</td>
<td>218 ± 23</td>
<td>241 ± 20</td>
<td>289 ± 6</td>
<td>269 ± 17</td>
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<td>18 ± 10</td>
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<tr>
<td>CD + ETOH, CD</td>
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<td>216 ± 9</td>
<td>221 ± 17</td>
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<td>17 ± 11</td>
<td>24 ± 9</td>
<td>20 ± 9</td>
<td>-</td>
<td>21 ± 6</td>
</tr>
</tbody>
</table>

1 ppm ± S.E.

2 Times
   1. Initial
   2. One month feeding
   3. After dosing
   4. Six weeks after dosing
   5. End of experiment

3 Significantly different from control values, p < 0.01.

4 Significantly different from control values, p < 0.05.
(papillomas) in each group.

Zinc levels decreased in the esophagus after MBN treatment in control diet animals (Tables XXIII and XXIV). These changes were not significant. Zinc levels were less in control pair fed rats compared to control ad libitum but not to a significant degree. These levels did not fluctuate due to dosing. Zinc deficiency caused a significant reduction in esophageal zinc levels. Treatment with MBN further reduced levels. In rats receiving zinc deficient diets through the dosing regimen and then returned to control diet, no increase in zinc levels was seen one month after repletion whereas an increase was seen two months after repletion. Not until 3 months post repletion did esophageal zinc levels return to normal values.

Zinc excess raised zinc levels somewhat. Ethanol had little effect on these levels. No significant differences were seen in any of the dietary groups at any of the time points for liver zinc. Esophageal tumor zinc levels were comparable to levels of surrounding tissue.

4. **Vitamin A Levels**

Vitamin A was measured in the serum and liver of rats consuming various diets, as shown in Table XXV. Six rats were tested in each group. Although serum levels of vitamin A were somewhat less in zinc deficient rats compared to control ad lib rats the reduced levels were within a normal range. There
<table>
<thead>
<tr>
<th>Group</th>
<th>Dietary Zinc</th>
<th>MBN</th>
<th>ETOH</th>
<th>13 cis RA</th>
<th>Before MBN</th>
<th>End of MBN</th>
<th>1 month post MBN</th>
<th>2 months post MBN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Liver 112 ± 14</td>
<td>111 ± 6</td>
<td>113 ± 7</td>
<td>118 ± 13</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>Esophagus 198 ± 23</td>
<td>220 ± 30</td>
<td>218 ± 14</td>
<td>201 ± 20</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Liver 108 ± 11</td>
<td>99 ± 10</td>
<td>103 ± 16</td>
<td>106 ± 17</td>
</tr>
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<td>Esophagus 202 ± 18</td>
<td>171 ± 24</td>
<td>191 ± 20</td>
<td>199 ± 24</td>
</tr>
<tr>
<td>3</td>
<td>60 p.f.</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Liver 101 ± 10</td>
<td>107 ± 14</td>
<td>111 ± 20</td>
<td>116 ± 17</td>
</tr>
<tr>
<td></td>
<td>to gr.5</td>
<td></td>
<td></td>
<td></td>
<td>Esophagus 195 ± 20</td>
<td>198 ± 36</td>
<td>184 ± 20</td>
<td>176 ± 12</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
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<td>Liver 94 ± 12</td>
<td>89 ± 9</td>
<td>92 ± 8</td>
<td>96 ± 11</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Esophagus 159 ± 21</td>
<td>138 ± 18</td>
<td>143 ± 17</td>
<td>132 ± 18</td>
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<td>-</td>
<td>Liver 99 ± 16</td>
<td>91 ± 11</td>
<td>108 ± 13</td>
<td>101 ± 14</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>Esophagus 143 ± 26</td>
<td>157 ± 27</td>
<td>151 ± 31</td>
<td>189 ± 14</td>
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<td>6</td>
<td>60</td>
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<td>-</td>
<td>Liver 104 ± 12</td>
<td>111 ± 16</td>
<td>106 ± 13</td>
<td>109 ± 12</td>
</tr>
<tr>
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<td>Esophagus 221 ± 13</td>
<td>171 ± 23</td>
<td>181 ± 19</td>
<td>172 ± 14</td>
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<tr>
<td>7</td>
<td>7</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Liver 92 ± 7</td>
<td>96 ± 11</td>
<td>91 ± 13</td>
<td>94 ± 11</td>
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<td>Esophagus 131 ± 26</td>
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<td>130 ± 30</td>
<td>138 ± 24</td>
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<tr>
<td>8</td>
<td>7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Liver 91 ± 11</td>
<td>88 ± 7</td>
<td>87 ± 11</td>
<td>82 ± 17</td>
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<td>Esophagus 142 ± 28</td>
<td>157 ± 27</td>
<td>171 ± 17</td>
<td>162 ± 13</td>
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</table>

1 ppm ± S.E.

2 Significantly different from control diet levels, p < 0.01.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Before MBN</th>
<th>After MBN</th>
<th>End of Experiment</th>
</tr>
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<td>114 ± 17</td>
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<tr>
<td></td>
<td>E</td>
<td>204 ± 19</td>
<td>218 ± 20</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>-</td>
<td>206 ± 17</td>
</tr>
<tr>
<td>CD, Pair fed</td>
<td>L</td>
<td>114 ± 13</td>
<td>117 ± 13</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>208 ± 11</td>
<td>213 ± 16</td>
</tr>
<tr>
<td></td>
<td>T</td>
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<td>-</td>
</tr>
<tr>
<td>ZD</td>
<td>L</td>
<td>98 ± 6</td>
<td>101 ± 14</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>151 ± 7</td>
<td>142 ± 13</td>
</tr>
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<td>T</td>
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<td>136 ± 11</td>
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<td>ZD, CD</td>
<td>L</td>
<td>91 ± 13</td>
<td>103 ± 12</td>
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<tr>
<td></td>
<td>E</td>
<td>148 ± 20</td>
<td>167 ± 18</td>
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<td>CD, ZD</td>
<td>L</td>
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<td></td>
<td>E</td>
<td>-</td>
<td>200 ± 11</td>
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<tr>
<td></td>
<td>T</td>
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<td>185 ± 21</td>
</tr>
<tr>
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<td>L</td>
<td>119 ± 21</td>
<td>109 ± 17</td>
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<td></td>
<td>E</td>
<td>248 ± 17</td>
<td>231 ± 13</td>
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<tr>
<td></td>
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<td>196 ± 17</td>
</tr>
<tr>
<td>CD, EX</td>
<td>L</td>
<td>-</td>
<td>121 ± 9</td>
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<td></td>
<td>E</td>
<td>-</td>
<td>241 ± 16</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>-</td>
<td>221 ± 13</td>
</tr>
</tbody>
</table>

1 µg/g tissue ± S.E.  
3 Significantly different from control, p<0.01.

2 L is liver.  
E is esophagus.  
T is tumor.
### TABLE XXV

**VITAMIN A LEVELS**

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Dietary Zinc ppm</th>
<th>Ethyl Alcohol</th>
<th>13-cis RA</th>
<th>Serum (µg/100ml) Before Dosing</th>
<th>1 month after dosing</th>
<th>Liver (µg/g) Before Dosing</th>
<th>1 month After Dosing</th>
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<tbody>
<tr>
<td>1</td>
<td>60</td>
<td>-</td>
<td>-</td>
<td>57 ± 6&lt;sup&gt;1&lt;/sup&gt;</td>
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<td>64 ± 14</td>
<td>59 ± 12</td>
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<td>2</td>
<td>60</td>
<td>+</td>
<td>-</td>
<td>52 ± 5</td>
<td>58 ± 6</td>
<td>51 ± 8</td>
<td>65 ± 7</td>
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<td>60, p.f. to gr.5</td>
<td>+</td>
<td>-</td>
<td>48 ± 5</td>
<td>47 ± 4</td>
<td>65 ± 12</td>
<td>71 ± 11</td>
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<td>4</td>
<td>7</td>
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<td>-</td>
<td>45 ± 3</td>
<td>42 ± 5</td>
<td>72 ± 13</td>
<td>78 ± 14</td>
</tr>
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<td>5</td>
<td>7, (60)</td>
<td>+</td>
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<td>41 ± 4</td>
<td>54 ± 6</td>
<td>73 ± 8</td>
<td>81 ± 17</td>
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<td>+</td>
<td>42 ± 7</td>
<td>49 ± 8</td>
<td>73 ± 12</td>
<td>64 ± 8</td>
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<td>7</td>
<td>+</td>
<td>+</td>
<td>54 ± 8</td>
<td>59 ± 12</td>
<td>81 ± 10</td>
<td>74 ± 9</td>
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</table>

<sup>1 ± S.E.</sup>
was no significant difference of serum zinc levels between zinc deficient and control pair fed rats, before or after dosing. No differences were found in the liver.

5. **pNitro Anisole Demethylase Activity**

The activity of p-nitroanisole demethylase activity in the liver, as measured by the production of p-nitophenol from p-nitroanisole, showed no differences between dietary groups except in those animals receiving ethanol with control diet (p < 0.05) (Table XVI).

B. **BIOCHEMICAL STUDIES**

Purification of DNA.

DNA was isolated by CsCl gradient and fractionated into 0.375 ml aliquots. DNA peak was located in fractions 11-15 as identified by both optical density and incorporated \(^3\text{H}\)-thymidine (Figure 10). Contamination of DNA by protein and RNA was measured at 2.5% and 3%, respectively, by BIORAD and ORCINOL. The 260:280 ratio, a measure of DNA purity was consistently 1.79 - 1.83. A ratio of 1.72 was minimum accepted level of purity.
<table>
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<th>p-nitro-1 phenol formed</th>
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<td>CD, AD LIB</td>
<td>278 ± 31</td>
</tr>
<tr>
<td>CD, p.f.</td>
<td>261 ± 29</td>
</tr>
<tr>
<td>ZD</td>
<td>281 ± 41</td>
</tr>
<tr>
<td>C/ETOH</td>
<td>361 ± 37</td>
</tr>
<tr>
<td>ZD/ETOH</td>
<td>341 ± 44</td>
</tr>
</tbody>
</table>

1 µg/g liver/hour

2 Significantly different from control, p<0.05
Figure 10. Optical Density and DPMs of DNA Gradient Fractions after Incorporation of $^{3}H$-thymidine.
1. **DNA Synthesis**

   a. **Incorporation of $^3$H-thymidine - Scintillation counting**

   DNA synthesis was examined in the esophagus and liver of rats consuming either a control ad lib, control pair-fed or zinc deficient diet. Synthesis was measured over the course of the experiment in untreated rats, rats receiving a single dose of MBN or six doses of MBN.

   The course of DNA synthesis in these 9 dietary-carcinogen groups was assessed according to four stages: (1) before carcinogen exposure; (2) immediately after carcinogen exposure; (3) one to two week period following stage two; and (4) chronic post dosing - months following carcinogen exposure.

   (1) **Before carcinogen exposure**

   Zinc deficiency, compared to control diet, caused significant enhancement in esophageal DNA synthesis after two weeks ($p < 0.005$) and four weeks ($p < 0.005$) of the dietary regimen -- the time immediately prior to carcinogen dosing (Table XXVII and Figure 11). Pair feeding significantly lowered synthesis at two weeks ($p < 0.05$) and lowered it at four weeks, although not significantly.

   There were no differences in DNA synthesis levels in the liver at two or four weeks (Table XXVII and Figure 12).

   The levels of synthesis attained during the four week
TABLE XXVII

INCORPORATION OF $^{3}$H-THYMIDINE (DPM/μg DNA)

**Dietary Period**

<table>
<thead>
<tr>
<th></th>
<th>Two weeks</th>
<th></th>
<th>Four weeks</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Esophagus</td>
<td></td>
<td>Esophagus</td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>63 ± 6</td>
<td></td>
<td>58 ± 7</td>
<td></td>
</tr>
<tr>
<td>PF</td>
<td>37 ± 3(^1)</td>
<td></td>
<td>38 ± 5</td>
<td></td>
</tr>
<tr>
<td>ZD</td>
<td>117 ± 10(^2)</td>
<td>152 ± 8(^2)</td>
<td>69 ± 6</td>
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</tbody>
</table>

**Four hours post dosing**

<table>
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<th>Four weeks</th>
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<th>Maximum</th>
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</thead>
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<tr>
<td></td>
<td>Esophagus</td>
<td></td>
<td>Enhancement</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td></td>
<td>(%) increase</td>
</tr>
<tr>
<td>CD</td>
<td>26 ± 3(^1) (55)</td>
<td>21 ± 6(^1) (71)</td>
<td>113 ± 8(^2), 4(193)</td>
</tr>
<tr>
<td>PF</td>
<td>22 ± 4(^1) (42)</td>
<td>26 ± 8(^1) (63)</td>
<td>93 ± 5(^3) (160)</td>
</tr>
<tr>
<td>ZD</td>
<td>18 ± 4(^1) (88)</td>
<td>24 ± 4(^1) (65)</td>
<td>152 ± 10(^2) (272)</td>
</tr>
</tbody>
</table>

\(^1\) Significantly different from control ad lib, at two weeks, p <0.05.

\(^2\) Significantly different from control ad lib, at two weeks, p <0.005.

\(^3\) Significantly different from control ad lib, at two weeks, p <0.01.

\(^4\) Significantly different from pair fed, maximum enhancement, p < 0.01.
Figure 11. Esophageal DNA synthesis in control ad lib, control pair fed and zinc deficient untreated rats 28 days before and after time of single dose.
Figure 12. Hepatic DNA synthesis in control ad lib, control pair fed and zinc deficient untreated rats 28 days before and after time of first dose.
feeding period for the three dietary groups in both the esophagus and liver were maintained throughout the course of the experiment in untreated animals with no significant fluctuations (Figures 13, and 14).

(2) Inhibition following carcinogen exposure.

Single dose. Esophageal DNA synthesis was significantly reduced in all three dietary groups 4 hours, 24 hours and 48 hours after a single dose of carcinogen (Table XXVII and Figure 15). At four hours synthesis in the zinc deficient group was reduced 87% from 152 to 18 DPMs, 55% in the control ad lib group from 58 to 26 DPMs and 43% in the pair fed group from 38 to 22 DPMs. There was no significant difference between dietary groups during this period of inhibition. The zinc deficient and pair fed groups were still significantly depressed at three days post dosing, the control ad lib diet was not.

In the liver all dietary groups were significantly depressed at 4 hours after a single dose. Beyond this time period there was no significant depression of dietary groups (Table XXVI and Figure 16).

Six doses. Esophageal DNA synthesis was significantly depressed (p < 0.01) at one and three days for all dietary groups (Figure 17). The control diet ad lib and zinc deficient diets were significantly depressed at five days; the zinc deficient group remained depressed at days 7 and 10. These levels were comparable to inhibition levels after a
**Figure 13.** Esophageal DNA synthesis in control diet ad lib, control pair fed and zinc deficient untreated rats from 1 day to 35 days after time of sixth dose.
Figure 14. Hepatic DNA synthesis in control diet ad lib, control pair fed and zinc deficient untreated rats from one to 35 days after time of sixth dose.
DPMs/µg DNA

- MBN
  • CD AD LIB
  △ CD PAIR FED
  □ ZINC DEF

DAYS AFTER SIXTH DOSE
(DAYS AFTER FIRST DOSE)
Figure 15. Esophageal DNA synthesis in control diet ad lib, control pair fed and zinc deficient rats, either untreated or after one dose MBN.
Figure 16. Hepatic DNA synthesis in control diet ad lib, control pair fed and zinc deficient rats either untreated or after single dose MBN.
Figure 17. Esophageal DNA synthesis in control diet ad lib, control pair fed and zinc deficient rats either untreated or after six doses of MBN.
single dose.

DNA synthesis in the liver was significantly depressed (p < 0.01) in all dietary groups at days 1 and 3 only post dosing (Figure 18).

(3) **Following inhibition.**

**One dose.** There was no significant enhancement of DNA synthesis in the esophagus or liver for any of the dietary groups (Figures 15 and 16) following inhibition. Esophageal synthesis in zinc deficient animals became enhanced compared to the untreated control diet group five days after a single dose (p < 0.01). This reflected a return to the enhanced level of untreated zinc deficient rats rather than a reaction specific to carcinogen treatment.

**Six doses.** Esophageal DNA synthesis in control ad lib rats was significantly enhanced from 58 DPMs basal to a maximum 113 DPMs (p < 0.005) 7 days after the sixth dose. Synthesis then dropped off but remained significantly enhanced at 10 days (p < 0.05) before returning to basal levels (Figure 17, table XXVII). Control pair feeding significantly increased synthesis from 38 DPMs to a maximum 93 DPMs (p < 0.01) at 14 days after the sixth dose. At 21 days, the next measurement, synthesis had returned to untreated values (Figure 17). Synthesis in the zinc deficient group became significantly enhanced (p < 0.05) compared to untreated control diet levels at day 14.
Figure 18. Hepatic DNA synthesis in control diet ad lib, control pair fed or zinc deficient rats either untreated or after six doses MBN.
There was no significant enhancement of hepatic DNA synthesis in any of the dietary groups, although levels did increase above untreated values, reaching a peak at day 14 for all three groups (Figure 18). Hepatic synthesis was enhanced a maximum of 15%, 9% and 31% for the control ad lib, control pair fed and zinc deficient groups, respectively, compared to 193%, 160% and 272% for esophageal synthesis.

(4) **Chronic Post Dosing.**

DNA synthesis in the control diet ad lib and control diet pair fed groups remained at basal levels for remainder of experiment after dropping from enhanced levels. In carcinogen treated zinc deficient rats synthesis returned to the enhanced levels of the untreated zinc deficient group 10 days after a single dose and 28 days after the six dosing regimen (Figures 15, 17 and 19). Figure 19 compares difference in pattern of return to enhanced levels after a single does and six doses in the zinc deficient group. Once enhanced, this enhancement continued for the remainder of the experiment.

b. **Incorporation of ^3H^,-thymidine - Autoradiography**

Autoradiographic slides from 5 animals on control ad lib diet and 5 animals on zinc deficient diet were counted for labeling at three different time points: 4 weeks on diet (immediately before dosing), 4 hours after a single dose and 7
Figure 19. Esophageal DNA synthesis after one and six doses of MBN in zinc deficient rats.
days after 6 doses. 1,000 cells from each esophagus were counted. A nucleus containing 5 or more grains was considered labeled. The results, shown in Table XXVIII, are expressed as labeled nuclei per 100 cells. After four weeks on diet there were approximately 2.5 times more cells labeled in the zinc deficient group than in the control diet group (23.6 vs. 9.8), comparable to the relationship in the scintillation counted results (Table XXVII, 117 + 10 vs. 63 ± 6 DPM/µg DNA). 4 hours post dosing reduced labeled cells in both groups to 4.1 ± 1.9 control diet and 6.8 ± 1.6 zinc deficient diet. At 7 days after the sixth dose the control diet had a labeling index of 16.7 ± 3 compared to 6.3 ± 2 in the zinc deficient group. This finding was consistent with results of the scintillation study which showed control diet significantly enhanced to almost twice untreated levels and zinc deficient synthesis inhibited.

c. Mitotic Counts

Mitotic figures were counted by the same procedure as autoradiographically labeled cells. Results (Table XIX) are expressed as mitotic figures per 100 cells counted. The relationship of mitotic figures at four weeks on diet was consistent with that seen in scintillation counting and autoradiography - 6.9 ± 3 in zinc deficient esophagi vs. 2.1 ± 1.5 in control diet, a ratio of 3:1. At four hours after a single dose mitotic figures were 1.1 ± 0.8 in control diet vs. 1.7 ± 0.9 in zinc deficient rats. Seven days after six doses there were 5.8 ± 1.6 mitotic figures in control diet rats vs.
## TABLE XXVIII

### AUTORADIOGRAPHY

<table>
<thead>
<tr>
<th></th>
<th>Four weeks</th>
<th>Four hours post single dose</th>
<th>7 days post six doses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Diet</td>
<td>9.8 ± 4(^1)</td>
<td>4.1 ± 1.9(^3)</td>
<td>16.7 ± 3(^5)</td>
</tr>
<tr>
<td>Zinc Deficient Diet</td>
<td>23 ± 5(^2)</td>
<td>6.8 ± 1.64(^4)</td>
<td>6.3 ± 2</td>
</tr>
</tbody>
</table>

\(^1\) Labelled/100 cells, 1000 cells counted per each of 5 animals; 5 grains or more per nuclei considered labeled.

\(^2\) Significantly different from control diet, same time pt. p < 0.01.

\(^3\) Significantly different from control diet at four weeks, p < 0.05.

\(^4\) Significantly different from zinc deficient diet, four weeks p < 0.005.

\(^5\) Significantly different from control diet, four weeks p < 0.01.
### TABLE XXIX

**MITOTIC COUNTS**

<table>
<thead>
<tr>
<th></th>
<th>Four weeks</th>
<th>Four hours post single dose</th>
<th>7 days post six doses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Diet</td>
<td>2.1 ± 1.51</td>
<td>1.1 ± 0.8</td>
<td>5.8 ± 1.63</td>
</tr>
<tr>
<td>Zinc Deficient Diet</td>
<td>6.9 ± 3</td>
<td>1.7 ± 0.92</td>
<td>2.1 ± 1.32</td>
</tr>
</tbody>
</table>

1 Mitotic Figures per 100 cells counted; 1000 counted per each of five animals.

2 Significantly different from zinc deficient diet at four weeks, p < 0.01.

3 Significantly different from control diet at four weeks, p < 0.05.
2.1 ± 1.3 in zinc deficient.

These data indicate that scintillation counting was an accurate and valid method of assessing DNA synthesis.

2. Binding of Methylbenzylnitrosamine to DNA

a. Binding of Methyl[\textsuperscript{14}C-benzyl]nitrosamine

This labeled compound was tested at a number of different dosage levels and time points as described in Methods section. Radioactivity was not detected in any of these tests indicating that either the benzyl label of the compound was not binding to DNA or that it was binding at a level below the sensitivity of the techniques and equipment employed, i.e., that the binding was at a level that emitted fewer DPMs than were detected as background. With 120-150 μg DNA, yielding 25-30 μg guanine, and a carcinogen with a specific activity of 20 mci/mm, there would have to be a minimum binding level of 2.5-3.5 adducts/6x10\textsuperscript{6} to produce cpms above the 20 cpms background. This experiment was not pursued further.

b. Binding of [\textsuperscript{14}C methyl]benzylnitrosamine

(1) Pilot study.

Six 300 gram rats on control diet were injected i.p. with 2.5 mg/kg body weight [\textsuperscript{14}C-methyl]benzylnitrosamine.
Rats were killed four hours later and esophagus was processed to obtain DNA. DNA was fractionated in 32 0.375 ml aliquots which were brought up to 1 ml with deionized water. Corresponding fractions from three animals were pooled to provide 3 ml for each fraction. These fractions were dialyzed to remove CsCl, and one ml was counted with addition of scintillation fluid. Fractions of the other three animals were pooled into six larger fractions (fractions 1-5, 6-10, 11-16, 17-22, 23-27, 28-32). Corresponding pools for the three rats were combined, dialyzed and counted.

Significant levels of detectable radioactivity were present exclusively in the DNA peaks of the samples (Figures 20 and 21). This indicated the likelihood of binding of the labeled compound to DNA at levels that could be measured by the experimental protocol.

(2) HPLC technique to separate adducts.

An HPLC technique was developed to separate adduct N-7 methylguanine, O6-methylguanine and 3-methyladenine from each other and from guanine and adenine. Using a Biorad cation exchange column, 500 mM ammonium formate pH 3.9, 57°C at flow rate of 0.7 ml/min these adducts were successfully separated with a minimum separation of two minutes or 1.4 ml between end of one peak and start of another. The entire run lasted approximately 120 minutes (Figure 22).

A solution containing known amounts of authentic standards was processed from the hydrolysis stage through the HPLC
Figure 20. Optical density and DPM's of DNA gradient from three control diet rats given 2.5 mg/kg $^{14}$C-methyl] benzyl nitrosamine (S.A. 20 mci/mm).
Figure 21. Optical density and DPM's of six pooled fractions of DNA gradient from three control diet rats given 2.5 mg/kg $[^{14}\text{C}-\text{methyl}]$ benzylnitrosamine (S.A. 20 mci/mm).
**Figure 22.** HPLC chromatogram of authentic standards guanine, adenine, 7-methyl guanine, O\(^6\)-methylguanine and 3-methyladenine. In 500 mM ammonium formate, pH 3.9, 57°C, flow rate 0.7 ml/min.
procedure to determine losses of compounds either due to instability or transfer techniques. Maximum losses were approximately 6.5% and were consistent for each compound, indicating no significant or preferential loss.

(3) Effects of carcinogen dose on binding levels

18 rats on control diet for one month were divided into 3 groups of six and given a single i.p. dose of [14C-methyl] benzyl nitrosamine of 1.5, 2.5, or 3.5 mg/kg body weight (S.A. 20 mci/mm). DNA of rats of each group was pooled and measured for amounts of O6-methyl guanine (Table XXX; figure 23) and N7 methyl guanine (Table XXX; figure 24). Both adducts increased linearly, suggestive of a dose-response relationship. There was approximately 10 times more N-7 methylguanine than O6-methylguanine.

(4) In Vitro adduct Decline

A group of rats was injected with [14C-M]BN and sacrificed 4 hours later. The DNA was isolated through the dialysis stage and then divided into three groups: one that was processed immediately, another that was maintained in pH 7.0 buffered solution at 37°C for 24 hours and processed and a third that was maintained in pH 7.0 buffered solution at 37°C for 48 hours and then processed. Levels of N-7 methyl guanine and O6-methylguanine were compared to those of animals sacrificed 4 hours, 24 hours or 48 hours after receiving a
**TABLE XXX**

**DOSE RESPONSE OF N-7 METHYLGUANINE AND O\textsuperscript{6}-METHYLGUANINE**\(^1\)

<table>
<thead>
<tr>
<th>Dose MBN mg/kg body weight</th>
<th>N-7 methylguanine</th>
<th>O\textsuperscript{6}-methylguanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>153</td>
<td>10</td>
</tr>
<tr>
<td>2.5</td>
<td>262</td>
<td>26</td>
</tr>
<tr>
<td>3.5</td>
<td>330</td>
<td>34</td>
</tr>
</tbody>
</table>

\(^1\) Adducts/G x 10\(^6\)
Figure 23. Dose response of O\textsuperscript{6}-methylguanine to 1.5, 2.5 and 3.5 mg/kg body weight MBN.
Figure 24. Dose response of N-7-methylguanine to 1.5, 2.5 and 3.5 mg/kg body weight.
single dose. As Table XXXI and Figure 25 shows, there was very little decline in adduct levels in vitro compared to the decline of adduct levels in vivo, indicating that the decline was due to an active in vivo process rather than a simple falling off or breakdown of DNA.

(5) **Carcinogen bind** to DNA after single dose of MBN

In esophageal DNA 7-methylguanine was bound at a level of 411 adducts/guanine x 10^6 in zinc deficient animals compared to 286 adducts/guanine x 10^6 in control diet rats (Table XXXII). O^6^-methyl guanine also bound at a higher level in zinc deficient rats than controls (55 adducts/guanine x 10^6 compared to 31 adducts/guanine x 10^6) (Figures 26 and 27). Pair feeding reduced the levels of both of these adducts as compared to control diet ad lib to 228 x 10^6 adducts/guanine and 23 adducts/guanine x 10^6 for 7-methylguanine and O^6^-methylguanine, respectively. Levels of both adducts in zinc deficient esophageal DNA were significantly different from levels in the pair fed group, p < 0.01. As the ratio of O^6^-:7-methylguanine shows (Table XXXII) the amount of the promutagenic base O^6^-methylguanine was greater in the zinc deficient group compared to the control diet groups on this relative basis as well as on an absolute basis.

3-methyladenine levels were greater in the zinc deficient group showing a pattern similar to that of the two other adducts. As the last two columns of Table XXXII show there
### TABLE XXXI

**IN VITRO AND IN VIVO DECLINE OF ADDUCTS**

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>N-7 methylguanine</th>
<th></th>
<th>O-6-methylguanine</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>In vitro</strong></td>
<td><strong>In vivo</strong></td>
<td><strong>In vitro</strong></td>
<td><strong>In vivo</strong></td>
</tr>
<tr>
<td>4</td>
<td>400</td>
<td>400</td>
<td>36</td>
<td>36</td>
</tr>
<tr>
<td>24</td>
<td>380</td>
<td>200</td>
<td>36</td>
<td>14</td>
</tr>
<tr>
<td>48</td>
<td>360</td>
<td>110</td>
<td>35</td>
<td>4</td>
</tr>
</tbody>
</table>

1 Adducts/G x 10^6
Figure 25. *In vitro* and *in vivo* decline of N-7-methylguanine and O6-methylguanine over 48 hour period.
### TABLE XXXII

**SINGLE DOSE OF 14C-MBN**

<table>
<thead>
<tr>
<th>Adducts / G x 10^6</th>
<th>7-meG</th>
<th>O6-meG</th>
<th>O6-meG</th>
<th>3mA</th>
<th>Guanine dpm/μm</th>
<th>Adenine dpm/μm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Esophagus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD (1641)</td>
<td>286.5</td>
<td>31.5</td>
<td>0.11</td>
<td>14</td>
<td>680</td>
<td>385</td>
</tr>
<tr>
<td>PF (869)</td>
<td>228 + 31</td>
<td>23.0 + 7</td>
<td>0.10</td>
<td>16</td>
<td>590</td>
<td>300</td>
</tr>
<tr>
<td>ZD (1605)</td>
<td>411 + 33</td>
<td>55.5 + 6</td>
<td>0.135</td>
<td>37</td>
<td>840</td>
<td>465</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>91</td>
<td>6.0</td>
<td>0.065</td>
<td>1.5</td>
<td>395</td>
<td>187</td>
</tr>
<tr>
<td>PF</td>
<td>82</td>
<td>2.6</td>
<td>0.031</td>
<td>ND</td>
<td>340</td>
<td>148</td>
</tr>
<tr>
<td>ZD</td>
<td>99</td>
<td>4.8</td>
<td>0.048</td>
<td>1.0</td>
<td>308</td>
<td>119</td>
</tr>
</tbody>
</table>

1 dpm

2 Significantly different from control pair fed, p < 0.01.

ND not detectable
Figure 26. HPLC chromatography of hydrolysed DNA from 4 control diet rats.
Figure 27. HPLC chromatograph of hydrolysed DNA from 6 zinc deficient rats that received 2.5 mg/kg body weight \(^{14}\text{C-methyl} \text{benzyl nitrosamine.}\)
was biosynthetic incorporation of the $^{14}$C-methyl group into guanine and adenine.

Levels of 7-methyl guanine were 3-4 times lower in the liver than the esophagus; levels of $O^6$-methylguanine were 5-10 times lower, indicating a much lower relative level of $O^6$-methylguanine as reflected by the ratios that were one-third to one-half those in the esophagus. There were no clear cut differences between dietary groups.

(6) Carcinogen binding after six doses MBN

The levels of 7 methylguanine, $O^6$-methyladenine and 3 methyl adenine were examined in the three dietary groups at 4 hours, 24 hours and 48 hours after the sixth dose (third labeled dose) of MBN. In the esophagus the levels of both 7-methylguanine and $O^6$-methylguanine for all three dietary groups increased at four hours (Table XXXIII) compared to four hours after a single dose. 7-methylguanine mG increased in a range from 25% - 36% and $O^6$-methylguanine from 6% - 15%, the greatest increase for both adducts in the zinc deficient group.

This greater increase in N-7 methylguanine compared to $O^6$-methylguanine is reflected in the reduced ratios $O^6$:N-7 at 4 hours after the last dose compared to four hours after a single dose. The ratio dropped more in the control diet groups than the zinc deficient group.

At both 24 and 48 hours $O^6$-methylguanine decreased at a more rapid rate than 7 methylguanine for all three dietary
TABLE XXXIII

ESOPHAGUS-SIX DOSES MBN (LAST 3 LABELED)

Adducts / G x 10^6

<table>
<thead>
<tr>
<th></th>
<th>7-meG</th>
<th>O6-meG</th>
<th>O6:7-meG</th>
<th>3meA</th>
<th>Guanine dpm/μm</th>
<th>Adenine dpm/μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 hr.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>360</td>
<td>33.3</td>
<td>0.09</td>
<td>17</td>
<td>2200 (↑223)</td>
<td>540 (↑40)</td>
</tr>
<tr>
<td></td>
<td>(↑25)*</td>
<td>(↑6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PF</td>
<td>303</td>
<td>26</td>
<td>0.086</td>
<td>11</td>
<td>1645 (↑178)</td>
<td>390 (↑30)</td>
</tr>
<tr>
<td></td>
<td>(↑33)</td>
<td>(↑13)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZD</td>
<td>558</td>
<td>64</td>
<td>0.114</td>
<td>44</td>
<td>1930 (↑130)</td>
<td>510 (↑10)</td>
</tr>
<tr>
<td></td>
<td>(↑36)</td>
<td>(↑15)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 hr.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>158</td>
<td>11.3</td>
<td>0.07</td>
<td>7</td>
<td>2910 (↑32)</td>
<td>460 (↑18)</td>
</tr>
<tr>
<td></td>
<td>(↓56)</td>
<td>(66)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>PF</td>
<td>118</td>
<td>7.2</td>
<td>0.06</td>
<td>3</td>
<td>1910 (↑16)</td>
<td>420 (↑8)</td>
</tr>
<tr>
<td></td>
<td>(↓61)</td>
<td>(72)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>ZD</td>
<td>284</td>
<td>28</td>
<td>0.098</td>
<td>14</td>
<td>2318 (↑20)</td>
<td>535 (↑5)</td>
</tr>
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<td></td>
<td>(↓49)</td>
<td>(55)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>48 hr.</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CD</td>
<td>63</td>
<td>2.7</td>
<td>0.043</td>
<td>ND</td>
<td>3590 (↑23)</td>
<td>585 (↑127)</td>
</tr>
<tr>
<td></td>
<td>(↓60)</td>
<td>(↓76)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PF</td>
<td>41</td>
<td>ND</td>
<td>--</td>
<td>ND</td>
<td>2290 (↑20)</td>
<td>460 (↑9)</td>
</tr>
<tr>
<td></td>
<td>(↓66)</td>
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</tr>
<tr>
<td>ZD</td>
<td>123</td>
<td>8.6</td>
<td>0.07</td>
<td>2</td>
<td>2735 (↑18)</td>
<td>550 (↑3)</td>
</tr>
<tr>
<td></td>
<td>(↓57)</td>
<td>(↓69)</td>
<td></td>
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</tr>
</tbody>
</table>

* percent increase or decrease from previous measurement

ND not detectable
groups. This is reflected in decreasing $O^6:N-7$ methylguanine ratios. Both 7-methylguanine and $O^6$-methylguanine levels dropped slowest in the zinc deficient group over the two time periods and fastest in the pair feeding group. This was also reflected in the $O^6 : N-7$ ratios. From 4 hours to 24 hours the ratio dropped 14% in the zinc deficient group, 22% in the control diet group and 30% in the pair fed group. From 24 hours to 48 hours the ratio dropped 28% in the zinc deficient group and 38% in the control diet group.

Radioactivity incorporated into guanine and adenine increased the most from 4 hours after the first dose to four hours after the sixth dose. This provided the greatest time for biosynthetic incorporation. Further increases were seen at 24 and 48 hours.

In the liver, 7 methylguanine and $O^6$-methylguanine levels increased at similar rates from 4 hours after a single dose to 4 hours after the sixth dose, so that $O^6:N-7$ methylguanine ratios did not change appreciably (Table XXXIV). There was no clear cut difference between dietary groups at this time point or at 24 or 48 hours after dosing.

The drop in adduct levels at 24 and 48 hours showed no consistent pattern. The pair feeding group showed the greatest drop in 7-methylguanine levels from 4 to 24 hours (63%), the zinc deficient group showed the least drop (45%). From 24 to 48 hours the zinc deficient group dropped the most (68%) compared to the control diet group which dropped the least (45%).

In the liver, $O^6$ methylguanine levels became undetectable
<table>
<thead>
<tr>
<th></th>
<th>7-meG</th>
<th>O6-meG</th>
<th>O6-7-meG</th>
<th>3meA</th>
<th>Guanine dpm/μm</th>
<th>Adenine dpm/μm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>4 hr.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>120</td>
<td>8</td>
<td>0.067</td>
<td>2</td>
<td>435</td>
<td>205</td>
</tr>
<tr>
<td></td>
<td>(↑30)</td>
<td>(↑25)</td>
<td></td>
<td></td>
<td>(↑20)</td>
<td>(↑10)</td>
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<tr>
<td>PF</td>
<td>104</td>
<td>4</td>
<td>0.038</td>
<td>2</td>
<td>391</td>
<td>164</td>
</tr>
<tr>
<td></td>
<td>(↑27)</td>
<td>(↑54)</td>
<td></td>
<td></td>
<td>(↑15)</td>
<td>(↑11)</td>
</tr>
<tr>
<td>ZD</td>
<td>113</td>
<td>5</td>
<td>0.044</td>
<td>1</td>
<td>347</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td>(↑14)</td>
<td>(↑.4)</td>
<td></td>
<td></td>
<td>(↑13)</td>
<td>(↑8)</td>
</tr>
<tr>
<td><strong>24 hr.</strong></td>
<td></td>
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* percent increase or decrease from previous measurement

ND not detectable
(below approx. 2-25 adducts/G x $10^6$) at 24 hours in the pair fed group and at 48 hours in the other two groups. This compared to an undetectable level of this adduct in the esophagus in only the pair fed group at 48 hours.
V. DISCUSSION

A. TUMOR STUDIES

These studies demonstrated that zinc deficiency, whether caused by 7 ppm zinc in a soy protein based diet or 3 ppm zinc in an egg white based diet, significantly enhanced esophageal tumor incidence in rats exposed to the esophagus-specific carcinogen methylbenzylnitrosamine (MBN). Tumor incidence was increased from a range of 31% - 40% in control ad lib diet to 75% - 88% in the zinc deficient diet. Tumor enhancement was evidenced at 4, 8, 12 and 29 weeks post dosing. The height-ened sensitivity of zinc deficient animals to MBN carcinogenicity was also shown by the increase in tumor incidence from 75% in the first study to 88% in the last two studies in response to an increased dosage of MBN from 2.0 mg/kg body weight to 2.5 mg/kg body weight. No change was seen in control diet rats.

Pair feeding control diet animals to the reduced food intake of zinc deficient animals significantly decreased tumor incidence to approximately half the level found in control diet ad libitum animals, from a range of 39%-31% to 16%-15%. This clearly indicated that reduced food intake characteristic of zinc deficient rats was not the cause of tumor enhancement, but rather exerted an opposite effect.

To better understand whether the tumor enhancing effect
of zinc deficiency was limited to the dosing stage or to the post dosing stage, rats were switched after dosing from a zinc deficient diet to a control ad lib diet or from a control ad lib diet to a zinc deficient diet. In each of the three studies in which one or both of these switches was made, tumor incidence in these switched groups was between that of control diet throughout and zinc deficient diet throughout, indicating that zinc deficiency during each of these stages contributed to the overall enhancement of zinc deficiency.

The similarity of the tumor incidences for both switched groups in the third study, 62% and 67%, provided the clearest evidence of this. Nevertheless, the relative contribution of the two periods to the overall enhancement is unclear.

The tumor enhancing effect of zinc deficiency, applied only up to and through the dosing period, was greatly affected by length of time on control diet during the post dosing period. In rats on zinc deficient diet for 8 weeks (4 weeks feeding, 4 weeks dosing) followed by 21 weeks of control ad lib diet, tumor incidence, although between that of control diet throughout and zinc deficient diet throughout, (51% vs 40% and 75%) was not significantly different from control diet. In the second study, in which 7 weeks on zinc deficient diet was followed by only 8 weeks on control diet, tumor incidence, although again in between that of the two diets throughout, (75% vs. 39% and 88%) was this time not significantly different from zinc deficient diet throughout. In the third study, in which 7 weeks on zinc deficient diet was followed by 12 weeks on control diet -- a post dosing time
period in between the 21 weeks of study one and 8 weeks of study two --tumor incidence was significantly greater than control diet throughout and significantly less than zinc deficient diet throughout.

As the time on the post dosing control diet, which followed the zinc deficient diet, was increased from 8 to 12 to 21 weeks, tumor incidence changed from a level approximating that of the zinc deficient diet throughout to one of the control diet throughout. This suggested that zinc deficiency was capable of establishing a primaly effect on tumor incidence during the dosing stage, and that the expression of the effect could be greatly altered by length of time on the control diet that followed. In the zinc deficient-throughout group, the continuation of zinc deficiency after dosing may have added little to tumor enhancement other than "protecting" or allowing complete expresion of the enhancement already established during dosing (which would not occur if a switch to control diet took place for a long enough period of time).

This type of effect is analogous to the classic initiation-promotion model established in the skin with DMBA-croton oil (Bereblum, 1959; Frei et al, 1964). Initiation is defined as events that cause irreversible genetic change which may or may not be expressed at a later time. Promotion is defined as events which follow initiation, which are reversible and which can enhance or trigger (alter) expression of genetic damage. In these studies zinc deficiency exerted an effect analagous to an initiator, that
effect expressed to the extent that a subsequent control diet was applied, or not applied.

Our protocol, however, does not actually fit an initiation-promotion scheme. The multiple dosing schedule over a number of weeks involves an overlap of initiation and promotion events, so that the two stages -- and effects on those stages -- cannot be clearly separated. This may be a reason for the powerful effect of zinc deficiency during this period; it actually affects both stages. But to the extent that hyperplasia -- enhanced DNA synthesis -- is the central promotional event, the dosing regimen of our studies effectively eliminates this until after dosing so that these two phases may be separated. This is discussed further in a following section. Whatever the effect of zinc deficiency during the dosing phase, it is clear that zinc deficiency applied during only the post dosing phase significantly enhances tumor incidence. This effect likely involves some aspect of tumor growth.

Zinc excess applied during either of these stages did not inhibit tumor incidence, which indicated that zinc did not possess anti-carcinogenic properties for this tumor system and that, therefore, the enhanced tumor incidence of a zinc deficient state could not be due to the absence of these properties. Zinc excess did exert a protective effect by slightly reducing the total number of tumors in the group with a similar tumor incidence, and reducing the number of esophagi with multiple tumors, but this effect would not account for the dramatic enhancement of tumor incidence in the zinc deficient
state. An alternative explanation is that the deficiency created a condition that enhanced tumor incidence. This would explain why sufficient zinc would lower tumor incidence from the deficient state and that additional zinc would offer no further protection.

Zinc deficient-induced esophageal hyperplasia is one condition that fits the data for two reasons: (1) hyperplasia is known to enhance tumor incidence; and (2) this effect of zinc deficiency is unique to the esophagus, the only tissue displaying tumor enhancement. In all other tissues zinc deficiency causes hypoplasia and tumor inhibition (McQuitty et al., 1970; Dewys and Pories, 1972; Poswillo and Cohen, 1970; Duncan et al., 1974; and Duncan and Dreositi, 1975). What appears to be a discrepancy in tumor data between our studies and those of others is quite consistent in terms of hyperplasia. The specific effects of this condition are discussed in a following section.

Alcohol consumption, a major etiological factor in esophageal carcinogenesis, was examined to compare its effects to those of zinc deficiency. Ethanol provided in the drinking water at levels of 4% or 10% consistently enhanced tumor incidence but not to a significant degree as in the zinc deficient groups. In combination with either control or zinc deficient diets ethanol consumption enhanced tumor incidence approximately 10% above the levels of the diets alone. Ethanol applied before and during dosing led to no enhancement in tumor incidence, suggesting that its enhancing effects occurred during the post dosing period. Animals consumed additional calories
from ethanol at the expense of reduced food intake, to maintain the level of calories consumed. This decreased nutrient:calorie ratio was a confounding factor that may have affected tumor production. It would be worthwhile to provide a liquid diet containing ethanol to better control the level of intake of all other nutrients in relation to calories.

The effect of the synthetic retinoid, 13-cis retinoic acid, on tumor growth was examined in rats at highest risk — on a zinc deficient diet and consuming ethanol. Other studies (Sporn et al., 1976; Squire et al., 1977) had demonstrated an ability of this retinoid to inhibit or reverse tumor growth. In our studies an opposite effect was observed. Adding this to the diet of these high risk rats after completion of dosing increased tumor incidence and caused greater disturbance of the esophageal epithelium including hyperplasia, hyperkerotis and more massive tumors that coalesced. Whether this effect was unique to esophageal carcinogenesis, to zinc deficiency or ethanol consumption or a combination of these factors is unclear and requires further examination.

In addition to enhancing total tumor incidence which consisted primarily of papillomas, zinc deficiency enhanced the number of carcinomas, which are considered a more advanced form of the disease. The percentage of tumors in each of the three studies that were carcinomas was approximately twice as great in the zinc deficient groups as in the control diet groups — 10%, 6% and 8% vs. 6%, 3% and 3%. Because the incidence of carcinomas increased within each dietary group with increasing length of the study, the higher incidence of car-
cinomas in the zinc deficient group can be viewed as an acceleration of this process.

Zinc deficient animals had more multiple numbers of tumors per rat than control; excess and pair fed rats had fewer high multiple tumors per rat. Zinc deficient rats also had larger tumors than control diet rats. Taken together this data indicates that zinc deficiency enhances tumor incidence, accelerates the carcinogenic process and increases both the number and size of tumors.

One possible reason for the enhancing effects of zinc deficiency was its proposed ability to induce a deficiency of vitamin A, a nutrient shown to have protective effects in other organs (Saffioti et al., 1967; Port et al., 1975; Cone and Nettlesheim, 1973; Nettlesheim et al., 1976; Smith et al., 1975). No significant deficiency developed in these studies which was consistent with the findings of others (Smith et al., 1976; Carney et al., 1976). Furthermore, the addition of 13-cis retinoic acid to the diet enhanced rather than inhibited tumorogenesis.

There was no significant effect of diet on the P-450 enzyme p-nitroanisol demethylase. This does not, however indicate that a zinc deficient diet might not affect relevant enzymes. We tested this enzyme in the liver. There may be other enzymes, either in the liver or specific to the esophagus, (Hodgson et al., 1980; Schweinsberg and Schott-Kollat, 1976) that might be affected by zinc deficiency.
B. Biochemical Studies

1. DNA Synthesis

The effects of the three diets, control ad lib, control pair fed and zinc deficient, on DNA synthesis in the esophagus (target organ) and liver (non target organ) was examined over the course of the carcinogenic process. During the first stage, the 4 week feeding period prior to carcinogen exposure, zinc deficiency significantly enhanced DNA synthesis; pair feeding significantly depressed DNA synthesis at 2 weeks and depressed it, but not significantly, at 4 weeks. These results correlated to subsequent tumor production. Enhanced synthesis can increase the vulnerability of cells and DNA to carcinogenic assault (Bereblum, 1959; Frei et al., 1964; Warwick, 1971). A decrease in synthesis, as seen in the pair fed group, which had the lowest tumor incidence, may therefore decrease the vulnerability of DNA to carcinogenic assault. There was no difference in hepatic synthesis between dietary groups.

Stage two examined DNA synthesis immediately after exposure to either 1 or 6 doses of MBN. Most carcinogens are capable of inhibiting DNA synthesis, but carcinogens or cocarcinogens specific for a certain tissue usually cause a greater inhibition than nontarget tissue carcinogens (Slaga et al., 1974). Mirvish (1982) has shown a strong correlation between esophageal specificity of carcinogens and ability to inhibit DNA synthesis. His studies showed that 10 times as much non-
esophageal carcinogen was required to inhibit DNA synthesis to the same degree as an esophageal carcinogen. Mirvish concluded that DNA inhibition in the esophagus could be used as a short term assay for determining the specificity of carcinogens or cocarcinogens for the esophagus.

The patterns of inhibition in these studies were consistent with these principals, based upon the tumor incidences. Synthesis was more depressed in the high tumor zinc deficient group than the control diet groups; synthesis was more depressed within each dietary group after 6 doses (carcinogenic) of MBN than after a single (non-carcinogenic) dose; and synthesis was more inhibited in the target tissue (esophagus) than the non target tissue (liver). The greater inhibition of DNA synthesis from zinc deficiency compared to control diet was consistent with the greater inhibition within the control diet group from a carcinogenic dose of MBN than from a single dose or the greater inhibition in the tumorigenic esophagus compared to the nontumorigenic liver. The action of zinc deficiency was analogous to carcinogenic or cocarcinogenic effects as shown in Mirvish's work and the patterns of DNA synthesis of this study. Because there was no enhanced cell death in the zinc deficient group compared to the other groups, this greater inhibition might be due to more carcinogen binding. This was given support in the binding studies, discussed in the following section.

The third stage examined DNA synthesis following inhibition. Complete carcinogens often enhance DNA synthesis after the inhibition. This enhancement is considered a potentially
integral part of the carcinogenicity of a compound since it can prevent adequate or accurate repair of lesions, thereby "fixing" the damage to DNA (Lewis and Swenberg, 1982). Therefore, not only the presence of enhancement, but its intensity and timing might be crucial to the carcinogenic potential of a compound.

There was no enhancing of DNA synthesis in either the esophagus or liver after a single (non-carcinogenic) dose of MBN for any of the dietary groups. After six doses esophageal DNA synthesis became significantly enhanced in control diet rats 7 days after dosing and in pair fed and zinc deficient rats 14 days after dosing. There was a slight but insignificant enhancement of synthesis in the liver. That this enhancement occurred after a carcinogenic dose but not after a single (non carcinogenic) dose and that it occurred in the target organ (esophagus) but not in the non-target organ (liver) was consistent with the thesis that DNA synthesis enhancement is an integral component of the carcinogenic potential of a chemical.

In this regard, the timing of enhancement in the control ad lib and control pair fed groups was consistent with tumor incidence levels. The pair fed group, producing half the tumor incidence of the control ad lib group, showed DNA synthesis enhancement a week after that of the control ad lib group. This meant that this group had an additional week for DNA repair - removal of promutagenic damage - which would be consistent with reduced tumor incidence.

This relationship did not exist for the zinc deficient
group, which had the highest tumor incidence but which also became significantly enhanced one week after the control ad lib group, the same time as the pair-fed group (with the lowest tumor incidence). There may be two reasons for this effect. First, although both groups became significantly enhanced at the same time, synthesis in the zinc deficient group was significantly inhibited up to the time of the enhancement, whereas synthesis was only inhibited for three days after dosing in the pair fed group and was then normal for 11 days prior to enhancement. To the extent that inhibition reflected carcinogen-DNA interactions, there was more bound carcinogen in the zinc deficient group than the pair fed group at the time of enhancement. There was also less time between significant inhibition and significant enhancement in the zinc deficient group than the pair fed group. This time period may be more important than the time between actual exposure and enhancement since carcinogens can bind to and inactivate proteins, including repair enzymes, as well as DNA, and so during the period of significant inhibition little repair would take place. The pair fed group had 11 days of normal cellular function prior to the critical enhancement.

The control ad lib diet had a lag time between significant inhibition and enhancement similar to the zinc deficient diet -- and so shorter than the pair fed group -- but less inhibition than the zinc deficient group, indicative of lower binding levels (and so less damage could be "fixed"). This would place control ad lib between the pair fed group and the zinc deficient group, consistent with the order of tumor inci-
dence levels.

A second explanation for the difference in tumor incidence of zinc deficient and pair fed animals which experience similar times of synthesis enhancement could be seen in the fourth stage of the carcinogenic process. Whereas the enhancements in the two control diet groups peaked quickly and then returned to the level of untreated controls, the 14 day zinc deficient enhancement -- which was an enhancement in relation to the level of untreated controls but an actual inhibition compared to levels of untreated zinc deficient rats -- continued to return to the level of enhancement characteristic of untreated zinc deficient animals, and remained at that level. Such increased DNA synthesis, not found in the pair-fed group, might enhance growth of tumors. It is unclear what would happen with DNA synthesis in zinc deficient rats switched to control diet after dosing since this was not examined.

Taken together these results showed a pattern of DNA synthesis that would explain an enhancing effect of zinc deficient diet and an inhibiting effect of control pair fed diet on this carcinogenic process.

One concern in the determination of DNA synthesis through the incorporation of $^3$H-thymidine was the accuracy of this method as indicative of de novo synthesis. As mentioned by Maurer (1981) incorporation of exogenous thymidine is a minor pathway for supply of thymidine compared to the salvage pathway; many conditions and agents can affect transport and incorporation of this source, and so indicate a change in synthesis, without actually affecting a change.
Although most of these potential hazards were not involved in our study, we ascertained the validity of scintillation counting by counting mitotic figures. This method indicated DNA synthesis independent of any label. As table XXIX in the Results section indicates, results from scintillation counting was consistent with mitotic counts, under a variety of conditions. This indicated that this was a valid measure of DNA synthesis.

2. Carcinogen Binding to DNA

Methylbenzylnitrosamine is considered the most potent of a class of asymmetrical nitrosamines that specifically induce esophageal tumors. Because of its asymmetry this carcinogen is capable of producing two different ultimate alkylating species: a benzylating cation and a methylating cation. There is no evidence to support significant metabolism to the benzylating species or binding of that group to esophageal DNA (Hodson et al., 1981). Our studies supported this finding. However, this does not preclude the possibility that some binding did take place (Chin, unpublished) which was beyond the sensitivity of our techniques and equipment. And it is possible that these minute levels of binding could be a significant factor in esophageal carcinogenesis.

Binding of the methyl cation to esophageal DNA has been shown in a number of studies (Fong, et al., 1978; Kraft and Tannenbaum, 1980; Hodgson et al., 1980). These studies demonstrated that in the esophagus, levels of the promutagenic
base O⁶-methylguanine, as well as N-7-methylguaine, were higher in the target tissue esophagus than in other nontarget tissues such as lung and liver. The dose response study we performed showed that higher levels of MBN -- which have already been shown to produce higher tumor incidences -- caused higher levels of both adducts. The other studies also showed the O⁶-methyl:7-methyl-guanine ratios were higher in the esophagus, further underlining a specificity of effect. O⁶-methylguanine has often been sited as the promutagenic adduct of significance in carcinogenesis (Pegg, 1977; Loveless and Hampton, 1969; Margison and Kleihuus, 1975). Although other more minor adducts may be involved, levels of O⁶-methylguanine, the ratio of O⁶-methylguanine: 7-methylguanine, and its slower decrease over time are all consistent with MBN esophageal carcinogenesis and suggest mechanisms by which this carcinogen -- and cofactors that affect this carcinogen -- may act.

The findings in our studies, including the effects of the different diets, were consistent with those of the other studies and pointed to the importance of O⁶-methylguanine in esophageal carcinogenesis in general and in the enhancing effects of the zinc deficient diet in particular.

Four hours after a single labeled dose of MBN there were significantly higher levels of both N-7- and O⁶-methylguanine in the esophagus than in the liver. Levels in the liver for both adducts did not vary much between dietary groups whereas in the esophagus adduct levels correlated to tumor incidence levels.
Adduct levels in the esophagus were lowest in the pair fed group and highest in the zinc deficient group. Although levels for both adducts were lower in the pair fed group than the ad lib group, they were proportionately so, so that the ratio of O\textsuperscript{6}-:7-methylguanine for the two groups was similar, 0.11 ad lib and 0.10 pair fed. This ratio fell within the range seen in other studies of animals on control diet (Lawley, 1976). In the zinc deficient group, not only were adduct levels higher, but O\textsuperscript{6}-methylguanine levels were proportionately much higher, reflected in a ratio of 0.135, 30% higher than that found in the control diet animals of this and other studies and consistent with significantly enhanced tumor incidence in zinc deficient-MBN esophageal carcinogenicity. The higher levels of binding in the control ad lib group compared to the pair fed group, without an increased ratio, suggested that there was simply a greater exposure to carcinogen in the ad lib group, perhaps due to greater DNA synthesis prior to exposure, but no other specificity of effect. The higher binding levels in the zinc deficient group, accompanied by the greater relative level of O\textsuperscript{6}-methylguanine, reflected in the increased ratio, suggested that in addition to increased overall exposure to carcinogen -- perhaps also due to enhanced synthesis -- there was a specific effect, such as reduced repair, of the promutagenic base.

In the liver not only were levels of both adducts greatly reduced, but O\textsuperscript{6}-methylguanine levels were proportionately much lower, so that ratios were one half to one third the level in the esophagus. This suggests that not only
was there less initial carcinogen exposure or binding but a specific effect of either less O⁶methylguanine formation or more rapid removal. This finding was consistent with other studies (Hodgson et al, 1980) and with the pattern of tumor incidences in this study.

After six doses of carcinogen, the last three labeled, levels of both N-7 methylguanine and O⁶methylguanine increased in both esophagus and liver, for all dietary groups. Increases in 7 methylguanine levels were comparable, approximately 30%. Levels of O⁶-methylguanine increased more in liver than esophagus, at the 4 hour post dosing time, except in the zinc deficient group which only increased 0.4%; the two control groups of the liver increased 30% and 54% compared to 6%, 13% and 15% in the esophagus. It is unclear why there would be a greater increase in the liver although levels were so low, a range of 2.6 - 6 adducts/guanine x 10⁶, that the increase to the 4-8/guanine x 10⁶ range may have been within the margin of error and not meaningful. Ratios in liver remained one third to one half the level in the esophagus.

The decreased ratios of N-7-methylguanine: O⁶-methylguanine from four hours after a single dose to four hours after 6 doses, despite an absolute increase in the levels of both adducts, indicated a slower rate of accumulation of O⁶-methylguanine than 7-methylguanine. The ratios decreased from 0.11 and 0.10 to 0.09 and 0.09 in the control diet groups and from 0.135 to 0.114 in the zinc deficient group. This suggested that although there was an overall accumulation of adducts due to repeated exposure, there was
also a falling off or active process of adduct removal that was more pronounced or specific for O6-methyl group than the 7-methyl group. Repeated dosing of MBN may have enhanced activity of enzymes responsible for activation of MBN to ultimate carcinogen but it may also have activated the repair system.

The falling off or removal of adducts continued at 24 and 48 hours after end of dosing, O6-methylguanine levels falling faster than 7-methylguanine levels, as reflected in decreasing ratios. In the esophagus, rate of removal of O6-methylguanine was slowest in the zinc deficient group. Rates of removal were much more rapid in the liver, O6-methylguanine levels becoming undetectable in the pair fed group at 24 hours and in the other two groups at 48 hours.

These data suggest a repair process specific for the O6 methyl group. In the esophagus, only the pair fed group had undetectable levels at 48 hours. Given that O6-methylguanine levels in the control ad lib group would probably become undetectable by 96 hours, a question must be raised as to the importance of the different times of synthesis enhancement as a reason for different tumor incidences since these enhancements in the control ad lib and pair fed groups occurred at 7 and 14 days — times well after levels of this promutagenic base were undetectable. It is important to note, however, the distinction between undetectable levels and lack of binding. Using a labeled compound of 20 mci/mm S.A. and approximately 120 μg of DNA (which produces approximately 20–30 μg of guanine that can be adducted) there would have to be a minimum
binding level of 1.8 - 1.8 adducts/guanine x 10^6 to produce sufficient radioactivity detectable above background levels of 20 cpm's. Much lower levels of adducts could be quite significant to the carcinogenic process but would be undetectable. That the rate of decrease was not a simple falling off of adduct, breakdown of DNA, or dilution due to synthesis was supported in two ways: (1) there was a differential rate of decline for the two adducts; and (2) the in vitro study showed little decline in adducts over the same 48 hour period.

Based upon the importance of the promutagenic base, O^6-methylguanine, in carcinogenesis in general and esophageal carcinogenesis in particular, the zinc deficient diet exerted two effects that would explain its tumor enhancing effects: (1) a higher absolute level of both adducts, as well as a higher relative level of O^6-methylguanine which represents an enhanced carcinogenic effect and (2) a slower rate of removal of the O^6-methylguanine, leaving more to be fixed as permanent damage. The first effect could be caused by greater synthesis. The higher level of O^6-methylguanine and its slower removal could be due either to the reduced activity of repair enzyme(s) that are zinc dependent (Phillips and Kindrell, 1980) or to inactivation of proteins by greater levels of carcinogen exposure due to enhanced synthesis. The results of this study fit well with, and are in part explained by, the results of the DNA synthesis studies, more synthesis causes more binding. The results of this study, in turn, also clarify the patterns seen in the DNA synthesis studies which
fit well with the binding of carcinogen, i.e. more binding causes greater subsequent DNA synthesis inhibition. Taken together these two studies help to clarify the tumor enhancing action of zinc deficiency on methylbenzylnitrosamine-induced esophageal carcinogenesis.
VI. CONCLUSIONS

1. Zinc deficiency significantly enhanced esophageal carcinogenesis induced by the esophagus-specific carcinogen, methylbenzyl nitrosamine. The overall enhancing effects of this deficit appear to be exerted during both the dosing and post dosing stages, although effects during the dosing stage may be more important. Zinc deficiency also increased the number of tumors per esophagus, the size of tumors and the percent of tumors that were carcinomas.

2. Zinc deficiency exerted a minor, protective effect on the carcinogenic process indicating that the dramatic tumor enhancing effects of zinc deficiency are likely not due to the absence of anti-carcinogenic effects of zinc.

3. Chronic consumption of alcohol in the drinking water at concentrations from 4% to 10% consistently enhanced tumor incidence in both the zinc deficient and control ad lib groups but not to a significant extent. One study showed the enhancing effect was primarily exerted during tumor growth.

4. The addition of 13-cis retinoic acid to the diet after dosing enhanced, rather than inhibited, tumor
incidence, although not to a significant degree. This treatment also caused greater morphological derangement including hyperplasia, hyperkeratosis and inflammation. Alcohol consumption may have been a confounding factor in this effect.

5. Zinc deficiency exerted no significant effect on vitamin A levels indicating the effect of zinc deficiency was not mediated by altered vitamin A levels.

6. Zinc deficient tumor enhancement was consistent with its enhancement of DNA synthesis prior to carcinogen exposure, which would make cells more vulnerable to carcinogen assault, and with its ongoing high levels of synthesis in the post dosing period which could enhance tumor growth. The pair fed inhibition of tumor growth was consistent with its inhibition of DNA synthesis prior to carcinogen exposure which could have reduced vulnerability to carcinogenic assault and by its delay in synthesis enhancement, compared to control diet ad lib, which could have provided more time for adduct removal and lesion repair.

7. Zinc deficiency enhanced adducts levels, particularly 6-methylguanine, and increased persistence of this promutagenic adduct, both effects of which could account for its tumor enhancement.
Pair feeding decreased levels of adducts and reduced persistence, particularly of O6-methylguanine, which could account for its inhibiting effect on MBN esophageal carcinogenesis. The effects of the diets on adduct formation were consistent with the effects observed on synthesis, i.e., enhancement or inhibition of synthesis caused more or less adducts. These adduct levels, in turn, were consistent with subsequent levels of DNA synthesis, i.e., more binding caused greater inhibition.
VII. SUGGESTIONS FOR FUTURE RESEARCH

1. Examine effects of zinc deficiency applied for progressively shorter periods of time, during dosing or post dosing to more clearly pinpoint time of effect.

2. Examine effects of zinc deficiency on MBN carcinogenesis in the presence of a DNA synthesis inhibitor, such as difloromethylornithine (DFMO), to better ascertain the importance of proliferation in zinc deficient-mediated tumor enhancement.

3. Examine effects of 13-cis RA on MBN esophageal carcinogenesis independent from ethanol consumption. This could be examined at higher levels than 67 ppm during both dosing and post dosing, with or without zinc deficiency. If feasible, measure levels of retinol binding protein in esophagus.

4. Examine the effects of alcohol on MBN carcinogenesis utilizing a liquid diet that would allow higher levels of alcohol but would control nutrient: caloric ratios.

5. Examine MBN metabolism directly to determine effect of dietary manipulations on P-450 system (rather than examining enzyme system).
6. Examine DNA synthesis at time points following the switching of diets and after each successive dose to determine how the patterns change during these critical phases.

7. Using more labeled carcinogen examine adduct formation during successive doses and at more time points after dosing to determine pattern of change from single to carcinogenic dose and how adducts from this carcinogenic dose persist into a period of DNA synthesis enhancement.
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APPENDIX A

HPLC DETERMINATION OF RETINOL IN SERUM

Introduction:

The method involves ethanol precipitation of serum proteins and extraction of retinol into hexane. The hexane extract is washed with water then dried, resuspended in ethanol and injected into the HPLC. Quantitation of retinol is achieved by determining the ratio of peak heights or areas of the retinol peak to a peak of retinyl acetate added to the serum sample at the 1st step of processing, and comparing this ratio to a set of ratios generated with pure, authentic retinol and retinyl acetate.

Specifics and Details

Clean glassware is required. 10 x 75 or 12 x 75 mm disposable culture tubes are used throughout the procedure. Standard solutions are made in ethanol, concentrations of 0.8 - 2.0 ng/µl (E¹ cm retinyl acetate 1550; retinol 1835 blanked against ethanol at 325α). Our standards are made up in 10 ml glass stoppered volumetric flasks covered with aluminum foil and are stored under N₂ in the refrigerator except when in use. (Also we have about 1/2 the fluorescent light fixtures disconnected in the lab and recently have pulled the blinds to 2/3 closed). Standard purity and concentration is checked daily by AU and chromatographic behavior. Standards are good for 7-10 days, we seldom use them longer than that.
100 µl of serum is pipetted into a tube, 20 µl retinyl acetate solution as is 80 µl EtOH and 1.0 - 1.5 ml nanograde hexane. EtOH concentrations higher than 50% result in lack of ROH extraction and low peak heights while concentrations less than 50% do not extract RAc fully and can lead to abnormally high standard curve slopes is added. For the larger hexane volume the 12 x 75 mm tubes are recommended. The mixture is vortexed for 20", with complete mixing and protein precipitation.

The solution is centrifuged in desk top centrifuge with plastic inserts plugged with paper placed into the centrifuge wells to adapt to the smaller tube diameter, for 5 minutes at 2200 RPM or greater.

The hexane phase is pipetted into another tube containing 100 µl distilled water, and vortex and centrifuge steps are repeated although shorter vortex and centrifuge times are acceptable at this step water volume is not critical.

The hexane layer is pipetted into a clean dry tube and dried under a stream of nitrogen. Immediately after the sample has reached dryness 22-23 µl ethanol are added and the solution vortexed, turning the tube on its side to solubilize retinol adhered to the walls of the tube. The solution is taken up in a 25 µl injection (blunt tip) syringe and injected. Final volume should be 20µl ± 1 µl.

**HPLC Conditions**
2.0 mg/min HPLC grade MeOH previously sonicated to degas is the mobile phase. UV detection is at 325 #. UV detector is set at time constant .3, and Chart .2-.5 AuFs. Chart speed is 5 mm/min, 10 mV. Find enclosed a representative chromatogram of a serum sample under these conditions.

Standard curves are made using the same amount of retinyl acetate as is used in the serum analysis, with varying amounts of retinol (e.g. 5#ROH + 20# RAc, 10 # ROH + 20 # RAc, 20 # ROH + 20 # RAc). As these standards are made in ethanol, no phase separation will occur between the ethanol phase and the hexane phase unless a small amount of (100 µl) of aqueous material, water or saline, is added. (See note on EtOH concentration in serum analysis, [EtOH] must be identical in standard curve samples). The standards are carried through the same processing steps as samples.
APPENDIX B

Krebs Ringer Buffer

<table>
<thead>
<tr>
<th>2 Liter</th>
<th>1 Liter</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>13.88 g</td>
<td>6.94 g</td>
<td>NaCl</td>
</tr>
<tr>
<td>0.708</td>
<td>0.354 g</td>
<td>KCl</td>
</tr>
<tr>
<td>0.324</td>
<td>0.162 g</td>
<td>K H₂PO₄</td>
</tr>
<tr>
<td>0.588</td>
<td>0.294 g</td>
<td>Mg 5O₄·7H₂O</td>
</tr>
<tr>
<td>1.80</td>
<td>0.90 g</td>
<td>Glucose (5 m mole)</td>
</tr>
</tbody>
</table>

Stir on magnetic stirrer with 100% CO₂ bubbling for 5 minutes then add

7.62     0.381 g  CaCl₂H₂O

continue bubbling CO₂ and stirring for 5 minutes then add

4.20     2.10 g  NaHCO₃

Store under CO₂ or Ca may precipitate. Check pH periodically use CO₂ bubbling to maintain pH 7.4
1) **HKM-sucrose (pH 7.0)**

85.6 gm sucrose

100 ml 0.5M HEPES (pH 7.0)

5.0 ml 1M MgCl₂

13.5 ml 2M KCl

adjust to 1000 ml with H₂O

Dissolve sucrose in approx. 800 ml Milli-Q water, add buffer and salt solutions, and adjust to final volume of 1000 ml.

2) **HKM-sucrose (pH 7.0) + TRITON X-100**

As above except 5 ml of TRITON X-100 (0.5%) are added with the buffer + salt solutions, and brought up to a final volume of 1000 ml with Milli-Q water.

3) **0.5M HEPES (pH 7.0)**

Dissolve 59.6 gm HEPES in approx. 450 ml Milli-Q water. Adjust pH to 7.0 by adding conc. HCl dropwise. Adjust volume to 500 ml with water and check pH with pH paper.

4) **1M MgCl₂**

Dissolve 40.7 gm anhydrous MgCl₂ in Milli-Q water and bring up to a final volume of 200 ml.

5) **2M KCl**

Dissolve 29.8 gm KCl in Milli-Q water and bring up to a final volume of 200 ml.
APPENDIX D
GRADIENT SOLUTIONS

A) Neutral Gradients

1) Lysing Buffer

1.0 ml Sarkosyl

2.5 ml 200 mM Na₂EDTA (pH 7.0)

10.0 ml 0.5M HEPES (pH 7.0)

adjust to 100 ml with H₂O

2) Cs₂SO₄-EDTA-HEPES

54.3 gm Cs₂SO₄

1.25 ml 200 mM Na₂EDTA (pH 7.0)

5.0 ml 0.5M HEPES (pH 7.0)

adjust to 50 ml with H₂O

Add Cs₂SO₄ and solutions, bring up to approx. 45 ml, and stir on low heat until dissolved. Adjust final volume to 50 ml with H₂O.

3) CsCl-EDTA-HEPES

252.6 gm CsCl

6.25 ml 200 mM Na₂EDTA (pH 7.0)

25 ml 0.5M HEPES (pH 7.0)

adjust to 250 ml with H₂O

4) 0.5M HEPES (pH 7.0)

Dissolve 59.6 gm HEPES in approx. 450 ml H₂O. Lower pH to 7.0 by dropwise addition of conc. HCl. Adjust volume to 500 ml with H₂O and recheck pH with pH paper.
APPENDIX E

To measure DNA, RNA and protein, DNA fractions are pooled to provide approximately 200 ug DNA per sample. A single diphenylamine, orcinol and biorad are performed on each 200 ug DNA sample; therefore, three of these samples are used to provide triplicates for each test. Prior to the test each pooled sample is dialysed to remove CsCl using millipore filter with suction. As fluid is removed more Hepes buffer is added. Six additions of buffer are made to complete dialysis. Each sample is brought up to volume of 4.0 ml with hepes buffer. For tests the following amounts are used: diphenylamine - 1.0 ml; orcinol-2:0 ml; and biorad 0.8 ml. Total - 3.8 ml.


Standard

Calf thymus DNA (Sigma, type V) was desiccated in the refrigerator for 2 days. An appropriate sample was weighed, transferred to a clean beaker, and dissolved in 1 N/PCA at 70°C. OD_{260} was measured to determine the amount and standard curve was constructed between 10 and 100 ugm DNA per tube.

DNA determination

1. 1 ml of DNA sample added to 1 ml of 2N PCA (to yield 2 ml of 1N PCA, as standards).

2. Add 2 ml of 4% diphenylamine in glacial acetic acid (prepared fresh). Mix well.
3. Add 0.1 ml aqueous acetaldehyde (kept cold)
   (2.0 ml acetaldehyde brought up to 979 ml with cold water,
   mixed thoroughly, and kept cold). Mix well.
4. Let stand overnight at room temperature.
5. Read O.D. at 595.
6. As blank use 1 ml hepes buffer in place of DNA.


Standards

standards in hepes buffer, measure O.D. 260
to determine amounts and construct standard curve between 2 and 30 ug
per tube (per 2 ml).

RNA Determination

1. Take 2 ml of DNA sample and 2 ml orcinol reagent.
2. Ampules are sealed with torch or covered with marbles.
   Mix well with vortex.
3. Immerse in boiling water for 35.0 minutes.
4. Remove and chill on ice.
5. Read O.D. 666 nm
6. 2 ml distilled water in place of sample used for blank.

Reagents.

Cupric ion reagent. 0.1500 gm CuCl₂·2H₂O was dissolved in 100.0
ml concentrated HCl. The solution is very stable.
Orcinol Reagent. 12.5 gm orcinol was dissolved in 95% ethyl alcohol to make 25 ml of 50% (w/v) stock solution. For preparation of working solution 2 ml stock solution was mixed with 100 ml of cupric ion reagent. Orcinol stock solution, if kept in dark and cooled, is stable.


Microscopy procedure.

Standards

Bovine serum albumin is diluted to appropriate levels to yield 1-10 ug protein per 0.8 ml.

Protein Determination

1. Place 0.8 ml of standards or sample in clean, dry test tubes.

   Place 0.8 ml sample buffer in test tube for blank.

2. Add 0.2 ml Dye Reagent Concentrate.

3. Vortex (avoid excess foaming)

4. After period 5 minutes - one hour read OD_{595}.

5. Plot OD_{595} versus concentration of standards

Calculation.

In orcinol and biorad assays, DNA in solution absorbs at the wavelength of measurement; adjustments must be made to subtract out this effect so as to determine actual amount of RNA and protein, respectively. In diphenylamine, neither RNA or protein absorbs so that reading of unknown from standard curve reflects actual amount of DNA and no adjustment is necessary.
Following is the procedure to adjust orcinol and biorad results to obtain actual amounts of RNA and protein, respectively.

1. Construct DNA standard curves from the orcinol and biorad assays. This will provide a linear relationship of absorbance of DNA in either the orcinol or biorad assay.

2. Once the amount of DNA in a sample is determined by the diphenylamins assay that amount can be read off either the orcinol or biorad DNA standard curves to indicate the amount of absorbance that the DNA in that sample generates from either of these assays.

3. Subtract the DNA—caused absorbance from the total absorbance of samples for each assay— orcinol or biorad.

4. This net absorbance is due to RNA in orcinol and protein in biorad. Read these net absorbances off appropriate standard curves — orcinol—RNA standard curve or biorad—protein standard curve — to obtain actual amounts of RNA and protein in each sample.
AUTORADIOGRAPHY TECHNIQUE

Slide Preparation

I. Dry sections in 37°C. oven for 1 hr.

II. Deparaffinize sectioning as follows under hood:
   1. 2 xylene - 5 mins. each
   2. 2 absolute alcohol - 2 mins. each
   3. 2 of 1:1 ether/absolute alcohol - 2 mins. each
   4. air dry sections - 15 mins.

Dark Room Preparation

1. Clean counter thoroughly.
2. Water bath 40-45°C.
3. Small beaker and dipping cylinder.
4. Clean, dry plain slides.
5. Turn on Duplex super safelight or safelight with Kodak wratten #2 filter.
7. Slides to be dipped.

Dipping

In darkroom with safelight only.
1. Place Kodak NTB 3 emulsion in small beaker and let liquify in water bath for 45 mins.
2. Very slowly, poor melted emulsion with dipping cylinder.
3. Use gauze square to wipe away the air bubble on top.
4. Dip plain slides to check if emulsion is bubble free.
5. Dip slides with sections. Wipe back of slides with kimwipes.
6. Drain slides against the wall on paper towel at 30° angle for 10 mins.
7. Place slide in staining rack and let dry slowly in refrigerator, inside darkroom, for 3-4 hrs. or overnight.
   **** Refrigerator guarded from opening.
8. Place slides in black boxes that have been prepared with a gauze package of drierite, then:
   1. Stack black boxes into 3 layer yellow box.
   2. Wrap black boxes with Aluminum foil.
   Store in refrigerator for appropriate exposure time (~ 2 wks. or more).

Developing
In darkroom with safelight only.
1. Transfer slides to staining rack.
2. Kodak D-19 developer - 17°C. \( \frac{2}{3} \) mins. (discard solution after use)
   Distilled water 3.8 liters at 38°C. while stirring slowly add the dry Kodak developer D-19. Stir until completely dissolved.

\( \frac{47}{57} \) grams of acid fixer/250 ml distilled water.
\( \frac{140}{140} \) " " " " /750 ml " " " "
Developing (cont'd)

5. Turn on room light. Wash slides in cool → room temp. running water --- 30 mins.

Staining

1. Mayer's hematoxylin - 2-3 minutes.
2. Tap water rinse - 6 changes.
3. Distilled water - rinse.
4. Distilled water - 20 minutes. Check for blue nuclei.
5. Water-soluble eosin - 30 seconds.
   (4 gm eosin Y, 800 ml distilled water, 4 drops glacial acetic acid. Add 1 thymol crystal).
6. Tap water - 3 changes.
7. 95% alcohol - 2 changes.
8. 100% alcohol - 2 changes.
9. Xylene - 2 changes.
10. Xylene - 5 minutes.
11. Coverslip with permont.
12. Use acid alcohol to clean the remaining emulsion on back of slides.
   Acid Alcohol = 1% HCl in 20% alcohol.

Note:

The volume of emulsion in gel form will reduce to half when melted. Clean small beaker and dipping cylinder with soapy water, acid alcohol and tap water.