

**Effect of Dietary Zinc Intake on Mammary Tumorigenesis
Induced by N-methyl-N-nitrosourea in Rats**

by

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ABSTRACT

The overall objective of this study was to investigate the effect of dietary zinc intake on mammary tumorigenesis induced by N-methyl-N-nitrosourea (MNU) in rats. In Experiment 1, twenty-one-day-old female Sprague-Dawley rats were randomly assigned to: severe-low-zinc (1mg zinc/kg diet, Z1), marginal-low-zinc (3mg zinc/kg diet, Z3), control (31mg zinc/kg diet, Z31), high-zinc (171mg zinc/kg diet, Z171), very-high-zinc (346mg zinc/kg diet, Z346), pair-fed-to-severe-low-zinc (PZ31-Z1) and pair-fed-to-marginal-low-zinc (PZ31-Z3) groups. On day one of the experiment, each dietary treatment group was further divided into sham- and MNU-treated (50 mg/kg body weight, i.p.) groups ($n = 6$). After 100 days, the plasma, liver, kidney, heart, lung, muscle, femur, small intestine, skin, normal mammary gland and mammary tumor were removed for zinc determination. Body zinc distribution as indicated by tissue zinc concentrations was mainly affected by dietary zinc intake, but not MNU treatment. MNU-induced mammary tumorigenesis resulted in an accumulation of zinc in mammary tumors. In Experiment 2, twenty-one-day-old female Sprague-Dawley rats were randomly assigned to: marginal-low-zinc (3mg zinc/kg diet, Z3), required-zinc (12mg zinc/kg diet, Z12), control (31mg zinc/kg diet, Z31), high-zinc (155mg zinc/kg diet, Z155), pair-fed-with-required-zinc-diet (PZ12) and pair-fed-with-control-diet (PZ31) groups. On day one of the experiment, the rats were injected with MNU (40 mg/kg body weight, i.p.). After 28 and 56 days, mammary gland whole mounts were prepared to detect microscopic lesions ($n=6$). The remaining 24 rats were maintained on the assigned diet for 98 days to assess mammary tumorigenesis. Tissue zinc concentrations were also determined and found to be essentially not affected by MNU-induced mammary tumorigenesis. Tumor incidence in Z3 rats was significantly lower than that in Z12, Z31 and

Z155 rats ($p < 0.05$) due, at least in part, to the reduced feed intake associated with zinc deficiency. Tumor incidence in Z3 rats was significantly lower than that in PZ12 rats ($p < 0.05$), but was the same as in PZ31 rats. Dietary zinc intake had no effect on the tumor multiplicity, tumor weight and tumor burden during MNU-induced mammary tumorigenesis. Overall, these results suggest that dietary zinc intake is critical in modulating palpable MNU-induced mammary tumor formation in rats when feed intake is reduced.

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1. INTRODUCTION

In Canada, it has been estimated that there will be 18,700 new cases of breast cancer and 5,400 deaths from breast cancer in 1999 (National Cancer Institute of Canada, 1999). Breast cancer remains the most frequently diagnosed cancer with high mortality rate among women in Canada. The high incidence and mortality rates of breast cancer represent a major health threat to Canadian women and a serious burden to our health care system. Despite the high incidence and mortality rates, the etiology of breast cancer is still unclear (Nandi *et al.*, 1995; Russo and Russo, 1996). The development of breast cancer is influenced by many factors, such as ionizing radiation, diet, reproductive history, and genetic and endocrinologic factors (Doll and Peto, 1981; Hunter and Willett, 1994; Ames *et al.*, 1995; Russo and Russo, 1996). Diet has been estimated to contribute to about 50% of breast cancer deaths in the United States (Doll and Peto, 1981; Ames *et al.*, 1995). However, the dietary factors responsible for breast cancer remain unclear.

Increasing evidence from both animal and human studies has linked zinc to mammary tumorigenesis. An increased uptake of ^{65}Zn by spontaneous mammary tumors in mice (Tupper *et al.*, 1955) and an inverse relationship between plasma zinc concentration and tumor burden in rats implanted with mammary adenocarcinoma (Philcox *et al.*, 1994) suggest a role of zinc during mammary tumorigenesis. An apparently higher zinc concentration in the breast cancer tissue as compared to that in the normal breast tissue in humans (Mulay *et al.*, 1971; Santoliquido *et al.*, 1976; Margalioth *et al.*, 1983; Rizk and Sky-Peck, 1984; Ng *et al.*, 1993; Borella *et al.*, 1997) also supports the same notion, although this line of evidence needs to be interpreted with caution. Despite the apparent association between zinc and

mammary tumorigenesis, the relationship between dietary zinc intake and mammary tumorigenesis is not known.

2. HYPOTHESIS AND RATIONALE

It is hypothesized that the level of dietary zinc intake, through its influences on the body zinc status and consequently the biological functions of zinc, exerts an effect on mammary tumorigenesis in rats. Zinc plays an essential role in many physiological functions such as cell proliferation and growth. Mammary tumorigenesis is a form of abnormal growth characterized by uncontrolled cell proliferation in the mammary gland. To support its high rate of cell proliferation, the mammary tumor requires a continuous supply of zinc. Since dietary zinc intake affects the body zinc status, which, in turn, influences the biological functions of zinc, it is possible that dietary zinc intake could ultimately influence mammary tumorigenesis in rats.

3. OBJECTIVES

The overall objective of this thesis was to investigate the effect of different levels of dietary zinc on mammary tumorigenesis induced by N-methyl-N-nitrosourea (MNU) in rats.

The specific objectives were:

1. to investigate the effect of MNU treatment on body zinc distribution in rats fed different levels of dietary zinc.
2. to compare the zinc concentrations between MNU-induced mammary tumors and mammary glands in rats.
3. to investigate the effect of dietary zinc intake on the development and growth of MNU-induced mammary tumors in rats.

4. LITERATURE REVIEW

4.1. Biological functions of zinc

Zinc is an essential nutrient for both animals and humans. In a 70-kg healthy adult male, it is estimated that the body contains about 3 grams of zinc with the majority of zinc found in the muscle, bone, skin and liver (Jackson, 1989). Total body zinc is primarily maintained through the regulation of intestinal absorption and endogenous excretion in response to dietary zinc intake (King and Keen, 1994). There is no known storage site of zinc in the body. Zinc status is commonly assessed by measuring the plasma zinc concentration. However, it is important to realize that plasma zinc concentration is also influenced by non-nutritional factors such as stress and infection (Jackson, 1989; Cousins, 1989).

Zinc plays an important role in many functions in the body (Vallee and Falchuk, 1993). Zinc is a cofactor for over 300 enzymes in which it plays a catalytic, structural or regulatory role. It is also involved in the stabilization of structures such as gene regulatory proteins. As an enzyme cofactor and a structural stabilizer, zinc is involved in the synthesis of nucleic acids and proteins and is critical in many physiological functions, such as cell proliferation and growth. For instance, as a cofactor for DNA polymerase, zinc is involved in DNA replication (Vallee and Galdes, 1984; Shankar and Prasad, 1998). Through its cofactor role in RNA polymerase and its structural role in the gene regulatory proteins such as transcriptional factors (Vallee and Falchuk, 1993), zinc is essential in the synthesis of RNA. Since zinc is involved in the stabilization of RNA (Vallee, 1959) and ribosomes (Tal, 1969; Hambidge *et al.*, 1986), zinc plays an important role in protein synthesis. Through its

function in the synthesis of nucleic acids and proteins, zinc is critical in cell proliferation and growth.

The effect of zinc deficiency on DNA and protein synthesis has been documented in both *in vivo* and *in vitro* systems. Stephan and Hsu (1973) reported that the DNA synthesis in zinc deficient rats was inhibited in the skin, liver and spleen. Recombined macrophages and lymphocytes from zinc deficient mice showed a lower mitogenic response than those from pair-fed mice *in vitro* (James *et al.*, 1987). Furthermore, DNA synthesis was also reduced in cells incubated in zinc deficient medium (Flynn, 1984) and in medium containing a cation chelator (Chesters *et al.*, 1989; Kanemaru *et al.*, 1992; Back *et al.*, 1993; MacDonald *et al.*, 1998). Supplementation of zinc to the medium containing a cation chelator increased DNA synthesis and such restoration of DNA synthesis was specific to zinc (Chesters *et al.*, 1989; Kanemaru *et al.*, 1992; Back *et al.*, 1993; MacDonald *et al.*, 1998). Kaji *et al.* (1994) showed that zinc deficiency reduced DNA and protein synthesis in vascular endothelial cells *in vitro*. Besides, the protein synthesis was also inhibited in the cell-free system using enzymes isolated from the liver of zinc deficient rats (Hicks and Wallwork, 1987).

At the cellular level, zinc deficiency results in reduced cell division (Clegg *et al.*, 1989). Dietary zinc deficiency reduced distal colon cell proliferation as compared to pair-fed control in rats (Lawson *et al.*, 1988). The rate of jejunal cell division in zinc deficient rats was also lower than that in pair-fed rats (Southon *et al.*, 1985). Kaji *et al.* (1994) showed that zinc deficiency decreased cell proliferation in vascular endothelial cells *in vitro*. The proliferation of cells of lymphoid origin (Molt-3, Raji, HUT-78) and myeloid (HL-60) origin was also reduced under zinc deficient conditions *in vitro* (Martin *et al.*, 1991; Prasad *et al.*, 1996). The increased cell doubling time in malignant lymphoblastoid cells (HUT-78)

incubated in zinc deficient medium was suggested to be related to the reduced activity of thymidine kinase which is involved in DNA synthesis (Prasad *et al.*, 1996).

When zinc intake in growing animals is mildly insufficient, the body zinc is conserved by reducing the growth rate (King, 1990). James *et al.* (1987) showed that dietary zinc deficiency reduced the final body weight in mice. Zinc deficient rats also gained less weight than pair-fed control rats (Southon *et al.*, 1985). Although it is well documented that zinc deficiency reduces the growth of animals, the mechanism by which it affects growth is still not clear (Prasad, 1995).

Growth is influenced by hormones such as growth hormone and insulin-like growth factor. Body zinc status has been shown to influence the levels of circulating hormones which are involved in the regulation of growth. Zinc deficiency resulted in a decrease in serum insulin (Dorup *et al.*, 1991; Roth and Kirchgessner, 1994) and serum insulin-like growth factor I (IGF-1) (Dorup *et al.*, 1991; Roth and Kirchgessner, 1994; McNall *et al.*, 1995; Ninh *et al.*, 1995) in rats. The decreased serum IGF-1 level was accompanied by a reduced hepatic IGF-1 mRNA level in zinc deficient rats (McNall *et al.*, 1995; Ninh *et al.*, 1995). Although the serum level of growth hormone was not changed (Dorup *et al.*, 1991; Ninh *et al.*, 1995), the number of hepatic growth hormone receptors was reduced in zinc deficient rats (Ninh *et al.*, 1995). Therefore, it has been speculated that dietary zinc deficiency lowers the number of hepatic growth hormone receptors which decreases the binding of growth hormone (Roth and Kirchgessner, 1994; Ninh *et al.*, 1995). The decreased growth hormone binding to its receptors, together with decreased insulin level, may lead to a reduction in IGF-1 synthesis and, hence, serum IGF-1 level. It has been suggested that such hormonal changes are associated with the growth retardation during zinc deficiency (Dorup

et al., 1991; Roth and Kirchgessner, 1994; McNall *et al.*, 1995; Ninh *et al.*, 1995). However, the injection of recombinant human IGF-1 into zinc deficient rats did not reverse the growth retardation induced by zinc deficiency (Browning *et al.*, 1998; Cha and Rojhani, 1998; Ninh *et al.*, 1998). This finding suggests that the growth retardation during zinc deficiency is not caused by a low circulating level of IGF-1, but the possibility of disrupted functions of IGF-1 receptor requires further clarification. In addition, several hormone receptors such as estrogen receptor, progesterone receptor and glucocorticoid receptor have been shown to contain zinc-binding domains (Vallee and Falchuk, 1993). Through its structural role in these hormone receptors, zinc could affect the interaction of hormones with their receptors and the subsequent cellular events. For instance, by affecting the binding of estrogen receptor to DNA, zinc may affect the functions of estrogen which include the stimulation of cell division and protein synthesis (Miller, 1996).

In addition to the zinc deficient effect, the effect of high zinc intake in animals and high zinc concentration in cell culture systems on DNA synthesis, cell proliferation and growth have also been studied. Kobusch *et al.* (1990) reported that DNA synthesis was increased in hepatocytes incubated in media with a zinc concentration of up to 40 μM . Prasad *et al.* (1996) showed that a medium containing 10 μM zinc supported the maximum growth of human malignant lymphoblastoid cells (HUT-78). Further increase in zinc concentration to 25 μM and 50 μM showed similar effects as that in 10 μM medium. Similarly, increasing dietary zinc intake above the requirement level does not necessarily enhance the growth of animals. Reeves (1995) showed that the body weight gain of rats fed a high-zinc diet (350 mg zinc/kg diet) was not different from that of the rats fed a control diet (50 mg zinc/kg diet). A diet containing 1350 mg zinc/kg diet had no effect on the body

weight gain in mice (Sato *et al.*, 1997). However, when animals were fed an excessive level of zinc (more than 5000 mg zinc/kg diet), growth was reduced (Hambidge *et al.*, 1986; Fox, 1989). Baker and Duncan (1983) showed that DNA synthesis in implanted hepatoma in rats fed a high-zinc diet (500 mg/kg diet) was reduced, along with reduced thymidine kinase and DNA polymerase activities (Duncan and Dreosti, 1976). Similarly, in murine malignant melanoma (BL-6) *in vitro*, an increase of zinc from 15 μM to 153 μM resulted in a reduction in cell proliferation (Perkins and Duncan, 1991).

Apoptosis is a form of cell suicide characterized by a reduction in cell volume, condensation of nuclear chromatin, blebbing of cell surface, formation of apoptotic bodies and DNA fragmentation (Sunderman, 1995; Fraker and Telford, 1997; Shankar and Prasad, 1998). Zinc has been shown to play a role in the modulation of apoptosis. Zinc deficiency induces apoptosis *in vivo* and *in vitro* (Sunderman, 1995). Although not many *in vivo* studies have been performed, Elmes (1977) showed that the number of apoptotic bodies was increased in the small intestine of zinc deficient rats as compared to the pair-fed controls. The apoptotic effect of zinc deficiency has been demonstrated in cell culture systems containing intracellular zinc chelators (McCabe *et al.*, 1993; Treves *et al.*, 1994). In addition, Martin *et al.* (1991) showed that the proportion of apoptotic cells (myeloid origin (HL-60) and lymphoid origin (Raji)) in zinc deficient medium was higher than that of the control. Telford and Fraker (1995) reported that media containing 80 – 200 μM zinc induced apoptosis in mouse thymocytes *in vitro*. On the other hand, high level of extracellular zinc in cell culture systems inhibits apoptosis (Flieger *et al.*, 1989). Martin and Cotter (1991) showed that an addition of 400 μM zinc to the culture medium reduced the percentage of apoptotic cells by half. However, because most of the studies that examined the relationship

between zinc and apoptosis were *in vitro* studies, the relevance of the results from *in vitro* systems to physiological conditions needs to be interpreted with caution.

In summary, through its function as an enzyme cofactor and a structural stabilizer, zinc is critical in DNA and protein synthesis. Zinc deficiency could result in lowered DNA and protein synthesis, reduced cell division and reduced growth. On the other hand, high dietary zinc intake in animals and high zinc concentration in culture media do not enhance growth and cell proliferation. Besides, excessively high zinc intake in animals could result in growth retardation. Apoptosis, which is the programmed cell death, could be induced by zinc deficiency while high zinc concentration inhibits apoptosis *in vitro*. Clearly, zinc exerts a great influence on cell proliferation and growth.

4.2. Mammary tumorigenesis

4.2.1. Human breast cancer

Although breast cancer is the most frequently diagnosed cancer with high mortality rate among Canadian women (National Cancer Institute of Canada, 1999), the etiology of breast cancer is still unclear (Nandi *et al.*, 1995; Russo and Russo, 1996). It is believed that breast cancer is influenced by many factors including diet, genetics, reproductive history and endocrinology (Doll and Peto, 1981; Hunter and Willett, 1994; Ames *et al.*, 1995; Nandi *et al.*, 1995; Russo and Russo, 1996).

Diet has been estimated to contribute to about 50% of breast cancer deaths in the United States (Doll and Peto, 1981; Ames *et al.*, 1995). High consumption of vegetables and fruits has been demonstrated to be associated with a lower risk of breast cancer (Franceschi *et al.*, 1995; Yuan *et al.*, 1995; Freudenheim *et al.*, 1996; Franceschi *et al.*, 1998; Zhang *et*

al., 1999). On the other hand, high consumption of meat is usually associated with a higher risk of breast cancer (Lee *et al.*, 1991; Yuan *et al.*, 1995; Ronco *et al.*, 1996). However, the dietary factors responsible for breast cancer are still unclear.

Caloric restriction has been shown to inhibit mammary tumorigenesis in animal studies (Kritchevsky *et al.*, 1984; Klurfeld *et al.*, 1989; Freedman, 1990; Mehta *et al.*, 1993). Klurfeld *et al.* (1989) showed that in 25% caloric restricted rats, the tumor incidence, tumor multiplicity, tumor weight and tumor burden were significantly lower than that in *ad libitum* rats. The tumor incidence in 40% caloric restricted rats was also significantly lower than that in *ad libitum* rats, but this inhibitory effect was not observed in intermittent caloric restricted rats (Mehta *et al.*, 1993). Despite the extensive studies in animals, it is difficult to investigate the effect of caloric restriction on breast cancer risk in humans (Hunter and Willett, 1994).

Although the relationship between dietary fat and breast cancer has been studied extensively, results are inconclusive. High level of fat consumption has been suggested to be related to an increased risk of mammary tumorigenesis in animals (Kritchevsky *et al.*, 1984; Freedman, 1990; Ghoshal *et al.*, 1994). This enhancing effect of high fat consumption on breast cancer risk is also supported by most of the case-control studies (Ronco *et al.*, 1996). However, cohort studies fail to demonstrate such enhancing effect of fat (Willett *et al.*, 1992; Hunter *et al.*, 1996). In addition to the amount of fat, results demonstrating the relationship between the type of fat and breast cancer are also inconsistent. For instance, saturated fat has been shown to be associated with a higher risk of breast cancer in some of human case-control studies (Ronco *et al.*, 1996; Favero *et al.*, 1998). However, saturated fat inhibits mammary tumorigenesis in animal studies (Hopkins *et al.*, 1981; Cohen *et al.*, 1986). Despite the debatable relationship between dietary fat and breast cancer, it is generally

believed that the enhancing effect of fat on the risk of mammary tumorigenesis is, at least in part, due to the high energy consumption associated with high fat intake (Welsch, 1994).

In addition, several factors such as vitamin A (Yuan *et al.*, 1995; Zhang *et al.*, 1999), vitamin C (Yang *et al.*, 1995; Freudenheim *et al.*, 1996) and vitamin E (Freudenheim *et al.*, 1996; Favero *et al.*, 1998) have been suggested to exert a protective effect against breast cancer. However, because of the limited and inconsistent findings (Kushi *et al.*, 1996; Jarvinen *et al.*, 1997; Verhoeven *et al.*, 1997), definite conclusions cannot be drawn (Hunter and Willett, 1994).

Clearly, although diet plays a role in the development of breast cancer, the dietary factors involved are still unclear.

4.2.2. Rodent mammary tumor models

There are various experimental models used for the studying of mammary tumorigenesis, such as transplanted mammary tumors and induced mammary tumors in rodents. The transplanted mammary tumor models are generally not appropriate to study the etiology of mammary tumorigenesis (Gould, 1995). In mice, mammary tumors are mainly induced by virus and these tumors are primarily hormone independent (Medina, 1982). On the other hand, rat mammary tumors, which are commonly induced by chemicals or radiation, are influenced by hormones (Russo *et al.*, 1990; Gould, 1995; Nandi *et al.*, 1995). The hormone dependency of rat mammary tumors resembles human breast cancer more closely than mouse mammary tumors. Hence, rat mammary tumor models are the most commonly used animal models to study mammary tumorigenesis.

In rats, chemical-induced mammary tumorigenesis is characterized by a higher incidence and shorter latency period as compared to radiation-induced mammary tumorigenesis (Huggins, 1979; Gould, 1995). Similar to human breast cancer, chemical-induced rat mammary tumors are influenced by dietary, genetic and endocrinological factors (Russo *et al.*, 1990; Russo and Russo, 1996). It is generally agreed that human breast cancer is originated from the terminal ductal structures. In rats, chemical-induced mammary tumors mainly arise from the terminal end buds, which are equivalent to the terminal ductal lobular units in humans. Furthermore, the histological classification of mammary tumors in rats and humans are similar. Most of the chemical-induced rat mammary tumors have their corresponding histological types in human breast cancer. In addition to the similarity in pathogenesis and histopathological classification, chemical-induced rat mammary tumors and human breast cancer are also similar in the expression of immunocytochemical markers. Because of these commonalities, chemical-induced rat mammary tumor models are considered to be useful experimental models for studying human breast cancer.

4.2.3. Chemical-induced mammary tumorigenesis

Chemical-induced tumorigenesis is a gradual process that involves three stages: initiation, promotion and progression (Figure 1) (Cooper, 1992; Archer, 1992; Archer, 1996). The first stage in tumorigenesis is the initiation stage which is an irreversible and rapid process. When a normal cell is exposed to an indirect-acting chemical carcinogenic initiator, the initiator can be either detoxified or metabolized to a reactive electrophilic intermediate which could bind to DNA. In case of a direct-acting chemical carcinogenic initiator, the initiator could bind directly to DNA. However, if the chemical carcinogenic initiator or the

reactive intermediate are detoxified and excreted, genetic alteration would not result. Otherwise, a DNA lesion-bearing cell is formed. If the DNA lesion-bearing cell undergoes DNA repair or cell death, tumor cells would not be formed. Otherwise, when the DNA lesion-bearing cell undergoes cell proliferation, the potentially repairable DNA lesion becomes permanent that results in the formation of an initiated cell. Under the influence of promoting agents, the initiated cell grows and undergoes rapid cell proliferation which leads to the formation of an actively proliferating cell population. In contrast to the initiation stage, promotion involves reversible changes over a long period of time. The mechanism of action of tumor promoters is poorly understood. Once the autonomous preneoplastic cells are formed, the expansion of the cell population is spontaneous and irreversible. Besides, additional mutations occur within the expanding tumor cell population leading to the formation of tumor cells with increased proliferative capacity, invasiveness and metastatic potential.

To induce mammary tumorigenesis in rats, N-methyl-N-nitrosourea (MNU) and 7,12-dimethylbenz(a)anthracene (DMBA) are the most widely used chemical carcinogens (Russo *et al.*, 1990; Russo and Russo, 1996). DMBA is a procarcinogen which requires activation to become carcinogenic. It is activated, possibly by cytochrome P450, in the mammary epithelial cells to polar metabolites which could bind to DNA resulting in DNA damage (Russo *et al.*, 1990; Russo and Russo, 1996). On the other hand, MNU is a direct-acting carcinogen which does not require activation.

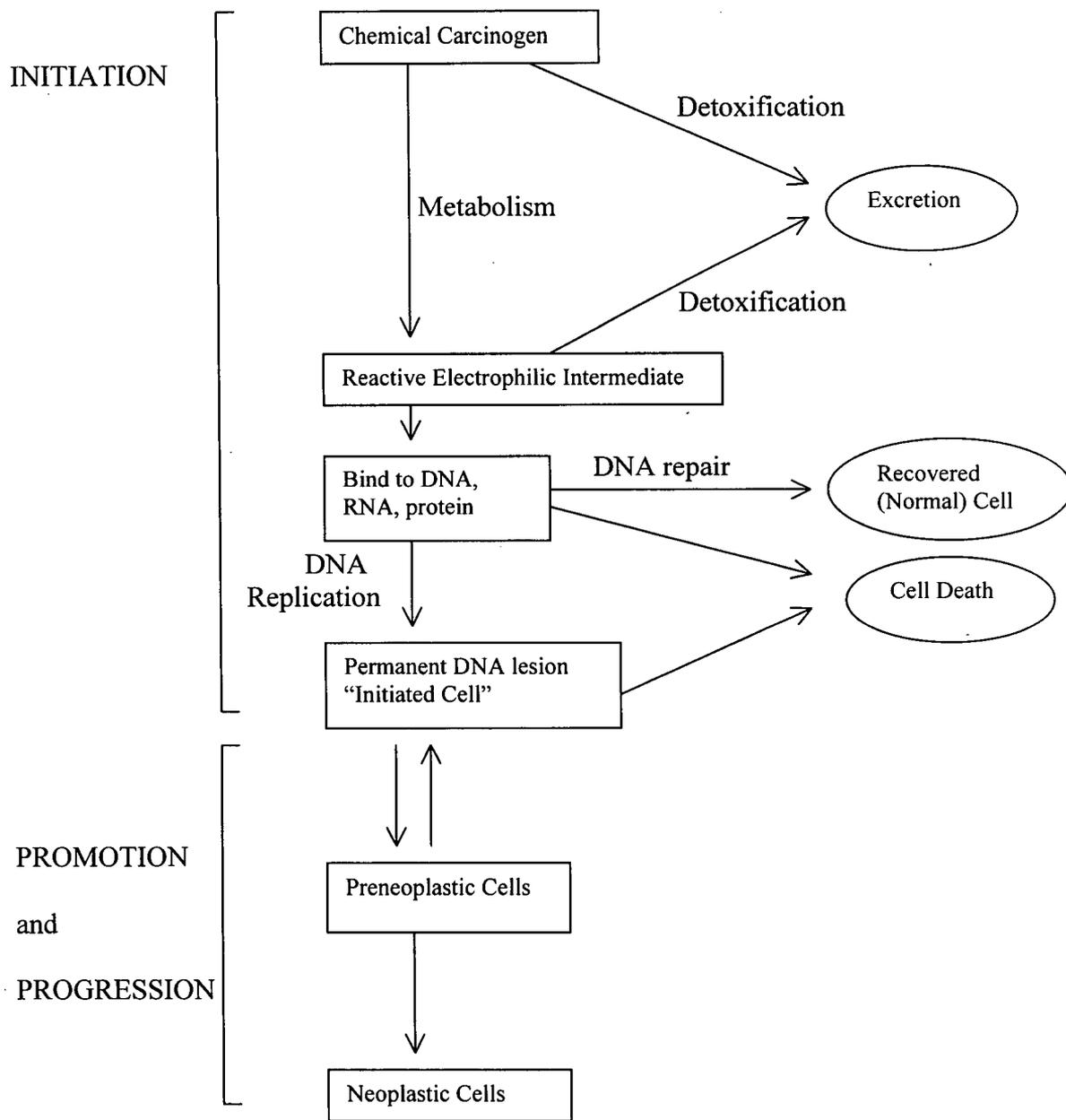


Figure 1. The mechanism of chemical-induced tumorigenesis. Adapted from Archer 1996.

4.3. Zinc and mammary tumorigenesis

4.3.1. Plasma/serum zinc concentration during mammary tumorigenesis

Altered plasma/serum zinc concentration during mammary tumorigenesis is observed in both animal and human studies. Breast cancer patients showed a lower serum zinc concentration than that in healthy individuals (Gupta *et al.*, 1991; Sharma *et al.*, 1994; Yucel *et al.*, 1994) and patients with benign breast diseases (Gupta *et al.*, 1991). Borella *et al.* (1997) reported that the plasma zinc concentration in breast cancer patients was also lower than that in healthy controls. In addition, the degree of hypozincemia appears to be influenced by the stage of breast cancer development. Patients with advanced breast cancer, but not those at the early stage of breast cancer, showed a significantly lower serum zinc concentration than that in healthy individuals (Gupta *et al.*, 1991). As compared to patients with non-metastatic breast cancer, patients with metastatic breast cancer had a lower plasma zinc concentration (Holtkamp *et al.*, 1990).

The hypozincemic effect of mammary tumorigenesis has also been shown in experimental animal models. Plasma zinc concentration in male rats implanted with mammary adenocarcinoma was lower than that in sham-treated controls (Mills *et al.*, 1984). Philcox *et al.* (1994) clearly demonstrated that an increase in tumor burden was correlated with a decrease in plasma zinc concentration in rats implanted with mammary adenocarcinoma. In rats with 4.3% tumor burden, plasma zinc concentration was 66% of that in tumor-free rats. As the tumor burden increased to 9.9%, plasma zinc concentration decreased to 51% of that in tumor-free rats. The lowest plasma zinc concentration was observed in rats bearing the highest tumor burden. In rats with 16.3% tumor burden, plasma zinc concentration further dropped to 46% of that in tumor-free rats. This inverse

relationship between tumor burden and plasma zinc concentration strongly suggests an association between zinc and mammary tumorigenesis.

However, a lack of hypozincemic effect of mammary tumorigenesis has also been reported in human studies. There was no significant difference in serum/plasma zinc concentrations between breast cancer patients and healthy individuals (Garofalo *et al.*, 1980; Piccinini *et al.*, 1996; Koksoy *et al.*, 1997), between patients with malignant breast cancer and those with benign breast lesions (Garofalo *et al.*, 1980; Koksoy *et al.*, 1997), and among patients at different stages of breast cancer (Garofalo *et al.*, 1980; Yucel *et al.*, 1994; Seven *et al.*, 1998). Furthermore, Gupta *et al.* (1991) reported that serum zinc concentration in patients diagnosed with early stage of breast cancer was elevated as compared to that in healthy individuals. Seven *et al.* (1998) also showed that plasma zinc concentration in breast cancer patients was higher than that in patients with benign breast disease.

Plasma zinc concentration is affected by both nutritional and non-nutritional factors (Jackson, 1989; King, 1990). Under normal circumstances, plasma zinc concentration is maintained through homeostasis. In case of mild zinc deficiency, the intestinal absorption efficiency of zinc increases, the endogenous loss of zinc decreases and the growth rate reduces to conserve tissue zinc. Zinc homeostasis can be re-established without changes in plasma zinc concentration during mild zinc deficiency. When dietary zinc intake is so low that zinc homeostasis cannot be established, plasma zinc concentration is reduced. Reduced total food consumption and/or reduced zinc content of food consumed would result in insufficient zinc intake. In addition to the nutritional factors, plasma zinc concentration can also be suppressed by non-nutritional factors such as stress, infection and inflammation.

Although the cause of the changes in plasma zinc concentration during mammary tumorigenesis is unknown, it is possible that such changes result from reduced total food consumption, reduced zinc content of food consumed, a possible non-nutritional factor, or a combination of these possible causes. Firstly, because the food intake and the use of supplements are not well documented in some of the human studies, the interpretation of the results from human studies mentioned earlier is not possible. In rats, the total feed intake between tumor-bearing and tumor-free rats were not significantly different (Mills *et al.*, 1984; Philcox *et al.*, 1994). Therefore, it is unlikely that the changes in plasma zinc concentration during mammary tumorigenesis result from a reduction in total food consumption.

Another possible cause of the changes in plasma zinc concentration during mammary tumorigenesis is the zinc content of food consumed. Mill *et al.* (1984) demonstrated that plasma zinc concentration was lower in tumor-bearing rats fed a zinc deficient diet as compared to that in tumor-bearing rats pair-fed with an adequate zinc diet. Such decrease in plasma zinc concentration in zinc deficient rats was due to a reduction in zinc content of food consumed. Therefore, plasma zinc concentration could be affected by dietary zinc level during mammary tumorigenesis.

Despite the influence of dietary zinc on plasma zinc concentration, non-nutritional factors might also affect plasma zinc concentration during mammary tumorigenesis. When the amount of total food intake and zinc content in the food consumed were the same, plasma zinc concentration in tumor-bearing rats was significantly lower than that in tumor-free rats (Mills *et al.*, 1984; Philcox *et al.*, 1994). In this case, because the level of dietary zinc was the same between tumor-bearing and tumor-free rats, the change in plasma zinc

concentration was probably due to the development of mammary tumors. As reviewed earlier, zinc plays a role in cell proliferation and tumor cells proliferate at a high rate. It is likely that mammary tumor cells require a constant supply of zinc to support their high rate of cell proliferation. Hence, it is possible that mammary tumorigenesis leads to a reduction in plasma zinc concentration.

4.3.2. Tissue zinc concentrations during mammary tumorigenesis

A few studies have investigated the effect of mammary tumorigenesis on zinc concentration in tissues other than plasma. Philcox *et al.* (1994) reported that an increase in tumor burden did not affect intestinal zinc concentration in rats implanted with mammary adenocarcinoma. On the other hand, the liver zinc concentration was elevated in rats bearing a tumor burden of 16.3% of body weight, but not in rats bearing tumor burden of 4.3% and 9.9% of body weight, as compared to that in tumor-free rats. Mills *et al.* (1984) showed that kidney and heart zinc concentrations were not significantly different between tumor-bearing and tumor-free rats. However, similar to the previous study, the liver zinc concentration in rats bearing an average of 21 g mammary adenocarcinoma was significantly higher than that in tumor-free rats. The cause of the increase in liver zinc concentration and its significance in mammary tumorigenesis are unclear.

4.3.3. Mammary tumor zinc concentration

Several human studies have shown that zinc concentration in the breast cancer tissue is significantly higher than that in the normal breast tissue when expressed on the basis of either wet-tissue weight (Santoliquido *et al.*, 1976; Margalioth *et al.*, 1983; Borella *et al.*,

1997) or dry-tissue weight (Mulay *et al.*, 1971; Santoliquido *et al.*, 1976; Rizk and Sky-Peck, 1984; Ng *et al.*, 1993). This higher breast cancer zinc concentration is possibly due to an increased uptake of zinc by the breast cancer tissue. However, it is well established that the normal breast tissue contains a considerable amount of fatty tissue whereas the breast cancer tissue is composed of tightly packed cancer cells (Cooper, 1992). It is also known that fatty tissue is generally low in zinc (van Rig and Pories, 1980) and body zinc is primarily associated with proteins (Walsh *et al.*, 1994). Therefore, it is possible that the elevated zinc concentration in the breast cancer tissue is a reflection of the compositional difference between normal breast tissue and breast cancer tissue. Thus, further studies are needed to investigate if zinc accumulates in the breast cancer tissue during breast cancer development.

A difference in zinc uptake between mammary tumors and mammary glands has been documented in mice. By injecting radioactive ^{65}Zn into mice bearing spontaneous mammary carcinoma, Tupper *et al.* (1955) showed that the level of ^{65}Zn in mammary tumors was higher than that in normal mammary glands starting from day 1 to day 14 after ^{65}Zn injection indicating an increased uptake and retention of ^{65}Zn by mammary tumors.

4.3.4. Dietary zinc intake and mammary tumorigenesis

Limited information is available regarding the effect of dietary zinc intake on mammary tumorigenesis. To date, only a few animal studies were performed using different experimental models and designs.

Mills *et al.* (1984) reported that low zinc intake (less than 4 mg zinc/kg diet) resulted in a 35% inhibition of mammary tumor growth in male rats implanted with mammary adenocarcinoma when compared to the pair-fed controls fed an adequate zinc diet (35-50 mg

zinc/kg diet). However, it is known that mammary tumorigenesis is affected by hormones such as estrogen and progesterone (Welsch, 1985; Nandi *et al.*, 1995). The implantation of mammary adenocarcinoma into male rats excludes the effect of ovarian hormones on the growth of such mammary tumors. In addition, since the zinc requirement of mammary tumors at various stages of mammary tumorigenesis may be different, the relevance of this finding is limited to implanted mammary tumors which represent established mammary tumors. Thus, although this study showed an inhibitory effect of low zinc intake on the growth of implanted mammary tumors in male rats, the effect of low zinc intake on mammary tumorigenesis under the influence of hormones is still unclear.

By using 7,12-dimethylbenz(a)anthracene (DMBA) to induce mammary tumors in female rats, Fischer *et al.* (1991) showed that there was no significant difference in the total number of mammary tumors developed between rats fed a high zinc diet (150 mg zinc/kg diet) and those fed an adequate zinc diet (30 mg zinc/kg diet). However, more sensitive parameters, such as tumor latency period and tumor incidence, were not reported in this study. Therefore, the effect of high zinc intake on mammary tumorigenesis remains unclear.

4.4. Summary

Although it has been suggested that diet influences the risk of breast cancer, the dietary factors responsible for breast cancer remain unclear. Zinc exerts many physiological functions including cell proliferation and growth promotion. Mammary tumorigenesis is characterized by uncontrolled cell proliferation. It is possible that zinc influences mammary tumorigenesis through its role in mammary tumor cell proliferation. Both animal and human studies provide evidence suggesting a possible linkage between zinc and mammary

tumorigenesis. An increased uptake of ^{65}Zn by spontaneous mammary tumors in mice (Tupper *et al.*, 1955) and an inverse relationship between mammary tumor burden and plasma zinc concentration in rats (Philcox *et al.*, 1994) strongly argue for a role of zinc during mammary tumorigenesis. Furthermore, an apparently higher zinc concentration in the breast cancer tissue as compared to that in the normal breast tissue (Mulay *et al.*, 1971; Santoliquido *et al.*, 1976; Margalioth *et al.*, 1983; Rizk and Sky-Peck, 1984; Ng *et al.*, 1993; Borella *et al.*, 1997) also supports the same notion, although this line of evidence needs to be interpreted with caution. Despite the apparent importance of zinc in mammary tumorigenesis and the possible linkage between zinc and mammary tumorigenesis, the effect of dietary zinc intake on mammary tumorigenesis is essentially unknown.

5. MATERIALS AND METHODS

5.1. Experiment 1

5.1.1. Diets

A modified egg-white based AIN-93G diet (31 mg zinc/kg diet) (Reeves *et al.*, 1993; Reeves, 1996) was used as the control diet (Tables I-1, Appendix I). Four experimental diets were formulated based on the modified egg-white based AIN-93G diet: severe-low-zinc diet (1 mg zinc/kg diet), marginal-low-zinc diet (3 mg zinc/kg diet), high-zinc diet (171 mg zinc/kg diet) and very-high-zinc diet (346 mg zinc/kg diet) (Tables I-1, Appendix I). All the nutrients in the experimental diets were the same except the zinc level.

5.1.2. Dietary treatments and animals

Twenty-one-day-old female Sprague-Dawley rats with an average initial body weight of 43 g (31 – 52 g) (Animal Care Center, UBC) were randomly divided into 7 dietary treatment groups ($n = 12$ rats/group): severe-low-zinc (Z1), marginal-low-zinc (Z3), control (Z31), high-zinc (Z171), very-high-zinc (Z346), pair-fed to severe-low-zinc (PZ31-Z1) and pair-fed to marginal-low-zinc (PZ31-Z3). Rats in Z1, Z3, Z31, Z171, and Z346 groups were fed the severe-low-zinc diet, marginal-low-zinc diet, control diet, high-zinc diet and very-high-zinc diet, respectively, for 100 days *ad libitum*. Dietary zinc deficiency is known to suppress feed intake in rats and reduced caloric intake reduces the risk of mammary tumorigenesis (American Cancer Society Research Workshop, 1993). Thus, pair-fed groups (PZ31-Z1 and PZ31-Z3) were included to overcome this potential confounding factor. Rats in PZ31-Z1 and PZ31-Z3 groups were fed the same amount of the control diet as was

consumed by the rats in Z1 and Z3 groups, respectively, during the previous 24 hours. On day one of the experiment, each rat in the pair-fed groups was given 5 g of the control diet.

Following thorough cleaning, feed jars and water bottles were treated with disodium ethylenediamine tetraacetate (EDTA) overnight and rinsed with double-deionized water before use. All rats had free access to double-deionized water. The rats were housed individually in stainless steel cages in a temperature- and humidity-regulated room with a 12-hour light-dark cycle.

5.1.3 Tumor induction

On day one of the experiment, each dietary treatment group was further divided into sham-treated and MNU-treated groups with 6 rats per group. MNU was dissolved in saline containing 0.05% acetic acid (pH 4) (Thompson and Adlakha, 1991). The MNU solution was prepared just before injection and used within 30 minutes after preparation. The solution was kept on ice and protected from light throughout the injection period. To induce mammary tumorigenesis, rats in the MNU-treated group were injected with MNU at a dose of 50 mg per kg body weight intraperitoneally (Russo *et al.*, 1990; Thompson *et al.*, 1995; Gould, 1995). Rats in the sham-treated group were injected with saline containing 0.05% of acetic acid intraperitoneally.

5.1.4. Assessment of zinc and copper status

Host weight gain, feed intake and plasma zinc concentration were used to assess body zinc status. Body copper status was assessed by determining the plasma and liver copper concentrations.

Body weight was monitored bi-weekly to assess the growth of rats. Host weight gain was calculated using the following formula:

$$\text{host weight gain} = (\text{final body weight} - \text{initial body weight}) - \text{tumor weight}$$

Feed intake of Z31, Z171 and Z346 rats were monitored twice weekly. Feed intake of Z1 and Z3 rats were monitored daily in order to determine the amounts of feed fed to the corresponding rat in PZ31-Z1 and PZ31-Z3 groups. At the end of the feeding trial, plasma was prepared by centrifugation of heparinized blood at 4°C at 2,000 rpm for 15 minutes and stored at -80°C until analysis.

5.1.5. Determination of tissue zinc and copper concentrations

At the end of the feeding trial, the rats were sacrificed and the liver, kidney, heart, lung, muscle, femur, small intestine, skin, normal mammary gland, and mammary tumor were removed for the determination of tissue zinc concentrations. The small intestine was rinsed thoroughly with double-deionized water. Copper concentration was determined in the liver. The tissues were stored at -80°C, except for the liver, mammary gland and mammary tumor which were stored in liquid nitrogen, until analyses.

All re-usable glasswares and crucibles were acid washed in 2 N nitric acid overnight to prevent potential contamination.

Tissues (0.5 - 1.0 g) were dried in acid-washed crucibles to a constant weight at 100°C overnight. The dried tissues were cooled in a desiccator. Then, the dried tissues were transferred quantitatively to acid-washed glass test tubes and wet ashed with 3 ml of

concentrated nitric acid at 70°C overnight (Clegg *et al.*, 1981). Each test tube was covered with an acid-washed glass marble to prevent evaporation. Upon complete digestion, the wet ashed tissue samples were transferred quantitatively to 5 or 10 ml acid-washed volumetric flasks and brought to volume with double-deionized water. The digested tissue samples were then transferred to new plastic vials. The digested tissue samples and plasma samples were diluted with 0.1 N nitric acid to an appropriate concentration for the determination of zinc and copper using flame atomic absorption spectroscopy (Atomic Absorption Spectrophotometer, model 2380, Perkin Elmer, Norwalk, CT, USA). The tissue zinc and copper concentrations were expressed on a per dry-tissue weight basis.

5.1.6. Determination of protein concentrations in mammary tumors and mammary glands

The protein concentrations in mammary tumors and mammary glands was determined following the method of Lowry *et al.* (1951). Briefly, mammary tumors and mammary glands (0.2 g) were homogenized with a total of 9.8 ml 1 M sodium hydroxide using the Polytron (PCU-2-110, Brinkmann) at room temperature. The homogenates were allowed to sit for 1 hour at room temperature. Then, the homogenates were diluted ten times with 1 M sodium hydroxide. The diluted homogenates (100 µl) were added to 1 ml of a 50:1 mixture of 2% sodium carbonate and 0.5% copper sulfate in 1% sodium tartrate. The mixtures were then incubated for 10 minutes at room temperature. After the incubation, 100 µl of diluted Folin-Ciocalteu phenol reagent (1:1) was added to the incubation mixtures and mixed immediately. After 30 minutes incubation, the absorbance of the final reaction mixtures at 750 nm was obtained using a spectrophotometer (UV/VIS Spectrophotometer, PU 8600,

Philips, Cambridge, Great Britain). The protein concentration of the samples was determined from the standard curve constructed using bovine serum albumin. Due to the low number of tumor-bearing rats and the small tumor size, mammary tumors and tumor-free mammary glands from three tumor-bearing rats in PZ31-Z3, Z31, Z171 and Z346 MNU-treated groups were analyzed. Mammary glands from three rats in PZ31-Z3, Z31, Z171 and Z346 sham-treated groups were also analyzed for the protein concentration.

5.1.7. Determination of lipid concentrations in mammary tumors and mammary glands

The lipid concentrations in mammary tumors and mammary glands was determined following the method of Folch *et al.* (1957). Briefly, mammary tumors and mammary glands (0.5 – 1.0 g) were homogenized with a total of 10 - 20 ml chloroform : methanol (2:1) using the Polytron. The mammary tumor and mammary gland homogenates were left overnight at room temperature for lipid extraction. The following day (day 2), the homogenates were vacuum filtered using Whatman #1 filter paper and transferred to graduated cylinders. Sodium chloride solution (0.73%) at a volume of 20% that of the extracts was added to the extracts and mixed. The mixture was left at room temperature overnight to allow for phase separation. On day 3, after recording the total volume of the lower phase, the upper phase was removed. The lower phase was transferred to a pre-weighed, desiccated aluminium dish and allowed to evaporate in the fume hood. Then, the aluminium dishes were placed in a drying oven at 100°C for 3 hours and cooled in a desiccator overnight. On day 4, the weight of aluminium dishes containing lipid extracts was recorded. Similar to the rationale for protein determination, mammary tumors and tumor-free mammary glands from three tumor-bearing rats in PZ31-Z3, Z31, Z171 and Z346 MNU-treated groups as well as mammary

glands from three sham-treated rats in PZ31-Z3, Z31, Z171 and Z346 groups were analyzed for the lipid concentration.

5.1.8. Assessment of the development of mammary tumors

Rats were palpated weekly for mammary tumors starting at week 4 after MNU injection. The mammary tumor development was assessed using the following parameters:

- 1) tumor incidence: percentage of rats that developed palpable mammary tumors in each dietary-MNU treatment group
- 2) number of palpable mammary tumors formed in each dietary-MNU treatment group
- 3) tumor multiplicity: the average number of palpable mammary tumors per tumor-bearing rat in each dietary-MNU treatment group
- 4) tumor latency period: the average time that the first mammary tumor became palpable in a tumor-bearing rat in each dietary-MNU treatment group

5.1.9. Statistical analysis

The differences among dietary treatment means and between MNU treatment means were analyzed using one-way analysis of variance followed by the least significant difference test ($p < 0.05$) (The SAS System for Windows Release 6.12).

5.2. Experiment 2

5.2.1. Diets

A modified egg-white based AIN-93G diet was used as the control diet (31 mg zinc/kg diet) (Tables I-2, Appendix I). Rats fed an egg-white based diet required 12 mg zinc per kg diet to support maximum growth when they were housed in a zinc-free environment (National Research Council, 1972). Since the zinc content of the AIN-93G diet is about three times the level that supports maximum growth, it appears to be important to include a diet containing the requirement level of zinc (12 mg zinc/kg diet). In addition, a marginal-low-zinc diet (3 mg zinc/kg diet) and a high-zinc diet (155 mg zinc/kg diet) were also formulated. These experimental diets were formulated based on the modified egg-white based AIN-93G diet. All the nutrients in the diets were the same except for the zinc level (Tables I-2, Appendix I).

5.2.2. Dietary treatments and animals

Twenty-one-day-old female Sprague-Dawley rats with an average initial body weight of 41 g (27 – 54 g) (Animal Care Center, UBC) were randomly assigned to one of the 6 dietary treatment groups ($n = 36$ rats/group): marginal-low-zinc (Z3), required-zinc (Z12), control (Z31), high-zinc (Z155), pair-fed with required-zinc diet (PZ12) and pair-fed with control diet (PZ31). Rats in Z3, Z12, Z31 and Z155 groups were fed the marginal-low-zinc, required-zinc, control and high-zinc diets, respectively, *ad libitum*. Rats in PZ12 and PZ31 groups were fed the same amount of the required-zinc diet and control diet, respectively, as was consumed by Z3 rats during the previous 24 hours. Six rats from each dietary treatment group were sacrificed on day 28 and 56 post-MNU injection. The remaining 24 rats in each

group were maintained on the assigned diet for a total of 98 days. Animal care procedure was the same as described in Experiment 1.

5.2.3. Tumor induction

Based on the literature (van Rig and Pories, 1980) and the results obtained from Experiment 1, sham-treatment does not induce mammary tumorigenesis. Therefore, in order to minimize the use of animals, sham-treated group was excluded from this experiment. The preparation and the handling of MNU solution were the same as described in Experiment 1. To minimize the suffering, the MNU dosage in this experiment was reduced from 50 mg/kg body weight, as used in Experiment 1, to 40 mg/kg body weight. All the rats were injected with MNU at this dosage intraperitoneally on day one of the experiment.

5.2.4. Assessment of zinc status

Host weight gain, feed intake, plasma zinc concentration and hematocrit were used to assess the body zinc status. Body weight was monitored monthly to assess the growth of rats. Host weight gain was calculated using the following formula:

$$\text{host weight gain} = (\text{final body weight} - \text{initial body weight}) - \text{tumor weight}$$

Feed intake of Z12, Z31 and Z155 rats were monitored twice weekly. Feed intake of Z3 rats was monitored daily in order to determine the amounts of feed fed to the rats in PZ12 and PZ31 groups. At the end of the feeding trial, plasma was prepared by centrifugation of heparinized blood at 4°C at 2,000 rpm for 15 minutes and stored at -80°C until analysis.

5.2.5. Assessment of the development and growth of mammary tumors

Rats were palpated weekly for mammary tumors starting at week 4 after MNU injection. The tumor incidence, number of palpable tumors, tumor multiplicity, tumor weight, tumor burden and tumor latency period were determined to assess the development and growth of mammary tumors.

On day 28 and 56 post-MNU injection, the right abdominal-inguinal mammary glands from 6 rats in each dietary treatment group were carefully excised for whole mounts preparation to detect the presence of microscopic lesions (Thompson *et al.*, 1995). Briefly, mammary glands were spread onto microscopic slides (75 X 50 mm) and covered with nylon fabric. The mammary glands were then fixed in 10% neutral buffered formalin overnight. The following day, the whole mounts were rinsed with double-deionized water for 15 minutes and dehydrated in a series of ethanols (70%, 95% and 100%) for 1 hour each. After dehydration, the whole mounts were placed in toluene until they were clear (3 hours and 40 – 97 hours for whole mounts prepared on day 28 and 56 post-MNU injection, respectively). The whole mounts were then rehydrated in a series of ethanols (100%, 95% and 75%) and water for 1 hour each, and stained in carmine solution (1 g/1000 ml double-deionized water) until staining was completed (3-6 days and 7-13 days for whole mounts prepared on day 28 and 56 post-MNU injection, respectively). Subsequent to staining, the whole mounts were dehydrated in a series of ethanols (70%, 95% and 100%) for 1 hour each and cleared in xylene for 3-6 hours. Finally, the whole mounts were removed from xylene, drained, sealed in heat seal pouches filled with 20 ml methyl salicylate, and left overnight. Excess methyl

salicylate and air were removed the next day. The whole mounts were examined under microscope with 16x magnification.

5.2.6. Determination of tissue zinc concentrations

At the end of the feeding trial, the rats were sacrificed and the femur, liver, small intestine, mammary gland and mammary tumor were removed. The small intestine was rinsed thoroughly with double-deionized water. The tissues were stored at -80°C , except for the mammary gland and mammary tumor which were stored in liquid nitrogen, until analysis. From each dietary treatment group, the tissues from 6 tumor-free rats and 6 tumor-bearing rats with the highest tumor burden were used for the determination of zinc. The procedure was the same as described in Experiment 1. The wet-ashed tissue samples were diluted with double-deionized water and the plasma samples were diluted with 0.1 N nitric acid to an appropriate concentration for the determination of zinc using flame atomic absorption spectroscopy.

5.2.7. Statistical analysis

The differences among dietary treatment means and between tumor-free and tumor-bearing means were analyzed using one-way analysis of variance followed by the least significant difference test ($p < 0.05$) (The SAS System for Windows Release 6.12), except for the differences in tumor incidence among dietary treatment groups which were analyzed using χ^2 test ($p < 0.05$).

6. RESULTS

6.1. Experiment 1

The specific objectives of Experiment 1 were:

1. To investigate the effect of MNU treatment on body zinc distribution in rats fed different levels of dietary zinc.
2. To compare the zinc concentrations between MNU-induced mammary tumors and mammary glands in rats.

6.1.1. Assessment of zinc status

Rats fed the severe-low-zinc diet developed zinc deficiency as indicated by growth retardation, cyclic eating pattern, dermal lesions on the palms and around the eye area, and alopecia. Only two out of the fourteen Z1 rats survived the feeding trial. Due to the low survival rate of Z1 rats (14%), Z1 and PZ31-Z1 rats were excluded from the biochemical analyses.

The symptoms of zinc deficiency, such as growth retardation, cyclic eating pattern and alopecia, were also observed in most of the Z3 sham-treated and Z3 MNU-treated rats. Feed intake of Z3 sham-treated rats was 61% that of Z31 sham-treated rats while feed intake of Z3 MNU-treated rats was 64% that of Z31 MNU-treated rats ($p < 0.05$) (Table 1). Host weight gain of Z3 sham-treated rats was 57% that of Z31 sham-treated rats ($p < 0.05$). Host weight gain of Z3 MNU-treated rats was 59% that of Z31 MNU-treated rats ($p < 0.05$). Feed intake : host weight gain ratio between Z3 sham-treated and Z31 sham-treated rats were not significantly different whereas the ratio in Z3 MNU-treated rats was significantly higher than that in Z31 MNU-treated rats. Plasma zinc concentrations in Z31 sham-treated and Z31

Table 1. Effect of dietary zinc intake and N-methyl-N-nitrosourea (MNU) treatment on feed intake, host weight gain and plasma zinc concentration in rats¹

	MNU	Dietary treatment groups				
		Z3	PZ31-Z3	Z31	Z171	Z346
Feed intake (g/rat/day)	-	10.3±1.2 ^b	10.2±1.2 ^b	16.8±1.5 ^a	15.9±0.7 ^a	15.7±1.0 ^a
	+	8.4±0.8 ^b	8.4±0.8 ^b	13.2±0.7 ^a	13.3±0.6 ^{a*}	14.1±0.6 ^a
Host weight gain ² (g/rat/day)	-	1.6±0.2 ^b	1.8±0.1 ^b	2.8±0.1 ^a	2.5±0.1 ^a	2.7±0.3 ^a
	+	1.3±0.2 ^c	1.6±0.1 ^{bc}	2.2±0.2 ^a	2.0±0.1 ^{ab*}	2.0±0.1 ^{ab*}
Feed intake/ Host weight gain	-	6.8±0.5	5.7±0.3	6.2±0.8	6.4±0.4	6.0±0.4
	+	7.1±0.7 ^a	5.2±0.2 ^c	6.0±0.3 ^{bc}	6.8±0.3 ^{ab}	7.1±0.2 ^{a*}
Plasma zinc (µg/ml)	-	0.8±0.2 ^b	1.9±0.1 ^a	2.0±0.1 ^a	1.9±0.1 ^a	1.7±0.1 ^a
	+	0.7±0.3 ^c	1.8±0.1 ^{ab}	2.0±0.0 ^a	1.4±0.1 ^{b*}	1.5±0.1 ^b

¹Values represent the mean ± SEM, *n*=6 rats (except Z3 sham-treated and MNU-treated, PZ31-Z3 sham-treated and MNU-treated, and Z31 MNU-treated groups, where *n*=5). The means among dietary treatments in each row not sharing a common letter are significantly different (*p*<0.05). The asterisk indicates a significant difference in the means between sham-treated and MNU-treated groups in each dietary treatment group (*p*<0.05). Z3: marginal-low-zinc; PZ31-Z3: pair-fed to Z3; Z31: control; Z171: high-zinc; Z346: very-high-zinc. MNU-: sham-treated; MNU+: MNU-treated.

²Host weight gain = body weight gain – tumor weight.

MNU-treated rats were 2.0 µg/ml. Plasma zinc concentrations in Z3 sham-treated and Z3 MNU-treated rats were 0.8 and 0.7 µg/ml, respectively, which were significantly lower than that in Z31 sham-treated and Z31 MNU-treated rats.

Since zinc deficiency suppressed feed intake, a pair-fed group was included in order to differentiate the effect caused by zinc *per se* and the effect caused by reduced feed intake associated with dietary zinc deficiency. Z3 sham-treated and Z3 MNU-treated rats gained similar amounts of weight as compared to PZ31-Z3 sham-treated and PZ31-Z3 MNU-treated rats, respectively. Since Z3 rats and PZ31-Z3 rats, regardless of MNU treatment, consumed a similar amount of feed and gained a similar amount of weight, the reduced host weight gain in Z3 rats as compared to Z31 rats was due to the reduced feed intake associated with dietary zinc deficiency. Feed intake : host weight gain ratio between Z3 sham-treated and PZ31-Z3 sham-treated rats were not significantly different. Z3 MNU-treated rats had a significantly higher feed intake : host weight gain ratio as compared to that in PZ31-Z3 MNU-treated rats. Plasma zinc concentrations in Z3 sham-treated rats was 42% that in PZ31-Z3 sham-treated rats while plasma zinc concentration in Z3 MNU treated rats was 39% that in PZ31-Z3 MNU-treated rats ($p < 0.05$). Therefore, with a similar host weight gain but reduced plasma zinc concentration as compared to PZ31-Z3 rats, Z3 sham-treated and MNU-treated rats developed marginal zinc deficiency.

Feed intake, host weight gain, feed intake : host weight gain ratio and plasma zinc concentrations in Z31, Z171 and Z346 sham-treated rats were not significantly different (Table 1). Similarly, feed intake and host weight gain in Z31, Z171 and Z346 MNU-treated rats were not significantly different. Feed intake : host weight gain ratio in Z31 MNU-treated was not significantly different from that in Z171 MNU-treated rats, but was significantly

lower than that in Z346 MNU-treated rats. Plasma zinc concentrations in Z171 and Z346 MNU-treated rats were 1.4 and 1.5 µg/ml, respectively, which were significantly lower than that in Z31 MNU-treated rats.

MNU treatment significantly suppressed host weight gain in Z171 and Z346 MNU-treated rats when compared to Z171 and Z346 sham-treated rats, respectively (Table 1). The 20% reduction in host weight gain in Z171 MNU-treated rats as compared to that in Z171 sham-treated rats could be attributed to the 16% reduction in feed intake in the former group. Feed intake of Z3, PZ31-Z3, Z31 and Z346 rats were not affected by MNU treatment. Feed intake : host weight gain ratio was not significantly different between sham-treated and MNU-treated rats within the same dietary treatment group, except in Z346 group. The feed intake : host weight gain ratio in Z346 MNU-treated rats was significantly higher when compared to that in Z346 sham-treated rats. MNU treatment caused a significant reduction in plasma zinc concentration in Z171 MNU-treated rats, but not in Z3, PZ31-Z3, Z31 and Z346 MNU- treated rats, as compared to their corresponding sham-treated rats.

6.1.2. Assessment of copper status

It has been shown that excessive intake of zinc would lead to a compromised copper status due to the reduction of copper absorption at the intestinal level (King and Keen, 1994). In order to determine whether the copper status was compromised in rats fed high-zinc diets in this study, plasma and liver copper concentrations were determined.

Plasma and liver copper concentrations were not significantly different among dietary treatment groups in sham-treated rats (Table 2). A similar result was shown in MNU-treated rats. In addition, comparing sham-treated rats to MNU-treated rats within the same dietary

Table 2. Effect of dietary zinc intake and N-methyl-N-nitrosourea (MNU) treatment on plasma and liver copper concentrations in rats¹

Tissues	MNU	Copper concentration				
		Z3	PZ31-Z3	Z31	Z171	Z346
Plasma ² ($\mu\text{g/ml}$)	-	1.4 \pm 0.1	1.5 \pm 0.1	1.5 \pm 0.0	1.5 \pm 0.1	1.5 \pm 0.1
	+	1.4 \pm 0.0	1.5 \pm 0.0	1.4 \pm 0.1	1.4 \pm 0.2	1.4 \pm 0.0
Liver ($\mu\text{g/g}$ dry weight)	-	21 \pm 3	22 \pm 2	17 \pm 1	17 \pm 1	18 \pm 1
	+	16 \pm 1	20 \pm 2	17 \pm 1	15 \pm 2	20 \pm 2

¹Values represent the mean \pm SEM, $n=6$ rats (except Z3 sham-treated and MNU-treated, PZ31-Z3 sham-treated and MNU-treated, and Z31 MNU-treated groups, where $n=5$). Z3: marginal-low-zinc; PZ31-Z3: pair-fed to Z3; Z31: control; Z171: high-zinc; Z346: very-high-zinc. MNU-: sham-treated; MNU+: MNU-treated.

²For the determination of plasma copper concentration, $n=2$ in Z3 MNU-treated group.

treatment group, plasma and liver copper concentrations were also not significantly different. Therefore, the copper status of Z171 and Z346 rats was not compromised.

6.1.3. Effect of dietary zinc intake and N-methyl-N-nitrosourea (MNU) treatment on body zinc distribution

In this study, body zinc distribution was determined by the zinc concentrations in the femur, liver, skin, small intestine, kidney, lung, muscle, heart, mammary gland and mammary tumor. Z3 rats had the lowest femur zinc concentration among all the dietary treatment groups (Table 3). Femur zinc concentration in Z3 sham-treated rats was 23% and 26% that in Z31 sham-treated and PZ31-Z3 sham-treated rats, respectively ($p < 0.05$). Femur zinc concentration increased as the dietary zinc level increased. Femur zinc concentration in Z171 sham-treated rats was 129% that in Z31 sham-treated rats. The increase in femur zinc concentration was more profound in Z346 rats. Femur zinc concentration in Z346 sham-treated rats was 208% that in Z31 sham-treated rats ($p < 0.05$). Similar to sham-treated rats, femur zinc concentration in Z3 MNU-treated rats was 24% and 26% that in Z31 MNU-treated and PZ31-Z3 MNU-treated rats, respectively. Femur zinc concentration in Z171 MNU-treated rats was 127% that in Z31 MNU-treated rats while femur zinc concentration in Z346 MNU-treated rats was 199% that in Z31 MNU-treated rats ($p < 0.05$). Clearly, femur zinc concentration was responsive to dietary zinc intake. In contrast, there was no significant difference in femur zinc concentration between sham-treated and MNU-treated rats in each dietary treatment group indicating that MNU treatment had no effect on femur zinc concentration in rats.

Table 3. Effect of dietary zinc intake and N-methyl-N-nitrosourea (MNU) treatment on tissue zinc concentrations in rats¹

Tissues	MNU	Zinc concentration ($\mu\text{g/g}$ dry weight)				
		Z3	PZ31-Z3	Z31	Z171	Z346
Femur	-	89 \pm 12 ^d	349 \pm 9 ^c	392 \pm 11 ^c	504 \pm 19 ^b	815 \pm 83 ^a
	+	88 \pm 18 ^d	340 \pm 8 ^c	370 \pm 11 ^{bc}	471 \pm 30 ^b	737 \pm 65 ^a
Liver	-	70 \pm 7 ^c	100 \pm 6 ^{ab}	85 \pm 3 ^b	88 \pm 3 ^{ab}	101 \pm 6 ^a
	+	75 \pm 5 ^c	94 \pm 3 ^{ab}	86 \pm 4 ^{bc}	87 \pm 6 ^{bc}	105 \pm 3 ^a
Skin	-	55 \pm 2 ^b	88 \pm 5 ^a	70 \pm 6 ^{ab}	67 \pm 2 ^b	61 \pm 8 ^b
	+	53 \pm 5 ^b	84 \pm 4 ^a	66 \pm 8 ^{ab}	68 \pm 4 ^{ab}	65 \pm 6 ^b
Small intestine	-	83 \pm 9	113 \pm 15	69 \pm 6	79 \pm 9	81 \pm 7
	+	80 \pm 8 ^b	122 \pm 8 ^a	120 \pm 14 ^{a*}	109 \pm 10 ^a	123 \pm 14 ^{a*}
Kidney	-	81 \pm 4 ^c	106 \pm 4 ^b	113 \pm 3 ^{ab}	119 \pm 5 ^a	118 \pm 2 ^a
	+	79 \pm 4 ^c	115 \pm 4 ^a	116 \pm 5 ^a	97 \pm 4 ^{b*}	99 \pm 7 ^{b*}
Lung	-	76 \pm 4	80 \pm 4	73 \pm 3	71 \pm 4	65 \pm 2
	+	81 \pm 4	79 \pm 3	80 \pm 3	83 \pm 5	74 \pm 2 [*]
Muscle	-	55 \pm 9	47 \pm 4	50 \pm 4	59 \pm 7	47 \pm 6
	+	57 \pm 5	51 \pm 2	56 \pm 6	56 \pm 6	51 \pm 4
Heart	-	70 \pm 2	71 \pm 3	70 \pm 1	72 \pm 1	73 \pm 3
	+	67 \pm 2	76 \pm 5	76 \pm 4	71 \pm 3	71 \pm 3
Mammary gland	-	9.2 \pm 2.0	11.0 \pm 2.1	5.4 \pm 1.0	7.0 \pm 1.2	5.6 \pm 1.0
	+	11.2 \pm 1.5	13.5 \pm 1.6	7.3 \pm 1.9	8.9 \pm 1.2	8.9 \pm 1.5

¹Values represent the mean \pm SEM, $n=6$ rats (except Z3 sham-treated and MNU-treated, PZ31-Z3 sham-treated and MNU-treated, and Z31 MNU-treated groups, where $n=5$). The means among dietary treatments in each row not sharing a common letter are significantly different ($p<0.05$). The asterisk indicates a significant difference in the means between sham-treated and MNU-treated groups in each dietary treatment group ($p<0.05$). Z3: marginal-low-zinc; PZ31-Z3: pair-fed to Z3; Z31: control; Z171: high-zinc; Z346: very-high-zinc. MNU-: sham-treated; MNU+: MNU-treated.

Liver zinc concentration in Z3 sham-treated rats was 30% lower than that in PZ31-Z3 sham-treated rats ($p < 0.05$) (Table 3). Liver zinc concentrations were not significantly different between Z31 and Z171 sham-treated rats, but liver zinc concentration in Z346 sham-treated rats was 19% higher than that in Z31 sham-treated rats ($p < 0.05$). Similar to sham-treated rats, Z3 MNU-treated rats had a 20% decrease in liver zinc concentration as compared to that in PZ31-Z3 MNU-treated rats ($p < 0.05$). Z346 MNU-treated rats had a 22% increase in liver zinc concentration as compared to that in Z31 MNU-treated rats. When liver zinc concentration was compared between sham-treated and MNU-treated rats with the same dietary treatment, they were not significantly different. Hence, liver zinc concentration was not affected by MNU treatment.

PZ31-Z3 sham-treated rats had a significantly higher skin zinc concentration than that in Z3 sham-treated rats (Table 3). Skin zinc concentrations in Z31, Z171 and Z346 sham-treated rats were not significantly different. Likewise, skin zinc concentration in PZ31-Z3 MNU-treated rats was also significantly higher than that in Z3 MNU-treated rats while skin zinc concentrations among Z31, Z171 and Z346 MNU-treated rats were not significantly different. Within the same dietary treatment group, skin zinc concentration in MNU-treated rats was not significantly different from that in sham-treated rats.

Dietary zinc intake did not affect small intestinal zinc concentration in sham-treated rats (Table 3). Z3 MNU-treated rats had a 34% reduction in small intestinal zinc concentration as compared to that in PZ31-Z3 MNU-treated rats ($p < 0.05$). Small intestinal zinc concentrations were not significantly different among Z31, Z171 and Z346 MNU-treated rats. MNU treatment resulted in a significant increase in small intestinal zinc concentrations in Z31 and Z346 rats. Small intestinal zinc concentration in Z31 MNU-

treated rats was 174% that in Z31 sham-treated rats while small intestinal zinc concentration in Z346 MNU-treated rats was 152% that in Z346 sham-treated rats ($p < 0.05$). Small intestinal zinc concentration in Z171 MNU-treated rats was also 38% higher than that in Z171 sham-treated rats; however, it did not reach statistical significance.

Z3 rats had the lowest kidney zinc concentration among all the dietary treatment groups (Table 3). Kidney zinc concentration in Z3 sham-treated rats was 24% lower than that in PZ31-Z3 sham-treated rats ($p < 0.05$). Kidney zinc concentrations were not significantly different among Z31, Z171 and Z346 sham-treated rats. On the other hand, kidney zinc concentration in Z3 MNU-treated rats was 31% lower than that in PZ31-Z3 MNU-treated rats. Kidney zinc concentration in Z171 and Z346 MNU-treated rats showed a 17% and 15% reduction, respectively, as compared to that in Z31 MNU-treated rats ($p < 0.05$). MNU treatment did not affect kidney zinc concentrations in Z3, PZ31-Z3 and Z31 rats. However, kidney zinc concentration in Z171 MNU-treated rats was 18% lower than that in Z171 sham-treated rats ($p < 0.05$). Similarly, kidney zinc concentration in Z346 MNU-treated rats was 16% lower than that in Z346 sham-treated rats ($p < 0.05$).

Lung zinc concentrations were not significantly different among sham-treated dietary treatment groups (Table 3). Similarly, in MNU-treated rats, lung zinc concentrations were also not significantly different among dietary treatment groups. Within the same dietary treatment group, lung zinc concentrations in sham-treated and MNU-treated rats were not significantly different, except in Z346 group. MNU treatment resulted in a significant increase in lung zinc concentration in Z346 MNU-treated rats as compared to that in Z346 sham-treated rats.

Zinc concentrations in the muscle, heart and mammary gland were not significantly different regardless of the dietary treatment and MNU treatment in rats (Table 3).

Zinc concentrations in MNU-induced mammary tumors were not significantly different among all the dietary treatment groups (Table 4).

6.1.4. Zinc concentrations in mammary tumors and mammary glands

6.1.4.1. Composition of mammary tumors and mammary glands

Mammary tumor zinc concentration was in the range of 83 – 102 $\mu\text{g/g}$ dry-tissue weight (Table 4) whereas mammary gland zinc concentration was 5.4 – 13.5 $\mu\text{g/g}$ dry-tissue weight (Table 3). Zinc concentration expressed on a per dry-tissue weight basis in the mammary tumor was 6-19 times higher than that in the mammary gland. It is well documented that mammary tumors are composed of tightly packed tumor cells while normal mammary tissue contains a considerable amount of fat (Cooper, 1992). It is also well established that body zinc is primarily associated with protein (Walsh *et al.*, 1994) while fatty tissue is generally low in zinc (van Rig and Pories, 1980). Therefore, it is important to clarify whether the apparently elevated mammary tumor zinc concentration as compared to that in mammary gland was due to the compositional difference between the two types of tissue or a true accumulation of zinc in the MNU-induced mammary tumor in rats.

The tissue composition was assessed by determining the moisture content, and the protein and lipid concentrations in the normal mammary gland from sham-treated rats (NMG) as well as tumor-free mammary gland (TFMG) and MNU-induced mammary tumor (Tumor) from MNU-treated tumor-bearing rats. Since dietary zinc intake had no effect on the moisture content, and the protein and lipid concentrations in NMG, TFMG and Tumor

Table 4. Effect of dietary zinc intake on zinc concentration in N-methyl-N-nitrosourea (MNU)-induced mammary tumors¹

	Zinc concentration ($\mu\text{g/g}$ dry weight)				
	Z3	PZ31-Z3	Z31	Z171	Z346
Mammary tumor	92	83 ± 11	90 ± 10	86 ± 8	102 ± 5

¹ Values represent the mean \pm SEM. Z3, $n=1$; PZ31-Z3, $n=5$; Z31, $n=8$; Z171, $n=7$; Z346, $n=4$. Z3: marginal-low-zinc; PZ31-Z3: pair-fed to Z3; Z31: control; Z171: high-zinc; Z346: very-high-zinc.

(Table III-1 – III-3, Appendix III) and the sample size was small ($n=3$), the results from different dietary treatment groups were pooled to compare the composition of mammary tumors and mammary glands (Table 5).

The moisture content, and the protein and lipid concentrations between NMG and TFMG were not significantly different indicating that MNU treatment had no effect on the composition of mammary glands (Table 5). However, the moisture content in Tumor (81%) was significantly higher than that in NMG (25%) and TFMG (30%). The protein concentration in Tumor was 7.8 and 5.8 times that in NMG and TFMG, respectively ($p < 0.05$). On the contrary, the lipid concentration in Tumor was significantly lower than that in NMG and TFMG. Clearly, the composition of mammary tumors and mammary glands were different as indicated by the significantly higher moisture content and protein concentration as well as the lower lipid concentration in mammary tumors as compared to that in mammary glands.

6.1.4.2. Accumulation of zinc in mammary tumors

Mammary tumor and mammary gland zinc concentrations, when expressed on the basis of wet-tissue weight, dry-tissue weight or tissue protein, were not affected by dietary zinc intake (Table III-4 – III-6, Appendix III). Due to the small sample size ($n = 3$), the results from different dietary treatment groups were pooled to compare the zinc concentrations in mammary tumors and mammary glands (Table 6).

Zinc concentrations, when expressed on a per wet-tissue or dry-tissue weight basis, were not significantly different between NMG and TFMG (Table 6). In contrast, zinc concentrations in both NMG and TFMG were significantly lower than that in Tumor. On a

Table 5. The composition of mammary glands and N-methyl-N-nitrosourea (MNU)-induced mammary tumors in rats with and without MNU treatment¹

Tissues	Moisture (%)	Protein (g/g dry weight)	Lipid (g/g dry weight)
NMG	25 ± 3 [#]	0.09 ± 0.01 [#]	0.81 ± 0.04 [*]
TFMG	30 ± 3 [#]	0.12 ± 0.02 [#]	0.75 ± 0.05 [*]
Tumor	81 ± 1 [*]	0.70 ± 0.02 [*]	0.35 ± 0.07 [#]

¹Values represent the mean ± SEM, *n*=12 rats (except for the lipid concentration in Tumor, *n*=11). The means among tissues in each column not sharing a common symbol are significantly different (*p*<0.05). NMG: normal mammary gland from sham-treated rats; TFMG: tumor-free mammary gland from MNU-treated tumor-bearing rats; Tumor: MNU-induced mammary tumor from MNU-treated tumor-bearing rats.

Table 6. Zinc concentrations in mammary glands and N-methyl-N-nitrosourea (MNU)-induced mammary tumors in rats with and without MNU treatment¹

Tissues	Zinc concentration		
	($\mu\text{g/g}$ wet weight)	($\mu\text{g/g}$ dry weight)	($\mu\text{g/g}$ protein)
NMG	$5 \pm 1^{\#}$	$8 \pm 1^{\#}$	$90 \pm 11^{\#}$
TFMG	$7 \pm 0^{\#}$	$10 \pm 1^{\#}$	$97 \pm 15^{\#}$
Tumor	$16 \pm 1^*$	$90 \pm 6^*$	$125 \pm 3^*$

¹Values represent the mean \pm SEM, $n=12$ rats. The means among tissues in each column not sharing a common symbol are significantly different ($p<0.05$). NMG: normal mammary gland from sham-treated rats; TFMG: tumor-free mammary gland from MNU-treated tumor-bearing rats; Tumor: MNU-induced mammary tumor from MNU-treated tumor-bearing rats.

per wet-tissue weight basis, zinc concentration in Tumor was 3.2 and 2.3 times that in NMG and TFMG, respectively ($p < 0.05$). On a per dry-tissue weight basis, zinc concentration in Tumor was 11 and 9 times that in NMG and TFMG, respectively ($p < 0.05$). The higher moisture content in Tumor is mainly responsible for the greater increase in zinc concentration in this tissue when expressed on a per dry-tissue weight basis (Table 5 and 6).

In addition to the higher moisture content, the protein concentration in Tumor was also significantly higher than that in NMG and TFMG (Table 5). It is well established that body zinc is primarily associated with protein (Walsh *et al.*, 1994). Therefore, it is important to determine if the apparently higher mammary tumor zinc concentration was a reflection of the higher protein concentration. On a per tissue protein basis, zinc concentrations between NMG and TFMG were not significantly different (Table 6). However, zinc concentration in Tumor was 1.4 and 1.3 times that in NMG and TFMG, respectively ($p < 0.05$). This significantly higher zinc concentration expressed on the basis of tissue protein in Tumor indicates an accumulation of zinc in MNU-induced mammary tumors in rats.

6.1.5. Effect of dietary zinc intake on the development of N-methyl-N-nitrosourea (MNU)-induced mammary tumors

MNU treatment, but not sham-treatment, resulted in the development of mammary tumors, regardless of the dietary zinc intake. The tumor incidence in Z3 MNU-treated rats (20%) was lower than that in PZ31-Z3 MNU-treated (60%), Z31 MNU-treated (80%), Z171 MNU-treated (50%) and Z346 MNU-treated (67%) rats (Table 7). Similarly, the number of palpable tumors in Z3 MNU-treated rats (1) was also lower than that in PZ31-Z3 MNU-treated (6), Z31 MNU-treated (8), Z171 MNU-treated (7) and Z346 MNU-treated (7) rats.

Table 7. Effect of dietary zinc intake and N-methyl-N-nitrosourea (MNU) treatment on the development of MNU-induced mammary tumors¹

	MNU	Dietary treatment groups				
		Z3	PZ31-Z3	Z31	Z171	Z346
Tumor incidence (%)	-	0	0	0	0	0
	+	1/5 (20)	3/5 (60)	4/5 (80)	3/6 (50)	4/6 (67)
Number of palpable tumors	-	-	-	-	-	-
	+	1	6	8	7	7
Tumor multiplicity ²	-	-	-	-	-	-
	+	1	2.0 ± 0.6	2.0 ± 0.7	2.3 ± 0.3	1.8 ± 0.5
Latency period ² (Weeks after MNU injection)	-	-	-	-	-	-
	+	12	11.0 ± 1.2	9.8 ± 1.3	7.3 ± 0.3	8.0 ± 1.1

¹There were 6 rats/group (except Z3 sham-treated and MNU-treated, PZ31-Z3 sham-treated and MNU-treated, and Z31 MNU-treated groups, where $n=5$). Z3: marginal-low-zinc; PZ31-Z3: pair-fed to Z3; Z31: control; Z171: high-zinc; Z346: very-high-zinc. MNU-: sham-treated; MNU+: MNU-treated.

²Values represent the mean ± SEM.

Furthermore, Z3 MNU-treated rats had the lowest tumor multiplicity and longest latency period among the dietary treatment groups. Together, these results suggest that marginal zinc deficiency suppresses the development of MNU-induced mammary tumors in rats. However, due to the small sample size and high variability of mammary tumorigenesis, no firm conclusion can be drawn from this observation and further studies with a larger sample size are required to investigate the effect of dietary zinc intake on the development and growth of mammary tumors.

6.2. Experiment 2

The specific objective of Experiment 2 was to investigate the effect of dietary zinc intake on the development and growth of MNU-induced mammary tumors in rats.

6.2.1. Assessment of zinc status

Feed intake of Z3 rats was 73% and 74% that of Z12 and Z31 rats, respectively ($p < 0.05$), whereas host weight gain of Z3 rats was 77% that of Z12 and Z31 rats ($p < 0.05$) (Table 8). Plasma zinc concentration in Z3 rats was 50% and 56% that in Z12 and Z31 rats, respectively ($p < 0.05$). However, when compared to PZ12 and PZ31 rats, Z3 rats gained a similar amount of host body weight. Hence, the reduced host weight gain in Z3 rats, as compared to Z12 and Z31 rats, was the result of reduced feed intake associated with dietary zinc deficiency. It is interesting to note that, although both feed intake and host weight gain of Z3 rats were not statistically significantly different from that of PZ12 and PZ31 rats, feed intake : host weight gain ratio in Z3 rats was significantly higher than that in their pair-fed controls. As compared to PZ12 and PZ31 rats, plasma zinc concentration in Z3 rats was significantly lower while the hematocrit level remained unaffected ($p < 0.05$). Therefore, similar host weight gain and hematocrit level, along with a lower plasma zinc concentration, as compared to PZ12 and PZ31 rats indicates that Z3 rats developed marginal zinc deficiency.

With a 29% feed restriction, PZ12 rats had a significantly lower host weight gain and feed intake : host weight gain ratio as compared to Z12 rats (Table 8). Similarly, feed intake, host weight gain and feed intake : host weight gain ratio were significantly lower in PZ31 rats as compared to Z31 rats. The plasma zinc concentration and hematocrit level were not

Table 8. Effect of dietary zinc intake on feed intake, host weight gain, plasma zinc concentration and hematocrit level in rats¹

	Dietary treatment groups					
	Z3	PZ12	PZ31	Z12	Z31	Z155
Feed intake (g/rat/day)	11.0±0.4 ^b	10.7±0.2 ^b	10.8±0.1 ^b	15.0±0.4 ^a	14.9±0.4 ^a	15.3±0.5 ^a
Host weight gain ² (g/rat/day)	1.7±0.0 ^b	1.8±0.0 ^b	1.8±0.0 ^b	2.2±0.1 ^a	2.2±0.1 ^a	2.3±0.1 ^a
Feed intake/ Host weight gain	6.6±0.1 ^a	5.9±0.1 ^b	6.0±0.1 ^b	7.0±0.2 ^a	6.9±0.2 ^a	6.8±0.3 ^a
Plasma zinc (µg/ml)	0.9±0.1 ^c	1.7±0.0 ^{ab}	1.7±0.0 ^{ab}	1.8±0.0 ^a	1.6±0.1 ^b	1.6±0.0 ^{ab}
Hematocrit (%)	43±1	42±1	43±1	42±1	43±1	42±1

¹Values represent the mean ± SEM, *n*=24 rats (except Z3, PZ12 and PZ31 groups, where *n*=25; Z155 group, where *n*=23; hematocrit in PZ31 group, where *n*=24). The means among dietary treatment groups not sharing a common letter are significantly different (*p*<0.05). Z3: marginal-low-zinc; PZ12: pair-fed with required-zinc diet; PZ31: pair-fed with control diet; Z12, required-zinc; Z31: control; Z155: high-zinc.

²Host weight gain = body weight gain – tumor weight.

affected by the reduction in feed intake. Therefore, feed restriction suppressed the growth of rats, but had no effect on the zinc status of rats.

Feed intake, host weight gain, feed intake : host weight gain ratio, plasma zinc concentration and hematocrit level were not significantly different between PZ12 and PZ31 rats (Table 8). Similarly, when Z12 rats were compared to Z31 rats, feed intake, host weight gain, feed intake: host weight gain ratio and hematocrit level were also not significantly different. However, plasma zinc concentration in Z31 rats was significantly lower than that in Z12 rats.

Feed intake, host weight gain, feed intake: host weight gain ratio, plasma zinc concentration and hematocrit level were essentially not different among Z12, Z31 and Z155 rats indicating that at a dietary zinc intake level between 12 and 155 mg zinc/kg diet had no effect on body zinc status in rats.

6.2.2. Effect of dietary zinc intake on the development and growth of N-methyl-N-nitrosourea (MNU)-induced mammary tumors

6.2.2.1 Detection of microscopic lesions

To provide an early detection of the development and growth of mammary tumors, determination of microscopic lesions in mammary gland whole mounts, which were prepared on day 28 and 56 after MNU injection, was performed. On day 28 after MNU injection, a microscopic lesion was detected in one of the mammary glands from Z3 rats (Table 9). On day 56 after MNU injection, there was no microscopic lesion observed in the mammary glands from Z3, PZ12 and PZ31 rats. In contrast, mammary glands from two of Z12 and Z31 rats and one of Z155 rats showed microscopic lesions. The total number of microscopic

Table 9. Effect of dietary zinc intake on the development of microscopic lesions detected in the mammary gland whole mounts prepared on day 28 and 56 after N-methyl-N-nitrosourea (MNU) injection¹

		Dietary treatment groups					
		Z3	PZ12	PZ31	Z12	Z31	Z155
Incidence	28	1/6	0/6	0/6	0/6	0/6	0/6
	56	0/6	0/6	0/6	2/6	2/6	1/6
Number of lesions	28	1	0	0	0	0	0
	56	0	0	0	3	5	2

¹ Z3: marginal-low-zinc; PZ12: pair-fed with required-zinc diet; PZ31: pair-fed with control diet; Z12, required-zinc; Z31: control; Z155: high-zinc.

lesions detected in the mammary glands from Z12, Z31 and Z155 rats were 3, 5 and 2, respectively.

Detection of microscopic lesions from mammary gland whole mounts provides an indication of an early developmental stage during mammary tumorigenesis. The results indicate that marginal zinc deficiency enhanced the development of microscopic lesions during the earlier stage of the mammary tumorigenesis. In contrast, dietary zinc intake at or above the requirement level for rats exerted an enhancing effect on mammary tumorigenesis at a later stage during the process. However, due to the very small sample size ($n = 6$), no firm conclusion could be drawn from this observation.

6.2.2.2 Development and growth of palpable N-methyl-N-nitrosourea (MNU)-induced mammary tumors

The tumor incidence in rats fed the assigned diets over the 98-day experimental period is shown in Figure 2. The tumor incidence in Z12, Z31 and Z155 rats were not significantly different over the experimental period. The tumor incidence in Z3 rats was significantly lower than that in Z12 and Z31 rats from week 8 after MNU injection to the end of the experiment. In order to differentiate whether the inhibitory effect of dietary zinc deficiency was due to a reduced feed intake associated with dietary zinc deficiency or zinc deficiency *per se*, comparisons were made between the pair-fed groups (PZ12 and PZ31) and the corresponding *ad libitum* groups (Z12 and Z31) as well as among Z3, PZ12 and PZ31 groups.

When PZ12 rats were compared to Z12 rats, the tumor incidence in PZ12 rats was significantly lower than that in Z12 rats from week 8 after MNU injection to the end of the

experiment (Figure 2). Similarly, PZ31 rats also showed a significantly lower tumor incidence than that in Z31 rats during the same period. The significant difference in tumor incidence between PZ12 and Z12 groups as well as PZ31 and Z31 groups indicates that reduced feed intake lowered tumor incidence.

Z3 rats had a similar tumor incidence as that in PZ12 rats till week 12 after MNU injection (Figure 2). From week 13 to the end of the experiment, the tumor incidence in Z3 rats was significantly lower than that in PZ12 rats. Similarly, the tumor incidence in PZ31 rats was also not significantly different from that in PZ12 rats till week 12 after MNU injection and was significantly lower than that in PZ12 rats from week 13 to the end of the experiment. The tumor incidence in PZ31 rats was not significantly different from that in Z3 rats over the experimental period. These results indicate that the reduced tumor incidence in Z3 rats from week 8 to week 12 after MNU injection was mainly due to the effect of reduced feed intake associated with dietary zinc deficiency.

At the end of the experiment, the tumor incidence in Z12 (75%), Z31 (83%) and Z155 (83%) rats were not significantly different (Figure 3). In contrast, the tumor incidence in Z3 rats was 12%, which was significantly lower than that in Z12, Z31 and Z155 rats. When PZ12 rats were compared to Z12 rats, the tumor incidence in PZ12 rats (44%) was significantly lower. Similarly, PZ31 rats had a tumor incidence of 16%, which was also significantly lower than that in Z31 rats. The significant difference in tumor incidence between pair-fed and their corresponding *ad libitum* groups clearly showed that reduced feed intake lowered tumor incidence. However, at the same feed intake level, the tumor incidence in Z3 and PZ31 rats were significantly lower than that in PZ12 rats. The tumor incidence significantly increased when dietary zinc intake increased from 3 to 12 mg zinc/kg diet, but

significantly decreased when dietary zinc intake was further increased from 12 to 31 mg zinc/kg diet. These observations indicate that dietary zinc intake modulated the tumor incidence in feed restricted rats and the reduced tumor incidence in Z3 rats was, at least in part, due to the reduced feed intake associated with dietary zinc deficiency.

The number of palpable tumors over the experimental period followed a similar pattern as the tumor incidence (Figure 4). The number of palpable tumors among Z12, Z31 and Z155 rats were not different, but were higher than that in Z3 rats from week 8 after MNU injection to the end of the experiment. At the end of the experiment, the number of palpable tumors in Z12, Z31 and Z155 rats were 49, 54 and 44, respectively, whereas Z3 rats had 8 palpable tumors (Figure 5). The number of palpable tumors in PZ12 and PZ31 rats were lower than that in Z12 and Z31 rats, respectively, from week 8 after MNU injection to the end of the experiment (Figure 4). Z3, PZ12 and PZ31 rats had similar number of palpable tumors until week 13 after MNU injection. At the end of the experiment, the number of palpable tumors in PZ12 rats was 17, which was higher than that in Z3 (8) and PZ31 (4) rats (Figure 5).

Dietary zinc intake had no effect on the tumor multiplicity, tumor weight, tumor burden and tumor latency period in rats (Table 10).

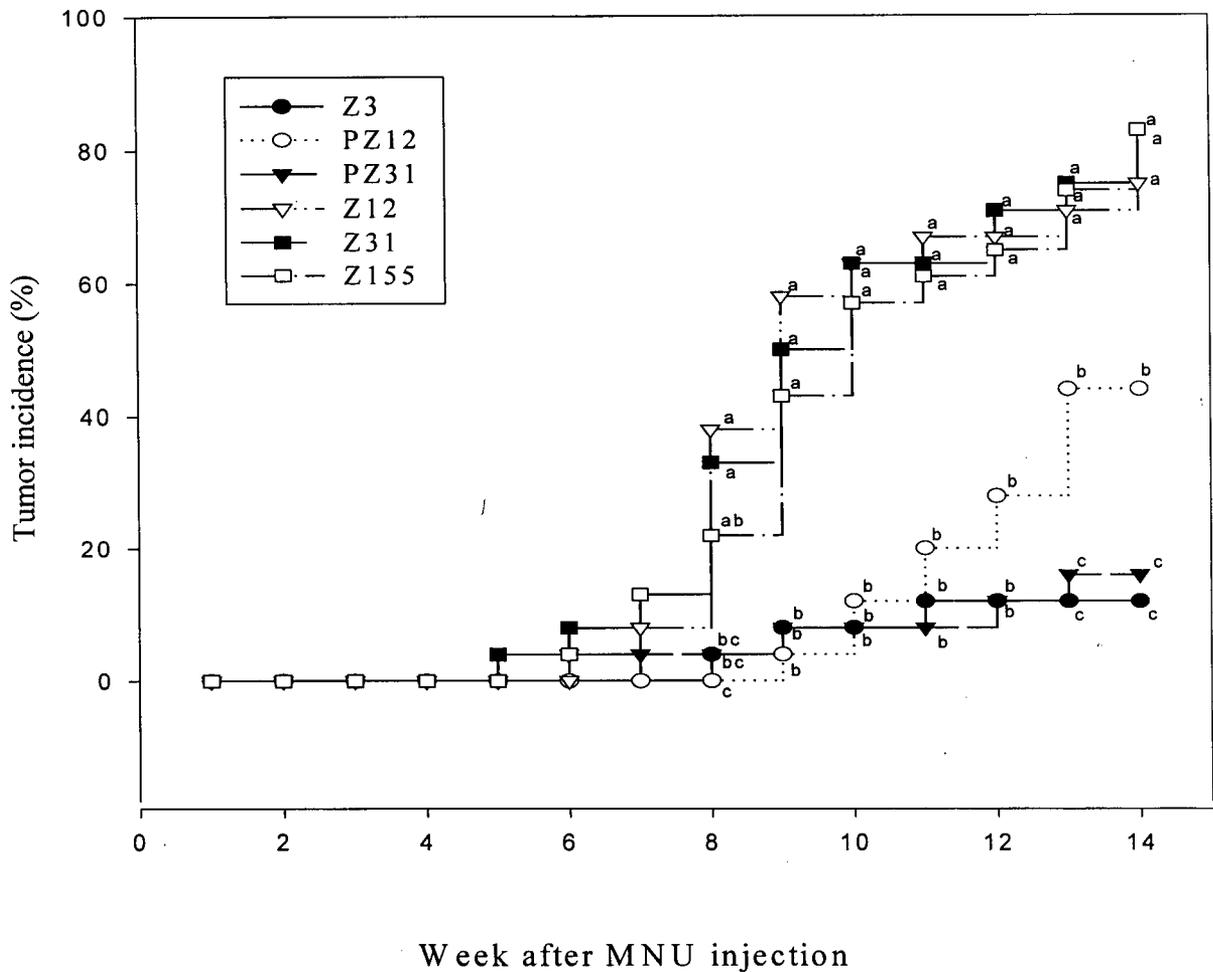


Figure 2. Effect of dietary zinc intake on the tumor incidence (%) in N-methyl-N-nitrosourea (MNU)-treated rats over the 98-day experimental period. The means among dietary treatment groups not sharing a common letter are significantly different ($p < 0.05$). The tumor incidence was not significantly different among dietary treatment groups from the beginning of the experiment to week 7 after MNU injection. Z3: marginal-low-zinc; PZ12: pair-fed with required-zinc diet; PZ31: pair-fed with control diet; Z12, required-zinc; Z31: control; Z155: high-zinc.

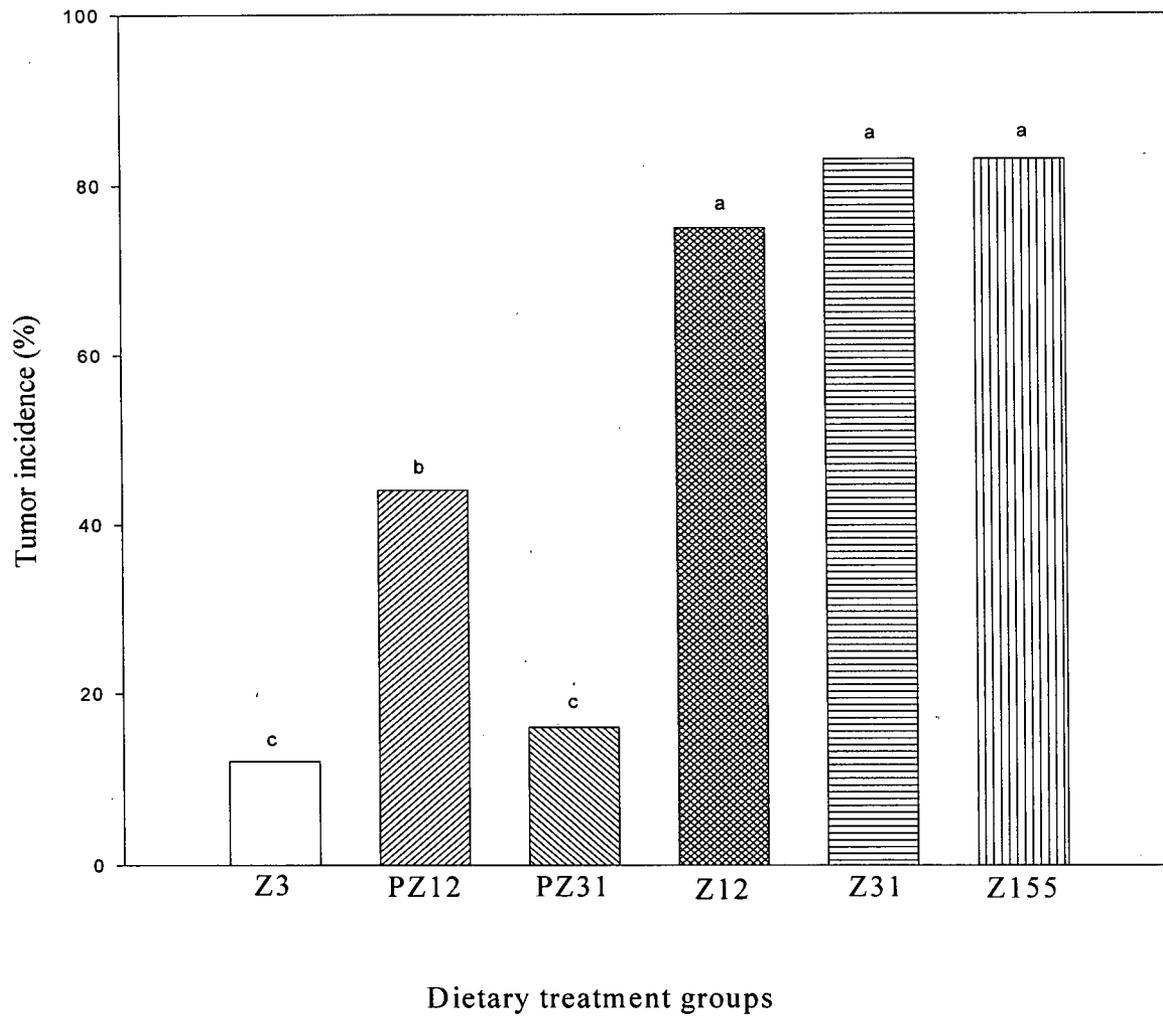


Figure 3. Effect of dietary zinc intake on the tumor incidence (%) in rats 14 weeks after N-methyl-N-nitrosourea (MNU) injection. The means among dietary treatment groups not sharing a common letter are significantly different ($p < 0.05$). Z3: marginal-low-zinc; PZ12: pair-fed with required-zinc diet; PZ31: pair-fed with control diet; Z12: required-zinc; Z31: control; Z155: high-zinc.

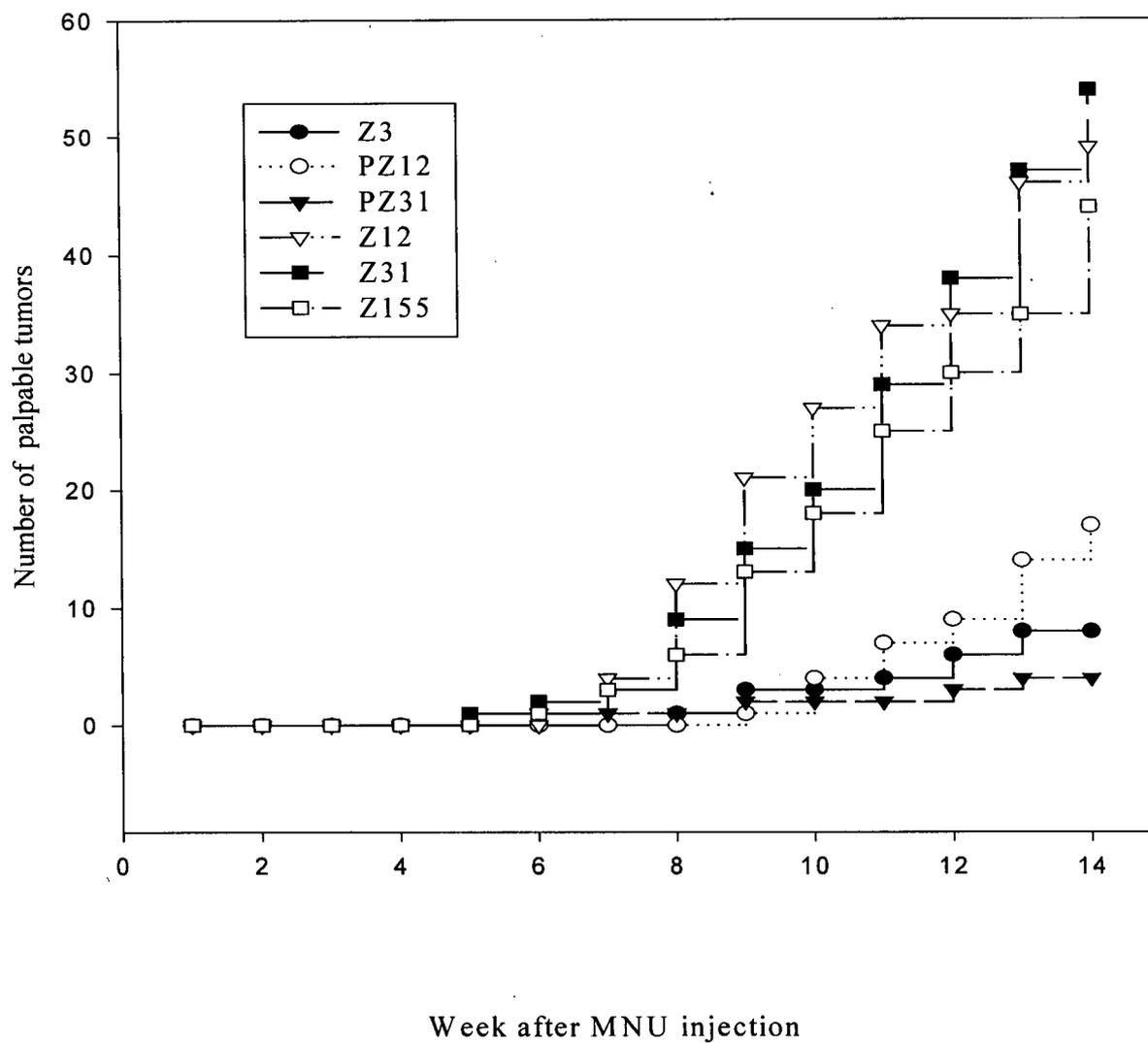


Figure 4. Effect of dietary zinc intake on the number of palpable tumors in N-methyl-N-nitrosourea (MNU)-treated rats over the 98-day experimental period. Z3: marginal-low-zinc; PZ12: pair-fed with required-zinc diet; PZ31: pair-fed with control diet; Z12, required-zinc; Z31: control; Z155: high-zinc.

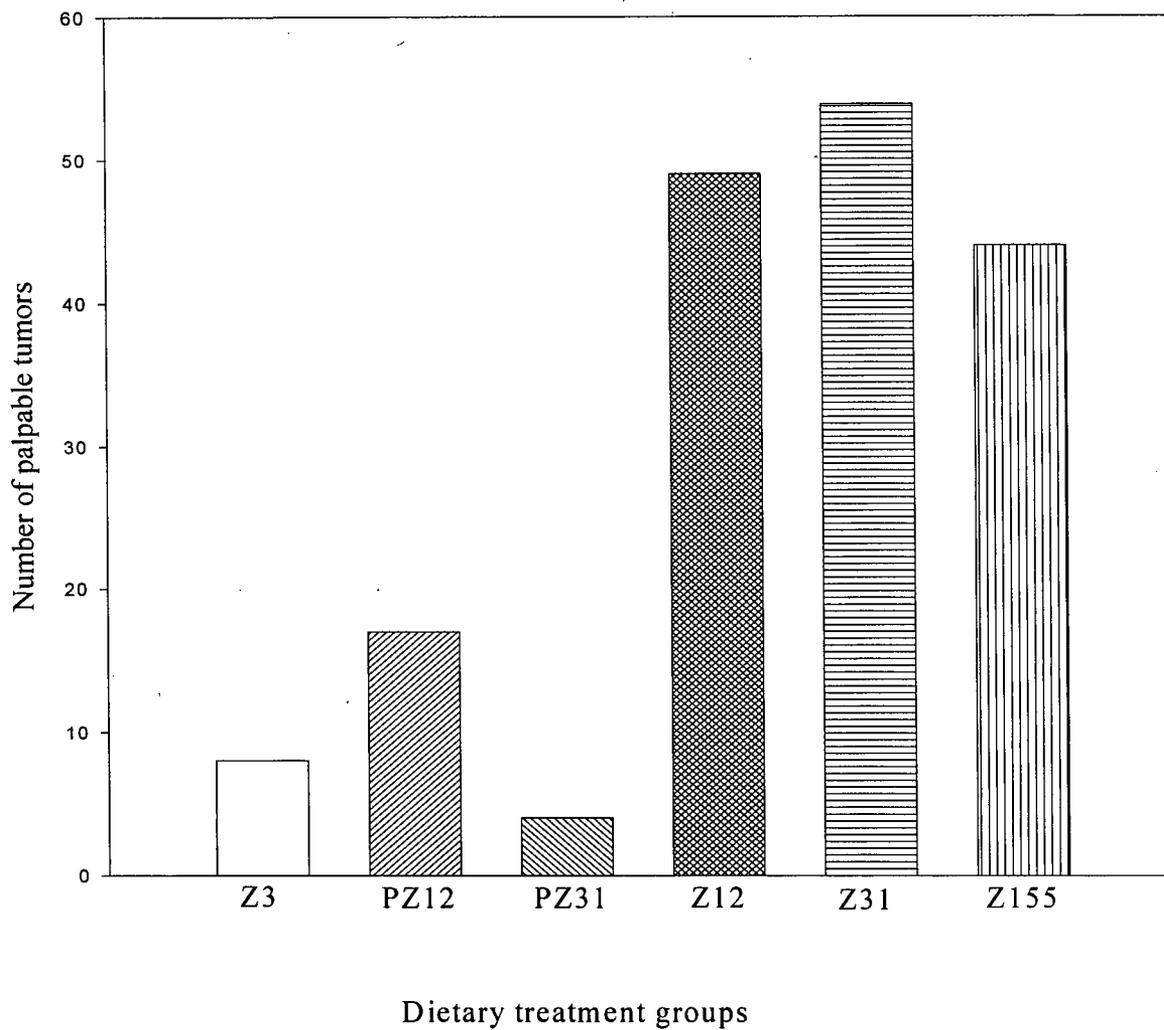


Figure 5. Effect of dietary zinc intake on the number of palpable tumors in rats 14 weeks after N-methyl-N-nitrosourea (MNU) injection. Z3: marginal-low-zinc; PZ12: pair-fed with required-zinc diet; PZ31: pair-fed with control diet; Z12, required-zinc; Z31: control; Z155: high-zinc.

Table 10. Effect of dietary zinc intake on the tumor multiplicity, tumor weight, tumor burden and tumor latency period in rats at week 14 after N-methyl-N-nitrosourea (MNU) injection.¹

	Dietary treatment groups					
	Z3	PZ12	PZ31	Z12	Z31	Z155
Tumor multiplicity ²	2.7±0.9	1.5±0.3	1.0±0.0	2.7±0.5	2.7±0.3	2.3±0.3
Tumor weight (g)	2.8±0.9	2.2±1.3	13.7±9.9	2.4±0.4	2.1±0.5	1.8±0.3
Tumor burden ³ (%)	3.6±1.6	1.5±1.0	6.3±4.6	2.8±0.8	2.2±0.7	1.7±0.3
Tumor latency period ⁴ (Weeks after MNU injection)	9.3±0.9	11.5±0.4	10.0±1.6	9.1±0.4	9.5±0.6	9.9±0.5

¹Values represent the mean ± SEM. Z3: marginal-low-zinc; PZ12: pair-fed with required-zinc diet; PZ31: pair-fed with control diet; Z12: required-zinc; Z31: control; Z155: high-zinc.

²Tumor multiplicity was the average number of palpable mammary tumors per tumor-bearing rat in the group.

³Tumor burden was expressed as a percentage of the total tumor weight in a tumor-bearing rat relative to the host weight.

⁴Tumor latency period was the average time that the first mammary tumor became palpable in a tumor-bearing rat in the group.

6.2.3. Effect of N-methyl-N-nitrosourea (MNU)-induced mammary tumorigenesis on tissue zinc concentrations

In order to determine the effect of MNU-induced mammary tumorigenesis on tissue zinc concentrations, tissues from 6 tumor-free rats and 6 tumor-bearing rats with the highest tumor burden in each dietary treatment group were used for the determination of zinc. The tumor burden in the tumor-bearing rats among the dietary treatment groups were not significantly different and the average tumor burden was calculated to be 4.5% (Table IV-1, Appendix IV).

Plasma zinc concentrations were not significantly different among PZ12, PZ31, Z12, Z31 and Z155 tumor-free rats (Table 11). Plasma zinc concentration in Z3 tumor-free rats was significantly lower than that in PZ12, PZ31, Z12, Z31, Z155 tumor-free rats. As compared to PZ12 and PZ31 tumor-free rats, plasma zinc concentration in Z3 tumor-free rats was 53% lower ($p < 0.05$). Similarly, plasma zinc concentrations in PZ12, PZ31, Z12, Z31 and Z155 rats tumor-bearing rats were not significantly different, but were significantly higher than that in Z3 tumor-bearing rats. Z3 tumor-bearing rats had plasma zinc concentration 37% and 41% lower than that in PZ12 and PZ31 tumor-bearing rats, respectively ($p < 0.05$). When plasma zinc concentrations were compared between tumor-free and tumor-bearing rats within each dietary treatment group, they were not significantly different.

In tumor-free rats, Z3 rats had the lowest femur zinc concentration. Femur zinc concentration in Z3 tumor-free rats was 80% and 81% lower than that in PZ12 and PZ31 tumor-free rats, respectively ($p < 0.05$) (Table 11). Femur zinc concentrations were not significantly different among PZ21, PZ31, Z12, Z31 tumor-free rats. Z155 tumor-free rats

Table 11. Effect of dietary zinc intake and N-methyl-N-nitrosourea (MNU)-induced mammary tumorigenesis on tissue zinc concentrations in rats¹

Tissues	Tumor	Zinc concentration ($\mu\text{g/g}$ dry weight)					
		Z3	PZ12	PZ31	Z12	Z31	Z155
Plasma ($\mu\text{g/ml}$)	-	0.8 ± 0.1^b	1.7 ± 0.1^a	1.7 ± 0.0^a	1.7 ± 0.1^a	1.5 ± 0.2^a	1.7 ± 0.2^a
	+	1.0 ± 0.1^b	1.6 ± 0.1^a	1.7 ± 0.1^a	1.8 ± 0.1^a	1.6 ± 0.1^a	1.7 ± 0.0^a
Femur	-	68 ± 9^c	333 ± 12^b	367 ± 13^b	339 ± 12^b	385 ± 18^{ab}	460 ± 19^a
	+	$166 \pm 15^{d*}$	335 ± 10^c	378 ± 22^b	$307 \pm 5^{c*}$	374 ± 6^b	473 ± 7^a
Liver	-	78 ± 7	89 ± 3	104 ± 7	97 ± 6	94 ± 3	97 ± 8
	+	82 ± 2	98 ± 6	100 ± 6	94 ± 5	104 ± 5	103 ± 2
Small intestine	-	91 ± 4	113 ± 5	113 ± 10	94 ± 7	99 ± 0	125 ± 19
	+	99 ± 8	$93 \pm 5^*$	110 ± 4	110 ± 6	116 ± 8	111 ± 7
Mammary gland	-	6.5 ± 0.6	5.8 ± 0.4	6.8 ± 0.6	7.3 ± 1.9	5.0 ± 1.0	5.9 ± 2.6
	+	8.7 ± 2.5	$7.8 \pm 0.6^*$	7.2 ± 0.1	7.9 ± 0.9	7.7 ± 2.1	7.3 ± 1.1

¹Values represent the mean \pm SEM, $n=6$ rats (except Z12 tumor-free group, where $n=4$; Z31 tumor-free group, where $n=2$; Z155 tumor-free group, where $n=3$; Z3 tumor-bearing group, where $n=3$; PZ31 tumor-bearing group, where $n=4$). Zinc concentrations were expressed on the basis of per dry-tissue weight, except for plasma ($\mu\text{g/ml}$). The means among dietary treatments in each row not sharing a common letter are significantly different ($p < 0.05$). The asterisk indicates a significant difference in the means between sham-treated and MNU-treated groups in each dietary treatment group ($p < 0.05$). Z3: marginal-low-zinc; PZ12: pair-fed with required-zinc diet; PZ31: pair-fed with control diet; Z12, required-zinc; Z31: control; Z155: high-zinc. Tumor-: tumor-free; Tumor+: tumor-bearing.

had femur zinc concentration 136% that in Z12 tumor-free rats ($p < 0.05$). Although not statistically significant, Z155 tumor-free femur zinc concentration was 119% that in Z31 tumor-free rats. Similarly, in tumor-bearing rats, femur zinc concentration in Z3 rats was significantly lower than that in all other dietary treatment groups. Femur zinc concentrations were not significantly different between PZ12 and Z12 tumor-bearing rats as well as PZ31 and Z31 tumor-bearing rats. Femur zinc concentration in PZ31 tumor-bearing rats was 13% higher than that in PZ12 tumor-bearing rats ($p < 0.05$). Similarly, Z31 tumor-bearing rats had a 23% higher femur zinc concentration than that in Z12 tumor-bearing rats ($p < 0.05$). Further increase in dietary zinc level resulted in an increase in femur zinc concentration. Femur zinc concentration in Z155 tumor-bearing rats was 154% and 126% that in Z12 and Z31 tumor-bearing rats, respectively ($p < 0.05$). Clearly, dietary zinc intake affected femur zinc concentration. In contrast, femur zinc concentrations in PZ12, PZ31, Z31 and Z155 rats were not affected by the development of MNU-induced mammary tumors. However, MNU-induced mammary tumorigenesis significantly lowered femur zinc concentration in Z12 rats and elevated femur zinc concentration in Z3 rats.

Liver zinc concentrations were not significantly different among dietary treatment groups in both tumor-free and tumor-bearing rats (Table 11). Liver zinc concentrations were also not significantly different between tumor-free and tumor-bearing rats in the same dietary treatment group.

Among the dietary treatment groups in both tumor-free and tumor-bearing rats, small intestinal zinc concentrations were not significantly different (Table 11). There was also no significant difference in small intestinal zinc concentrations between tumor-free and tumor-

bearing rats in the same dietary treatment group, except in PZ12 group. Small intestinal zinc concentration in PZ12 tumor-bearing rats was 82% that in PZ12 tumor-free rats ($p < 0.05$).

Mammary gland zinc concentrations were not significantly different among dietary treatment groups in both tumor-free and tumor-bearing rats (Table 11). In addition, mammary gland zinc concentrations were also not significantly different between tumor-free and tumor-bearing rats in the same dietary treatment group, except in PZ12 group. PZ12 tumor-bearing rats had a mammary gland zinc concentration 34% higher than that in PZ12 tumor-free rats ($p < 0.05$).

Zinc concentrations in MNU-induced mammary tumors were not significantly different among all the dietary treatment groups (Table 12) indicating that dietary zinc intake had no effect on MNU-induced mammary tumor zinc concentration.

Table 12. Effect of dietary zinc intake on zinc concentration in N-methyl-N-nitrosourea (MNU)-induced mammary tumors¹

	Zinc concentration ($\mu\text{g/g}$ dry weight)					
	Z3	PZ12	PZ31	Z12	Z31	Z155
Mammary tumor	89 \pm 24	91 \pm 3	113 \pm 9	90 \pm 4	94 \pm 3	92 \pm 10

¹ Values represent the mean \pm SEM, $n=6$ rats (except Z3 group, where $n=3$; PZ31 group, where $n=2$). Z3: marginal-low-zinc; PZ12: pair-fed with required-zinc diet; PZ31: pair-fed with control diet; Z12, required-zinc; Z31: control; Z155: high-zinc.

7. DISCUSSION

7.1. Effect of dietary zinc intake on body zinc distribution

In the present study, body zinc distribution was determined by zinc concentrations in the femur, liver, plasma, skin, small intestine, kidney, lung, muscle, heart and mammary gland. Marginal zinc deficiency (3 mg zinc/kg diet) lowered plasma, femur, liver, kidney and skin zinc concentrations in sham-treated rats. Heart, muscle, lung, small intestine and mammary gland zinc concentrations were not affected by marginal zinc deficiency. These observations are supported by previous reports. Hicks and Wallwork (1987) showed that zinc deficient rats (0.4 mg zinc/kg diet) had a significantly lower femur zinc concentration than that in pair-fed rats. In rats fed a severe zinc deficient diet (less than 1 mg zinc/kg diet), plasma and femur zinc concentrations were significantly lower than that in pair-fed rats (100 mg zinc/kg diet) while liver, kidney and brain zinc concentrations were not affected (Villet *et al.*, 1997). Mills *et al.* (1984) reported that zinc deficiency (< 4 mg zinc/kg diet) caused a significant decrease in plasma and kidney zinc concentrations, but not liver and heart zinc concentrations, in rats.

In addition to the effect of dietary zinc deficiency, high zinc intake also affects body zinc distribution. In the present study, high zinc intake (171 and 346 mg zinc/kg diet) increased femur zinc concentration in sham-treated rats. In rats fed the very-high-zinc diet (346 mg zinc/kg diet), liver zinc concentration was also elevated in sham-treated rats. In contrast, plasma, kidney, heart, lung, muscle, skin, small intestine and mammary gland zinc concentrations were not affected by high zinc intake. Similarly, Fischer *et al.* (1991) showed that plasma and liver zinc concentrations were not affected by high zinc intake (150 mg/kg

diet) whereas femur zinc concentration was significantly higher in rats fed a high zinc diet than that in the controls.

Under normal circumstance, total body zinc and tissue zinc concentrations are homeostatically controlled (Jackson, 1989; King, 1990). As a result, body zinc distribution is maintained through homeostatic regulation. During zinc deficiency, the body conserves tissue zinc by enhancing the efficiency of zinc absorption, decreasing the excretion of zinc and reducing the growth rate of animals. In case of mild zinc deficiency, by adjusting the absorption, excretion and growth rate, zinc homeostasis can be re-established and tissue zinc is conserved. Consequently, body zinc distribution is not affected. However, in case of marginal and severe zinc deficiency, zinc homeostasis cannot be maintained by the adjustment of absorption, excretion and growth rate. Zinc is mobilized from some tissues, which are known as the exchangeable pool of zinc, to support the functions of other tissues. The exchangeable pool of zinc consists, at least in part, of bone, plasma and liver. Hence, in the present study, zinc concentrations in the femur, plasma and liver were reduced in zinc deficient rats while muscle, heart, lung, small intestine and mammary gland zinc concentrations were not affected. In case of high zinc intake, the body decreases the efficiency of absorption and increases the fecal excretion of zinc to maintain the normal level of body zinc (King and Keen, 1994). When zinc homeostasis cannot be maintained, zinc concentrations in some tissues such as bone and liver increase (Fox, 1989) as seen in the present study. Taken together, body zinc distribution, which reflects zinc homeostasis, was affected by dietary zinc intake.

7.2. Effect of N-methyl-N-nitrosourea (MNU) treatment and mammary tumorigenesis on body zinc distribution

In contrast to the effect of dietary zinc intake, both MNU treatment and MNU-induced mammary tumorigenesis essentially had no effect on body zinc distribution in rats fed different levels of dietary zinc in the present study. Since body zinc distribution reflects zinc homeostasis, it appears that MNU treatment and MNU-induced mammary tumorigenesis did not affect zinc homeostasis in rats.

A few studies have investigated the effect of mammary tumor growth on zinc concentration in various tissues. Philcox *et al.* (1994) showed that in rats inoculated with mammary adenocarcinoma, the growth of mammary tumors resulted in a significant reduction in plasma zinc concentration as compared to that in tumor-free rats. As tumor burden increased from 4.3% to 16.3%, the degree of hypozincemic effect was enhanced. In contrast, zinc concentrations in liver, muscle and intestine were essentially not affected by the tumor burden. The liver, muscle and intestine zinc concentrations in rats with a 4.3% tumor burden were the same as that in tumor-free rats. However, with similar muscle and intestine zinc concentrations, rats with a 16.3% tumor burden had a significantly higher liver zinc concentration than that in tumor-free rats. This higher liver zinc concentration coincided with an increase in liver metallothionein concentration. Mills *et al.* (1984) also reported that plasma zinc concentration was significantly lower while liver zinc concentration was significantly higher in rats implanted with mammary adenocarcinoma than that in sham-treated tumor-free rats.

The apparent inconsistency between the present and above studies could be due to the difference in tumor burden that the rats had and the animal model used to study mammary

tumorigenesis. Firstly, Philcox *et al.* (1994) showed that an increase in tumor burden was associated with a reduction in plasma zinc concentration in rats. As tumor burden increased from 4.3% to 9.9%, the reduction in plasma zinc concentration increased from 34% to 49% of that in tumor-free rats. The largest decrease in plasma zinc concentration (64% lower than that in tumor-free rats) and the only increase in liver zinc concentration was observed in rats with 16.3% tumor burden. Although the tumor burden was not reported in Mills *et al.* (1984) study, the average tumor weight in control and zinc deficient rats were 21 g and 14 g, respectively. In contrast, the average tumor burden in the present study was 4.5% and the average tumor weight was 2.4 g, which were much lower than that reported by Philcox's and Mills' groups. Hence, it is likely that the unaltered plasma and liver zinc concentrations in tumor-bearing rats in the present study were related to the low tumor burden. Secondly, the other authors used an implanted mammary adenocarcinoma rat model, which represents the growth of established tumors. In contrast, mammary tumors were induced by MNU in the present work to study the formation and growth of MNU-induced mammary tumors. Therefore, in addition to the difference in tumor burden, the differences in the nature and the possible zinc requirement between implanted mammary tumors and MNU-induced mammary tumors could also explain the apparent discrepancy between the findings in the present and the other studies.

7.3. Mammary tumor zinc concentration

Several human studies have shown that zinc concentration in breast cancer tissue is significantly higher than that in normal breast tissue when expressed on the basis of wet-tissue weight (Santoliquido *et al.*, 1976; Margalioth *et al.*, 1983; Borella *et al.*, 1997) or dry-

tissue weight (Mulay *et al.*, 1971; Santoliquido *et al.*, 1976; Rizk and Sky-Peck, 1984; Ng *et al.*, 1993). In agreement with the findings of the above investigators, the present study also showed that MNU-induced mammary tumor zinc concentration was significantly higher than that in the mammary gland when expressed on the basis of wet- or dry-tissue weight regardless of the level of dietary zinc.

It is well known that mammary tumors are composed of tightly packed tumor cells while normal breast tissue contains a considerable amount of fat (Cooper, 1992). The compositional difference between mammary tumors and mammary glands is supported by the present study that the MNU-induced mammary tumors contained a significantly higher protein concentration and a significantly lower lipid concentration than that in mammary glands.

Because the protein concentration in mammary tumors is high and body zinc is primarily associated with protein (Walsh *et al.*, 1994), it is more reasonable to compare the zinc concentrations between the mammary tumor and mammary gland on the basis of tissue protein. On a per tissue protein basis, the present study showed that MNU-induced mammary tumor zinc concentration was significantly higher when compared to that in the mammary gland indicating an accumulation of zinc in the mammary tumor. This observation is in agreement with the study by Tupper *et al.* (1955). By injecting radioactive ^{65}Zn into mice bearing spontaneous mammary carcinoma, they found that the level of ^{65}Zn in mammary carcinoma was significantly higher than that in normal mammary glands from day 1 to day 14 after the injection. This finding demonstrated that the mammary tumor tissue took up and retained more ^{65}Zn than the normal mammary gland. The study by Tupper *et al.*

(1955), together with the elevated mammary tumor zinc concentration in the present study, suggests that zinc plays a role during mammary tumorigenesis.

Zinc is essential in cell division and growth (Clegg *et al.*, 1989; Vallee and Falchuk, 1993). Tumorigenesis is characterized by uncontrolled cell proliferation (Archer, 1992). Therefore, it is possible that the accumulation of zinc in mammary tumors suggests a role of zinc in mammary tumor cell division.

Metallothionein is a protein which has a high capacity for binding zinc (Bremner, 1991; National Cancer Institute Workshop, 1993; Vallee and Falchuk, 1993; Walsh *et al.*, 1994). Although the metal content may vary, metallothionein binds up to 7 moles of zinc per mole of protein. The suggested functions of metallothionein include cell proliferation and transport of zinc between proteins. It has been shown that certain types of tumor contain high level of metallothionein (National Cancer Institute Workshop, 1993; Pattanaik *et al.*, 1994). Hence, it is possible that the elevated level of zinc in MNU-induced mammary tumors reflects a high level of metallothionein, which might play a role in tumor cell proliferation. Further studies to elucidate the possible role of zinc as well as the level and the possible role of metallothionein in mammary tumors are suggested.

7.4. Effect of dietary zinc intake on mammary tumorigenesis

The present study showed that marginal zinc deficiency (3 mg zinc/kg diet) lowered the tumor incidence and the number of palpable tumors in rats as compared to that in rats fed diets containing 12 – 155 mg zinc/kg diet *ad libitum* starting from week 8 after MNU injection to the end of the experiment. The tumor incidence is a measure of the risk of mammary tumor development while the number of palpable tumors is a measure of the

formation of mammary tumors. A significantly lower tumor incidence and number of palpable tumors in marginal zinc deficient rats as compared to the *ad libitum* rats indicates that marginal zinc deficiency lowered the risk of developing MNU-induced mammary tumors and inhibited the formation of MNU-induced mammary tumors.

Because dietary zinc deficiency suppressed feed intake, and reduced caloric intake reduces the risk of mammary tumorigenesis (American Cancer Society Research Workshop, 1993), it is important to differentiate the effect of reduced feed intake associated with dietary zinc deficiency and the effect of zinc deficiency *per se*. Firstly, in the present study, the tumor incidence in feed restricted (PZ12 and PZ31) rats was significantly lower than that in their corresponding *ad libitum* (Z12 and Z31) rats starting from week 8 after MNU injection to the end of the experiment. The number of palpable tumors also followed the same pattern. These results indicate that the risk of developing MNU-induced mammary tumors was inhibited by a 30% feed restriction. Similarly, Blask *et al.* (1986) showed that 30% feed restriction starting at week 3 after DMBA injection resulted in a 13% reduction in the tumor incidence 15 weeks after the initiation of the dietary treatment. The formation of new mammary tumors was also lower in the feed restricted rats than that in the controls.

The mechanism of the inhibitory effect of feed restriction on the risk of mammary tumor development is not well understood. It has been documented that mammary tumorigenesis is influenced by hormones such as growth hormone, insulin, insulin-like growth factor I (IGF-1) and prolactin (Welsch, 1985; Nandi *et al.*, 1995). However, feed restriction results in a reduction in the serum levels of growth hormone (Restrepo and Armario, 1989), insulin (Restrepo and Armario, 1989), and IGF-I (McNall *et al.*, 1995; Ninh *et al.*, 1995), which might affect mammary tumorigenesis. In addition, the rate of cell

proliferation is another determinant of mammary tumorigenesis. Lok *et al.* (1990) reported that a 20% - 40% energy restriction inhibited mammary cell proliferation. The authors also suggested that "calorie restriction may affect both the rate of cellular proliferation in the mammary gland directly and apparently also through the effects of pituitary secretion of prolactin and growth hormone". Furthermore, Zhu *et al.* (1999) showed that energy restriction inhibited cell proliferation and induced apoptosis in mammary gland lesions. Hence, it is possible that feed restriction lowers the levels of hormones involved in mammary tumorigenesis, together with suppressed rate of cell proliferation and increased apoptosis, resulting in an inhibition of palpable mammary tumor formation.

In order to study the effect of zinc deficiency *per se*, comparison is made between zinc deficient and pair-fed rats. In the present study, when Z3 rats were compared to pair-fed (PZ12 and PZ31) rats, the tumor incidence among the groups were not significantly different from the beginning of the experiment to week 12 after MNU injection. During the same period of time, the number of palpable tumors formed in these rats was also similar. Therefore, during the first 12 weeks after MNU injection, the effect of marginal zinc deficiency on the risk of MNU-induced mammary tumor development was mainly due to the reduced feed intake associated with dietary zinc deficiency.

From week 13 after MNU injection to the end of the experiment, the significantly lower tumor incidence and lower number of palpable tumors in Z3 rats when compared to that in PZ12 rats suggest that marginal zinc deficiency reduces the risk of MNU-induced mammary tumor development and inhibits the formation of MNU-induced mammary tumors.

Zinc is important to the synthesis of nucleic acids and proteins; consequently, it is critical to cell proliferation and growth (Chesters, 1989). During zinc deficiency, the

synthesis of DNA (Stephan and Hsu, 1973; James *et al.*, 1987) and protein (Hicks and Wallwork, 1987) are reduced. Cell division is also suppressed during zinc deficiency (Southon *et al.*, 1985; Lawson *et al.*, 1988). Hence, it is possible that during zinc deficiency, the rate of mammary cell division is suppressed. Consequently, the rate of division of mammary cells bearing DNA lesions might be low which results in a lower number of palpable mammary tumors formed. In addition, zinc deficiency also causes a decrease in serum insulin (Dorup *et al.*, 1991; Roth and Kirchgessner, 1994) and IGF-I levels (Dorup *et al.*, 1991; Roth and Kirchgessner, 1994; McNall *et al.*, 1995; Ninh *et al.*, 1995). As mentioned earlier, these hormones are involved in mammary tumorigenesis (Welsch, 1985; Nandi *et al.*, 1995). Therefore, it is possible that zinc deficiency reduces the serum level of these hormones, which are involved in mammary tumorigenesis, resulting in a reduction in the formation of palpable mammary tumors. Furthermore, it has been shown that apoptosis is induced in cells treated with intracellular zinc chelators (McCabe *et al.*, 1993; Treves *et al.*, 1994) and cells incubated in low level of extracellular zinc in cell culture studies (Sunderman, 1995; Fraker and Telford, 1997). Hence, it is also possible that zinc deficiency induces apoptosis in DNA lesion-bearing mammary cells which results in the formation of fewer mammary tumors. Therefore, zinc deficiency may reduce the rate of mammary cell proliferation, lower the serum levels of hormones involved in mammary tumorigenesis and induce apoptosis leading to a reduction in the formation of palpable mammary tumors.

As zinc intake increased from 3 to 12 mg zinc/kg diet in pair-fed rats in the present study, the risk of developing mammary tumors was enhanced from week 13 after MNU injection to the end of the experiment as mentioned earlier. However, further increase in zinc intake from 12 to 31 mg zinc/kg diet in pair-fed rats lead to a reduction in the risk of

mammary tumor development. The mechanism by which zinc exerts its effect on mammary tumor formation in these feed restricted rats is not known. It is possible that the elevated zinc intake in feed restricted rats may potentiate the immune function above basal level (Shankar and Prasad, 1998), which inhibits the formation of mammary tumors. In addition, zinc has been suggested to act as an antioxidant (Villet *et al.*, 1997). Although this function of zinc is not well characterized (Bray and Bettger, 1990; Walsh *et al.*, 1994), the suppression of mammary tumor formation in feed restricted rats with higher zinc intake may be related to the antioxidant role of zinc. Overall, the results suggest that the level of zinc intake is critical in modulating the risk of developing MNU-induced mammary tumors in rats when the feed intake is reduced. Furthermore, the inhibitory effect of marginal zinc deficiency on the risk of developing MNU-induced mammary tumors was, at least in part, due to the reduced feed intake associated with dietary zinc deficiency from week 13 after MNU injection to the end of the experiment.

In the present study, the risk of MNU-induced mammary tumor development and the formation of MNU-induced mammary tumors in Z155 rats were the same as that in Z12 and Z31 rats. This is in agreement with Fischer *et al.* (1991) who also reported that the number of tumors developed in rats fed a high-zinc diet (150 mg zinc/kg diet) was not significantly different from that in the control rats 16 weeks after DMBA administration.

As suggested earlier, zinc may play a role in mammary tumor cell division. Zinc deficiency may reduce the rate of DNA lesion-bearing mammary cell division which leads to a reduction in the formation of palpable mammary tumors. However, high zinc intake may not necessarily accelerate the rate of mammary cell division or the formation of palpable mammary tumors. It is well known that body zinc is homeostatically regulated (Jackson,

1989; King, 1990). As zinc intake increases, the efficiency of absorption decreases while that of excretion increases. As a result, body zinc is maintained and the biological functions of zinc are unaltered until homeostasis can no longer be maintained. As mentioned earlier, zinc concentrations in the liver, intestine and mammary gland were not significantly different among Z12, Z31 and Z155 rats in the present study. Therefore, it is possible that the biological functions of zinc in Z155 rats were not different from that in Z12 and Z31 rats. Consequently, the formation of palpable MNU-induced mammary tumors was not significantly different among these groups.

In contrast to the tumor incidence, the tumor weight, tumor burden and tumor multiplicity were not significantly different among all the dietary treatment groups in the present study suggesting that the growth and development of palpable MNU-induced mammary tumors in tumor-bearing rats are not affected by feed restriction or dietary zinc intake.

Unlike the results from the present study, feed restriction has been shown to inhibit the growth of mammary tumors. Blask *et al.* (1986) showed that 30% feed restriction starting at week 3 after DMBA injection significantly lowered the mammary tumor size and tumor multiplicity 15 weeks after the initiation of the dietary treatment. When feed intake was restricted to 42% of that consumed by rats fed *ad libitum* starting 12-14 weeks after DMBA injection, the mammary tumor size in feed restricted rats was significantly lowered (Heuson and Legros, 1972). When 50% feed restriction was started 2 months after DMBA injection, the mammary tumor size and the number of mammary tumors per rat were significantly lowered in feed restricted rats as compared to the control rats 15 weeks after the initiation of the dietary treatment (ThyagaRajan *et al.*, 1993). The discrepancy in tumor size

and tumor multiplicity between the present study and the other studies could be attributed to the differences in the extent of feed restriction, the initiation time of feed restriction and the type of mammary tumors.

Although limited information regarding the relationship between dietary zinc intake and mammary tumor growth is available, Mills *et al.* (1984) reported that the growth of implanted mammary tumors was 35% inhibited in male rats fed a zinc deficient diet (less than 4 mg zinc/kg diet) as compared to the pair-fed controls. The discrepancy between the above and the present study could be due to the differences in the tumor size and the tumor model used. Firstly, the average tumor weight was 14 g and 21 g in zinc deficient and control rats, respectively, in Mills *et al.* (1984) study whereas the average tumor weight was 2.4 g in the present study. Secondly, the above study examined the growth of implanted mammary tumors. The implantation of mammary tumors in male rats excludes the effect of ovarian hormones that are involved in mammary tumorigenesis. The present study used MNU to induce mammary tumorigenesis in order to study the formation of palpable mammary tumors as well as the growth of established mammary tumors.

Despite the limited information regarding the effect of dietary zinc deficiency on mammary tumor growth, the relationship between zinc deficiency and the growth of various types of tumor has been studied. Zinc deficiency inhibited the growth of Walker 256 carcinosarcoma implanted in rats (DeWys *et al.*, 1970; McQuitty *et al.*, 1970; Mills *et al.*, 1981). The growth of P388 leukemia was also inhibited in zinc deficient mice (Barr and Harris, 1973). Similarly, zinc deficiency inhibited the growth of Lewis lung carcinoma implanted in mice (DeWys *et al.*, 1972). However, because of the possible difference in zinc requirement of different tumors (van Rig and Pories, 1980), extrapolation of these results to

the present study is not feasible. Nevertheless, the consistent results obtained suggest a general inhibitory effect of zinc deficiency on tumor growth.

In addition, the relationship between excessively high zinc intake and the growth of tumors other than mammary tumors has also been studied. In rats fed diets containing excessively high levels of zinc (500 – 2500 mg zinc/kg diet), the growth of transplanted hepatoma was inhibited (Duncan *et al.*, 1974). The DNA synthesis in hepatoma implanted in rats fed excessively high zinc diets (more than 500 mg/kg diet) was reduced (Duncan and Dreosti, 1976; Baker and Duncan, 1983). Perkins and Duncan (1991) reported that an addition of zinc from 15 μM to 153 μM to the medium resulted in a reduction in cell proliferation in murine malignant melanoma (BL-6) *in vitro*. Although these studies showed an inhibitory effect of excessively high zinc level on the growth of hepatoma *in vivo* and melanoma *in vitro*, the effect of excessively high zinc intake (e.g. more than 50 times that of the required level) on mammary tumorigenesis has not been studied. Hence, it would be interesting to examine the possibility of such inhibitory effect of excessively high zinc intake on mammary tumorigenesis.

In the present study, the tumor latency period was not significantly different among all the dietary treatment groups 14 weeks after MNU injection. The tumor latency period, which indicates the average time that the first mammary tumor becomes palpable in a rat in a dietary treatment group, is a useful parameter when the tumor incidence among all the treatment groups is the same. The longer the tumor latency period, the lower the risk of mammary tumorigenesis. However, in the present study, because the tumor incidence varied among dietary treatment groups, the tumor latency period was difficult to interpret. For instance, the tumor latency period in Z3 rats was not significantly different from that in Z31

rats. Since the tumor latency period indicates the risk of mammary tumorigenesis, a similar tumor latency period suggests a similar risk of mammary tumorigenesis in Z3 and Z31 rats. However, the tumor incidence in Z3 rats (12%) was lower than that in Z31 rats (83%) which means that a lower percentage of zinc deficient rats developed mammary tumors. By the time that 83% of Z3 rats develop mammary tumors, the tumor latency period would be longer than that in Z31 rats. Because a longer tumor latency period means a lower risk of mammary tumorigenesis, the risk of mammary tumor development in Z3 rats would be lower. Hence, taking into account the varied tumor incidence in the present study, it is difficult to draw conclusion with regard to the tumor latency period.

7.5. Overall conclusions and future directions

Body zinc distribution, as shown by zinc concentrations in the femur, liver, plasma, skin, small intestine, kidney, lung, muscle, heart and mammary gland, was mainly affected by dietary zinc intake in rats. MNU treatment essentially did not affect body zinc distribution in rats regardless of the dietary zinc level. During MNU-induced mammary tumorigenesis, plasma, liver, femur, small intestine and mammary gland zinc concentrations were also essentially not affected in rats bearing an average tumor burden of 4.5%. The higher zinc concentration when expressed on the basis of tissue protein in MNU-induced mammary tumors as compared to that in mammary glands indicates an accumulation of zinc in the mammary tumor and suggests a role of zinc during MNU-induced mammary tumorigenesis.

The level of zinc intake was critical in modulating the formation of palpable MNU-induced mammary tumors in rats when the feed intake was reduced. The suppressed risk of

palpable MNU-induced mammary tumor development during marginal zinc deficiency was, at least in part, due to the reduced feed intake associated with dietary zinc deficiency. However, the growth of palpable MNU-induced mammary tumors was not affected by dietary zinc intake.

In the present study, twenty-one-day-old female rats were injected with MNU. It has been shown that an injection of MNU in rats on day 21 of age allows the induction of tumorigenic response over a relatively short time interval (Thompson *et al.*, 1995). However, since 21 days old rats are not sexually mature, mammary tumorigenesis following MNU injection occurs in the absence of ovarian hormones. Because ovarian hormones play a role in mammary tumorigenesis (Welsch, 1985), the biology of these mammary tumors may be different from the tumors developed when MNU is injected after the rats reach sexual maturity. In addition to MNU injection, dietary treatment was also initiated on day 21 of age in rats in the present study. Since both MNU injection and dietary treatment were administered at the same time, the present study did not differentiate the effect of dietary zinc intake on the initiation or the promotional stage of mammary tumorigenesis. Future experiments could be designed to address the effect of dietary zinc intake on either the initiation or promotional stage of mammary tumorigenesis. Further studies to investigate the effect of dietary zinc intake on mammary tumorigenesis induced by MNU under the influence of ovarian hormones are also suggested.

Based on the results that MNU-induced mammary tumors had a higher zinc concentration when expressed on a per tissue protein basis as compared to that in mammary glands, it becomes important to investigate the biological significance of this accumulation of zinc during mammary tumorigenesis. In addition, it also appears to be important to elucidate

the role of zinc in the modulation of mammary tumor formation in rats when the feed intake is reduced.

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APPENDIX I
Dietary composition

Table I-1. Composition of diets used in Experiment 1.

Ingredients	severe-low-zinc	marginal-low-zinc	control	high-zinc	very-high-zinc
	g/kg diet				
Cornstarch	582.5	572.5	572.5	572.5	572.5
Egg white	200.0	200.0	200.0	200.0	200.0
Sucrose	100.0	100.0	100.0	100.0	100.0
Soybean oil	70.0	70.0	70.0	70.0	70.0
Mineral premix ¹	35.0	35.0	35.0	35.0	35.0
Vitamin premix ²	10.0	10.0	10.0	10.0	10.0
Zinc premix ³	0.0	10.0	10.0	10.0	10.0
Choline bitartrate	2.5	2.5	2.5	2.5	2.5

¹The mineral premix provided the following (g/kg diet): calcium phosphate, dibasic 13.17; potassium citrate, monohydrate 7.98; calcium carbonate, anhydrous 2.92; sodium chloride 2.28; sodium chloride 2.28; potassium sulfate 1.59; magnesium oxide 0.84; ferric citrate 0.21; manganese carbonate 0.11; cupric carbonate 0.03; chromium potassium sulfate, 12 hydrate 9.8×10^{-3} ; sodium selenite 3.5×10^{-3} ; potassium iodate 3.5×10^{-3} ; sucrose 5.85.

²The vitamin premix provided the following (g/kg diet): dl- α -tocophenol acetate (250 IU/g) 0.30; nicotinic acid 0.03; cyanocobalamin (0.1% in mannitol) 2.5×10^{-2} ; d-Ca pantothenate 1.6×10^{-2} ; retinyl acetate (500,000 IU/g) 8×10^{-3} ; pyridoxine HCl 7×10^{-3} ; thiamin HCl 6×10^{-3} ; riboflavin 6×10^{-3} ; cholecalciferol (400,000 IU/g) 2.5×10^{-3} ; d-biotin 2.2×10^{-3} ; folic acid 2×10^{-3} ; menadione 5×10^{-4} ; sucrose 9.59.

³The zinc premix provided 2, 30, 170 and 345 mg zinc to each kg of marginal-low-zinc, control, high-zinc, and very-high-zinc diets, respectively.

Table I-2. Composition of diets used in Experiment 2.

Ingredients	marginal-low-zinc	required-zinc	control	high-zinc
	g/kg diet			
Cornstarch	572.5	572.5	572.5	572.5
Egg white	200.0	200.0	200.0	200.0
Sucrose	100.0	100.0	100.0	100.0
Soybean oil	70.0	70.0	70.0	70.0
Mineral premix ¹	35.0	35.0	35.0	35.0
Vitamin premix ²	10.0	10.0	10.0	10.0
Zinc premix ³	10.0	10.0	10.0	10.0
Choline bitartrate	2.5	2.5	2.5	2.5

¹The mineral premix provided the following (g/kg diet): calcium phosphate, dibasic 13.17; potassium citrate, monohydrate 7.98; calcium carbonate, anhydrous 2.92; sodium chloride 2.28; sodium chloride 2.28; potassium sulfate 1.59; magnesium oxide 0.84; ferric citrate 0.21; manganese carbonate 0.11; cupric carbonate 0.03; chromium potassium sulfate, 12 hydrate 9.8×10^{-3} ; sodium selenite 3.5×10^{-3} ; potassium iodate 3.5×10^{-3} ; sucrose 5.85.

²The vitamin premix provided the following (g/kg diet): dl- α -tocophenol acetate (250 IU/g) 0.30; nicotinic acid 0.03; cyanocobalamin (0.1% in mannitol) 2.5×10^{-2} ; d-Ca pantothenate 1.6×10^{-2} ; retinyl acetate (500,000 IU/g) 8×10^{-3} ; pyridoxine HCl 7×10^{-3} ; thiamin HCl 6×10^{-3} ; riboflavin 6×10^{-3} ; cholecalciferol (400,000 IU/g) 2.5×10^{-3} ; d-biotin 2.2×10^{-3} ; folic acid 2×10^{-3} ; menadione 5×10^{-4} ; sucrose 9.59.

³The zinc premix provided 2, 11, 30 and 154 mg zinc to each kg of marginal-low-zinc, required-zinc, control and high-zinc diets, respectively.

APPENDIX II

Materials

Manganese carbonate and sodium selenite were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA).

Magnesium oxide, potassium citrate, potassium sulfate and xylene were obtained from BDH Inc. (Toronto, ON).

Egg white was obtained from Dysts Inc. (Bethlehem, PA, USA).

Acetic acid, calcium carbonate, carmine, chloroform, copper sulfate, chromium potassium sulfate, cupric carbonate, disodium ethylenediamine tetraacetate, ethanol, methanol, potassium iodate, potassium citrate, nitric acid, sodium carbonate, sodium chloride, sodium hydroxide, sodium tartrate, toluene and zinc sulfate were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

Cornstarch, sucrose, d-biotin, d-Ca pantothenate, cholecalciferol, cyanocobalamin, folic acid, menadione, nicotinic acid, pyridoxine HCl, retinyl acetate, riboflavin, thiamin HCl, dl- α -tocopherol acetate were obtained from ICN Biochemicals Inc. (Aurora, OH, USA).

Soybean oil was obtained from Maximum Nutrition Ltd. (Toronto, ON) and Canasoy (Vancouver, BC).

Bovine serum albumin, calcium phosphate, choline bitartrate, ferric citrate, methyl salicylate and N-methyl-N-nitrosourea were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Folin-Ciocalteu phenol reagent was obtained from VWR Scientific (West Chester, PA, USA).

APPENDIX III

**The composition of mammary glands and
N-methyl-N-nitrosourea (MNU)-induced mammary tumors**

Table III-1. Moisture content in mammary glands and N-methyl-N-nitrosourea (MNU)-induced mammary tumors in sham-treated and MNU-treated rats fed diets containing various levels of zinc¹

Tissues	Moisture content (%)			
	PZ31-Z3	Z31	Z171	Z346
NMG	32 ± 6 ^{a,#}	20 ± 2 ^{a,#}	25 ± 5 ^{a,#}	23 ± 7 ^{a,#}
TFMG	32 ± 3 ^{a,#}	23 ± 7 ^{a,#}	29 ± 6 ^{a,#}	35 ± 5 ^{a,#}
Tumor	78 ± 2 ^{a,*}	83 ± 2 ^{a,*}	80 ± 1 ^{a,*}	84 ± 1 ^{a,*}

¹Values represent the mean ± SEM, *n*=3 rats. The means among dietary treatments in each row not sharing a common letter and among tissues in each column not sharing a common symbol are significantly different (*p*<0.05). PZ31-Z3: pair-fed to marginal-low-zinc; Z31: control; Z171: high-zinc; Z346: very-high-zinc. NMG: normal mammary gland from sham-treated rats; TFMG: tumor-free mammary gland from MNU-treated tumor-bearing rats; Tumor: MNU-induced mammary tumor from MNU-treated tumor-bearing rats.

Table III-2. Protein concentrations in mammary glands and N-methyl-N-nitrosourea (MNU)-induced mammary tumors in sham-treated and MNU-treated rats fed diets containing various levels of zinc¹

Tissues	Protein concentration (g/g dry weight)			
	PZ31-Z3	Z31	Z171	Z346
NMG	0.10 ± 0.04 ^{a, #}	0.09 ± 0.04 ^{a, #}	0.08 ± 0.01 ^{a, #}	0.09 ± 0.02 ^{a, φ}
TFMG	0.12 ± 0.02 ^{a, #}	0.10 ± 0.04 ^{a, #}	0.11 ± 0.05 ^{a, #}	0.15 ± 0.02 ^{a, #}
Tumor	0.67 ± 0.02 ^{a, *}	0.68 ± 0.05 ^{a, *}	0.72 ± 0.01 ^{a, *}	0.69 ± 0.03 ^{a, *}

¹ Values represent the mean ± SEM, *n*=3 rats. The means among dietary treatments in each row not sharing a common letter and among tissues in each column not sharing a common symbol are significantly different (*p*<0.05). PZ31-Z3: pair-fed to marginal-low-zinc; Z31: control; Z171: high-zinc; Z346: very-high-zinc. NMG: normal mammary gland from sham-treated rats; TFMG: tumor-free mammary gland from MNU-treated tumor-bearing rats; Tumor: MNU-induced mammary tumor from MNU-treated tumor-bearing rats.

Table III-3. Lipid concentrations in mammary glands and N-methyl-N-nitrosourea (MNU)-induced mammary tumors in sham-treated and MNU-treated rats fed diets containing various levels of zinc¹

Tissues	Lipid concentration (g/g dry weight)			
	PZ31-Z3	Z31	Z171	Z346
NMG	0.67 ± 0.08 ^{a,*}	0.81 ± 0.04 ^{a,*}	0.86 ± 0.03 ^{a,*}	0.89 ± 0.08 ^{a,*}
TFMG	0.73 ± 0.04 ^{a,*}	0.77 ± 0.21 ^{a,*}	0.70 ± 0.11 ^{a,*#}	0.79 ± 0.02 ^{a,*}
Tumor	0.60 ± 0.36 ^{a,*}	0.20 ± 0.04 ^{a,#}	0.48 ± 0.06 ^{a,#}	0.19 ± 0.08 ^{a,#}

¹ Values represent the mean ± SEM, *n*=3 rats (except Tumor from PZ31-Z3 group, where *n*=2). The means among dietary treatments in each row not sharing a common letter and among tissues in each column not sharing a common symbol are significantly different (*p*<0.05). PZ31-Z3: pair-fed to marginal-low-zinc; Z31: control; Z171: high-zinc; Z346: very-high-zinc. NMG: normal mammary gland from sham-treated rats; TFMG: tumor-free mammary gland from MNU-treated tumor-bearing rats; Tumor: MNU-induced mammary tumor from MNU-treated tumor-bearing rats.

Table III-4. Zinc concentration expressed on a per wet weight basis in mammary glands and N-methyl-N-nitrosourea (MNU)-induced mammary tumors in sham-treated and MNU-treated rats fed diets containing various levels of zinc¹

Tissues	Zinc concentration ($\mu\text{g/g}$ wet weight)			
	PZ31-Z3	Z31	Z171	Z346
NMG	$7 \pm 1^{a,\#}$	$5 \pm 1^{a,\#}$	$6 \pm 1^{a,\#}$	$4 \pm 1^{a,\phi}$
TFMG	$8 \pm 0^{a,\#}$	$6 \pm 2^{a,\#}$	$6 \pm 0^{a,\#}$	$7 \pm 1^{a,\#}$
Tumor	$15 \pm 2^{a,*}$	$17 \pm 1^{a,*}$	$17 \pm 1^{a,*}$	$16 \pm 0^{a,*}$

¹ Values represent the mean \pm SEM, $n=3$ rats. The means among dietary treatments in each row not sharing a common letter and among tissues in each column not sharing a common symbol are significantly different ($p<0.05$). PZ31-Z3: pair-fed to marginal-low-zinc; Z31: control; Z171: high-zinc; Z346: very-high-zinc. NMG: normal mammary gland from sham-treated rats; TFMG: tumor-free mammary gland from MNU-treated tumor-bearing rats; Tumor: MNU-induced mammary tumor from MNU-treated tumor-bearing rats.

Table III-5. Zinc concentration expressed on a per dry weight basis in mammary glands and N-methyl-N-nitrosourea (MNU)-induced mammary tumors in sham-treated and MNU-treated rats fed diets containing various levels of zinc¹

Tissues	Zinc concentration ($\mu\text{g/g}$ dry weight)			
	PZ31-Z3	Z31	Z171	Z346
NMG	$10 \pm 2^{a, \#}$	$6 \pm 1^{a, \#}$	$8 \pm 2^{a, \#}$	$6 \pm 2^{a, \#}$
TFMG	$11 \pm 1^{a, \#}$	$8 \pm 3^{a, \#}$	$9 \pm 1^{a, \#}$	$11 \pm 2^{a, \#}$
Tumor	$72 \pm 15^{a, *}$	$101 \pm 10^{a, *}$	$85 \pm 8^{a, *}$	$102 \pm 6^{a, *}$

¹ Values represent the mean \pm SEM, $n=3$ rats. The means among dietary treatments in each row not sharing a common letter and among tissues in each column not sharing a common symbol are significantly different ($p<0.05$). PZ31-Z3: pair-fed to marginal-low-zinc; Z31: control; Z171: high-zinc; Z346: very-high-zinc. NMG: normal mammary gland from sham-treated rats; TFMG: tumor-free mammary gland from MNU-treated tumor-bearing rats; Tumor: MNU-induced mammary tumor from MNU-treated tumor-bearing rats.

Table III-6. Zinc concentration expressed on a per tissue protein basis in mammary glands and N-methyl-N-nitrosourea (MNU)-induced mammary tumors in sham-treated and MNU-treated rats fed diets containing various levels of zinc¹

Tissues	Zinc concentration ($\mu\text{g/g}$ protein)			
	PZ31-Z3	Z31	Z171	Z346
NMG	107 \pm 24 ^{a,*}	95 \pm 29 ^{a,*}	94 \pm 22 ^{a,*}	65 \pm 17 ^{a,*}
TFMG	97 \pm 17 ^{a,*}	106 \pm 51 ^{a,*}	108 \pm 38 ^{a,*}	75 \pm 18 ^{a,*}
Tumor	120 \pm 11 ^{a,*}	134 \pm 2 ^{a,*}	123 \pm 6 ^{a,*}	122 \pm 5 ^{a,*}

¹ Values represent the mean \pm SEM, $n=3$ rats. The means among dietary treatments in each row not sharing a common letter and among tissues in each column not sharing a common symbol are significantly different ($p<0.05$). PZ31-Z3: pair-fed to marginal-low-zinc; Z31: control; Z171: high-zinc; Z346: very-high-zinc. NMG: normal mammary gland from sham-treated rats; TFMG: tumor-free mammary gland from MNU-treated tumor-bearing rats; Tumor: MNU-induced mammary tumor from MNU-treated tumor-bearing rats.

Appendix IV

**The tumor burden in N-methyl-N-nitrosourea (MNU)-treated tumor-bearing rats
whose tissues were used in the determination of tissue zinc concentrations**

Table IV-1. The tumor burden in N-methyl-N-nitrosourea (MNU)-treated tumor-bearing rats whose tissues were used in the determination of tissue zinc concentrations¹

	Z3	PZ12	PZ31	Z12	Z31	Z155
Tumor burden ² (%)	3.6 ± 1.6 ^a	2.6 ± 1.8 ^a	6.3 ± 4.6 ^a	6.4 ± 1.4 ^a	5.1 ± 1.7 ^a	3.3 ± 0.5 ^a

¹ Values represent the mean ± SEM, *n*=6 rats (except Z3 group, *n*=3; PZ31 group, *n*=4). The means between dietary treatment groups not sharing a common letter are significantly different (*p*<0.05). Z3: marginal-low-zinc; PZ12: pair-fed with required-zinc diet; PZ31: pair-fed with control diet; Z12: required-zinc; Z31: control; Z155: high-zinc.

²Tumor burden was expressed as a percentage of the total tumor weight in a tumor-bearing rat relative to the host weight.