EXPERIMENTAL COLON CANCER: EFFECTS OF ROUTE OF 1,2-DIMETHYLHYDRAZINE (DMH) ADMINISTRATION, CABBAGE CONSUMPTION AND PRIOR EXPOSURE TO DMH

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ABSTRACT

EXPERIMENTAL COLON CANCER: EFFECTS OF ROUTE OF 1,2-DIMETHYLHYDRAZINE (DMH) ADMINISTRATION, CABBAGE CONSUMPTION AND PRIOR EXPOSURE TO DMH

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Three studies were conducted to examine different aspects of 1,2-dimethylhydrazine (DMH) induced carcinogenesis.

The first study examined the DMH model of carcinogenesis for studying colon tumor liver metastases, a critical problem in cases of human colorectal cancer. The present study is the first to investigate the metastastic potential of intrarectal DMH administration. Findings from this study suggest that in male Sprague-Dawley rats, the dose of DMH influences tumor site while the route of DMH administration influences tumor metastases. However, metastases was primarily limited to the mesentery and therefore, intrarectal administration of DMH is not an effective method for inducing colon tumor liver metastases. Alternative animal models need to be developed.

The second study was undertaken because of recommendations by two scientific bodies to increase the consumption of cruciferous vegetables as a means of reducing the risk of cancer. Two separate experiments examined effects of cabbage consumption on DMH induced colon

carcinogenesis in CF1 male mice. In the first experiment, cabbage consumption significantly increased both colon tumor incidence and number. Results of the second experiment suggested that cabbage exerts its effect on carcinogenesis only during promotion. Cabbage consumption did not induce xenobiotic metabolizing enzymes, indicating they were not involved in the enhanced carcinogenesis. Recommendations to increase cruciferous vegetable consumption may have been made prematurely.

The last study was initiated after observing that mice appear to develop an increase in tolerance to DMH toxicity. Findings showed that prior exposure with 30 mg of DMH/kg body weight results in a two fold increase in the medium lethal concentration for DMH. In addition, in comparison to control mice, mice pretreated with DMH exhibited decreased levels of DNA methylation in response to a test dose of DMH. The increased tolerance and decreased methylation in pretreated mice appears to be mediated through a change in DMH metabolism. The altered metabolic pattern may result from the decreased levels of hepatic cytochrome P450 in pretreated mice, indicating this enzyme complex may play a role in DMH metabolism. Findings suggest that the commonly used DMH dosing regimen to initiate tumors may warrant re-evaluation.

DEDICATION

To my parents, Carmen and Eileen Messina for giving me the love and encouragement I needed to pursue my educational goals.

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INTRODUCTION

In the United States, one of four Americans will develop cancer and one of five will die from it. Cancer is the second leading cause of mortality in this country, killing an estimated 500,000 people annually (1). Health surveys indicate that the threat of cancer is uppermost in the minds of the American public. Although the public fear of cancer has increased in recent years, the age adjusted cancer incidence and mortality rates, with the exception of cancer of the respiratory tract, have remained about the same (1).

Two aspects of cancer control have become increasingly evident. First, treatment success depends greatly on early detection. As example, early detection of colon and rectal cancer has been shown to reduce the mortality from these malignancies (2). Second, dietary modification may represent a significant means of cancer prevention. On the basis of epidemiologic data, it has been estimated that 35% of cancer is diet related (3). Consequently, there has been an increased emphasis on identifying dietary components influencing carcinogenesis.

Cancer of the colon is one of the types of cancer thought to be influenced by diet. There are approximately 500,000 new cases of colorectal cancer in the world yearly (4). Epidemiologic findings have indicated that a high fat and low fiber diet is related to an increased risk for

colon cancer (5, 6), whereas several case control studies have found consumption of cruciferous vegetables to be associated with a decreased risk (6-8).

To enhance our understanding and to establish a causal relationship between a particular dietary component and colon cancer, animal models have been developed. For inducing experimental colon cancer, 1,2-dimethylhydrazine (DMH) (9) and DMH derivatives (10) are frequently used. Studies of experimentally induced colon cancer may provide insights how to significantly reduce both the incidence of and mortality from human colon cancer.

CHAPTER 1 ... REVIEW OF LITERATURE

REVIEW OF LITERATURE

Epidemiologists believe that 60 to 90% of human cancer is related to enviromental factors (11); therefore, a significant percentage of cancer may be potentially avoidable. Interest in the relationship between diet and cancer has been stimulated by epidemiologic studies in which large differences in cancer incidence rates have been found among countries (12, 13). Findings from these studies, along with data from migration studies, have suggested that these differences are largely diet related. For instance, the U.S. population has a low incidence of stomach cancer and a high incidence of colon and breast cancer (1). In contrast, the Japanese have a high incidence of stomach cancer and a low incidence of colon and breast cancer (14).

Correlations have been found between per capita fat intake and the national rate of breast cancer mortality and between per capita fat intake and mortality from colon cancer (12). One problem with stating conclusions based on these data, is that there are many differences among countries, not just differences in dietary customs. For example, the association that exists between per capita fat intake and the incidence of breast and colon cancer, can also be seen between gross national product and breast and colon cancer (12).

The extent to which genetic disposition contributes to

differences in cancer incidence among countries can generally be determined by studies of migrant populations and of secular trends within countries. In general, populations migrating from an area with one pattern of cancer incidence rates will acquire incidence rates characteristic of their new location (15). In the case of Japanese immigrants to the United States, within approximately two generations, the colorectal cancer incidence approaches that of the United States level (16, 17). The rapid rise in colorectal cancer incidence among immigrants rules out the possibility that genetics is a main factor for differences among colon cancer among countries. The increase in cancer incidence closely paralleles adoption of the host countries dietary patterns.

EXPERIMENTAL COLON CANCER

Colonic tumors induced by DMH, or DMH derivatives, azoxymethane and methylazoxymethanol, are the most commonly used experimental models of colon cancer. These compounds, when injected subcutaneously, induce primarily large intestinal tumors which are similar in nature to those seen in human colon cancer (18). Therefore, the DMH model is in most respects, a good model for studying human colon cancer (19). The major difference between DMH induced colon cancer and human colon cancer, is the lack of metastases. In cases of human colonic cancer, approximately

1/2 of the patients have evidence of lymphatic metastases and 1/3 have evidence of hematogenous metastases at the time of presentation. With respect to lymphatic metastases, the usual pattern is an orderly progression of tumor through the lymphatic network from the pericolonic lymph nodes and progressively up the perivascular lymphatics (20). The usual route of hematogenous metastases is initially to the liver and subsequently to the lung (21). Most studies using DMH to induce colon cancer have produced a very low incidence of metastases. The metastases that do occur are generally found in the regional lymphatics or on the peritoneal surface. Whereas liver metastases are commonly found in the human disease, they are rarely, if ever, found in DMH models. Human colon tumor liver metastasis is a critical problem in the treatment of colon cancer. Thus far, the DMH model of carcinogenesis does not appear suitable for studying metastasis. Presently, attempts are being made to develop animal models designed for the study of colon tumor liver metastases (22, 23).

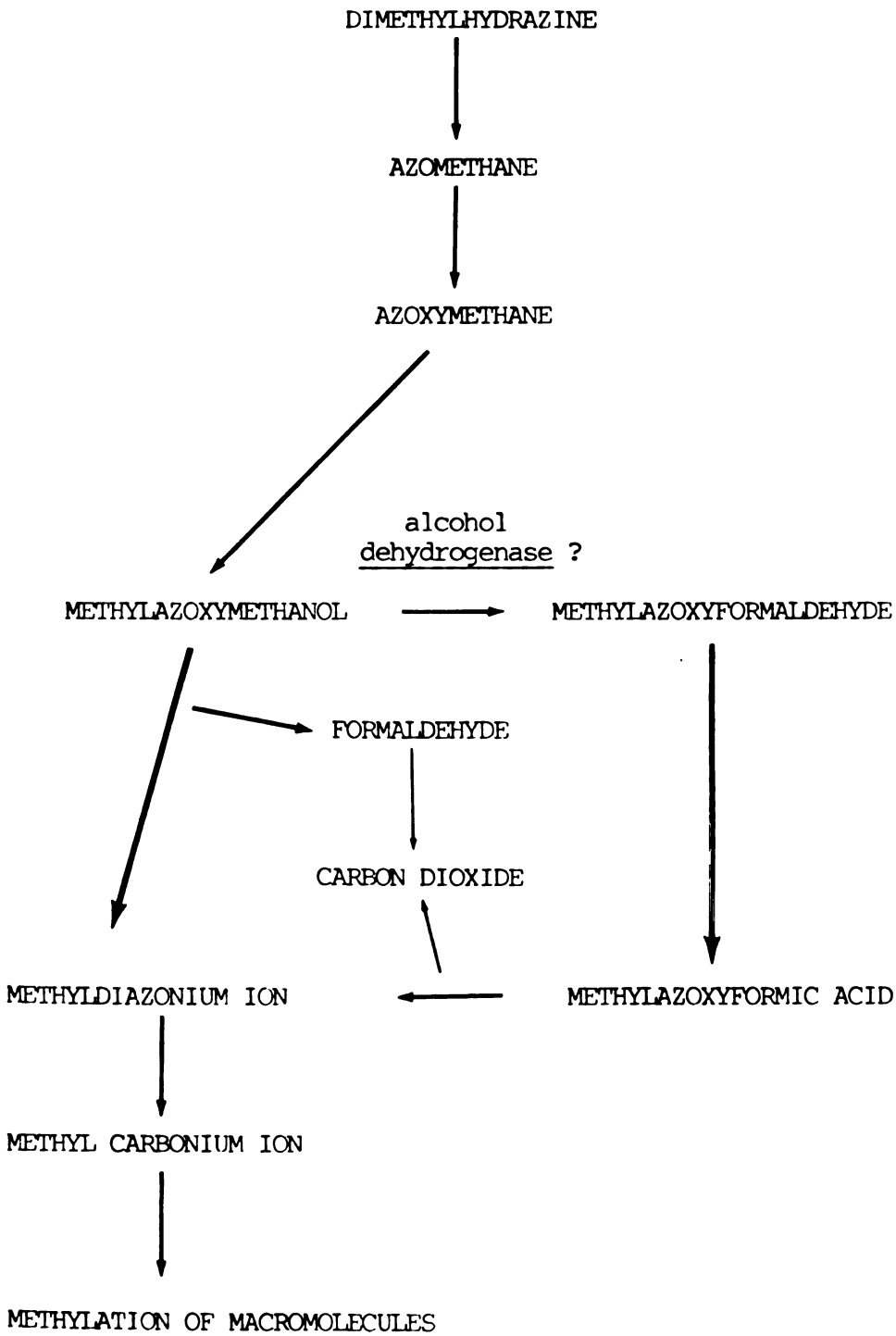
Dimethylhydrazine Metabolism

In order to exerts its effects, DMH must be metabolically activated. It is believed that DMH induces carcinogenesis via DNA methylation (24, 25). DMH metabolism has been studied extensively, although a complete understanding of its mode of action has not yet

been achieved. As intially proposed by Druckey, in 1969, the procarcinogen DMH (figure 1), is activated in vivo to azomethane, azoxymethane and methylazoxymethanol to yield formaldehyde and the reactive methyldiazonium ion (24, 25). The spontanous breakdown of the methyl diazonium ion results in the formation of a methyl carbonium ion, which is thought to be the actual methylating species. After the injection of labeled DMH in vivo, radioactive azomethane and carbon dioxide are exhaled, the latter compound presumably resulting from the spontaneous breakdown of formaldehyde (26, 27). Labeled azoxymethane and methylazoxymethanol are excreted in the urine (28) and labeled methyl groups bind to DNA and other cellular macromolecules (26, 27). Although methylazoxymethanol breaks down spontanously (29, 30), it is believed that an enzymatic conversion must also be involved to account for the narrow range of organotropism of this carcinogen.

Two theories explaining DMH organospecificity have been proposed. Weisburger suggested that DMH is metabolised to methylazoxymethanol in the liver, conjugated with glucuronic acid, transported via the bile to the gut where it undergoes hydrolysis by intestinal flora, thereby directly exposing the colon to this carcinogen (31). However, evidence strongly refuting this hypothesis comes from studies showing that the biliary metabolites of DMH account for approximately only 1% of the total administered dose (32) and that these metabolites incubated with or

Figure 1. METABOLISM OF 1,2-DIMETHYLHYDRAZINE



(From Fiala, ES., Inhibition of Tumor Induction and Development, Zedeck, MS and Lipkin M, Eds., Plenum Press, New York. 1981, 37.)

without β-glucuronidase are non-mutagenic (33). Perhaps more damaging to the credibility of this scheme, are studies showing that segments of the colon surgically removed from the fecal stream are as equally subject to DMH induced carcinogenesis as is the intact colon (34). This suggests that DMH and/or its metabolites reach the colon via the blood stream.

The second theory explaining the organospecificity of DMH, focuses on the specific metabolic capability of the colon. Cytosolic fractions of various rat tissues as well as solutions of purified horse liver alcohol dehydrogenase (ADH) are able to catalyze the reduction of NAD using methylazoxymethanol as a substrate (35). The ability to stimulate methylazoxymethanol reduction of NAD in various tissues is reported to be directly correlated with their sensitivity to methylazoxymethanol-acetate induced carcinogenesis. The protein responsible for this reaction is reportedly similar to ADH. Schoental, in 1973 proposed that ADH may catalyse the oxidation of methylazoxymethanol to methylazoxyformaldehyde (figure 1), but this compound has not yet been isolated (36). ADH would contribute to the organospecificity of DMH only if there was a colon specific isoenzyme, since ADH is ubiquitous. However, a recent report by Fiala et al., showing that methylazoxymethanol is metabolized to a methylating species equally in two strains of deer mice, one with and the other without ADH, indicates that, at least in this animal, ADH

is not important in DMH metabolism (37). Furthermore, pyrazole, a known inhibitor of both DMH metabolism and ADH, was equally effective in both types of mice. It is clear from this discussion, that the enzyme(s) responsible for the complete metabolism of DMH are as yet unknown, as is an explanation for the organospecificity.

NUTRITION AND COLON CANCER

The colon and rectum are among the most frequent sites of cancer development in affluent societies with a Westernized life-style. Internationally, the U.S., Canada, the countries of northwestern Europe, Australia, and New Zealand have the highest colon cancer incidence rates (38). Although numerous dietary factors have been examined for their relationship with colon cancer, two components, dietary fat and fiber, appear to be the strongest candidates responsible for an increase in the incidence of colon cancer.

DIETARY FAT

Epidemiology

Wynder and Shigematsu were the first to suggest that dietary fat intake may be related to the international variations in colon cancer (39). Several years later, Armstrong and Doll, found a strong correlation ($r = 0.85$

for both men and women) between total meat intake and the world wide incidence of colon cancer (12). Similarly, in a study involving 38 countries, animal fat, total meat and red meat, but not vegetable fat, were strongly correlated with colon cancer incidence (40). In Japan, recent increases in fat consumption (41) have been associated with a striking increase in rates of colon cancer (42). Seventh Day Adventists, who consume little or no meat, were found to have 1/7 the colon cancer incidence of the control population, a finding which supports the notion of a fat-associated risk for colon cancer (43).

Consumption of meat, an important source of fat, was associated with large-bowel cancer among Japanese Hawaiian colon cancer patients in a study by Haenszel et al. (44), although no such relationship was found by this investigator in Japan (45). Potter and McMichael, in an Australian study, also found a positive correlation between colorectal cancer and protein intake (2-3 fold increased risk) (46). In addition to protein, caloric intake, which the authors suggest may be indicative of a high fat intake, was also related to an increased risk. Kune et al., comparing the dietary intake of 715 colorectal cancer patients with that of 727 age matched controls in Melbourne (Australia), found total fat intake to be positively associated with colon cancer incidence in both males and females (6). However, beef intake was a risk factor only in males, while a high intake of pork and fish was

protective in both sexes.

A recent Canadian study reported a higher intake of total fat, saturated fat, and cholesterol in colon cancer patients, although patients also consumed more total calories (47). Tajima and Tominaga, in a study involving 93 cases of colo-rectal cancer and 186 controls at the Aichi Cancer Center Hospital in Japan, found that eating a Western style breakfast for 10 years or more made a significant contribution to the risk of colon cancer, but decreased the relative risk for stomach and rectal cancer (48). Interestingly, a Puerto Rican study found patients with colon cancer had a higher intake of both fat and fiber than controls (49).

In contrast to the above findings, several studies have found neither fat or meat intake to be a risk factor for colon cancer. Phillips and Snowdon examined the consumption pattern for 21 different foods and for coffee and percent of desirable weight in relation to mortality rates from colon cancer in over 25,000 Seventh-Day Adventists (50). They found that consumption of meat or poultry was not significantly related to death from either colon or rectal cancer. However, egg consumption did show a positive relationship with colon cancer. Case-control studies of colon cancer in Israel (8) and the United States (51), were unable to establish an increased risk for fat or meat intake (Israeli study) or for meat intake (American study). In fact, Graham et al., found that when comparing

190 colon cancer patients with 600 controls, frequent meat eaters had a relative risk for colon cancer of about 1/3 that of those who rarely ate meat (7). Similarly, Stemmerman et al., found that saturated fat intake, rather than being a risk factor, was found to be associated with a decreased risk for colon cancer (52).

In summary, the preponderance of the epidemiologic data suggests that fat and/or meat intake is associated with an increased risk for colon cancer (5, 6, 12, 39-44, 46-49). However, several studies do not support this relationship (7, 8, 50-52). The inconsistency among these studies is most likely due to the complexity involved in conducting epidemiologic studies, with many uncontrolled or poorly controlled variables. At the present time, based on the available information, it is appropriate to conclude that dietary fat intake is a risk factor for colon cancer.

Animal studies

Early animal studies supported a positive association between dietary fat and experimental colon cancer. Reddy et al., found that rats fed a 5% corn oil diet had a greater incidence of DMH induced colon tumors than those fed 5% lard (53). When fed at the 20% level, overall tumor incidence was significantly enhanced, but the type of fat was not a factor. Dietary fat in rodents has also been shown to enhance colon cancer induced by azoxymethane, methylazoxymethanol, and methylnitrosourea (54, 55).

However, as has been pointed out in a number of reviews, these early studies employed diets which were not isocaloric and therefore, differences may not have been specifically related to fat intake per se, but rather to an increased caloric intake (56), or to differences in vitamin, mineral or protein intake. Consumption of a diet high in beef fat increased large intestinal tumor incidence when fed after azoxymethane administration, i.e, promotion (55). However, if animals were fed this diet before or during carcinogen administration, tumor incidence was not effected. Reddy and Maeura observed an increase in azoxymethane induced colon carcinogenesis in rats consuming diets containing 20% corn or safflower oil, as compared with animals consuming diets containing 5% oil (57). Reddy and Maruyama, investigated the effects of diets comprised of 5%, 13.6% or 23.5% corn oil or lard during or following the administration of azoxymethane (58). Corn oil increased tumorigenesis only when fed after the carcinogen was administered. When fed prior to and during azoxymethane administration, there was no effect. In contrast, both the 13.6% and 23.5% lard diets increased tumorigenesis when fed prior to and during carcinogen administration, i.e., initiation. The highest level of lard also enhanced tumorigenesis when fed during promotion. The effect of fat during initiation may be attributable in part, to the influence of fat on the metabolism of DMH by colonic microsomes (59).

In contrast to much of the above data, Nauss et al., found that diets composed of either 24% corn oil, beef tallow or Crisco had no effect on DMH induced carcinogenesis (60), a finding which concurs with work of Glauert and Bennink (61).

In summary, findings from animal studies, in general have found fat intake to be positively correlated with colon cancer (53-55, 57, 58). Although, as one might expect, there are studies which do not support this relationship, but they are relatively few in number (60, 61).

DIETARY FIBER

The relationship between fiber intake and colon cancer is particularly complex because there are several types of fiber which differ with respect to their effect on this disease. Overall, the data indicates that the less fermentable and more insoluble fibers such as lignin, cellulose and hemicellulose offer protection, whereas easily fermentable and very soluble fiber either offer no protection or are associated with tumor enhancement.

Epidemiology

Burkitt suggested that countries consuming a diet rich in fiber have a low incidence of colon cancer, whereas those eating refined carbohydrates, with little fiber have

a higher incidence of this disease (62). According to a recent comprehensive review by Jacobs, 69% of the human epidemiologic studies have demonstrated an inverse association between high fiber diets and colonic cancer (63). Of greater significance, is the fact that when these studies were catagorized according to the type of fiber, an inverse association between cereal fiber and colon cancer was found in 80% of the cases, whereas fruit and vegetable consumption showed no association in 75% of the cancers. However, two British studies found that the components of fiber containing foods exhibiting the highest negative correlation with colon cancer include the pentose fraction and uronic acids (64, 65).

A comparison between the incidence of colon cancer and fiber consumption in two Scandanavian countries, found that individuals from Denmark, residents of which have a four fold higher colon cancer incidence then Finland, consumed approximately 50% less total fiber (66). A follow up study found a very strong inverse relationship between cereal consumption and colon cancer incidence in four different areas in Denmark and Finland (67). Similarly, cereal fiber was found to be the only source of fiber negatively associated with colon cancer incidence among 38 countries (40). Cereal fiber had a higher negative correlation with mortality from colon cancer than that of crude fiber. Finally, Walker et al., observed a negative association between fiber intake and the incidence of colon cancer

among South Africans (68).

Generally, findings from case control studies have not found fiber to be protective against colon cancer (47, 69-71). Among those case control studies in which fiber has been shown to be protective, are reports showing an inverse relationship between vegetable consumption and colon cancer (45, 72). The combination of a high fiber-high vegetable diet was found to be associated with a decreased risk for colon cancer by Kune et al (6). However, several studies have failed to find an association between vegetable consumption and colon cancer (69, 73). Furthermore, in one study, colon cancer patients consumed more vegetables than controls (44), while in another study, there was a strong indication that cabbage consumption increased the relative risk for colon cancer (47).

The contrasting findings between case control studies and those studies comparing colon cancer incidence and fiber consumption among countries indicates the uncertainity surronding this issue. Although the association between fiber intake and colon cancer has been discussed for many years, it appears the basis for this contention is quite weak. Until new findings are presented, it is not possible to say with a high degree of certainty that fiber is protective against colon cancer.

Animal Studies

One advantage to using animal models is that it allows

for testing various fiber fractions independently. However, in doing this, important interelationships among dietary fibers may be overlooked. Additionally, interpretation of the data becomes difficult when there are differences among species.

Generally, wheat bran has been shown to reduce experimental colon cancer in mice (74) and rats (75-78), but Clapp et al., found that in mice consuming one of four kinds of wheat bran, tumor incidence ranged from 44% to 72% as compared to an 11% tumor incidence in control mice (no fiber) (79). Furthermore, Jacobs has recently shown that wheat bran supplementation enhances colon carcinogenesis in Sprague-Dawley rats and attributes this effect to a decreased colonic pH, resulting from an increase in bacterial fermentation (80, 81).

In general, the addition of cellulose to the diets of rats has had a protective effect on DMH induced carcinogenesis (82, 83), although Ward et al., reported that the addition of cellulose had no effect on azoxymethane induced colon cancer (84). Freeman et al., found that rats consuming a 4.5% or a 9% cellulose diet had approximately one half the tumor frequency and tumor number as the fiber free group (82). No difference in these parameters was noted between the two cellulose groups. Cellulose exerted its inhibitory effect on tumor frequency during the period of carcinogen administration. In this study, dietary pectin had no effect on carcinogenesis,

although several studies have found that pectin enhances DMH induced carcinogenesis (85, 81).

In summary, animal studies investigating the relationship between fiber intake and experimental colon cancer do not support a protective effect of fiber. For each fiber, almost without exception, are studies showing opposite effects on colon carcinogenesis.

BILE ACIDS AND COLON CANCER

Diet may influence colon carcinogenesis by altering bile acid concentration in the large intestine. Bile acids have a range of effects on the intestinal cells of the colon, including hyperplasia (86) and disruption of cell membrane (87). Fecal bile acid excretion is quantitatively related to dietary fat intake (88) and it has been suggested that dietary fiber may exert a protective effect against colon cancer by diluting the concentration of bile acids (89). When administered intrarectally following a dose of the colon carcinogen N-methyl-N'-nitrosoguanidine, sodium deoxycholate, lithocholic acid, taurodeoxycholic acid, sodium cholate, and sodium chenodeoxycholate all increase tumor frequency (90-92). Fecal bile acid levels have been shown to be increased in populations with high rates of colon cancer (93, 94), in individual patients with colon cancer (95, 96) and in patients with colonic polyps (97). Fecal bile acid concentration in Denmark, which has

a high incidence of colon cancer, was found to be higher then in Finland, which has a low incidence of colon cancer (67). Of interest, was the finding that daily fat intake was not related to colon cancer incidence. The protective factor in this study, appeared to be the increased fecal bulk of the Finland population.

In contrast to the above data, findings from several studies have failed to show a relationship between bile acids and colon cancer (66, 98). Tanida et al., found that the amount of dysplasia and the number of polyps present in the colon, correlated with a higher level of bile acid excretion (99). However, patients with the largest polyps excreted less total bile acids (99). Consequently, the authors of this study concluded that the contribution of bile acids in the development of adenomatous polyps in Japanese subjects was insignificant. Finally, Breure et al., determined total and individual bile acid concentration and found no difference between 23 patients with colonic carcinoma and 21 controls (100).

Findings presented generally suggest that high fecal bile acid concentrations correlated with increased colon cancer incidences. Fecal bile acid concentration is influenced by both fat and fiber intake - increasing dietary fat increases bile acid concentration while increasing dietary fiber decreases bile acid concentration.

CRUCIFEROUS VEGETABLES AND CANCER

A select committee on Diet, Nutrition and Cancer, sponsored by the National Research Council, in 1982 recommended increased consumption of cruciferous vegetables as a means to reduce the incidence of cancer (101). At the present time, this position is endorsed by the American Cancer Society (102). The human data supporting an inverse association between cabbage consumption and colon cancer comes from case control studies (6-8, 45, 51, 72, 103). The only study comparing cabbage consumption and colon cancer incidence among countries failed to find an association (40).

Epidemiology

In a study of 190 male and female colon cancer patients and 600 age matched controls, an increased risk for colon cancer was found to be associated with a decreased consumption of vegetables, in particular cabbage, but also including brussels sprouts and broccoli (7). The relative risk of those individuals consuming the highest amounts of cabbage as compared to those consuming little or none, was approximately one-third. No relationship between fiber and colon cancer was noted. An earlier study by this same investigator, also found an increased risk for both male and female colon cancer patients as the frequency of cabbage consumption decreased (51). These results are in

agreement with Bjelke, who found less cabbage consumption among colorectal cancer patients in comparison to age and sex matched controls (103).

In an Australian study, not only was a high fiber-high vegetable diet protective, but cruciferous vegetable consumption as well (6). Modan et al., in an Israeli study, determined the frequency of consuming 243 different foods and beverages in 198 colon cancer and 77 rectal cancer cases and a comparable number of controls (8). Cancer patients consumed significantly less from the fiber group, which included 73 different foods with a high fiber content. Cabbage, in particular, was one of only 10 foods which, when evaluated separately, was consumed significantly less frequently than both the "neighborhood" and surgical controls. Manousos et al., found that colon cancer patients reported significantly less consumption of vegetables, especially cabbage, beets, lettuce and spinach (72). There was an eight fold increased risk between those consuming a high meat-low vegetable diet and those consuming a high vegetable-low fat diet.

Haenzel et al., in a study involving 588 patients with colorectal cancer and 1176 hospitalized controls, found a negative association between consumption of hakusal (cabbage) and colon cancer in Japan (45). However, these authors found no such correlation in Hawaiin subjects (44). Similarly, Miller et al., reported that cruciferous vegetables had only a minor protective effect against colon

cancer in females and no protective effect for males (104). Finally, Tajima and Tominaga, reported a two fold increase in relative risk for colon cancer in patients consuming cabbage frequently (3-4 times/week), although the risk associated with cabbage consumption was not statistically significant (48).

In summary, most case controls studies have found cabbage consumption to be protective against colon cancer (6-8, 45, 51, 72, 103), whereas only a few studies failed to find a correlation between cabbage consumption and colon cancer (44, 48, 104). The only study comparing cabbage consumption and colon cancer incidence among countries, did not find an association (40). In conclusion, on the basis of the human data, it appears cabbage consumption is associated with a decreased relative risk for colon cancer.

Animal Studies

Several animal studies have shown diets containing cruciferous vegetables and/or compounds isolated from cruciferous vegetables to reduce experimentally induced cancers. Wattenberg et al., found that when rats were fed a diet containing 25% cauliflower one week following dimethylbenzanthracene administration they had a decreased mammary tumor frequency in comparison to animals consuming the control diet (105). Boyd et al., determined the level of α fetoprotein, which is used as a marker for cancer, in Fischer 344 rats 5 weeks after the administration of the

liver carcinogen, aflatoxin B_1 (106). Rats consuming a 20% freeze dried cauliflower diet, had one half the level of fetoprotein as rats administered aflatoxin B_1 and consuming the basal diet. Similarly, the number of aflatoxin B_1 induced hepatic gamma-glutamyl transaminase foci, an indication of preneoplasia, was significantly lower in male Fischer rats fed a 20% brussels sprouts diet for 3 weeks prior to and for two weeks during carcinogen administration in comparison to the basal group without brussel sprouts (107).

In addition to using the whole vegetable, glucosinolate derivatives, compounds isolated from cruciferous vegetables which are thought to possess anti-carcinogenic properties, have also been shown to inhibit carcinogenesis (108, 109). Hydrolysis of indolylmethyl glucosinolate, the parent compound, produces three glucosinate derivatives: indole-3-carbinol (I3C), indole-3-acetonitrile and 3,3'-diindolymethyl (110). I3C is the most frequently tested derivative. I3C has been found to significantly reduce the frequency of dimethylbenzanthracene mammary cancer in Sprague Dawley female rats (109), benzo(a)pyrene induced gastric cancer in female ICR/Ha mice (111) and aflatoxin B_1 induced liver cancer in fingerling rainbow trout (112). However, when I3C was fed to fingerling trout during promotion only, the frequency of liver cancer was enhanced. When this compound was fed during initiation only, tumor frequency was inhibited (113).

When looking specifically at the effect of cabbage on experimentally induced cancers and/or the effect of I3C on DMH induced colon cancer, it becomes readily apparent that very little work has been done in this area. There are only two published studies in which the effect of cabbage consumption on DMH induced carcinogenesis was examined (114, 115). However, these studies provide very little insight into this relationship. Srisangnam et al., employed a strain of mice resistant to DMH induced colon carcinogenesis, with the result that no colon tumors were produced (114). In the study by Srisangnam et al., tumors were found primarily in the spermatic cord, and to a lesser extent in the kidney and liver. The incidence of spermatic tumors was higher in mice consuming diets composed of either 10% and 20% dried cabbage, while tumor incidence in the 40% cabbage group was reduced. However, in no case were differences between the cabbage groups and controls statistically significant. Temple et al., found cabbage consumption had no effect on DMH induced colon carcinogenesis (115). However, in this study, diets contained only 13% fresh weight cabbage, a level which may have been too low to exert an effect.

Although several case control studies suggested cabbage was protective against colon cancer, Pence et al., found that I3C supplementation enhanced DMH induced colon carcinogenesis in male Fischer 344 rats (116). In this study, a 2 x 4 factorial design was used to examine the

main and interactive effects of diets containing 15% wheat bran, 1% cholesterol with cholic acid, 20% beef tallow and 0.1% I3C. Test diets were fed throughout the experiment. Wheat bran decreased tumorigenesis while I3C was found to be the single main effect most responsible for increasing both tumor incidence and number. I3C was also found by Autrup et al., to enhance the binding of DMH to colon DNA (117). In this study, nontumorous human colon tissues were placed in culture for 24 hr after which time labeled DMH was added along with one of the test compounds under investigation. After a 24 hr incubation period, the amount of radioactive DNA was determined. The findings indicated that I3C enhanced binding to DNA by 66% over explants incubated without I3C.

Diets composed of approximately 10% cabbage fed to Syrian hamsters treated with a single dose of the pancreatic carcinogen, N-nitrosobis(2-oxopropyl)amine administered at 8 weeks of age, resulted in an increase in tumor number (118). Animals fed a high fat-cabbage diet had twice the number of tumors as animals fed a high fat diet without cabbage. The effect of consuming a 25% cabbage diet on aflatoxin B_1 induced liver carcinogenesis and liver levels of α fetoprotein was examined by Boyd et al (119). In the cabbage fed group, total tumor frequency and α fetoprotein level was significantly lower in comparison to the control group. However, the authors of this study acknowledged two confounding variables which may

have biased the results; cabbage fed animals consumed less food and had significantly lower body weights. Finally, results from an unpublished study by Wattenberg, cited in another paper by this author, show that Sprague Dawley rats placed on a diet composed of 10% cabbage one week after administration of dimethylbenzanthracene, had a decreased mammary tumor incidence in comparison to the basal group (93% vs 64%) (105).

Several authors have suggested that cruciferous vegetables and/or glucosinolate derivatives exert their effects on carcinogenesis through enzyme induction. For example, Pence et al., related the effect of I3C on DMH induced carcinogenesis to an increase in aryl hydrocarbon hydroxylase (AHH) (116). Wattenberg has attributed the inhibitory effect of cruciferous vegetables to an increase in AHH (109) and also to an increase in glutathione S-transferase (111). The decrease in α fetoprotein levels in rats administered aflatoxin B_1 and consuming a diet containing 25% cauliflower was attributed to an increase in hepatic aminopyrine N-demethylase (106). However, in contrast, Nixon et al., found that I3C supplementation inhibited aflatoxin induced liver cancer without increasing mixed function oxidase (112).

Procarcinogens require metabolism or activation to become carcinogens. In many cases, increasing hepatic cytochrome P450 leads to enhanced carcinogenesis. Frequently, when cytochrome P450 is increased, other

enzymes, referred to as phase II enzymes, are also elevated. Phase II enzymes, such as glutathione S-transferase and UDP-glucuronosyl transferase act to increase the excretion of xenobiotics, a process which is generally viewed as protective to the organism. Consequently, induction of enzymes that results from the consumption of cruciferous vegetable can not be simply viewed as either beneficial or harmful. The effect of enzyme induction on carcinogenesis depends on the specific carcinogen and the extent to which the relevant enzymes are effected. For a thorough discussion of the relationship between cytochrome P450 (phase I) and phase II enzymes, and carcinogenesis, the reader is referred to a review by Wattenberg (105).

In summary, animal studies have shown cabbage, cruciferous vegetables and/or glucosinolates to be protective against a number of non-colonic, experimentally induced cancers (105-109, 111, 112). There is however, some evidence indicating that carcinogenesis can also be enhanced (116, 118, 119). Furthermore, there is evidence indicating that I3C supplementation enhances experimental colon cancer (116).

RATIONALE FOR CONDUCTING DISSERTATION

Each of the three studies which comprise the present work, was undertaken so as to improve our understanding of

DMH induced colon carcinogenesis. The first study, "Effects of Route of 1,2-Dimethylhydrazine (DMH) Administration on Experimental Colon Cancer" had two aims: 1) to determine the feasability of using intrarectal administration of DMH as a means for inducing colon tumor liver metastases and 2) to determine the extent to which route of administration influences DMH induced carcinogenesis. As pointed out in the literature review, researchers are actively attempting to develop an animal model suitable for studying colon tumor liver metastases.

The second study, "Effects of Cabbage Consumption on 1,2-Dimethylhydrazine Induced Colon Carcinogenesis in CF1 Male Mice" was initiated because of the public recommendations by two scientific organizations to increase cruciferous vegetable consumption. Much of the justification for these recommendations is based on case control studies showing an inverse association between cabbage consumption and colon cancer. However, no animal studies have been done which either clearly prove or disprove this relationship.

Finally, the third study, "Effects of Prior Treatment with 1,2-Dimethylhydrazine (DMH) on DMH Induced DNA Methylation" was undertaken as a result of observations about DMH toxicity made during the cabbage and colon cancer experiments. A complete understanding of the mode of action of DMH has not yet been achieved. Increasing our understanding of DMH metabolism, may provide insights into the nature of colon cancer.

CHAPTER 2 .... EFFECTS OF ROUTE OF 1,2-DIMETHYLHYDRAZINE (DMH) ADMINISTRATION ON EXPERIMENTAL COLON CANCER

INTRODUCTION

1,2-dimethylhydrazine dihydrochloride (DMH) is frequently used to induce experimental colon cancer in both mice (9, 120) and rats (121, 122). The type of tumor induced by DMH resembles that seen in human colon cancer (18). However, the multiple liver metastases which occurs in approximately 20% of the human colorectal cancer cases has generally not been reported in laboratory animals administered DMH (123). Presently, researchers are attempting to develop an animal model to study the spread of colon cancer to the liver.

It has not been established whether the route of DMH administration influences metastases, although there is evidence suggesting it may modulate tumor number and location (124-127). For example, Toth et al. found that Swiss mice injected subcutaneously with DMH developed colon tumors (127); colon tumors did not develop in response to DMH administered intrarectally (128) or orally via the drinking water (126). Differences in tumor location and incidence were shown by Toth et al. to be a function of route, rather than the amount of DMH absorbed (130). A second factor influencing DMH induced carcinogenesis is the presence or absence of intestinal microflora. Reddy et al., reported a 21% colon tumor incidence in female germ-free Fischer rats administered DMH in comparison to a 93% tumor incidence in conventional animals (130). In contrast, when a DMH

derivative, azoxymethane, was administered, germ-free animals had an increased colon tumor incidence (93%) compared to conventional animals (60%) (130). It is difficult to ascertain the means by which the intestinal bacteria influence colon carcinogenesis by comparing germ-free and conventional animals, since the lack of microflora profoundly affects the intestinal mucosa in a variety of ways (131). Changes in both colon structure and/or metabolism could have an impact on colon carcinogenesis. Additional consideration should be given to the likelihood that some bacterial metabolism of DMH is feasible, given the extensive metabolic capability of the intestinal microflora (132).

We studied the influence of route of DMH administration on DMH induced carcinogenesis and tumor metastases to determine the feasibility of using DMH to induce colon tumor liver metastasis. Three groups of rats were either injected subcutaneously with DMH or given intrarectal instillations over a period of 20 weeks. Eight weeks following the final DMH administration, necropsies were performed on all animals and tumor type and location were noted.

MATERIALS AND METHODS

Animal conditions.-Male Sprague-Dawley rats (Harlan Sprague Dawley, Inc., Madison, WI) weighing approximately 200 g were placed into one of four groups. Animals were housed at 23°C in solid-bottom plastic cages (3 rats per cage) with wood

shavings for bedding. Rats were fed stock diet (Wayne Rodent Blox, Wayne Pet Food Division Continental Grain Co., Chicago, IL) and water ad libitum thoughout the experiment. Room lights were on from 0700 to 1900 hours daily.

Experimental design.-One group of animals was injected subcutanously with 20 mg of DMH/kg body weight; two other groups were administered intrarectally 20 and 40 mg of DMH/kg body weight, respectively. The control group received vehicle only. Immediately prior to administration, DMH was dissolved in 0.1 M sodium phosphate buffer and the pH adjusted to 7.4. All animals were administered 200 ul of either the DMH solution or vehicle (buffer only) between 0800 and 1000 hours once per week for 20 weeks. Subcutaneous injections were given behind the neck. Intrarectal instillations were given by inserting a plastic tube attached to a tuberculin syringe 8 cm through the anus. Prior to administration, fecal matter was removed by gently massaging the anal area. To aid in absorption, after withdrawing the plastic tube, rats were held with head down at a 45° angle for 30 seconds to limit rectal expulsion of the DMH solution. Eight weeks following the final administration of DMH, the experiment was terminated and animals were killed by CO_2 asphyxiation. Post-mortem examinations were performed on all rats. Location of all macroscopic tumors in the colon and rectum were recorded. Viscera were carefully examined for evidence of metastases and representative samples of all

affected organs were examined histologically. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 6 microns and stained with hematoxylin and eosin.

RESULTS

No difference in body weight was observed among the groups (means $\pm$ SD = 503 $\pm$ 28, 450 $\pm$ 31, 498 $\pm$ 38, 468 $\pm$ 29 for the control, 20 mg subcutaneous, and 20 and 40 mg intrarectal groups, respectively). Tumors were not observed in the control group. Almost without exception, intestinal tumors in all three groups receiving DMH were classified histologically as carcinomas. The incidence of large intestinal tumors in the 20 mg intrarectal group was lower ($P<0.05$) than in each of the other two groups, which had a similar colon tumor incidence (table 1). Additionally, the number of colon tumors per tumor bearing rat was lower ($P<0.05$) in the 20 mg intrarectal group than in the 20 mg subcutaneous group, but not in comparison to the 40 mg intrarectal group. No difference in the incidence of small intestinal tumors or number was noted among the groups (table 1).

In the 40 mg intrarectal group (table 2), the incidence of tumor metastases (53%) was greater ($P<0.05$) than in the 20 mg intrarectal group (0%), but not statistically different from the 20 mg subcutanous group (27%). In the 40 mg

Table 1. Tumor frequency in rats given 20 weekly administrations of DMH

Group[a]	Large intestine				Small intestine			
	Incidence (%)	Tumors No.	per tumor bearing rat[b]	Total	Incidence (%)	Tumors No.	per tumor bearing rat[b]	Total
20 mg (11) subcutaneous	100[c]	11	4.7[c] ± 2.8	52	46	5	2.0 ± 1.0	10
20 mg (12) intrarectal	33	4	1.3 ± 0.5	5	25	3	1.3 ± 0.6	4
40 mg (15) intrarectal	93[c]	14	2.2 ± 1.3	32	47	7	1.4 ± 0.5	10
Control (15)	0	0	---	--	0	0	---	--

[a] Effective number of rats in each group shown in parenthesis.

[b] Values represent means ± SD.

[c] Significantly different from 20 mg intrarectal group $P<0.05$.

Table 2. Tumor metastases in rats given 20 weekly administrations of DMH

Group[a]	Incidence of metastases (%)	No.	Description
20 mg (11) subcutaneous	27	3	Primarily mesentery, pancreatic lymph nodes and pancreas
20 mg (12) intrarectal	0	0	----------
40 mg (15) intrarectal	53[b]	8	Primarily mesentery, abdominal cavity, hepatic lymph nodes and pancreas

[a] Effective number of rats in each group shown in parenthesis.
[b] Significantly different from 20 mg intrarectal group $P<0.05$.

intrarectal group, 8 of 14 animals with colon tumors had metastases; 4 animals had metastases which originated from the small intestine, 3 from the large intestine and in one case it was not possible to determine the site of origin. In the 20 mg subcutaneous group, 3 animals exhibited varying degrees of metastases. Metastases in two of these animals originated from the colon while in the third, it was not possible to determine the site of origin. No metastases was observed in the 20 mg intrarectal group. Metastases were found primarily in the mesentery and mesenteric lymph nodes and to a lesser extent in the pancreas, lung, spleen, diaphragm, thorax and hepatic lymph nodes. One animal in the subcutaneous group developed tumor nodules in the liver; in the intrarectal group, one animal developed tumor nodules in the hilus of the liver. Overall, metastases originating from the colon were more wide spread than from the small intestine.

DISCUSSION

This experiment was undertaken in order to compare two different routes of DMH administration on DMH induced carcinogenesis and tumor metastases. There exists a need for developing an animal model suitable for studying colon tumor liver metastases, since in cases of human colorectal cancer this represents a critical secondary medical complication. The basis for using intrarectal instillations was two fold:

1) data on metastases induced by DMH administered intrarectally are lacking and 2) prior to absorption, DMH would first be exposed to the intestinal microflora and to the colon before undergoing hepatic metabolism. These two factors may influence colon carcinogenesis.

In the present study, subcutaneous injection was more effective in producing colon tumors than intrarectal administration. Colon tumor number and incidence in the 20 mg subcutaneous group were significantly greater ($P<0.05$) then in the 20 mg intrarectal group (table 1). No difference in colon tumor number was noted between the 40 mg intrarectal and 20 mg subcutaneous groups, even though the intrarectal group received twice the level of carcinogen. In fact, the number of colon tumors per tumor bearing rat in the 20 mg subcutaneous group was greater than that of the 40 mg intrarectal group, although this difference was not statistically significant. Clearly, these findings indicate the greater efficiency of subcutaneous injection to induce colon tumors. In contrast to the findings here, data taken from two separate studies by Reddy et al., indicated no difference between colonic tumor incidence in female Fischer rats induced by intrarectal and subcutaneous administration (130, 133). However, DMH induced carcinogenesis has been shown to differ among species, strain and sex (134), a fact which may have contributed to the difference between the studies by Reddy et al. and the present one. The difference between the two routes of administration in this study may

have been due to a greater amount of the DMH being made available to the animal through subcutaneous injection. Colonic absorption of DMH has been shown to be highly pH dependent (135). In order to maximize intrarectal DMH absorption in this study, the pH of the DMH solution was carefully adjusted to pH 7.4.

According to Toth el., route of administration, rather then the amount of DMH administered is the critical factor for determining tumor location (129). However, the present study suggests that, at least in Sprague-Dawley rats, the dose of DMH also influences tumor site. As the dose of DMH administered intrarectally was increased from 20 to 40 mg/kg, small intestinal tumor number increased 2.5 fold while large intestinal tumor number increased over 6 fold. A similar trend was observed with respect to tumor incidence. As the dose was increased, the incidence of small intestinal tumors increased slightly less than two fold while the incidence of large intestinal tumors increased almost threefold. The importance of dose is also supported by comparing the 20 mg intrarectal group and the 20 mg subcutaneous groups. The 20 mg subcutaneous group had only 2.5 times the number of small intestinal tumors as the 20 mg intrarectal group, but over 10 times the number of large intestinal tumors. It is probable, that because of poorer absorption in the intrarectal group, the amount of DMH made available to rats in the subcutaneous group was greater, even though the administered dose was identical. It must be noted however, that this last

comparison is also consistent with the notion that route of administration influences tumor site. Support for the importance of dose also comes from the observation that only in the 20 mg intrarectal group were there animals with small intestinal tumors without also having colon tumors. These data suggest that the colon is relatively more responsive to an increased dose of DMH than is the small intestine. As the dose of DMH was increased, the ratio of large intestinal to small intestinal tumor incidence and number increased dramatically. Clearly, because of the limited number of animals in each group and because only 3 different groups were involved in this study, any conclusions based on these data must be drawn with caution.

In contrast to the importance of dose in determining tumor site, tumor metastases appears to be influenced by the route of DMH administration. Of the 5 animals with small intestinal tumors in the 20 mg subcutaneous group, small intestinal tumor metastases possibly occured in one animal. In contrast, of the 7 animals with small intestinal tumors in the 40 mg intrarectal group, small intestinal tumor metastases was observed in 4 and possibly 5 animals. As to why small intestinal tumors metastasized when induced by intrarectal administration and not by subcutaneous injection, is not clear. Possibly, there is a difference in DMH metabolism between these two forms of administration. The colon has been shown to be capable of metabolizing DMH (136) and it is feasible that the intestinal microflora may also

play a role in this regard (132); intrarectal instillation may have provided more of an opportunity for this to occur.

The incidence of DMH induced metastases is often cited as being approximately five percent (137); although, several researchers have reported much higher rates (122, 138-140). Metastases in these studies generally did not involve the liver, as was the case in the present study. An alternative means of inducing colon tumor liver metastases in experimental animals, such as injecting human and murine colon tumor cell lines into the spleen of nude mice have produced promising results (141, 142).

In conclusion, results suggest that the dose level of DMH is a factor in determining tumor location and that the route of DMH administration influences small intestinal tumor metastases. Intrarectal administration of 40 mg of DMH/kg produced the greatest incidence of metastases but offers no advantage over subcutaneous injection for studying colon tumor liver metastases.

CHAPTER 3 .... EFFECTS OF CABBAGE CONSUMPTION ON 1,2-DIMETHYLHYDRAZINE (DMH) INDUCED COLON CARCINOGENESIS IN CF1 MALE MICE

INTRODUCTION

Colon cancer is a major form of cancer among industrialized countries (143). In the United States, colon cancer kills an estimated 50,000 people annually (1). Both genetic and environmental factors contribute to the incidence of this disease, with diet being cited as the most important environmental factor (3, 144). Among those dietary factors thought to be protective against colon cancer is cruciferous vegetables (146). Wattenberg, who conducted much of the initial animal work, found that supplementation with compounds isolated from cruciferous vegetables reduced 7,12-dimethylbenz(a)anthracene (DMBA) induced mammary cancer and benzo(a)pyrene induced stomach cancer (109). Diets containing cauliflower or cabbage were also found to reduce DMBA induced breast tumor incidence and number (105). In 1982, a report by the National Research Council, Committee on Diet, Nutrition and Cancer, recommended increased consumption of cruciferous vegetables as a means to decrease human cancer incidence (100). The American Cancer Society also supports this position (101).

The strongest epidemiologic data between cruciferous vegetables and cancer is between cabbage consumption and colon cancer. Several case control studies have found a decreased risk for colon cancer with an increase in cabbage consumption (6-8, 45, 51, 71, 103), although other studies did not find such a relationship (44, 48, 104).

The limited work examining the effect of cabbage consumption on 1,2-dimethylhydrazine (DMH) induced experimental colon cancer does not support the epidemiologic data. One study, used a strain of mice resistant to DMH induced colon carcinogenesis, with the result that no colon tumors were induced (114). In a second study, the lack of significant effect due to cabbage consumption, may have been because of the low level of cabbage employed in this study (115). More significantly, diets supplemented with indole-3-carbinol (I3C), a glucosinolate found in cabbage, increased DMH induced colon carcinogenesis in male F344 rats (116). Also, when I3C was incubated along with labeled DMH, the amount of radioactivity associated with DNA in explants of human colon tissue increased (117). In addition to the work with DMH, cabbage consumption by Syrian hamsters has been shown to enhance experimentally induced pancreatic cancer (118).

It is apparent that recommendations to increase cruciferous vegetable consumption were made without adequate supporting data from animal studies. The purpose of this study was to examine the effect of cabbage consumption on DMH induced colon carcinogenesis in CF1 male mice. Two separate experiments were conducted; findings from this study do not support a protective role for cabbage against colon carcinogenesis in the DMH model.

MATERIALS AND METHODS

Animal conditions.-CF1 male mice were used for all experiments and were housed in stainless steel hanging wire cages. In the first colon cancer experiment, five week old mice weighing 10-12 g were housed 3-4 per cage. Otherwise, 8-10 week old mice were housed individually and weighed approximately 30 grams. The animal room was temperature (23°C) and humidity controlled and was on a 12 hr light and dark cycle. Food and water were fed ad libitum.

Cabbage preparation.-Cabbage was incorporated into the diets in the following manner. After removing the outer leaves, the cabbage head (green cabbage, California grown) was cut into quarters and the center core discarded. The cabbage was finely shredded, steam blanched for 2 minutes, cooled by cold running water and placed in a refrigerator. The cabbage was then dried in a convection oven for 15 hrs at 55°C. Dehydration was complete within 7 days after arrival. The dried cabbage was ground into a fine powder and incorporated into diets at two month intervals. Diets and the powdered cabbage were kept refrigerated. Freeze dried cabbage was prepared similarly to the oven dried cabbage, except that after blanching, the cabbage was stored at -20°C and then lyophilized for 48 hours.

Diet formulation.-Cabbage, on a dry weight basis was

estimated to be 30% fiber (Paul and Southgate, 146). One-half of the fiber was assumed to be fermentable. For calculation purposes, we assummed the non-fiberous part of the cabbage to contain 4 kcal/gram. Therefore, each gram of cabbage on a dry weight basis, after discounting the fiber content, contained 2.8 kcal. The kcal and non-fermentable fiber gained through the addition of cabbage was balanced by removing appropriate amounts of sucrose and cellulose from the control diet. Each of the diets, was formulated such that the ratio of protein, fat, minerals and vitamins to kcal was the same and except for the positive control group, such that there was an equal amount of non-fermentable fiber in all groups (table 1). No correction was made for the negligible amount of vitamins, minerals, or protein in the cabbage, nor for the potential energy arising from the absorption and metabolism of organic acids produced by the bacterial fermentation of the fiber.

Carcinogen administration.-1,2-Dimethylhydrazine dihydrochloride (Aldrich Chemical Co.) was dissolved in 1 mM EDTA and neutralized with sodium bicarbonate immediately prior to administration. For both colon cancer experiments, each animal received 8 weekly subcutaneous injections of 30 mg of DMH/kg body weight in approximately 200 ul.

Colon cancer experiments.-In the first experiment, mice were fed one of 6 diets starting three weeks prior to carcinogen

Table 1. Composition of Diets

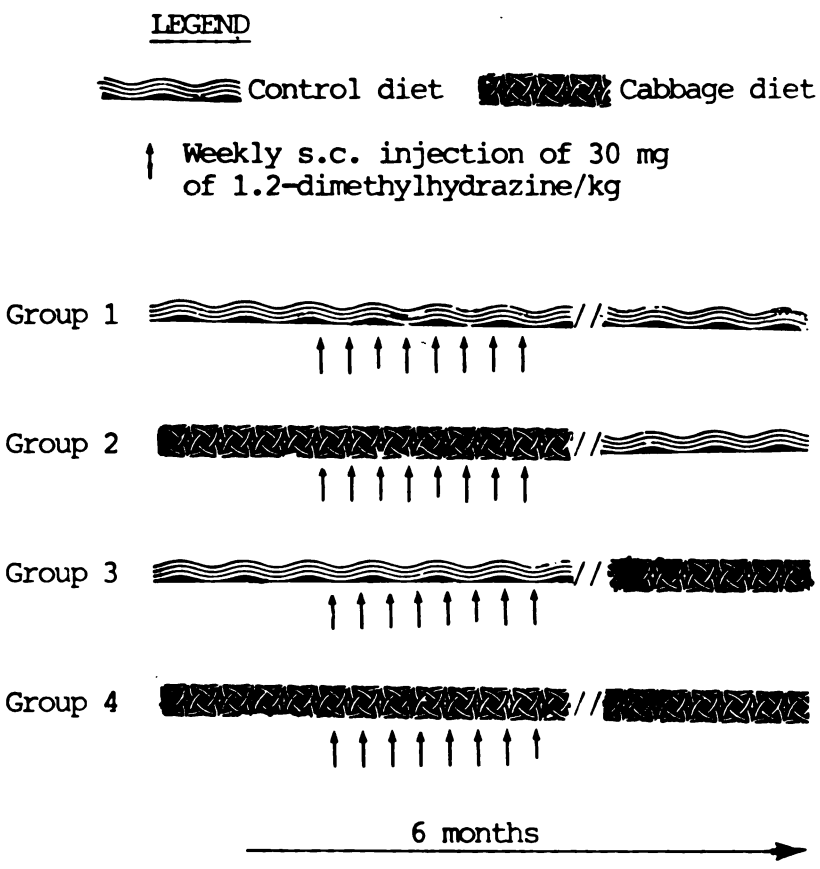
		g dried cabbage/100 grams diet				
Ingredient	0% fib/cab	0	6.7	13.4	20.1	26.8
Casein	25.0	23.7	23.7	23.7	23.7	23.7
Corn oil	8.0	7.9	7.9	7.9	7.9	7.9
Beef Tallow	16.7	15.8	15.8	15.8	15.8	15.8
Sucrose	43.8	41.5	35.8	30.1	24.4	18.7
Cellulose	0.0	5.0	4.0	3.0	2.0	1.0
Cabbage	0.0	0.0	6.7	13.4	20.1	26.8
Mineral*	4.5	4.3	4.3	4.3	4.3	4.3
Vitamins*	1.3	1.2	1.2	1.2	1.2	1.2
Methionine	0.4	0.4	0.4	0.4	0.4	0.4
Choline	0.3	0.2	0.2	0.2	0.2	0.2
total	100.0	100.0	100.0	100.0	100.0	100.0

*AIN-76 ™ Mineral and Vitamin mixes

administration (table 1). Nine months following the initial injection of DMH, animals were killed by CO_2 asphyxiation. In the second experiment, four groups of mice and two diets, the control and the 13.4% cabbage diet were employed. The experimental protocol is shown in figure 1. Groups 1 and 4, starting three weeks prior to carcinogen administration, were fed the control and cabbage diet, respectively. Groups 1 and 4 remained on these diets throughout the study. Group 2, the initiation group, and group 3, the promotion group, received the cabbage and control diets respectively, starting three weeks prior to carcinogen administration. Fourty-eight hours following the final injection of DMH, groups 2 and 3 were switched to the control and cabbage diets for the remainder of the experiment. Six months following the first DMH injection, mice were killed by CO_2 asphyxiation. For both experiments, immediately after killing, the large intestine, from the ileocecal junction to the anus was removed. Colons were opened longtitudinally, flushed with saline, pinned flat and placed in neutral buffered formalin. The sites of suspected tumors were recorded. Macroscopically abnormal tissue was embedded in paraffin, sectioned at 6 microns and stained with hematoxylin and eosin. Colons were coded such that tumor identification by the pathologist was done without knowledge of the dietary treatment.

Enzyme experiment.-Group 1, received the control diet and group 2, the 13.4% oven dried cabbage diet. Group 3 received

Figure 1 Experimental Design (Experiment 2)



the same diet as group 2, except that the cabbage was freeze dried, as described under cabbage preparation. To induce enzymes in the positive control, group 4, mice received an i.p. injection of 80 mg of sodium phenobarbitol/kg body weight for 3 consecutive days prior to being killed (147). Group 4 was fed the control diet. All animals were fed their respective diets for three weeks.

Immediately after being killed, livers were removed, rinsed with cold saline and homogenized in four volumes of buffer (0.1 M tris buffer, (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol and 250 mM sucrose). After centrifuging the crude homogenate for 20 min at 9,000 x g at 4°C, aliquots of the supernatant (S9) were removed and frozen at -20°C until analyzed. The remaining supernatant was then centrifuged at 100,000 x g for 75 min at 4°C. After removing the cytosol, the microsmal pellet was resuspended in a 10 mM tris HCl (pH 7.4), 1 mM EDTA and 20% glycerol buffer. The intestinal S9 fraction was obtained in the following manner. The large intestine was placed on a glass plate on ice with the mucosal side up. Mucosal cells were collected by gently scraping the mucosal surface with the edge of a microscope slide 3 times. The mucosal cells from 2 animals were combined and homogenized in 9 volumes of the tris-sucrose buffer. Cytosolic, microsomal and S9 fractions were frozen at -20°C until analyzed.

Hepatic cytochrome P450 content was determined by the method of Omura and Sato, using 91 mM^{-1} cm^{-1} as the molar

extinction coefficient for the dithionite-reduced cytochrome P450 complex (148). Liver alcohol dehydrogenase activity was measured in a Beckman Lambda 4B spectrophotometer by monitoring the rate of NAD conversion to NADH at 340 nm (149). Microsomal ethoxycoumarin O-deethylase activity was determined by a modification of the fluorimetric method of Greenlee and Poland, as described by Chang and Bjeldanes (150). The concentration of 7-OH-coumarin was determined fluorometrically (excitation 368 nm, emmission 456 nm) using a Varian SF-330 spectrofluorimeter. Sample quantification was based upon a calibration curve of 7-OH-coumarin, which was exposed to the identical procedure as samples. Glutathione S-transferase activity was measured colorimetrically using O-dinitrobenzene as substrate (151). Finally, UDP-glucuronosyltransferase activity was assayed spectrophotometrically using 2-aminophenol as substrate (152). Microsomal, cytosolic, and S9 protein concentration was determined by a modification of the Lowry procedure as described by Markwell et al (153).

RESULTS

In the first colon cancer experiment (table 2), the 20.1% cabbage fed group had more tumors per mouse (5.7 $\pm$ 5.3 vs 2.4 $\pm$ 2.1) and the 26.8% cabbage fed group a higher tumor incidence (96% vs 72%) than the control group ($P<0.05$). No other differences among the groups were noted when individual

Table 2. Tumor occurence in large intestines of mice treated with DMH and fed one of 6 diets

Group[a]	Body Wt(g)	Mice with colon tumors		All colon tumors	
		No	Percent	Per mouse	Per tumor bearing mouse
Positive control (13)	45 ± 6.8	10	77	2.2 ± 1.9	2.8 ± 1.7
0% Cabbage (31)	44 ± 5.4	22	72	2.4 ± 2.1	3.4 ± 1.7
6.7% Cabbage (18)	45 ± 4.3	17	94	4.1 ± 3.4	4.3 ± 3.4
13.4% Cabbage (11)	41 ± 8.3	11	100	4.1 ± 2.4	4.1 ± 2.4
20.1% Cabbage (19)	42 ± 5.1	16	84	5.7[c] ± 5.3	6.4[c] ± 5.2
26.5% Cabbage (24)	40 ± 4.4	23	96[c]	4.3 ± 3.7	4.5 ± 3.6
Combined[b] cabbage (72)	42 ± 5.4	67	93[c]	4.6[c] ± 3.9	4.9[c] ± 3.9

[a]Positive control lacked both cellulose and cabbage. Percent cabbage based on dry wt, g/100 g diet (for diet descriptions see table 1). Effective number of mice shown in parentheses. Values represent means ± SD.

[b]Values represent all cabbage consuming mice.

[c]Significantly different from control group $P<0.05$.

comparisons were made. Approximately 15% and 85% of the total tumors were classified as adenocarcinomas and adenomas, respectively. There was no difference in this parameter among groups. Because no difference in tumor number or incidence was observed among the cabbage groups, these groups were combined and compared to the control group. Cabbage fed mice had a higher tumor incidence (93% vs 72%) and more tumors per mouse (4.6 $\pm$ 2.4) than control mice ($P<0.05$).

In the follow up experiment (table 3), there were no statistically significant differences among the groups. However, there was an indication of an increased tumor number in groups 3 and 4. Approximately 10% and 90% of the total tumors were classified as adenocarcinomas and adenomas, respectively. There was no difference in this parameter among groups.

Cabbage consumption did not influence enzyme activity (table 4). In comparison to the control group, mice administered sodium phenobarbitol had increased levels of hepatic UDP-glucuronosyltransferase ($P<0.05$), glutathione S-transferase ($P<0.01$), cytochrome P450 ($P<0.01$) and ethoxycoumarin O-deethylase ($P<0.01$).

DISCUSSION

Our studies demonstrate that cabbage consumption enhances DMH induced colon carcinogenesis and that cabbage likely mediates its effect during promotion. In the first

Table 3. Tumor occurance in large intestines of mice treated with DMH and fed the control or cabbage diet

Group[a]	Body Wt(g)	Mice with colon tumors		All colon tumors	
		No.	Percent	Per mouse	Per tumor bearing mouse
Group 1 (control) (33)	43 ± 5.4	13	40	2.2 ± 1.5	0.9 ± 1.5
Group 2 Initiation (21)	42 ± 4.7	11	52	2.1 ± 1.1	1.1 ± 1.3
Group 3 Promotion (33)	42 ± 5.7	16	49	3.8 ± 2.9	1.8 ± 2.8
Group 4 Init-Prom (30)	41 ± 5.3	15	50	3.2 ± 2.1	1.6 ± 2.2

[a]Mice consumed either the control or 13.4% oven dried cabbage diet (table 1). Groups 1 and 4 consumed the control and and cabbage diets respectively throughout the experiment. Groups 2 and 3 consumed the cabbage and control diets respectively three weeks prior to and during the 8 weeks of carcinogen administration. Fourty-eight hours following the final injection of DMH, groups 2 and 3 were switched to the control and cabbage diets. Effective number of mice shown in parentheses.

Values represent means ± SD. There were no significant differences among groups ($P>0.05$)

Table 4. Effects of dietary treatment on levels of hepatic cytochrome P450 (P450), UDP-glucuronosyltransferase (UDPGT), ethoxycoumarin O-deethylase (ECD), alcohol dehydrogenase (ADH), and hepatic (GST) and intestinal glutathione S-transferase (IntGST)

	Enzyme activities[a] in					
Group	P450	UDPGT	ECD	ADH	GST	IntGST
Group 1 (Control)	0.36 ± 0.02	1.78 ± 0.15	4.28 ± 0.36	6.18 ± 0.30	738 ± 56	198 ± 38
Group 2 (Oven dried cabbage)	0.40 ± 0.04	1.79 ± 0.20	4.95 ± 0.33	6.12 ± 0.44	936 ± 94	182 ± 14
Group 3 (Freeze dried cabbage)	0.34 ± 0.02	2.11 ± 0.28	4.80 ± 0.17	5.92 ± 0.34	766 ± 29	237 ± 29
Group 4 (Sodium phenobarbitol)	1.42[b] ± 0.10	2.97[c] ± 0.26	6.92[b] ± 0.66	6.81 ± 0.45	1202[b] ± 100	166 ± 24

[a]Expressed as follows: P450 - nmoles cytochrome P450/mg protein; UDPGT - nmoles formed/mg protein/min, using aminophenol as substrate; ECD - nmoles 7-OH-coumarin formed/mg protein/min; ADH - nmoles NADH formed/mg protein/min; GST and IntGSH - umoles formed/mg protein/min, using O-dinitrobenzene as substrate. Values are means ± SD. Number of determinations for control, oven dried cabbage and freeze dried cabbage groups for all assays except IntGST was 12 and for IntGST was 6. Number of determinations for sodium phenobarbitol group was 7 for all assays except IntGSH and for IntGSH was 4.

[b]Significantly different from the control group $P<0.01$.

[c]Significantly different from the control group $P<0.05$.

experiment, in comparison to the control group, the 26.8% cabbage group had a significantly higher tumor incidence and the 20.1% cabbage group significantly more tumors per mouse. Overall, cabbage fed mice had significantly more tumors per mouse and a higher tumor incidence than control mice. Because there was difference among the cabbage groups in experiment 1, the 13.4% cabbage diet was chosen as an intermediate level and used in the follow up experiment. To decrease tumor incidence, animals were killed 6 months, rather then 9 months (as in experiment 1), following the first injection of DMH. Although there were no statistically significant differences in tumor frequency among the groups in experiment 2, there was an indication of an increase in tumor number in group 3, which received the cabbage diet after carcinogen administration i.e., during promotion, and in group 4, which received cabbage throughout the experiment i.e., during initiation and promotion.

These data conflict with findings from case control studies showing a protective effect of cabbage against colon cancer (6-8, 45, 51, 71, 103). However, the present study agrees with reports that I3C enhances DMH induced colon carcinogenesis in F344 rats (116) and increases DNA methylation in explants of colon tissue (117). Additional support, although less convincing because of a lack of statistical significance, comes from findings by Srisangnam et al., that mice consuming diets comprised of 10% and 20% cabbage had an increased number of DMH induced spermatic

tumors (114) and by Temple and El-Khatib, that cabbage fed female mice had twice the incidence of DMH induced colon tumors as controls (115).

Support can also be found for the the suggestion of the present study that cabbage exerts its tumor enhancement effect during the promotion phase of carcinogenesis. Nixon et al., found that I3C supplementation during promotion enhanced aflatoxin B_1 induced liver cancer in fingerling trout (154). And Birt et al., observed that I3C increased ornithine decarboxylase activity in mouse epidermis treated with 12-O-tetradecanoylphorbol-13-acetate (155).

This study demonstrates that cabbage consumption has no effect on several xenobiotic metabolizing enzymes in liver or large intestine of CF1 male mice. These enzymes have been cited for their role in carcinogenesis (156, 157), but a specific role in DMH induced carcinogenesis has not been established. However, cytochrome P450 has been implicated in DMH metabolism (37) and Pence et al., attributed the enhancing effect of I3C on DMH induced carcinogenesis to an increase in aryl hydrocarbon hydroxylase (AHH) (116). AHH was not determined, although cytochrome P450 content, which was measured, often parallels AHH activity. The lack of effect of cabbage indicates these enzymes did not play a role in the enhanced carcinogenesis in cabbage fed mice, an observation in line with our finding that cabbage likely influences DMH induced colon carcinogenesis only during promotion. Other studies have also reported changes in

carcinogenesis in response to consumption of cruciferous vegetable and/or I3C consumption without observing a change in enzyme activities (112, 114, 158-160).

The lack of enzyme induction by cabbage feeding agrees with findings from several studies. Diets fed to C57B16 mice containing 20% cabbage failed to increase hepatic cytochrome P450 (115, 161), AHH or epoxide hydrolase (161). Uotila et al, fed diets containing 2.5% and 100% cabbage to Spraque-Dawley rats and saw no effect on either ethoxycoumorin O-deethylase or UDP-glucuronosyltransferase at either level, although there was a 60% increase in epoxide hydrolase activity at the 100% cabbage level (162). Liver glutathione S-transferase activity did increase significantly (3.5 fold) in response to a 50% cabbage diet fed to Coturnix quail (163), but this effect was not observed in ICR/Ha mice fed 20% cabbage (164).

The finding that cabbage consumption does not reduce and may actually enhance experimentally induced colon cancer is significant because of public recommendations to increase the consumption of cruciferous vegetables. The effects of cabbage and/or glucosinolates on carcinogenesis appears to be carcinogen specific. Consequently, making recommendations based on the results with just a few carcinogens affecting only a limited number of tissues was premature. Also, since cabbage consumption appears to enhance the promotion phase of colon carcinogenesis, which is less carcinogenic specific, the consumption of large amounts of cabbage is contrainicated

because of the potential to promote colon cancer. Although cabbage is not likely to produce harmful effects in the amounts generally consumed, toxic effects are produced when cabbage is consumed in large amounts. Allyl isothiocyanate, a naturally occuring compound found in cabbage was found to be fetotoxic in Holtzman rats (166), to cause transitional cell papillomas in urinary bladders of male F344 rats (166) and to be mutagenic in the Ames test (167). Metabolites of glucosinolates have produced poor growth, hepatic and renal lesions (168), decreased B-carotene levels (169) and decreased protein synthesis in laboratory animals (170). Finally, the consumption of large amounts of cabbage has been responsible for development of goiter in humans (171). Because public recommendations to increase cruciferous vegetable consumption conflict with much of the animal work, continued research of this issue is needed.

CHAPTER 4 .... EFFECTS OF PRIOR TREATMENT WITH 1,2-DIMETHYLHYDRAZINE (DMH) ON DMH INDUCED DNA METHYLATION

INTRODUCTION

1,2-Dimethylhydrazine dihydrochloride (DMH) is frequently used to induce experimental colon cancer in both mice (9, 120) and rats (121, 122). It is believed that DMH induces tumors through DNA methylation. In order to exert its effects, DMH must be metabolically activated. As initially proposed by Druckey et al., the parent compound DMH is converted into a methyl carbonium ion, the ultimate carcinogenic agent (24, 25). In the metabolism of DMH, two gaseous metabolites, azomethane and carbon dioxide are produced. These gases provide a convenient method for evaluating DMH metabolism (172). Several compounds have been shown to inhibit the metabolism of DMH or of DMH derivatives (173-175). Inhibition of metabolism, leads to a reduction in DNA methylation (173, 176) and tumorigenesis (177, 178). Although there exists a considerable body of knowledge on DMH metabolism, a complete understanding remains elusive.

We observed in preliminary studies, that pretreatment with DMH decreases the toxicity of a subsequent treatment with this carcinogen. We found we could produce almost 100% survival in mice given a dose of DMH two to three times the normal lethal dose, if these mice were first exposed to a series of gradually increasing doses of DMH. In order to evaluate the mechanisms underlying this phenonomon, we offered two hypotheses: 1) sensitive mice

are eliminated after the initial injections of DMH and 2) mice adapt metabolically to reduce DMH toxicity. To test the first hypothesis, we determined the medium lethal concentration (LC_{50}) for DMH in two groups of mice, one of which was previously exposed to DMH. To test the second hypothesis, we examined the effect of pretreatment on DMH metabolism. Because alterations in DMH metabolism should be reflected by changes in the amount of DNA methylation, we also examined levels of 7-methylguanine and O^6-methylguanine in response to a test dose of DMH. Finally, we measured levels of hepatic cytochrome P450 to determine whether this enzyme complex changes in response to pretreatment.

MATERIALS AND METHODS

Animal conditions-For all experiments, 8-10 week old male CF1 mice (Harlan Co., Madison, WI) weighing approximately 30 grams were housed individually in stainless steel hanging wire cages. The animal room was temperature (23°C) and humidity controlled and was on a 12 hour light and dark cycle. All mice were fed a semipurified diet (modified AIN-76 diet) and water ad libitum (179). The composition of the diet when expressed as g/100 diet was as follows: casein, 23.7%; DL-methionine, 0.4%; sucrose, 41.5%; cellulose, 5.0%; corn oil, 7.9%; beef tallow, 15.8%; mineral mix, 4.3%; vitamin mix 1.2% and choline chloride,

0.2%. Choline chloride and DL-methionine were purchased from Sigma Chemical Co. (St. Louis, MO) and casein, cellulose, mineral and vitamin mix from U.S. Biochemical Co. (Cleveland, OH).

Carcinogen administration. For all injections, DMH (Aldrich Chemical Co.; Milwaukee, WI.) was dissolved in 1 mM EDTA and neutralized with sodium bicarbonate immediately prior to administration. DMH was injected s.c. in a volume of approximately 200 ul between 0800 and 1000 hours.

Medium lethal concentration (LC_{50}).-The LC_{50} for DMH was determined in two groups of mice. One group, composed of 90 mice, referred to as pretreated-30 (PT-30), was given a s.c. injection of 30 mg of DMH/kg body weight one week prior to conducting the LC_{50}. The second group, containing the control mice, was not previously exposed to DMH. Eighty-five of the surviving eighty-nine PT-30 mice were divided equally into 5 groups and given a s.c. injection of DMH at a level of either 40, 55, 70, 85 or 100 mg/kg body weight. Fifty control mice were divided equally into 5 groups and given a s.c. injection of DMH at a level of either 28, 32, 36, 40 or 44 mg/kg body weight. One animal from each dose was injected with DMH before a second animal at that same level was injected. Animals alive 7 days following the test dose were counted as survivors. The LC_{50} for DMH was calculated using the probit transformation

least likelihood method (180).

DMH metabolism. DMH metabolism was studied in three groups of mice: the control group, a second group referred to as pretreated-20 (PT-20) and a third group, referred to as pretreated-100 (PT-100). PT-20 mice received three weekly s.c. injections of 20 mg of DMH/kg body weight. PT-100 mice received four weekly stepwise s.c. injections of 30, 50, 75 and 100 mg of DMH/kg body weight. The final injection of DMH during the pretreatment period, for both the PT-20 and PT-100 mice was given 7-10 days prior to studying DMH metabolism. DMH metabolism was studied in control and PT-100 mice at two levels of DMH. Mice were injected with either 20 mg of [^{14}C]DMH (250 uCi/mmol, New England Nuclear, Boston, MA) or 100 mg of [^{14}C]DMH (50 uCi/mmol) per kg body weight. PT-20 mice received only the lower dose of [^{14}C]DMH.

Immediately after injection of the labeled DMH, mice were placed in an air tight chamber. Exhaled air was drawn by vacuum through a series of four flasks for a period of six hours. The collection system was essentially that as described by Fiala et al. (172). The first two flasks, designed for azomethane collection, each contained 350 ml of pure ethanol. The second flask was surrounded by dry ice. Aliquots of 0.4 ml were taken from each of these two flasks every 60 minutes for the first three hours. Contents of the second flask were replaced with fresh pre-cooled ethanol each hour. The third and fourth flasks were

for carbon dioxide collection and each contained 350 ml of 1 M NaOH. At the end of the 6 hour collection period, 0.2 ml aliquots were taken from the flasks containing NaOH. In all cases, aliquots were immediately placed in glass scintillation vials containing 15 ml ACS scintillation fluid (Amersham Co, Arlington Heights, IL). Total radioactivity was determined using a Beckman liquid scintillation counter. Results, based on radioactivity, are expressed as the percentage of total DMH exhaled as azomethane and carbon dioxide.

DNA methylation. Nine hours following a s.c. injection of 100 mg of DMH/kg body weight, PT-100 and control mice were killed via carbon dioxide asphyxiation. Livers were immediately removed, rinsed in cold saline and stored at -80°C. DNA was isolated by a modification of the method of Marmur (181). Livers were thawed and homogenized in 8 volumes of saline citrate buffer (0.15 M NaCl, 0.015 M trisodium citrate pH 7.0) and then centrifuged at 8,700 x g for 15 min at 0°C. The resulting pellet was dispersed in 8 volumes of 1 M NaCl and after adding 10% sodium dodecyl sulfate to a final concentration of 0.07% was placed on ice for 45 min. After the addition of 1/2 volume of chloroform:isoamylalcohol (10:2), tubes were vortexed for 7 min and then centrifuged for 10 min at 12,000 x g at 0°C. After repeating the chloroform:isoamylalcohol extraction step, 50 ug of RNAse/ml (bovine pancreatic ribonuclease (Type 1-A) Sigma Chemical Co., St. Louis, MO) was added to

the aqueous layer. A third extraction step followed the 30 min RNA digestion. The DNA was precipitated by the addition of 2 volumes of ethoxyethanol and then washed three times in 2 ml 95% ethanol. After drying under nitrogen for 30 min, the DNA was frozen at -20°C.

DNA methylation was quantitated using a modification of a procedure described by Herron and Shank (182). The isolated DNA was hydrolyzed in 1 N HCl at 37°C for 18 hours. The hydrolyzed DNA was solubilized in mobile phase (5 mg/ml) and approximately 0.2 mg DNA per injection was separated via HPLC using a strong cation exchange column (Partisil - 10 SCX, 25 cm x 4.5 mm id, Whatman Inc. Clifton, New Jersey), a mobile phase of 0.1 M ammonium phosphate, pH 2, and a flow rate of 2.0 ml per minute. Elution of the methylated bases, 7-methylguanine and O^6-methylguanine was monitored spectrofluorimetrically (excitation 295, emission 370) and guanine was detected spectrophotometrically at 276 nm. Calibration curves of concentration versus peak area for guanine, 7-methylguanine and O^6-methylguanine were used to establish regression equations for sample calculation.

Hepatic cytochrome P450. Immediately after carbon dioxide asphyxiation, livers from control and PT-100 mice were removed and homogenized in 4 volumes of buffer (0.1 M tris buffer (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol and 250 mM sucrose). After centrifuging the crude homogenate for 20

minutes at 9,000 x g at 4° C, the resulting supernatant was centrifuged at 100,000 x g for 75 minutes at 4° C. The microsomal pellet was resuspended in a 10 mM tris buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol. Hepatic cytochrome P450 content was determined by the method of Omura and Sato using 91 mM^{-1} cm^{-1} as the molar extinction coefficient for the dithionite-reduced cytochrome P450 complex (148). Microsomal protein concentration was determined by a modification of the Lowry procedure as described by Markwell et al (153).

RESULTS

Mice which received one injection of 30 mg of DMH/kg body weight 7 days prior to being tested for the LC_{50}, had a 2 fold higher LC_{50} ($P<0.05$) than the control mice (table 1). The LC_{50} for PT-30 mice was 77 mg/kg $\pm$ 1 versus 37 mg/kg $\pm$ 1 for control mice. Mice receiving a lethal dose of DMH generally died between 36 and 48 hours following the DMH injection.

DMH metabolism, as indicated by azomethane and carbon dioxide exhalation, is shown in table 2. In mice previously treated with DMH, the amount of exhaled azomethane increased and the amount of carbon dioxide derived from DMH decreased relative to control mice. When administered a test dose of 20 mg of [^{14}C]DMH/kg body weight, PT-20 and PT-100 mice exhaled approximately 1.5

Table 1. Effect of pretreatment with DMH on the medium lethal concentration for DMH in mice

Group[a] and Treatment	Dose (mg DMH/kg Bwt)	Mortality (%)[b]		LC_{50}[c]
Control	28	0	(0/10)	
	32	0	(0/10)	
	36	30	(3/10)	37 ± 1
	40	90	(9/10)	
	44	90	(9/10)	
PT-30	40	0	(0/17)	
	55	29	(5/17)	
	70	47	(8/17)	77[d] ± 1
	85	58	(10/17)	
	100	70	(12/17)	

[a] Control mice were not previously exposed to DMH. PT-30 mice received a s.c. injection of 30 mg DMH/kg body weight one week prior to the LC_{50} determination.

[b] Percentage of mice that died. Value in parenthesis is the number of mice that died per total in group.

[c] Calculated medium lethal concentration (mg DMH/kg body weight) ± 95% confidence interval.

[d] Significantly different from controls $P<0.05$.

Table 2. Percentage of DMH metabolized to azomethane and carbon dioxide in control and DMH-pretreated mice after injection with [^{14}C]DMH

Groups[a] and Treatment	[^{14}C]DMH (mg/kg Bwt)	Azomethane production (%)[b]	Carbon Dioxide production (%)[b]
Expt 1. (N=5)			
Control	20	14 ± 2.5	37 ± 5.8
PT-20	20	23[c] ± 1.2	25[d] ± 1.8
PT-100	20	28[d] ± 6.6	22[d] ± 1.6
Expt 2. (N=6)			
Control	100	21 ± 1.9	20 ± 2.0
PT-100	100	34[c] ± 1.5	9[c] ± 1.7

[a]Control mice were not previously exposed to DMH. Prior to the test dose, PT-20 mice received three weekly injections of 20 mg of DMH/kg and PT-100 mice 4 weekly stepwise doses of 30, 50, 75 and 100 mg of DMH/kg body weight.
[b]Values represent means ± SD
[c]Significantly different from controls $P<0.05$.
[d]Significantly different from controls $P<0.01$.

($P<0.05$) and 2.0 times ($P<0.05$) more azomethane respectively, than control mice. The amount of [^{14}C]DMH metabolized to carbon dioxide was decreased by approximately 33% and 40% in PT-20 and PT-100 mice respectively, ($P<0.01$). When administered 100 mg of [^{14}C]DMH/kg body weight, PT-100 mice exhaled 1.5 times more azomethane ($P<0.01$) and approximately 50% less carbon dioxide derived from DMH than control mice ($P<0.01$).

Levels of 7-methylguanine and O^6-methylguanine were determined in the livers of control and PT-100 mice after receiving a s.c. injection of 100 mg of DMH/kg body weight. Livers of PT-100 mice had 50% less 7-methylguanine and O^6-methylguanine than control mice ($P<0.01$, table 3). The ratio of the two bases was similar in both groups.

PT-100 mice had approximately 33% less hepatic cytochrome P450/mg microsomal protein than control mice ($P<0.05$) (table 4). Additionally, the ratio of liver weight to body weight was reduced by about 20% ($P<0.05$). Because of the smaller livers and lowered cytochrome P450/mg of microsomal protein, total cytochrome P450 content in PT-100 mice was decreased by approximately 50% ($P<0.05$).

DISCUSSION

DMH toxicity was investigated in this study after observing in preliminary studies, that pretreatment with DMH decreases the toxicity of a subsequent treatment with

Table 3. Effect of pretreatment with DMH on the formation of 7-methylguanine and O^6-methylguanine in livers of mice administered DMH

Group and Treatment[a]	No. of mice	7MGua:Gua[bc] (ng:ug)	O^6MGua:Gua[bc] (ng:ug)	7MGua:O^6MGua
Control	6	5.80 ± 0.61	1.04 ± 0.13	5.6 ± 0.7
PT-100	8	2.77[d] ± 0.40	0.49[d] ± 0.06	5.7 ± 0.9

[a]Control mice were not previously exposed to DMH. Prior to test dose, PT-100 mice received received 4 weekly stepwise doses of 30, 50, 75 and 100 mg of DMH/kg body weight.
[b]7MGua:Gua = 7-methylguanine:guanine; O^6MGua:Gua = O^6-methylguanine:guanine.
[c]Values represent means ± SD
[d]Significantly different from controls $P<0.01$.

Table 4. Effect of pretreatment with DMH on hepatic cytochrome P450 content[a]

Group[b] and Treatment	Liver wt:Bwt (g/100 g)	Cytochrome P450[c]	Total P450[d]
Control	6.6 ± 0.60	0.78 ± 0.11	4.41 ± 0.61
PT-100	5.2[e] ± 0.60	0.51[e] ± 0.09	2.36[e] ± 0.41

[a] Values represents means ± SD (N=9)
[b] Control mice were not previously exposed to DMH. Prior to the assay, PT-100 mice received 4 weekly stepwise doses 30, 50, 75 and 100 mg of DMH/kg body weight.
[c] nmoles hepatic cytochrome P450/mg microsomal protein.
[d] Total nmoles hepatic cytochrome P450.
[e] Significantly different from controls $P<0.05$.

this carcinogen. The two fold increase in the LC_{50} for DMH in PT-30 mice confirmed these observations. The LC_{50} of 37 mg/kg for control mice is similar to that reported by Chang of 35 mg/kg for CF1 female mice (183). Because only one of ninety mice died during pretreatment, we rejected our hypothesis that the decreased toxicity was due to the elimination of sensitive mice. Rather, because of the increased radioactive azomethane and decreased carbon dioxide exhalation in PT-20 and PT-100 mice, we accepted our second hypothesis. i.e., that mice adapt metabolically to reduce DMH toxicity. As the test dose of [^{14}C]DMH was increased from 20 mg to 100 mg/kg body weight, the percentage of [^{14}]DMH metabolized to azomethane increased while the percentage metabolized to carbon dioxide decreased. The effect of dose on DMH metabolism, as seen in the present study, has been reported by other investigators (172, 173).

The decreased carbon dioxide production in PT-20 and PT-100 mice suggested that these mice would exhibit less DNA methylation in response to a given dose of DMH. In the metabolism of DMH, as initially proposed by Druckey et al., the amount of carbon dioxide derived from DMH reflects methyl carbonium ion production (24, 25). The methyl carbonium ion is thought to be the agent responsible for DNA methylation and causation of tumors. Therefore, we determined the levels of 7-methylguanine and O^6-methylguanine. O^6-methylguanine is thought to be a

critical lesion for producing mutagenesis/carcinogenesis via DNA methylation (184-186) and was shown by Cooper et al., to correlate with the degree of sensitivity to DMH induced carcinogenesis in mice (187). However, this contrasts with findings by James and Autrup, where no difference in the amount of O^6-methylguanine was noted between two strains of mice with different degrees of senstivity to DMH (188). The target organ for DMH induced carcinogenesis is the colon, although overall, initially, more methylation occurs in liver (190, 191).

In agreement with our DMH metabolism data, which indicated a reduced metabolism to the active methylating species, PT-100 mice had 50% less methylation than controls ($P<0.01$). The reduced levels of 7-methylguanine and O^6-methylguanine are likely the result of a decrease in initial methylation and not due to enhanced DNA repair in PT-100 mice, because O^6-methylguanine-DNA methyltransferase, the protein responsible for removing the methyl group from O^6-methylguanine, has thus far been shown not to be inducible in mice (189). Our data suggest that: 1) the increased tolerance to DMH toxicity may be a result of a decrease in macromolecule methylation and 2) pretreatment may reduce the carcinogenic effect of DMH.

We did not determine colonic DNA methylation in this study. However, James and Autrup found less methylation in the colon of mice previously treated with DMH (188). There was approximately 50% less colonic methylation in animals

given 4 weekly doses of 45 mg of DMH/kg body weight and then administered a test dose at this same level in comparison to controls, which received only the test dose.

If the conversion of azomethane to methyl carbonium ion were inhibited, azomethane would accumulate, resulting in an increased exhalation, as was seen in PT-20 and PT-100 mice. Fiala et al., suggested that cytochrome P450 may be involved in DMH metabolism (37). In addition to the suggestion by Fiala et al., Autrup et al., found that phenobarbitol added to colon tissue explants along with [^{14}C]DMH increased the amount of radioactivity associated with DNA (117). The significance of this finding may be considerable, given that in this same system, disulfiram decreased the amount of radioactivity associated with DNA. Phenobarbitol has been found to increase (30) and disulfiram to decrease (177) DMH induced carcinogenesis. Phenobarbitol administration in vivo increases cytochrome P450 content (147). Therefore, we decided to measure hepatic cytochrome P450 content. The level of cytochrome P450 in PT-100 mice was approximately 50% lower in comparison to controls ($P<0.05$, table 4). These data suggest that the increased azomethane exhalation in PT-100 mice may have been a result of a decreased cytochrome P450 content.

In summary, the findings from this study are: 1) mice pretreated with DMH are less sensitive to DMH toxicity, as indicated by an increased LC_{50} for DMH; 2) administering

gradually increasing doses of DMH increases tolerance more than one single dose; 3) pretreatment alters DMH metabolism, as exhibited by an increase in the percentage of azomethane and a decrease in the percentage of carbon dioxide derived from DMH; 4) in comparison to control mice, mice previously treated with DMH exhibit decreased levels of hepatic DNA methylation in response to a test dose of DMH and 5) prior treatment with DMH decreases hepatic cytochrome P450, a finding which suggests this enzyme complex may be involved in DMH metabolism.

The reduced DNA methylation in mice previously treated with DMH suggests that the latter doses of this compound may have a reduced tumor initiating effect. When administered 20 mg of [^{14}C]DMH/kg body weight, radioactive carbon dioxide production in PT-20 mice (25%) was only slightly higher than in PT-100 mice (22%) (table 2). Therefore, since carbon dioxide production is indicative of potential methylation, it is likely PT-20 mice, which underwent a pretreatment regimen similar to that which is used to initiate tumors would also exhibit less DNA methylation in response to a test dose of DMH. Temple and El-Khatib, after observing an increased tolerance to DMH toxicity in Swiss mice (no data provided; 192), used a DMH dosing regimen to initiate tumors which consisted of s.c. injections of 23, 31, 42 and 4 doses of 56 mg/kg, rather than serial injections at the same level (115). However, before reaching conclusions about the optimum DMH dosing

regimen for tumor initiation; studies need to precisely determine the relationship between the total amount of DMH administered and the extent of colonic DNA methylation. If the number of DMH injections can be decreased without significantly reducing the initiating effects of this carcinogen, then a clearer separation between initiation and promotion can be achieved and the research value of the DMH model of carcinogenesis will be enhanced.

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

The primary objective of the first study, was to establish whether intrarectal instillation of DMH could be used as a model to study colon tumor liver metastases in Sprague-Dawley rats. DMH, when injected subcutanously, induces only very limited colon tumor metastases. Intrarectal administration of DMH had not been examined in this regard. Findings from this study suggest that route of DMH administration influences tumor metastases. In the group of rats administered 40 mg of DMH/kg body weight, tumor metastases occured in 8 of the 15 animals (53%). In contrast, in the group of rats injected s.c. with 20 mg of DMH/kg body weight, tumor metastases occured in only 3 of the 11 (27%) animals with colon tumors. The higher incidence of metastases was not a function of dose, since colon tumor frequency was similar in both groups. The enhanced metastases in the intrarectal group, was due to a higher rate of small intestinal tumor metastases. The clinical significance of this finding would appear to be limited since the incidence of human small intestinal cancer is rare. More significantly, metastases, when induced by either intrarectal administration or subcutaneous injection was primarily limited to the mesentery. Therefore, neither form of DMH administration provides a suitable method for studying colon tumor liver metastases.

The finding that route of administration influences metastases raises the possibility that DMH metabolism when

given by intrarectal instillation differs from that when injected subcutanously. Intrarectal administration may provide an opportunity for bacterial metabolism of DMH. In addition, DMH is first be exposed to the colon, which is capable of metabolizing DMH, before undergoing hepatic metabolism (136). Clearly, subcutaneous administration does not reflect the manner in which environmental carcinogens are absorbed. This study emphasizes the potential role of extrahepatic carcinogen metabolism.

In the second study, effects of cabbage consumption on DMH induced colon carcinogenesis was investigated in CF1 male mice. The need to examine this issue was clear. Recommendations to increase cruciferous vegetable consumption as a means of reducing cancer risk were based primarily on case control studies, which indicated in particular, that cabbage was protective against colon cancer (6-8, 45, 51, 71, 103). Prior to the present study, there were no studies in which the effect of cabbage consumption on experimently induced colon cancer could be adequately assessed. Two separate experiments were conducted. Results suggest that cabbage enhances, rather then inhibits carcinogenesis. In comparison to controls (0% cabbage), cabbage consuming mice had a significantly greater ($P<0.05$) tumor incidence (93% vs 72%) and more tumors per mouse (4.6 ± 3.9 vs 2.4 ± 2.1). Findings from the follow up experiment, indicate that cabbage consumption appears to mediate its influence only during the promotion phase of carcinogenesis, a suggestion which is

supported by the literature (154, 155). Cabbage consumption had no effect on xenobiotic metabolizing enzymes, indicating that these enzymes did not play a role in the enhanced carcinogenesis in cabbage fed groups.

The significance of this study is considerable for at least two reasons. First, the most pronounced effect of diet on human carcinogenisis is likely via its influence on promotion. Consequently, dietary components influencing the promotion phase of carcinogenesis, as appears to be the case with cabbage, are generally of greater importance than those influencing initiation. Second, if results of this study are confirmed, then public recommendations to increase cruciferous vegetable consumption may be unwarranted. Research into the manner in which cabbage influences the promotion phase of the DMH model of carcinogenesis needs to be conducted to determine the applicability of these findings to human colon cancer.

The last study was initiated after observing an increased tolerance to DMH toxicity in CF1 male mice previously exposed to this carcinogen. Results indicate that with pretreatment, DMH metabolism is altered such that DNA methylation is reduced. The reduced methylation indicates that the carcinogenic effect of DMH is also deminished, since DMH is thought to induce tumors via methylation. The implications of this finding are significant. Because repeated injections of DMH are commonly used to induce tumors, the initiation and promotion phases of carcinogenesis

in the DMH model can not be clearly separated. Consequently, the DMH model is somewhat inadequate for testing dietary effects on promotion only. It may be possible, based on findings of this work, to reduce the number of injections of DMH without a corresponding decrease in tumor initiation. This will provide a better opportunity to evaluate the effects of dietary treatments on the promotion phase of experimentally induced colon cancer.

In addition to the reduced level of methylation in response to pretreatment with DMH, animals also exhibited altered DMH metabolism. The percentage of DMH metabolized to azomethane increased while the percentage metabolized to carbon dioxide decreased with pretreatment. The decreased hepatic cytochrome P450 in pretreated mice suggest this enzyme complex may play a role in the altered DMH metabolism, and thus, indirectly in the decreased DNA methylation. The finding that hepatic cytochrome P450 may be involved in DMH metabolism may lead to an improved understanding of how certain dietary factors influence DMH induced colon carcinogenesis.

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