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INFLUENCE OF DIETARY FAT AND FIBER ON COLON CARCINOGENESIS

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ABSTRACT

INFLUENCE OF DIETARY FAT AND FIBER
ON COLON CARCINOGENESIS

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The overall objectives were to study 1) the effect of dietary factors on chemically-induced colon carcinogenesis and 2) the mechanisms by which diet influences colon carcinogenesis.

First, the effect of dietary agar and fat on 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis in mice was examined. Increasing dietary fat did not affect colon carcinogenesis, but feeding agar doubled the number of tumors induced. Dietary agar decreased fecal bile acid concentrations; therefore, agar's effect was not mediated by bile acids.

Agar could enhance colon carcinogenesis by directly altering the colon mucosa. Therefore, the colon mucosal topography, using scanning electron microscopy, was examined in rats fed control or agar diets. In some animals fed agar, the surface mucosa contained numerous folds and clefts which appeared to be discontinuities in the mucosa.

The next experiments examined the effect of diet on colon tumor promotion. The effect of varying colon bile acid levels on colon epithelial cell proliferation in DMH- or

saline-injected rats was examined. Bile acids levels were varied for 4 weeks by intrarectally injecting deoxycholic or lithocholic acid, or by varying the fat or fiber (wheat bran, agar, or carrageenan) content of the diet. None of the treatments altered the DNA synthesis rate, but the bile acid injections, dietary agar, and dietary wheat bran increased the number of cells per crypt. DMH increased the DNA synthesis rate.

The final experiments examined the effect of diet on colon tumor initiation, using the colon carcinogen DMH. DMH was first incubated with isolated rat colon epithelial cells, which metabolized it to gaseous metabolites, other metabolites in the media, and products which bound to DNA. The effects of dietary fat, wheat bran, and agar on the in vivo metabolism of DMH in rats were then examined. The metabolism of DMH to products which bound to colon epithelial cell DNA was not altered by diet even though the serum level of DMH plus its metabolites was increased by high-fat diets and decreased by dietary wheat bran. A diurnal variation was also seen in DMH metabolism.

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INTRODUCTION

Cancer of the colon is the second leading form of cancer and the second leading cause of deaths from cancer in the United States. In 1982, it is estimated that 123,000 new cases will be diagnosed in the United States (American Cancer Society, 1981).

Environmental factors, such as diet, are thought to influence the development of colon cancer. Epidemiological studies have shown that a high-meat and a low-fiber intake are associated with a high incidence of colon cancer (Correa and Haenszel, 1978). Therefore, it is desirable to study colon carcinogenesis in animal models to determine the mechanisms by which the disease develops.

My objective in this study was to examine the effects of nutrition on chemically-induced colon carcinogenesis in animal models. My specific aims were 1) to examine the effects of dietary changes (in fat and fiber) on 1,2-dimethylhydrazine-induced colon carcinogenesis and 2) to examine mechanisms by which diet may influence colon carcinogenesis. These mechanisms include 1) a direct effect on the colon mucosa by fiber, 2) an effect on tumor promotion, and 3) an effect on tumor initiation.

CHAPTER 1....REVIEW OF LITERATURE

REVIEW OF LITERATURE

Mechanisms of Carcinogenesis

Carcinogenesis in the Human

Cancer is the second leading cause of death in the United States. One in four Americans will develop cancer during his or her lifetime; one in five will die from it (American Cancer Society, 1981).

Because cancer is such a serious health problem, the determination of its cause and prevention is extremely important. At present, epidemiologists believe that 60 to 90% of human cancers are caused by environmental agents (Miller, 1978). Worldwide, the incidence of cancer in different tissues and organs varies widely from country to country (Doll, 1969). For example, the U.S. population has a low incidence of stomach cancer and a high incidence of colon and breast cancer. In contrast, the Japanese have a high incidence of stomach cancer and a low incidence of colon and breast cancer. When people migrate from one country to another, their descendents tend to develop the same types of cancers as the native population (Cairns, 1975).

The main environmental agents which are thought to cause cancer are radiation and chemicals. Ultraviolet radiation from sunlight induces skin cancer in humans. And early radiologists who were exposed to large amounts of radiation developed cancer at a high rate. But in general radiation is not thought to affect the development of cancers which cause the greatest mortality in man. The effect of low-level radiation on human cancer is an unanswered - and controversial - question (Upton, 1982).

The induction of cancer by chemicals has been seen often in humans. In the late 1700's, Percival Pott, a British physician, found that men who had worked as chimneysweeps had a high incidence of scrotal cancer (Pott, 1775). Pott attributed this to exposure to soot. Chimneysweeps in northern Europe, meanwhile, had a low incidence of scrotal cancer because of their greater cleanliness (Butlin, 1892). In the 1800's, German workers in dye factories who were exposed to large amounts of dyes had a high incidence of bladder cancer (Clayson, 1962). In this century, people who smoke cigarettes have a much higher incidence of lung cancer than nonsmokers (Robbins, 1974).

Chemical Carcinogenesis

In animals, chemical carcinogenesis was first observed in the early 1900's, when the application of coal tar to rabbit or mouse skin induced tumors (Yamagiwa and

Ichikawa, 1918; Tsutsui, 1918). Subsequently, chemicals isolated from coal tar, the polycyclic aromatic hydrocarbons (PAH), were found to induce skin tumors in mice in the early 1930's. Dibenz(a,h)anthracene was the first chemical to induce tumors (Kennaway and Hieger, 1930); other PAH - benzo(a)pyrene, 3-methylcholanthrene, and 7,12-dimethylbenz (a)anthracene - were also found to be carcinogenic (Cook, Hewett, and Hieger, 1933; Clayson, 1962). Since then, many classes of chemicals have been shown to induce tumors: aromatic amines, alkylating agents, dialkylnitrosamines, and many others (Miller, 1978).

The mechanism by which these chemicals induce cancer has been the subject of much research. The metabolism of these chemicals is important in the carcinogenic process. Although some carcinogens are direct-acting, most need to be metabolized to some other form to exert their carcinogenicity (Weisburger, 1978; Wright, 1980). Most are lipophilic and need to be metabolized to more water-soluble forms to be excreted. The pro-carcinogens are generally metabolized by mixed-function oxidases in the smooth endoplasmic reticulum to electrophilic forms (the ultimate carcinogens). These electrophiles then react with nucleophilic sites in the cell, such as the nitrogen and oxygen atoms in DNA and RNA and the nitrogen, oxygen, and sulfur atoms in proteins, to form DNA-, RNA-, and protein-bound adducts. Several carcinogens studied in detail, such as

benzo(a)pyrene and 2-acetylaminofluorene, are metabolized to proximate carcinogens and then further to electrophilic ultimate carcinogens, which then bind to cellular macromolecules. And when these carcinogens are injected into animals they form adducts with cellular nucleophiles in target and other tissues. Without metabolism, these compounds are non-reactive and do not bind to DNA, RNA, or protein (Miller, 1978; Weisburger, 1978).

Because DNA is the genetic material of the cell, the binding of carcinogen metabolites to DNA is very important. The somatic mutation theory of carcinogenesis states that alteration of genetic material in the cell is necessary for the induction of cancer. Alternatively, the epigenetic hypothesis states that cancer is caused without genetic changes (Straus, 1981).

The epigenetic hypothesis is supported by several experiments which show the reversion of tumor cells back into normal, differentiated tissue (Goldstein et al., 1964; Nameroff et al., 1970; Pierce and Wallace, 1971; Mintz and Illmensee, 1975). In the most prominent of these (Mintz and Illmensee, 1975), cells from a mouse teratocarcinoma were transplanted into blastulas from mice of another strain. These subsequently developed into chimeric mice in which many of its tissues were derived from the mouse teratocarcinoma cells. So tumor tissue, at least in some cases, can revert back to normal tissue. These

experiments imply that the conversion of normal cells to cancer cells is not necessarily caused by a permanent genetic change.

The somatic mutation hypothesis, on the other hand, states that the development of cancer is dependent on genetic alterations. The reaction of chemical carcinogens with DNA is most likely to lead to a mutation. But modification of a protein which is involved in DNA repair or of RNA that is integrated into DNA could also alter cellular DNA (Miller, 1978).

Evidence that a mutation in DNA leads to the development of cancer is strong. Chemical carcinogens, or their reactive metabolites, have been shown to be mutagenic in a number of systems. Bacterial mutagenesis systems have shown nearly all chemical carcinogens, after metabolism, to be mutagenic (Ames et al., 1975; McCann et al., 1975). And when transforming DNA is treated with ultimate carcinogens, it loses its ability to transform bacterial cells (Maher et al., 1968).

But the question remains as to how these alterations are brought about. For alkylating agents, a methyl, or other alkyl, group binds to a nucleophilic site in DNA, such as the O^6 of guanine (Lawley, 1976). This can lead to mispairing of DNA during DNA replication because of altered hydrogen bonding. PAH and aromatic amines, on the other hand, leave a large, bulky molecule attached to DNA.

After treatment of bacteria with 2-acetylaminofluorene, base-pair substitution, frame-shift, and deletion mutations have been seen (Miller, 1978). In mammalian cells, the type of mutation induced by such agents is not yet known.

Since DNA-carcinogen adducts can lead to mutation, the repair of these lesions by the cell is extremely important. If the defective nucleotide can be replaced before DNA replication, no mutation or harm to the cell is likely to occur. But if the DNA repair enzymes in the cell are lacking, miscoding and mutation of DNA are likely to occur, thus increasing the risk of cancer. This is seen in the disease Xeroderma pigmentosum, where DNA damage (thymine dimers) induced by ultraviolet light cannot be repaired by the cell (Cleaver et al., 1975). People with this disease have a very high incidence of skin cancer. Another example is seen in experiments in the fish Poecilia formosa. Exposure of its cells to ultraviolet light in vitro followed by their injection into another fish leads to the development of thyroid tumors (Hart et al., 1977). But if the cells are exposed to visible light before inoculation, allowing DNA repair (via photoreactivation) to occur, no tumors develop. Finally, in alkylation-induced carcinogenesis, correlations have been seen between the lack of removal of O^6-ethylguanine or O^6-methylguanine residues in a tissue and the subsequent development of tumors in that tissue (Goth and Rajewsky, 1974; Margison and Kleihues,

1975).

The Two-Stage Theory of Carcinogenesis

The two-stage theory divides carcinogenesis into two parts: initiation and promotion. This model was first demonstrated in mouse skin by Berenblum (1941) and Mottram (1944). In tumor initiation, a single subcarcinogenic dose of a chemical carcinogen (such as a PAH) is applied. Initiation is often suggested to consist of the binding of a carcinogen to DNA, followed by DNA replication, which fixes the mutation. Promotion, on the other hand, requires repeated applications over a period of several weeks or months. The classical promoter is croton oil; the active agent in croton oil is 12-0-tetradecanoylphorbol-13-acetate (TPA). Initiation is not reversible whereas promotion is. Neither the administration of an initiator nor repetitive doses of a promoter alone will lead to tumor formation, nor will the administration of the promoter before the initiator; the application of the initiator must be followed by repeated doses of the promoter (Diamond et al., 1980).

Initiation-promotion has also been demonstrated in other tissues. In rats, a small dose of 2-acetylamino-fluorene followed by long-term treatment with phenobarbital leads to a high incidence of liver tumors (Peraino et al., 1971; Peraino et al., 1973). Experiments in other tissues,

such as the mammary gland, bladder, and colon, also suggest a two-stage etiology (Armuth & Berenblum, 1974; Hicks et al., 1975; Reddy et al., 1978).

Whereas the molecular mechanisms of initiation have been examined in detail, those of promotion are less well understood. Promoters are thought to cause expression of the gene which has been altered by initiation. If this hypothesis is correct, the identification of the altered gene is imperative (Diamond et al., 1980).

To explore this hypothesis, it is necessary to examine the cellular effects of promoters. When a promoter is applied to a tissue (or tissue culture), the amount of DNA, RNA, and protein synthesis increases, and the mitotic rate increases (Boutwell, 1974). But before these changes occur, there is a large increase in ornithine decarboxylase (ODC) activity (Boutwell, 1978). Boutwell has proposed that ODC is the crucial protein in promotion. According to this theory, the gene which codes for the degradation of the enzyme is the gene altered during initiation. When a promoter is applied, the levels of ODC would stay elevated longer, increasing the level of polyamines (which increase cell division) in the cell. The increase seen in ODC activity, however, could be caused by some other change in the cell, rather than directly by the promoter.

Nutrition and Colon Carcinogenesis

Epidemiology of Colon Cancer

Colon cancer is the second leading cause of death from cancer in the United States. In 1982, it is estimated that 123,000 people in the U.S. will develop colorectal cancer and that 57,000 will die from it (American Cancer Society, 1981).

The incidence of colon cancer varies widely from one country to another. In general, the United States, Canada, and northern Europe have a high incidence, whereas Africa, Asia, and South America have a low incidence (Doll, 1969).

The majority of colon tumors have no hereditary pattern and are thought to be associated with environmental factors. A small number of cases of colon cancer are associated with the dominately inherited condition, familial polyposis of the colon (Dodd, 1977). But studies with migrant populations suggest that the environment is much more important (Haenszel et al., 1973; Correa and Haenszel, 1978). When people migrate from one country to another, their descendents tend to develop colon cancer at the same rate as the native inhabitants. For example, Japanese living in Japan have a low incidence of colon cancer. But Japanese who migrate to Hawaii or to the continental United States develop a high incidence of colon cancer after a few generations.

One environmental factor which may affect the development of colon cancer is diet. In general, populations which consume high levels of fiber and low levels of fat and protein have a low incidence of colon cancer (Correa and Haenszel, 1978). Conversely, low levels of fiber and high levels of fat and protein in the diet are associated with high colon cancer incidences. Low intakes of dietary fiber and high intakes of dietary fat and protein are usually associated with high intakes of meat or other animal products and low intakes of vegetable products. When people move from one country to another, they (and their descendents) gradually adopt the diet of the native population. At the same time, their colon cancer incidence also changes to that of the native population. For example, Japanese who move to the United States gradually change to a Western high-meat diet over several generations (Correa and Haenszel, 1978).

Chemically-Induced Colon Carcinogenesis

Although epidemiological studies in humans provide clues to the etiology of colon cancer, its exact cause cannot be determined in this way. Animal models, therefore, have been developed. Because no strain of rat or mouse develops colon cancer spontaneously with a high frequency, chemicals are used to induce colon tumors similar to those which are seen in humans.

Several chemicals (or classes of chemicals) induce colorectal tumors: 3-methylcholanthrene; methylnitrosourea (MNU); 3-methyl-4-aminobiphenyl and its analogs; methyl (acetoxymethyl) nitrosamine; N-methyl-N'-nitro-N-nitroso-guanidine (MNNG); 3-methyl-2-aminonaphthalene; and 1,2-dimethylhydrazine (DMH) and its derivatives (Weisburger et al., 1977; Berman et al., 1979). DMH and its derivatives azoxymethane (AOM) and methylazoxymethanol (MAM) have been the most widely used because they more specifically induce colon tumors (Weisburger, 1973; Fiala, 1977). MAM and cycasin, the glucuronide of MAM, were first found to be carcinogenic in the colon, as well as in other organs, by Laquer (1965). Subsequently, DMH was shown to be carcinogenic (Druckrey et al., 1967); DMH, moreover, is more specific than MAM for the colon (Weisburger, 1973). DMH has since been shown to induce colon tumors in a variety of species using several methods of injection.

DMH-induced colon carcinogenesis has been studied most in rats and mice. In mice, induction of tumors by DMH is strain-specific: CF_1, Swiss, BALB/c, and ICR/Ha mice are susceptible to DMH-induced colon carcinogenesis, whereas DBA/2 and C57BL/Ha mice are resistant (Thurnherr et al., 1973; Evans et al., 1974; Toth et al., 1976; Deschner and Long, 1977; Clapp et al., 1979). In CF_1 mice, DMH induces adenocarcinomas and areas of dysplasia in the colon (Deschner and Long, 1977); in rats, it induces adenomas

and adenocarcinomas (Newberne and Rogers, 1973; Sunter et al., 1978).

The mechanism by which DMH and its derivatives induce colon cancer has been the subject of much research. DMH has to be metabolically activated to induce cancer: no tumors are seen at the site of its injection (e.g. subcutaneous, intraperitoneal), unless it is injected into the colon (Toth et al., 1976; Sunter et al., 1978). After a series of metabolic reactions, DMH is thought to be converted to the methyldiazonium ion (Fiala, 1977). This electrophilic molecule then methylates nucleophilic sites in the cell: DNA, RNA, and protein (Miller, 1978). After radioactive DMH is injected into an animal, labeled methyl groups bind to DNA in the colon, as well as in other tissues (Rogers and Pegg, 1977; Likhachev et al., 1977). Binding was measured at both the O^6 and N^7 positions of guanine. Since it can lead to miscoding and mutation, binding at the O^6 position is considered to be the more critical (Miller, 1978). Alkylating agents also bind to other positions in DNA (Miller, 1978).

The mechanism by which DMH (or its metabolites) reaches the colon epithelial cells is not clear. When DMH was administered by subcutaneous injection, Hawks and Magee (1974) found that less than 1% of an injected dose is secreted into the bile. This suggests that DMH reaches the colon through the blood. But Pozharisski et al. (1975),

after the subcutaneous injection of labeled DMH, found high radioactivity in the bile and in the contents of the stomach, duodenum, ileum, ascending colon, and descending colon. So the means by which DMH reaches the colon cannot be stated with certainty at this time.

A pathway has been hypothesized by which DMH is metabolized to its ultimate carcinogen (Fiala, 1977). After injection, DMH is metabolized to azomethane (AM), which is a gas. Radioactive AM can be detected in exhaled air after labeled DMH is injected (Fiala et al., 1976b; Fiala et al., 1977). Next, AM is metabolized to AOM and then to MAM. Both of these compounds have been detected in the urine after the injection of labeled DMH (Fiala et al., 1976a; Fiala et al., 1977; Fiala et al., 1978). When the compounds disulfiram, diethyldithiocarbamate, or carbon disulfide are administered simultaneously with DMH, the amount of AOM and MAM excreted into the urine decreases, and the amount of AM exhaled increases dramatically (Fiala et al., 1977); the binding of DMH metabolites to cellular constituents also decreases after disulfiram injection (Swenberg et al., 1979). These experiments imply that these compounds block the metabolism of AM to AOM.

Next, MAM is metabolized to the methyldiazonium ion, which then methylates cellular nucleophiles. MAM is first thought to be metabolized to the corresponding aldehyde by alcohol dehydrogenase (ADH). When MAM is incubated with

ADH, carbonium ions are produced (Feinberg and Zedeck, 1980). Apparently the aldehyde form of MAM is unstable and decomposes rapidly. MAM also degrades spontaneously, but slower than when ADH is present. In addition, when pyrazole, an inhibitor of ADH, is injected into rats simultaneously with AOM, the metabolism of AOM to CO_2 is inhibited greatly (Fiala et al., 1978). Therefore, it appears that ADH is necessary for the maximal metabolism of DMH.

Although the pathway by which DMH is metabolized is fairly well established, the tissues in which its metabolism takes place are not. The colon has the ability to metabolize DMH; it is metabolized by both rat and human colon organ cultures to products which bind to DNA (Autrup et al., 1978; Autrup, 1980). But these studies do not determine whether DMH is metabolized by colon epithelial cells or by other tissue types in the culture. DMH might also be partially metabolized in vivo by other tissues, such as the liver, before reaching the colon.

The colon also metabolizes other xenobiotics. Rat and human colon organ cultures metabolize benzo(a)pyrene (BP) 7,12-dimethylbenz(a)anthracene, aflatoxin B_1, and dimethylnitrosamine to products which bind to DNA (Autrup et al., 1978; Autrup, 1980). The colon has a microsomal drug metabolizing system which hydroxylates drugs and PAH (Fang and Strobel, 1978a). This system metabolizes BP and 2-aminoanthracene to mutagenic products (Fang and Strobel,

1978b). Since BP is not carcinogenic in the colon (Toth, 1980), the importance of this system in colon carcinogenesis is not known.

Nutrition and Chemically-Induced Colon Carcinogenesis

The effect of nutrition on experimental colon cancer has been examined by varying different dietary components during the chemical induction of colon tumors. Typically DMH or its derivatives AOM or MAM are used to induce the tumors. Dietary components which have been examined include fat, protein and fiber.

Raising the dietary fat level has been shown to enhance the development of chemically-induced colon tumors in several studies in rats. Reddy et al. (1974a) found that increasing the dietary fat level from 0.5% to 5% or 20% enhanced DHM-induced colon carcinogenesis. The type of fat (animal vs. vegetable) does not alter the enhancing effect (Reddy et al., 1976a). And high-fat diets enhance colon carcinogenesis when any one of four carcinogens - AOM, DMH, MAM acetate, or MNU - is used (Reddy et al., 1977c; Bull et al., 1979). In addition, adding beef fat to a stock diet enhanced AOM-induced colon carcinogenesis (Nigro et al., 1975). It should be noted, however, that in the above studies, fat was not substituted for carbohydrate isocalorically: it was either substituted on a weight basis (gram for gram) or added to a stock diet. Therefore, it

cannot be concluded with certainty that the high-fat diet was responsible for the effect noted.

The effect of dietary protein has seldom been examined. Topping and Visek (1976) showed that increasing the level of dietary protein increased the incidence of DMH-induced colon tumors. But Clinton et al. (1979) found that changing the protein source (from soy to raw or charcoal-broiled beef) did not alter DMH-induced colon carcinogenesis.

The effect of dietary fiber on chemically-induced colon carcinogenesis varies with the particular fiber and the carcinogen used. Dietary wheat bran has been shown to inhibit chemically-induced colon carcinogenesis in several studies (Wilson et al., 1977; Barbolt and Abraham, 1978; Watanabe et al., 1979; Barbolt and Abraham, 1980); although Bauer et al. (1979) found that it had no effect. Dietary cellulose inhibited DMH-induced colon carcinogenesis in two studies (Freeman et al., 1978; Freeman et al., 1980); but Ward et al. (1973) found that it did not affect AOM-induced colon carcinogenesis.

The role of other fibers in the development of colon tumors is unclear. Dietary alfalfa enhances MNU-induced colon carcinogenesis but does not affect AOM-induced colon carcinogenesis (Watanabe et al., 1979). Pectin inhibits AOM-induced colon carcinogenesis, does not affect MNU-indiced colon carcinogenesis, and enhances DMH-induced colon carcinogenesis (Watanabe et al., 1979; Bauer et al.,

1979; Bauer et al., 1980). Guar gum does not affect the development of DMH-induced tumors (Bauer et al., 1980).

Finally, some dietary fibers clearly enhance colon carcinogenesis. Carrageenan, a sulfated polysaccharide, enhances both AOM- and MNU-induced colon carcinogenesis (Watanabe et al., 1978). In addition, carrageenan - after degradation to a lower molecular weight polysaccharide - induces colorectal tumors (Wakabayashi et al., 1978) and rectal squamous metaplasia (Fabian et al., 1973) in rats. Native carrageenan, however, does not induce tumors when fed to rats or hamsters (Rustia et al., 1980).

Mechanisms By Which Diet Alters Colon Carcinogenesis

Diet could alter colon carcinogenesis by 1) direct ingestion of carcinogens (initiators and promoters), 2) alteration of gut bacteria, 3) alteration of the levels of certain metabolites (which may affect colon carcinogenesis) in the colon and 4) direct modification of the colon mucosa.

Although humans consume trace amounts of carcinogenic chemicals (or chemicals which can be converted into carcinogens), it is not known if their ingestion influences the development of colon cancer. Humans ingest PAH, mainly from burnt or smoked food (Barnett, 1976; Lijinsky and Shubik, 1964; Lijinsky and Ross, 1967). 3-methylcholanthrene induces colorectal cancer, but BP does not

(Weisburger, 1973; Toth, 1980). The injection of nitrates and nitrites may lead to the formation of nitrosamines in the stomach (Issenberg, 1976; Fraser et al., 1980). Aflatoxins also may be found in food, but they induce liver, not colorectal cancer (Miller and Miller, 1976; Wilson, 1979).

Recently several investigators have reported the presence of mutagens in cooked food. Sugimura and Nagao (1979) found mutagens in the charred surface of fish and meat. They subsequently found that mutagens are produced in the pyrolysates of various amino acids. Of these, the ones from tryptophan were the most mutagenic; mutagens from serine and glutamate also had high activity. The mutagens from tryptophan and glutamate pyrolysates have been purified and are aromatic amines. In addition, mutagens are formed in beef when it is fried, broiled or boiled; they are also formed when beef stock is boiled for a long period of time (Commoner et al., 1978; Vithayathil et al., 1978; Pariza et al., 1979; Spingarn and Weisburger, 1979). Spingarn et al. (1980) isolated a mutagen from fried beef which is different from the ones in amino acid pyrolysates. At present, the exact structure has not been determined but the compound has been shown by mass spectroscopy and NMR to be an aromatic amine. Other foods have also been found to contain mutagens: fried potatoes; baked, toasted, and fried bread; coffee and tea; charred

vegetables; and charred sugar (Nagao et al., 1979; Sugimura and Nagao, 1979; Spingarn et al., 1980).

The significance of these findings is unknown at this time. But since beef intake is correlated with the colon cancer incidence (Correa and Haenszel, 1978), the presence of a mutagen in cooked or burnt beef provides a possible mechanism.

Alternatively, diet could alter colon carcinogenesis by changing the types of bacteria or their enzyme activities in the colon. A different bacterial population (or a bacterial population with altered enzyme activities) might produce co-carcinogenic materials in the colon. Hill et al. (1971) reported that populations at high risk for colon cancer excreted more Bacteroides and total anaerobic bacteria. But other studies have found no differences between individuals at high or low risk for colon cancer (Finegold et al., 1974; Moore and Haldeman, 1975; Goldberg et al., 1977). And patients with colon cancer do not have a different intestinal flora than control non-cancer patients (Mastromarino et al., 1978; Finegold et al., 1975).

Few studies have examined the effect of experimental diets on the human fecal flora. Reddy et al. (1975) found that increasing the fat content of the diet increased the number of anaerobic microflora. But altering the protein or fiber content does not change the fecal microflora

(Speck et al., 1970; Fuchs et al., 1976; Hentges et al., 1977).

Other studies suggest that differences in the metabolic activities of intestinal bacteria may be more important than the taxonomic groupings. The enzyme β-glucuronidase, which hydrolyzes glucuronide conjugates, is present in higher amounts in the feces of rats or humans fed high-fat diets (Reddy et al., 1974b; Reddy et al., 1977a). Patients with colon cancer or colon polyps also have higher levels of the bacterial enzymes cholesterol dehydrogenase and 7-α-dehydroxylase (Mastromarino et al., 1978). These enzymes may be important in degrading bile acids and cholesterol in the colon.

A third mechanism by which diet may influence colon carcinogenesis is by altering the levels of various metabolites in the colon. Bile acid metabolism, which is altered by dietary fat and fiber, is thought to be important in colon carcinogenesis.

When the intake of dietary fat is increased, the requirement for bile acids in the small intestine - to emulsify the fat - is increased. Thus, more bile acids are secreted into the duodenum (Danielsson, 1963). Although most of the bile acids are reabsorbed in the ileum, larger amounts reach the colon when dietary fat increases. People who eat high-fat diets have greater fecal bile acid excretion than people who eat low-fat

diets (Reddy et al., 1977b). In the colon, bile acids are deconjugated and then degraded to secondary bile acids (and possibly other compounds) as the result of bacterial activity (Reddy et al., 1977b; Weisburger et al., 1977). Thus, an increased dietary fat intake leads to increased bile acid levels in the colon and greater bile acid degradation. These changes correlate with a higher incidence of colon cancer in man (Reddy et al., 1977b; Weisburger et al., 1977).

Likewise, dietary fiber may alter colon carcinogenesis by influencing bile acid metabolism. Dietary fiber is not digested or absorbed in the small intestine and enters the colon unaltered (Cummings, 1976). In the colon fiber lowers bile acid concentrations by increasing the total bulk and by absorbing water (Mitchell and Eastwood, 1976). In addition, because of the fiber, the fecal mass moves through the colon faster; this is thought to reduce the amount of time that the intestinal cells are exposed to the fecal materials (Burkitt, 1971). Of course, the magnitude of these effects varies with the particular fiber which is fed. A fiber which absorbs a high amount of water, such as wheat bran, will have more of a diluting and laxative effect than one which does not, such as lignin.

In addition, some fibers bind bile acids in the small intestine. Lignin and alfalfa bind bile acids very well

in vitro, whereas cellulose and wheat bran do not (Story and Kritchevsky, 1976). A greater bile acid binding ability would lead to a greater amount of bile acids delivered to the colon. Although this increases the concentration of bile acids slightly, the fiber would lower bile acid concentrations overall because of water absorption and the bulk of the fiber.

The concentration and degradation of bile acids in the colon is important because certain bile acids are thought to act as tumor promoters in the colon. When administered intrarectally after an earlier dose of MNNG, sodium deoxycholate, lithocholic acid, taurodeoxycholic acid, sodium cholate, and sodium chenodeoxycholate all increase the frequency and number of colon tumors which develop (Narisawa et al., 1974; Reddy et al., 1976b; Reddy et al., 1977d).

In vitro studies generally show bile acids to influence carcinogenesis. Lithocholic acid, but not sodium deoxycholate, inhibits metabolic cooperation in cultured cells (Umeda et al., 1980), which suggests that it is a promoter. The inhibition of metabolic cooperation may be a factor in tumor promotion (Yotti et al., 1979). High concentrations of lithocholic acid also induce strand breaks in DNA of cultured cells (Kulkarni et al., 1980). Finally, enteric bacteria metabolize cholic and deoxycholic acids to products which bind to DNA (Zachariah et al., 1977). Bile acids,

however, are not mutagenic in the Ames Salmonella-microsome mutagenicity test (Silverman and Andrews, 1977).

If dietary protein does in fact alter colon carcinogenesis, it likely does so by increasing the levels of amino acids which enter the colon. Once in the colon, amino acids can be metabolized by bacteria to other compounds, which could possibly be carcinogenic. Most research has focused on the amino acid tryptophan. Fecal bacteria can convert tryptophan to indole using the enzyme tryptophanase (Chung et al., 1975). This enzyme is higher in the feces of rats fed high-meat diets than in those fed low-meat, low-protein diets (Chung et al., 1975). This could result in greater concentrations of indole and other, more degraded, metabolites of tryptophan in the colon, which could be important because indole and other tryptophan metabolites are carcinogenic or co-carcinogenic in bladder tumorigenesis (Rauschenbakh et al., 1963; Bryan, 1969; Oyasu et al., 1972). None of the major tryptophan metabolites produced in the colon, however, are mutagenic in the Ames Salmonella mutagenicity test (Bowden et al., 1976).

Finally, diet could affect colon carcinogenesis by directly modifying the colon mucosa. Using scanning electron microscopy, Cassidy et al. (1980, 1981) found that dietary fibers cause structural deviations in the colon mucosa. Alfalfa and bile acid binding resins -

colestipol, DEAE-Sephadex, and cholestyramine - caused the most deviations as compared to rats fed lab chow. Because fibers causing the most changes also have high bile acid binding capacities, fiber's effect on the colon mucosa may be mediated through bile acids.

CHAPTER 2 . . . . ENHANCEMENT OF 1,2-DIMETHYLHYDRAZINE-INDUCED COLON CARCINOGENESIS IN MICE BY DIETARY AGAR

INTRODUCTION

Agar, a gum which forms a firm gel at low concentrations, is used in food products as a gelling agent and is used pharmaceutically as a laxative (Selby and Wynne, 1973). Agar is a sulfated polysaccharide derived from the red-purple seaweeds (class Rhodophyceae) and consists primarily of galactose and 3,6-anhydro-galactose, along with glucuronic acid and a significant proportion of half-ester sulfates (Selby and Wynne, 1973; Cummings, 1976). Agar is not digested by humans and thus may be considered a dietary fiber (Cummings, 1976).

Although agar is consumed regularly by humans, few studies have examined the effects of feeding agar to experimental animals. Several studies have reported the effect of dietary agar on cholesterol metabolism (Tsai et al., 1976; Kelley and Tsai, 1978). However, studies examining the effect of agar on intestinal parameters are scarce. Watt and Marcus (1978) showed that Danish agar, after degradation by acid, induces ulcerative colitis when fed to guinea pigs.

Carrageenan, another sulfated polysaccharide derived from a seaweed, is deleterious to the colon (Watt and Marcus, 1973). Degraded and native carrageenan induce ulcerative colitis when administered orally to several species (Watt and Marcus, 1973; Grasso et al., 1973). Also, degraded carrageenan induces colorectal tumors

(Wakabayashi et al., 1978) and rectal squamous metaplasia (Fabian et al., 1973) when fed to rats, while native carrageenan enhances methylnitrosourea (MNU)- and azoxymethane (AOM)-induced colon carcinogenesis in rats (Watanabe et al., 1978).

Because of structural similarity between agar and carrageenan, we investigated the effect of dietary agar on 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis in CF_1 mice fed two different levels of dietary fat. DMH induces colon tumors organospecifically after injection into experimental animals (Newberne and Rogers, 1973; Deschner and Long, 1977). The tumors induced are histologically similar to colon tumors seen in humans (Newberne and Rogers, 1973; Deschner & Long, 1977). We also determined the levels of fecal neutral sterols and bile acids to learn if the effect of agar may be related to the effects of these chemicals, since fecal steroids have been hypothesized to play a cocarcinogenic role in colon carcinogenesis (Reddy et al., 1978). We found that agar enhanced DMH-induced colon carcinogenesis and decreased fecal steroid concentrations.

MATERIALS AND METHODS

Chemicals. Agar (bacto-agar) was obtained from Difco Laboratories, Detroit, MI; DMH was obtained from Aldrich Chemical Co., Milwaukee, WI; bile acid and neutral sterol standards were from Steraloids, Inc., Wilton, NH.

Colon Carcinogenesis. Four week old male CF_1 mice (ARS Sprague-Dawley, Madison, WI) were housed individually in stainless steel hanging wire cages. The animal room was temperature- and humidity-controlled and was on a 12-hour light-dark cycle. One hundred twelve mice were randomly assigned to one of four diets (Table 1): 1) a fiber-free control diet; 2) an agar diet, which consisted of the control diet plus 8% added agar (i.e. 8 gm. of agar were added to 100 gm. of the control diet); 3) a high-fat diet; and 4) a high-fat, agar diet, which consisted of the high-fat diet plus 8% added agar. The diets had a constant energy to protein ratio (we assumed that no calories were derived from agar). Vitamins were added to the diet to meet at least 150% of the NRC requirements (NAS/NRC, 1972). Water and food were provided ad libitum.

After one week on the diets, mice were given weekly subcutaneous injections of DMH. Before injection, DMH dihydrochloride (1 mg/ml) was dissolved in saline, and was adjusted to pH 6.8. Twenty-three mice in each dietary group were injected with 20 mg of DMH dihydrochloride per kg body weight for 20 weeks. Another group (5 mice/group) on the same diet received an equivalent amount of saline. The mice were weighed weekly during the experiment. Twenty-one weeks after the start of the experiment and one week after the last DMH injection, the mice were killed by cervical dislocation. The large intestine from the

Table 1. Composition of Diets (Percent of Diet)

	Control	Agar	High-Fat	High-Fat, Agar
Casein	20.0	18.5	25.0	22.7
Methionine	0.3	0.3	0.4	0.3
Safflower oil	2.0	1.8	2.5	2.3
Vitamin mix[1]	1.2	1.1	1.5	1.4
Mineral mix[2]	6.0	5.6	7.5	6.8
Glucose	70.5	65.3	43.1	39.2
Agar		7.4		9.1
Tallow			20.0	18.2
Total ingredients	100.0	100.0	100.0	100.0
kcal/g protein	18.7	18.8	18.8	18.8

[1]Vitamin mix contained (per 100g of mix): retinol acetate, 8333 IU; vitamin D, 2125 IU; a-tocopherol acetate, 330.0 mg; choline, 16.67 g; thiamin-HCl, 50.0 mg; riboflavin, 62.5 mg; pyridoxine, 20.8 mg; niacin, 150.0 mg; biotin, 4.2 mg; folic acid, 100.0 mg; vitamin B_{12}, 83.3 ug; inositol, 25.0 mg; p-aminobenzoic acid, 166.7 mg; menadione, 100.0 mg; ascorbic acid, 416.7 mg.

[2]Draper et al., 1964.

ileocecal valve to the anus was opened, examined visually for lesions, and placed in 10% buffered, neutral formalin. All suspected tumors were removed, embedded in paraffin, sectioned at six microns, and then stained with hematoxylin and eosin for microscopic examination. The lesions were classified according to the degree of cellular hyperplasia, dysplasia, or anaplasia and as to the presence or absence of spread below the basement membrane. Data was analyzed using the X^2 test for tumor incidence and analysis of variance for tumor number (Gill, 1978).

Fecal Neutral Sterol and Bile Acid Analysis. Twenty-four hour fecal collections for neutral sterol and bile acid analysis were made in the 10th and 11th weeks after the start of DMH injections. Neutral sterols were extracted from dried fecal samples from each mouse by a slight modification of the procedure described by Evrard and Janssen (1968). Sterols were extracted with petroleum ether after saponification with 20% KOH in ethylene glycol. ^{3}H-Cholesterol was added as an internal standard to account for incomplete recoveries and 5-a-cholestane was added to the extracted sterols as an internal standard for gas-liquid chromatography (GLC). The neutral sterols were silylated (Grundy et al., 1965) and the trimethyl silylyl (TMS) ethers were separated by GLC with a Varian Model 3700 gas chromatograph equipped

with a flame ionization detector. The GLC conditions were as follows: injector temperature = 250°C, detector temperature = 270°C, column temperature = 255°C. The neutral sterols were separated on a 1.8 m long, 3.2 mm diameter stainless steel column packed with 3% OV-17 on 100/120 Gas Chrom Q. Cholesterol, coprostanol, and coprostanone standards were silylated and chromatographed with 5-a-cholestane as an internal standard. The sterols were quantitated with a Varian CDS 111 integrator.

Bile acids were extracted from feces as described by Grundy et al. (1965). The fecal pellets from 4-5 mice were pooled for analysis. ^{14}C-Glycocholic acid was added as an internal standard to account for incomplete recoveries. After extraction, bile acids were separated from fatty acids by the method of Makita and Wells (1963). Bile acids were methylated by the addition of excess etheral diazomethane. The methyl esters of the bile acids were then dried, silylated, and separated by GLC. 5-a-Cholestane was used as the internal standard for GLC. GLC conditions were the same as above except that the column temperature was held initially at 210°C for 10 minutes, then increased 12°/minute to 270°C and held at 270°C until the last TMS ether was eluted. The bile acid TMS ethers were separated on a 1.8 meter long, 3.2 mm diameter stainless steel column packed with 3% SP-2100 on 100/120 Supelcoport. We have reported bile

acids as the sum of all bile acids rather than as individual bile acids because of the possible oxidation of hydroxyl groups while drying the fecal samples and because of the production of artifacts during alkaline hydrolysis (Lepage et al., 1978). The fecal steroid data was analyzed using standard analysis of variance statistics (Gill, 1978).

RESULTS

Colon Carcinogenesis. Mice which were injected with DMH gained less weight during the experiment than controls, while mice which were fed high-fat diets gained more weight than mice fed low-fat diets ($p < 0.05$). Addition of agar to the diet did not affect weight gain. During the experiment, one mouse from each dietary group died.

Only animals receiving DMH injections developed colorectal tumors (Table 2). The tumor incidence in the mice fed the high-fat, agar diet was greater than the tumor incidence in the mice fed the agar diet ($p < 0.05$). The tumor incidence among the other dietary groups was similar. The incidence of malignant tumors was slightly greater ($p < 0.2$) in mice fed the agar or high-fat diets as compared to mice fed the control diet; however, the malignant tumor incidence did not vary between the high-fat, agar diet and the agar or high-fat diet. The number of tumors per mouse and the number of tumors per tumor-

Table 2. Colon Tumor Incidence and Classification in Mice Injected with 1,2-Dimethylhydrazine

	Dietary Treatment			
	Control	Agar	High-Fat	High-Fat, Agar
Mice with tumors, %[1]	77	73	86	95
Mice with malignant tumors, %	50	68	68	77
Tumors/mouse[2]	1.9±0.4[3]	4.2±1.4	2.3±0.5	6.1±1.8
Tumors/tumor-bearing mouse				
Total[2]	2.4±0.5	5.7±1.8	2.7±0.5	6.4±1.9
Hyperplasia	0.1±0.1			0.1±0.1
Dysplasia	0.8±0.2	0.6±0.3	0.9±0.3	1.2±0.3
Adenocarcinoma[2]	1.5±0.5	5.1±1.8	1.8±0.4	5.1±1.9

[1]Mice fed the high-fat, agar diet had a greater tumor incidence than mice fed the agar diet ($p < 0.05$).

[2]Mice fed diets containing agar had more tumors ($p < 0.05$).

[3]Results are means ± SEM.

bearing mouse were not increased in the mice fed high-fat diets (as compared to mice fed low-fat diets) but were increased significantly in the agar-fed mice ($p < 0.05$). All malignant tumors were adenocarcinomas, and were confined to the epithelial layer (carcinoma-in-situ). Dysplastic and hyperplastic lesions were also seen. The dysplastic group was considered premalignant whereas the hyperplastic lesions were considered to be an initial response to DMH. Most tumors were found in the lower half of the colon and were often located in close proximity to lymphoid nodules. In some agar-fed mice, large numbers of tumors were concentrated in a small area.

Feeding agar had the general effect of decreasing fecal steroid concentrations (Table 3). We have reported neutral sterol concentrations as the sum of all the neutral sterols since degradation of neutral sterols was similar between groups. Fecal neutral sterol and bile acid concentrations were significantly reduced in mice fed agar ($p < 0.05$). Neutral sterol concentrations were higher in agar-fed mice on high-fat diets as compared to agar-fed mice on low-fat diets ($p < 0.05$); however, the fat content of the diet did not affect neutral sterol concentrations in mice fed agar-free diets. The dietary fat level did not affect fecal bile acid concentrations.

DISCUSSION

This experiment shows that dietary agar enhances the

Table 3. Fecal Neutral Sterol and Bile Acid Concentrations of CF_1 Mice Fed High- or Low-Fat Diets With or Without Added Agar

	Total Neutral Sterol Concentration[1] (mg/g)	% Degradation of Neutral Sterols[2,3]	Total Bile Acid Concentration[4] (mg/g)	Feces Excreted (g/day)
DMH-Treated				
Control	14.4±1.4[5]	29±1	22.1±8.4	0.23±.01
Agar	2.9±0.1	30±1	12.0±3.5	0.79±.05
High-fat	14.4±0.6	35±4	14.4±3.4	0.45±.01
High-fat, agar	6.4±0.3	21±1	14.4±1.4	0.92±.04
No DMH Treatment				
Control	14.2±1.9	31±2	27.6±4.8	0.24±.02
Agar	2.8±0.5	29±2	11.5±3.4	0.61±.06
High-fat	13.7±0.8	26±2	24.2±4.3	0.36±.05
High-fat, agar	6.8±0.4	23±2	12.8±2.9	0.82±.05

[1]Neutral sterol concentrations were lower in mice fed agar and were higher in mice fed the high-fat, agar diet as compared to mice fed the agar diet with low-fat content ($p < 0.05$).

[2]$$\% \text{ Degradation} = \frac{(\text{Coprostanol} + \text{Coprostanone})}{(\text{Cholesterol} + \text{Coprostanol} + \text{Coprostanone})} \times 100$$

[3]No significant difference.

[4]Bile acid concentrations were decreased in mice fed agar ($p < 0.05$).

[5]Results are means ± SEM.

development of DMH-induced colon tumors in male CF_1 mice. Although agar-fed mice showed only a slight increase ($p < 0.2$) in the incidence of malignant tumors, the number of tumors per mouse, number of tumors per tumor-bearing mouse, and number of adenocarcinomas per tumor-bearing mouse were all greater ($p < 0.05$) in mice fed agar as compared to mice not fed agar. The increase in the malignant tumor incidence is relatively modest, but the 2-fold difference in tumor number is similar to significant differences reported in other studies which have examined the effect of diet on colon carcinogenesis. For example, Reddy, Weisburger & Wynder (1974a) reported an increase from 0.78 to 1.63 tumors per rat when the dietary fat level was increased from 4% to 20%, and Barbolt and Abraham (1978) found that dietary bran significantly decreased the number of tumors per rat from 6.4 to 1.8 in DMH-induced colon carcinogenesis.

The effect of other dietary fibers on chemically-induced colon carcinogenesis varies with the particular fiber. Carrageenan, as stated earlier, enhances both AOM- and MNU-induced colon carcinogenesis (Watanabe et al., 1978). The role of other dietary fibers in chemically-induced colon carcinogenesis is unclear. Dietary alfalfa enhances MNU-induced colon carcinogenesis, but does not affect AOM-induced colon carcinogenesis (Watanabe et al., 1979). Pectin inhibits AOM-induced colon carcinogenesis

(Watanabe et al., 1979) does not affect MNU-induced colon carcinogenesis (Watanabe et al., 1979), and enhances DMH-induced colon carcinogenesis (Bauer et al., 1980). Other dietary fibers have been shown to inhibit chemically-induced colon carcinogenesis. Dietary wheat bran has been shown to inhibit chemically-induced colon carcinogenesis in several studies (Wilson et al., 1977; Barbolt and Abraham, 1978; Watanabe et al., 1979). Dietary cellulose has been shown to inhibit DMH-induced colon carcinogenesis in two studies (Freeman et al., 1978; Freeman et al., 1980); however, Ward et al. (1973) found that dietary cellulose did not affect AOM-induced colon carcinogenesis. These fibers are thought to inhibit colon carcinogenesis by reducing the concentration of bile acids, neutral sterols, or other materials in the colon. Several bile acids have been shown to act as tumor promoters in rat colon (Narisawa et al., 1974; Reddy et al., 1976b; Reddy et al., 1977d). However, dietary agar in the present study lowered the fecal neutral sterol and bile acid concentrations. Therefore, dietary agar must have enhanced tumorigenesis by another mechanism.

Dietary agar may enhance colon carcinogenesis by acting directly on the colon mucosa. Cholestyramine, an ion-exchange resin which has been shown by scanning electron microscopy to produce discontinuities in the epithelial layer in the colon when fed to rats (Cassidy et

al., 1980), also enhances chemically-induced colon carcinogenesis (Nigro et al., 1973).

Mice which were fed the high-fat diets had only a slightly higher ($p < 0.2$) malignant tumor incidence and had no change in tumor number as compared to mice fed low-fat diets. This finding disagrees with several other studies, which have shown that chemically-induced colon carcinogenesis is enhanced when the fat content of the diet is raised. Reddy et al. (1974a) found that increasing the dietary fat level from 0.5% or 5% to 20% enhances DMH-induced colon carcinogenesis. The type of fat (animal vs vegetable) does not alter the enhancing effect of high levels of dietary fat (Reddy et al., 1976a), and high-fat diets enhance colon carcinogenesis when any one of four carcinogens - DMH, AOM, MNU, or methylazoxymethanol acetate - is used (Reddy et al., 1977c; Bull, et al., 1979). Nigro et al. (1975) showed that adding beef fat to a stock diet enhances AOM-induced colon carcinogenesis. In our experiment, the observation that high-fat diets did not raise fecal neutral sterol and bile acid concentrations may explain why the high-fat diets did not enhance colon carcinogenesis. Also, we isocalorically substituted fat for carbohydrate, while the other studies substituted fat for carbohydrate on a weight basis (gram for gram) or added fat to a stock diet.

Another possible reason for differences between our

study and previous studies was that our study was done in mice while most other studies have been done in rats. The induction of colon tumors in mice by DMH has been less well studied than in rats, especially in studies which have examined nutrition and DMH-induced colon carcinogenesis. The induction of tumors in mice by DMH appears to be strain-specific. CF_1 (Thurnherr et al., 1973; Deschner and Long, 1977), Swiss (Toth et al., 1976), BALB/c (Clapp et al., 1979), and ICR/Ha (Evans et al., 1974) mice are susceptible to DMH-induced colon carcinogenesis, while DBA/2 and C57BL/Ha mice are resistant to DMH-induced colon carcinogenesis (Evans et al., 1974). In CF_1 mice, DMH induces adenocarcinomas and areas of dysplasia in the colon (Deschner and Long, 1977). In rats, DMH induces adenomas and adenocarcinomas in the colon (Newberne & Rogers, 1973; Sunter et al., 1978). Although studies examining the effects of BHA and BHT on chemically-induced colon carcinogenesis have been done with mice (Clapp et al., 1979; Wattenberg & Sparnins, 1979), all previous studies examining the effects of fat, fiber, and protein have been done with rats.

Several conclusions can be drawn from this study. First, dietary agar enhanced DMH-induced colon carcinogenesis in male CF_1 mice. When agar was fed, the tumor incidence was slightly higher and the number of tumors per mouse was doubled. Second, agar's effect was not mediated

by neutral sterols or bile acids - the fecal concentrations of these metabolites were lower when agar was fed.

This study shows that the ingestion of agar can cause adverse effects in the colon. Therefore, the consumption of agar by humans may be deleterious, and the biological effects of agar should be examined further. Also, this study further points out the heterogeneity of dietary fibers. Dietary fibers should not be treated as a homogenous group, particularly since components and substituents of the fiber determine their biological properties.

CHAPTER 3 . . . . DIETARY AGAR-INDUCED CHANGES IN COLON TOPOGRAPHY AS SEEN WITH SCANNING ELECTRON MICROSCOPY

INTRODUCTION

Agar, a gum derived from the red-purple seaweeds, is used in food products as a gelling agent and pharmaceutically as a laxative (Selby & Wynn, 1973). Because agar is not digested by humans, it may be considered a dietary fiber (Cummings, 1976).

I previously found that dietary agar enhances the development of 1,2-dimethylhydrazine (DMH)-induced colon tumors in mice. But I also found that dietary agar decreased the fecal concentrations of bile acids and neutral sterols, which are thought to play a role in the development of colon cancer. So agar likely does not affect colon carcinogenesis by altering sterol metabolism. Agar, however, could directly affect the colon mucosa. In an earlier study, Danish agar, after degradation by acid, induced ulcerative colitis when fed to guinea pigs (Watt and Marcus, 1978). Because ulcerative colitis in humans is a risk factor in the development of colon cancer, agar may act via this mechanism.

In this study, to further examine the effect of dietary agar on the colon mucosa, I investigated its effect on the colon mucosal topography as seen by scanning electron microscopy.

MATERIALS AND METHODS

250 gram male Sprague-Dawley rats (Spartan Research

Animals, Haslett, MI) were housed individually in stainless steel hanging wire cages. The animal room was temperature- and humidity-controlled and was on a 12-hour light/dark cycle. 10 rats were randomly assigned to 1 of 2 diets: a control diet or an agar diet, which consisted of the control diet plus 8% added agar (Table 4). The diets had a constant energy to protein ratio - we assumed that no calories were derived from agar. Vitamins were added to the diet to meet at least 150% of the NRC requirements. Water and food were provided ad libitum.

After feeding the diets for 6 weeks, we sacrificed the rats by overexposure to ether gas. A 1 cm colon segment 2 cm from the anus was removed, pinned on cellular polystyrene, rinsed with saline, and fixed for 2 hours in 4% phosphate-buffered glutaraldehyde, pH 7.2. The specimens were then dehydrated in a graded series of ethanol solutions and critical point-dried. The surface of the tissue was coated with gold in a vacuum evaporator and an ISI Super III scanning electron microscope was used to examine the tissues.

RESULTS AND DISCUSSION

Rats on either diet showed similar food intakes and weight gains.

The surface of the colon epithelium in rats fed the control diet was flat, with circular (Figure 1) or slit-like (Figure 2) openings corresponding to crypt

Table 4. Composition of Diet (Percent of Diet)

	Control	Agar
Casein	20.0	18.5
Methionine	0.3	0.3
Safflower oil	4.0	3.7
Vitamin mix[1]	1.2	1.1
Mineral mix[2]	4.0	3.7
Glucose	70.5	65.3
Agar	0	7.4
Total Ingredients	100.0	100.0

[1]See Table 1, Chapter 2.

[2]See Draper et al., 1964.

openings. Cellular boundaries could often be seen. Goblet cells appeared either as "holes" or as plugs of mucus in the epithelium. At higher magnification (Figure 3), microvilli were seen on the surface of the epithelial cells.

In rats fed the agar diet for 6 weeks, the colon mucosal surface contained structural deviations. Numerous folds and clefts were seen in the surface - more than could be accounted for by crypt openings alone (Figures 4 and 5). At higher magnification (Figure 6), these clefts appeared to be discontinuities in the epithelial lining. Microvilli could also be seen at higher magnification.

The changes produced by dietary agar may be related to the enhancement of DMH-induced colon carcinogenesis by dietary agar which was previously seen. Disruptions in the colon surface could allow fecal constituents which may be promoters (such as bile acids) to enter the epithelial cells in greater amounts. These disruptions might also induce a greater amount of proliferation in the crypts to replace damaged cells on the surface of the colon.

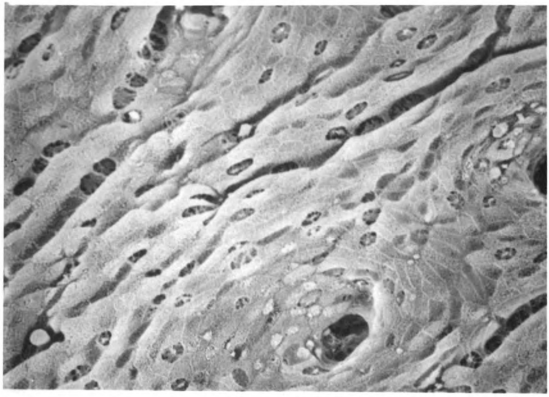
Cassidy et al. (1980, 1981) have shown other fibers to produce structural deviations in the colon mucosa. Fibers which produce a low amount of structural deviations, such as wheat bran or cellulose, tend to inhibit chemically-induced colon carcinogenesis (Barbolt and Abraham, 1978; Barbolt and Abraham, 1980; Freeman et al., 1978; Freeman

et al., 1980; Wilson et al., 1977). Cholestyramine, which enhances chemically-induced colon carcinogenesis (Nigro et al., 1973) produces a high amount of structural deviations in the colon mucosa. Fibers which produce a high amount of structural deviations also have a high bile acid binding capacity (and vice versa). Agar, however, produced abnormalities in the colon mucosa even though it decreases colon bile acid concentrations. Since agar does not increase bile acid excretion, it must have a low bile acid binding capacity. The effect of agar, therefore, was likely from the direct action of agar on the colon mucosa.

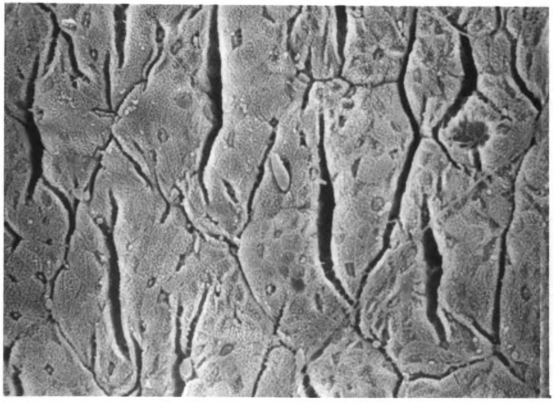
Although the number of rats per dietary treatment was small and the evaluation of the scanning electron micrographs was qualitative, these results are important because they represent a mechanism for the enhancement of colon carcinogenesis by agar. Further studies are needed to look into the biochemical mechanisms by which these changes are produced and the biochemical alterations in the colon which these changes produce.

Figure 1. Scanning Electron Microscopy of Colon from a Rat Fed the Control Diet. 700x. Crypts are visible as circular openings.

Figure 2. Scanning Electron Microscopy of Colon from a Rat Fed the Control Diet. 700x. Crypts appear as slit-like openings.



1

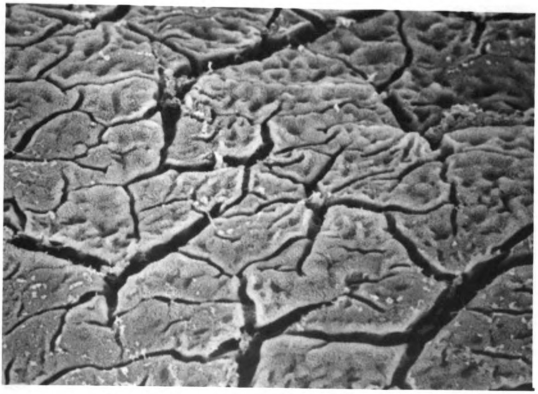


2

Figure 3. Scanning Electron Microscopy of Colon from a Rat Fed the Control Diet. 3000x.

Figure 4. Scanning Electron Microscopy of Colon From a Rat Fed the Agar Diet. 700x. Numerous folds and clefts are present.

3

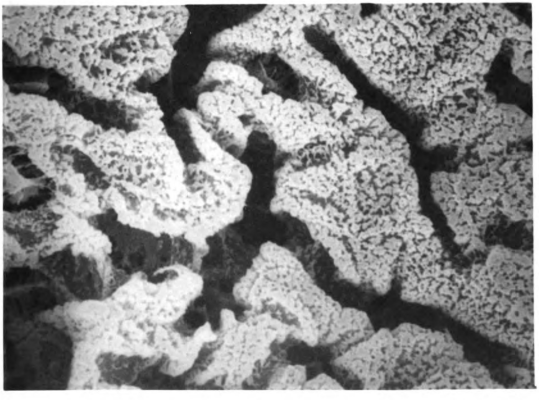


4

Figure 5. Scanning Electron Microscopy of Colon from a Rat Fed the Agar Diet. 700x. Numerous folds and clefts are present.

Figure 6. Scanning Electron Microscopy of Colon from a Rat Fed the Agar Diet. 3000x.

5



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CHAPTER 4 . . . INFLUENCE OF DIET, INTRARECTAL BILE ACID INJECTIONS, AND 1,2-DIMETHYLHYDRAZINE ON RAT COLON EPITHELIAL CELL PROLIFERATION

INTRODUCTION

Bile acids are thought to play a promoting role in the etiology of colon cancer. When fecal bile acid excretion is raised by increasing the fat content of the diet (Reddy et al., 1974), the incidence of chemically-induced colon tumors is increased in most studies (Reddy et al., 1974a; Reddy et al., 1976a; Reddy et al., 1977c), although other studies see no effect (Chapter 2; Brown, 1981). And when certain fibers, such as wheat bran, are added to the diet, both the fecal bile acid concentrations and the incidence of chemically-induced tumors are decreased (Barbolt and Abraham, 1978; Freeman et al., 1980; Reddy et al., 1980; Watanabe et al., 1979; Wilson et al., 1977). In addition, intrarectally injecting bile acids after an earlier dose of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) increases the development of colon tumors in rats (Narisawa et al., 1974; Reddy et al., 1976b; Reddy et al., 1977d). These studies suggest that bile acids are acting as tumor promoters in chemically-induced colon carcinogenesis.

Tumor promoters are thought to act by increasing proliferation in cells which have previously been treated with an initiator (i.e. a carcinogen) (Diamond et al., 1980). If bile acids are acting as tumor promoters, they should increase proliferation in colon epithelial cells after the previous administration of a colon carcinogen, such as 1,2-dimethylhydrazine (DMH).

In this study the effect of altering colon bile acid concentrations on colon epithelial cell kinetics was examined. Bile acid concentrations were altered by varying the fat or fiber content of the diet or by intrarectally injecting deoxycholic acid or lithocholic acid. Three different fibers were fed: wheat bran, which inhibits chemically-induced colon carcinogenesis, and agar and carrageenan, which enhance it (Barbolt and Abraham, 1978; Watanabe et al., 1978; Watanabe et al., 1979; Wilson et al., 1977). In the dietary experiment, fecal bile acid concentrations were determined to verify the effects of diet on fecal bile acid levels. And in both studies, DMH was injected in half the animals to determine if it is necessary for the action of the bile acids.

MATERIALS AND METHODS

Chemicals. Deoxycholic acid, murocholic acid, 3α, 7α-ol, 12-one-5B-cholanic acid, and 3α-ol, 7,12-one-5B-cholanic acid were from Steraloids, Inc., Wilton, NH; DMH was from Aldrich Chemical Co., Milwaukee, WI; carrageenan, lithocholic acid, and cholic acid were from Sigma Chemical Co., St. Louis, MO; standard Wheat Bran was from the American Association of Cereal Chemists, St. Paul, MN; agar was from Difco Laboratories, Detroit, MI; [methyl-^{3}H]-thymidine (69.4 Ci/mmole) was from ICN Pharmaceuticals Inc., Irvine, CA; tauro[carbonyl-^{14}C]cholic acid (54 mCi/mmole) was from Amersham/Searle, Arlington Heights, IL.

Effect of DMH and Intrarectal Bile Acid Injections on Colon Epithelial Cell Proliferation. Weanling male Sprague-Dawley rats (Spartan Research Animals, Haslett, MI) were housed in groups of 5 to 7 and were given an open-formula, cereal-based diet and water ad libitum. The animal room was temperature- and humidity-controlled and was on a 12-hour light-dark cycle.

One day after receiving the rats, I injected twenty rats subcutaneously with 200 mg of DMH dihydrochloride per kg body weight. Before injection, DMH was dissolved in saline (20 mg/ml) and adjusted to pH 6.8 with NaOH. Sixteen of the rats were injected with an equivalent amount of saline.

One week after the DMH injections, intrarectal injections were started. Rats were divided into three groups: one-third received intrarectal injections of deoxycholic acid, one-third received lithocholic acid, and one-third received saline. Before injection, bile acids were dissolved in saline (50 mg/ml) which had been adjusted to pH 9.5 with NaOH. The animals were injected with a 1 ml syringe fitted with a 5 cm. long stainless-steel animal-feeding bio-medical needle (Popper and Sons, Inc., New Hyde Park, NY; 18 ga. x 2). The needle was inserted 5 cm into the colon and 0.2 ml of the solution (10 mg of the bile acid) was injected. The rats were injected five days per week for 4 weeks. The day after

the last injection, the rats were sacrificed. One hour before sacrifice, the rats were injected intraperitoneally with ^{3}H-thymidine (0.5 uCi per gram body weight). After the rats were sacrificed (by overexposure to ether gas), the colon was removed and rinsed with water. The colon was measured and a 1 cm segment one-tenth of the distance from the anus to the ileocecal valve (about 2 cm) was removed and fixed in 10% buffered neutral formalin. The tissue was embedded in paraffin and sectioned at 6 um. After rehydration, the sections were dipped in Kodak NTB-2 nuclear track emulsion. After a four-week exposure time at 4^{o}C, the slides were developed in Dektol and then stained with Harris' hematoxylin and eosin. For each rat, the number of cells and the number of labeled cells were determined in 25 crypts. A cell was considered labeled if the nucleus was covered with five or more grains.

Effect of Diet and DMH on Colon Epithelial Cell Proliferation. Weanling male Sprague-Dawley rats were housed as described earlier. One day after receiving the rats, I injected half with 200 mg/kg of DMH dihydrochloride as described earlier.

One week after the DMH injections, the rats were started on purified diets. During the dietary period, the rats were housed individually in stainless-steel, hanging-wire cages. Five diets were fed: a low-fat, low-fiber control diet; a high-fat diet; a wheat bran diet; an agar

TABLE 5. Composition of Diets (Percent of Diet)

	Control	High-fat	Wheat bran	Agar	Carrageenan
Casein	20.0	27.5	15.9	18.5	18.5
Methionine	0.3	0.4	0.3	0.3	0.3
Choline	0.2	0.3	0.2	0.2	0.2
Vitamin mix	1.0	1.4	0.9	0.9	0.9
Mineral mix	4.0	5.5	3.7	3.7	3.7
Corn oil	5.0	37.0	4.0	4.6	4.6
Glucose	69.5	27.9	54.4	64.4	64.4
Wheat bran	---	---	20.6	---	---
Agar	---	---	---	7.4	---
Carrageenan	---	---	---	---	7.4

[1]American Institute of Nutrition, 1976.

[2]Draper et al., 1964.

diet; and a carrageenan diet (Table 5). Twelve rats were placed on each diet: six which had been injected with DMH and six which had not. I assumed that no calories were derived from agar or carrageenan. Wheat bran (which was found to contain 36% neutral detergent fiber) was added to give the same level of fiber as the agar and carrageenan diets. The amount of corn oil, casein, and glucose in the diet were adjusted to account for the amount of fat, protein, and carbohydrate in the wheat bran to make the diet isocaloric and isonitrogenous. Water and food were provided ad libitum for a 28 day feeding period. On the 28th day, the rats were sacrificed and the tissues were processed as described earlier.

On the 27th and 28th days of the dietary period, 24-hour fecal collections were made for bile acid analysis. The two collections from each rat were pooled and ground to form a powder. The fecal powder from two rats was pooled for analysis. ^{14}C-taurocholic acid was added as an internal standard to allow correction for incomplete recoveries. Bile acids were extracted from feces by a modification of the method described by Evrard and Janssen (1968). Feces were saponified directly in 20% KOH in ethylene glycol at 220^{o}. The samples were gassed with nitrogen before and during saponification to prevent oxidation of the bile acids. After saponification, neutral sterols were extracted with petroleum ether; next,

after acidification with concentrated HCl, bile acids were extracted 3 times with diethyl ether and twice with ethyl acetate. After methylation with etheral diazomethane, bile acids were separated from fatty acids and bile pigments by thin-layer chromatography (TLC) as described by Grundy et al. (1965). The bile acid solutions were applied to silica gel TLC plates. The plates were first developed in hexane: diethyl ether (70:30) to elute fatty methyl esters. Next, the plates were developed in ethyl acetate:acetone (70:30) to just below the fatty methyl ester band. The area of the plates containing the bile acids (just below the fatty methyl esters to just above the origin, which contained the bile pigments) was scraped off the plates into a test tube. After elution from the gel with chloroform:methanol (2:1), the bile acids were applied to a second TLC plate, which was developed in chloroform-acetone-methanol (70:25:5) as described by Madsen et al. (1976). After development, the plate was divided into three zones: a lower zone, with mainly cholic acid; a middle zone, with mainly hyodeoxycholic acid; and an upper zone with mainly deoxycholic and lithocholic acids. Twenty percent of each bile acid fraction was used for liquid scintillation spectroscopy to determine extraction efficiencies. Extraction efficiencies ranged from 40% to 75%; I assumed constant extraction efficiencies for all bile acids.

5-α-cholestane was added to the remaining 80% of the bile acids as an internal standard for gas-liquid chromatography (GLC). The bile acids were silylated (Grundy et al., 1965) and the TMS ethers were separated by GLC with a Varian Model 3700 gas chromatograph equipped with a flame ionization detector. The bile acids were separated on a 1.8 meter long, 3.2 mm diameter stainless-steel column packed with 3% SP-2100 on 100/120 Supelcoport. The injector temperature was 250° and the detector temperature was 270°. The column temperature was held initially at 210° for 10 minutes, then increased 3° per minute to 270°, and held at 270° until the last TMS ether had eluted. The following bile acids were used for standards: deoxycholic acid, murocholic acid, chenodeoxycholic acid, 3α,7α-ol, 12-one-5B-cholanic acid, 3α-ol, 7,12-one-5B-cholanic acid, lithocholic acid, cholic acid, and hyodeoxycholic acid. The bile acids were quantitated with a Varian CDS-111 integrator.

Statistics. Data was analyzed using 2-way analysis of variance. When significant f values were detected, treatment means were compared to the control mean using Bonferroni t statistics. If no significant interactions were seen, data for the DMH- and saline-injected rats was grouped together when analyzing for differences due to diet or intrarectal bile acid injections; if significant interactions were seen, data for DMH- and saline-injected

rats was analyzed separately. Differences between DMH- and saline-injected rats could be detected by the analysis of variance, if no significant interactions were seen (Gill, 1978).

RESULTS

Effect of DMH and Intrarectal Bile Acid Injections on Colon Epithelial Cell Proliferation.

Rats which were injected with DMH gained less weight than those not injected ($P < .01$); the final body weight of the rats injected with DMH was 208 $\pm$ 7 grams, whereas the average weight for controls was 263 $\pm$ 9 grams. Bile acid injections did not affect weight gain.

Table 6 shows the number of epithelial cells per crypt, the number of labeled cells, and the labeling index as affected by DMH or bile acid injections. A labeled cell is one which underwent DNA synthesis during the time period between ^{3}H-thymidine injection and sacrifice of the rat, and the labeling index is the percent of cells undergoing DNA synthesis. Rats injected with DMH had significantly more cells per crypt ($P < .05$), labeled cells per crypt ($P < .01$), and a higher labeling index ($P < .01$). Lithocholic acid injections increased the number of cells per crypt ($P < .05$), but did not affect the labeling index or the number of labeled cells per crypt. Deoxycholic acid injections slightly increased the number of cells per crypt, but again did not alter the labeling index or the

Table 6. Effect of 1,2-Dimethylhydrazine and Bile Acid Injections on Colon Epithelial Cell Kinetics

	No. of Cells per Crypt[1]	No. of Labeled Cells per Crypt[1]	Labeling Index[1,2]
DMH-treated Rats[3]			
Control(6)	72.5±2.4[4]	8.5±0.4	11.8±0.6
Deoxycholic acid(7)	79.0±2.6	9.7±0.5	12.4±0.5
Lithocholic acid(7)	80.7±2.9[5]	8.5±1.0	10.4±0.9
Saline-treated Rats			
Control(6)	69.0±1.4	6.2±0.5	9.1±0.7
Deoxycholic acid(5)	72.5±3.2	6.6±0.3	9.2±0.7
Lithocholic acid(5)	75.6±2.3[5]	7.5±0.8	10.0±0.8

[1]The values in DMH-treated rats were different from values from saline-treated rats ($P<.05$ by analysis of variance).

[2]Labeling Index = (No. of Labeled Cells/Total Cells) x 100

[3]The number of rats per treatment is indicated in parentheses.

[4]Results are means ± SEM

[5]Values are different from values in control (i.r. saline-injected) rats ($P<.05$ by Bonferroni t-test). Values from DMH-treated rats and saline-treated rats (for a particular intrarectal injection) were combined because no significant interactions were seen in the analysis of variance.

number of labeled cells per crypt.

Effect of Diet and DMH on Colon Epithelial Cell Proliferation. As indicated above, rats injected with DMH gained less weight and weighed less at the end of the experiment (302 ± 5 grams) than those not injected (316 ± 4 grams) ($P < .05$); diet did not affect weight gain.

The effects of diet and DMH injections on the total number of cells per crypt, number of labeled cells, and labeling index are shown in Table 7. Rats which were injected with DMH had slightly more cells per crypt and labeled cells per crypt, but the labeling index was not changed. Generally, dietary treatments did not affect colon epithelial cell proliferation. Dietary agar, however, did increase the total number of cells per crypt ($P < .05$). Dietary wheat bran slightly increased the total cell number.

Effect of Diet and DMH on Fecal Bile Acid Concentration, Excretion, and Metabolites. Table 8 shows the effect of diet and DMH on total fecal bile acid concentrations and excretion. All three fiber diets significantly lowered fecal bile acid concentrations ($P < .01$); dietary fat and DMH injections, however, did not affect them. Bile acid excretion, on the other hand, was significantly lower in rats which had received DMH injections ($P < .05$). Dietary wheat bran significantly increased bile acid excretion ($P < .05$); the other diets had no effect.

Table 7. Effect of 1,2-Dimethylhydrazine Injections and Diet on Colon Epithelial Cell Kinetics.

	No. of Cells per Crypt	No. of Labeled Cells per Crypt	Labeling Index
DMH-treated Rats			
Control	69.2±6.6 [1]	6.4±1.1	9.1±1.1
High-fat	67.5±2.4	5.1±0.4	7.6±0.7
Wheat bran	72.2±2.1	6.4±0.3	9.0±0.7
Agar	73.8±8.4 [2]	7.1±0.8	9.5±0.6
Carrageenan	72.6±3.0	6.5±0.7	8.9±0.6
Saline-treated Rats			
Control	57.6±2.1	3.5±0.5	6.0±0.9
High-fat	65.2±2.9	6.0±0.9	9.1±1.2
Wheat bran	71.8±2.1	4.5±0.7	6.3±0.9
Agar	75.5±5.2 [2]	6.5±1.6	8.7±1.8
Carrageenan	64.7±3.8	6.3±0.2	9.8±0.4

[1]Results are means ± SEM.

[2]Values are different from values in rats fed the control diet ($P < .05$ by Bonferroni t-test). Values from DMH-treated rats and saline-treated rats (for a particular diet) were combined because no significant interactions (between diet and DMH injections) were seen in the analysis of variance.

Table 8. Fecal Bile Acid Concentrations & Excretion in Rats Fed Various Diets

	Bile Acid Concentration (mg/g feces)	Bile Acid Excretion[1] (mg/day)
DMH-treated Rats		
Control	19.9±4.4 [2]	10.5±2.4
High-fat	19.4±1.3	14.7±0.9
Wheat bran	8.2±1.0 [3]	20.4±3.0 [3]
Agar	1.9±0.2 [3]	6.5±1.0
Carrageenan	2.7±0.5 [3]	9.9±2.2
Saline-treated Rats		
Control	17.5±4.5	10.7±2.3
High-fat	16.2±2.4	14.6±2.7
Wheat bran	7.7±1.4 [3]	27.0±5.2 [3]
Agar	5.9±0.8 [3]	18.6±3.5
Carrageenan	3.1±0.3 [3]	12.8±1.9

[1]Rats injected with DMH had lower bile acid excretion ($P<.05$ by analysis of variance).

[2]Results are means ± SEM.

[3]Values are different from values in rats fed control diet ($P<.05$ by Bonferroni t-test). Values from DMH-treated rats and saline-treated rats (for a particular diet) were combined for the Bonfferoni t-test because no significant interactions were seen (between diet and DMH injections) in the analysis of variance.

The individual bile acid pattern varied somewhat with diet, especially in saline-treated rats (Table 9). The proportion of cholic acid was altered significantly, in saline-treated rats but not in DMH-treated rats. In rats fed the high-fat, wheat bran, and carrageenan diets, the percent of bile acids present as cholic acid was lower than in rats fed the control diet ($P<.05$); rats fed agar had a slightly lower percentage ($P<0.1$) than controls. In rats fed the wheat bran diet, the percent of lithocholic acid was greater and the percent of deoxycholic acid was less than controls, in both DMH- and saline-treated rats ($P<.05$). In rats fed the high-fat and carrageenan diets, deoxycholic acid was the major metabolite, but it was not significantly higher than in controls. Hyodeoxycholic acid was the major metabolite in saline-treated rats fed the agar diet.

DISCUSSION

These experiments show that, when colon bile acid concentrations were altered for a four-week period, colon epithelial cell kinetics were not greatly affected. When bile acid levels in the colon were altered by diet (as shown by bile acid analysis) or bile acid intrarectal injections, neither the number of labeled cells per crypt nor the labeling index changed. The total number of cells per crypt, however, was increased significantly by lithocholic acid and dietary agar, and increased slightly

Table 9. Effect of Diet and 1,2-Dimethylhydrazine on Individual Fecal Bile Acid Metabolites

	Individual Bile Acids (% of Total)				
	Cholic	Lithocholic	Deoxycholic	Hyodeoxycholic	Others
DMH-treated Rats					
Control	17.6±7.7[1]	2.5± 0.0	28.0±10.0	17.5± 1.5	34.4± 6.8
High-fat	15.1±6.4	7.8± 1.1	35.0± 7.7	11.1± 3.2	31.1± 1.9
Wheat bran	12.1±3.1	34.4±15.0[3]	7.9± 4.7[3]	12.0± 3.6	33.6± 4.8
Agar	26.4±8.6	5.6± 1.4	16.2± 5.3	15.9± 2.6	35.8± 8.3
Carrageenan	3.9±1.6	10.9± 5.5	35.1± 8.5	10.0± 5.7	40.2± 4.6
Saline-treated Rats					
Control	28.3±6.6	3.9± 0.9	25.1± 3.4	14.3± 3.9	28.5±11.4
High-fat	6.9±3.2[2]	12.3± 2.3	37.4± 5.6	24.3± 3.6	19.2± 1.8
Wheat bran	3.6±1.7[2]	53.9±13.4[3]	4.0± 1.8[3]	3.6± 0.4	34.8±12.7
Agar	7.7±2.0	2.0± 0.5	6.8± 2.6	36.3±16.2	47.2±15.7
Carrageenan	2.5±0.1[2]	7.8± 1.5	35.5±14.8	7.6± 2.3	46.6±14.0

[1]Results are means ± SEM.

[2]Values are different from values from rats fed the control diet ($P<.05$ by Bonferroni t-statistics). Values from DMH- and saline-treated rats were analyzed separately because a significant interaction between diet and type of injection was seen in the analysis of variance.

[3]Values are different from values from rats fed the control diet ($P<.05$ by Bonferroni t-statistics). Values from DMH- and saline-treated rats (for a particular diet) were combined for analysis because no significant interactions were seen in the analysis of variance.

by deoxycholic acid and dietary wheat bran. These increases may indicate a low response to that particular treatment - perhaps a slight hyperplasia. Since both experiments exposed the rats to an altered bile acid level in the colon for a relatively long period of time, an acute initial effect, such as a higher labeling index, may have subsided after 4 weeks, as the rats adapted to the different bile acid concentrations. A prior DMH injection does not appear to be necessary for the action of the bile acids (i.e. no statistical interactions were seen). Thus, the changes in epithelial cell number took place whether or not initiation had occurred.

Dietary agar and wheat bran increased cell number independently of the total bile acid concentration. Total bile acid concentrations were lower in rats fed dietary agar or wheat bran. This implies that these fibers may be acting directly on the colon mucosa. Carrageenan, the other fiber fed, had no effect on colon epithelial cell kinetics even though it lowered fecal bile acid concentrations. Dietary fat did not affect either parameter.

Individual fecal bile acid metabolites were altered by diet, especially in rats which did not receive DMH. In all fiber-fed rats which did not receive DMH, fecal bile acids were more extensively degraded than in rats fed the control diet. This may be produced by a change in bacterial populations or bacterial enzyme activities

caused by the introduction of these plant fibers into the colon. Because of the large amount of fermentable material which is introduced, bacterial enzyme activities would likely change in order to exploit this food source. Similarly, changes caused by dietary fat may have resulted from an increase in the amount of fat or other materials entering the colon.

Despite these changes, it is unclear whether individual fecal bile acid concentrations altered colon epithelial cell kinetics. In rats fed agar, individual bile acid changes were seen, but the metabolite concentrations were still below control levels. In rats fed wheat bran, the large increase seen in lithocholic acid levels could possibly be responsible for the slight increase in colon epithelial cell number. But the total bile acid concentration was still less than half that seen in rats fed the control diet.

Bile acid excretion was altered by DMH injection and by dietary wheat bran. Wheat bran increased total fecal bile acid excretion ($P<.05$). This finding implies that bile acids bound to wheat bran in the intestine. Rats injected with DMH had lower bile acid excretion. This could be caused by liver toxicity from DMH injections or it could be related to the weight loss seen in the DMH-injected rats.

In other studies, varying bile acid levels in the

colon has produced an acute response. When bile acids are prevented from reaching the colon by a bile fistula, colon epithelial cell proliferation is reduced after two days (Deschner and Raicht, 1979). And when the amount of bile acids in the colon is increased by feeding 0.2% cholic acid for 3 days, colon epithelial cell proliferation is increased (Deschner et al., 1981). In addition, colon ornithine decarboxylase and S-adenosyl-methionine activities are increased by intrarectal bile acid injections (Takano et al., 1981).

Over a longer period of time, however, the effect of changing the bile acid concentration in the colon is less clear. After a two-week time period, dietary wheat bran increases the number of epithelial cells in colon crypts (Jacobs & Schneeman, 1981). Deschner et al., (1981) found that, when 0.2% cholic acid is added to the diet, cell proliferation is still increased after 26 weeks. But Cohen et al. (1980), in a similar experiment, found that feeding 0.2% cholic acid does not affect proliferation after 5 or 19 weeks. Our experiments support the view that the colon gradually adapts to changes in bile acid concentration over a period of time. Changes in the DNA synthesis rate may have returned to base values by the time the experiment was ended.

The injection of a large dose of DMH increased colon epithelial cell proliferation, especially in the first

experiment. In earlier experiments, DMH has been shown to have varying effects on cell proliferation. Immediately after DMH injection, DNA synthesis in colon epithelial cells decreases and many of the cells degenerate, because of the toxicity of DMH (Chan et al., 1976; Chang, 1981; Sunter et al., 1981). Next, an increase in cell proliferation is seen as the dead cells are replaced (Chan et al., 1976; Chang, 1981; Sunter et al., 1981). If the dose of DMH is low enough (and the treatment period is short enough), the number of cells in each crypt will return to baseline levels (Richards, 1981). But when DMH is injected repeatedly over a long period of time, crypt lengths do not return to normal (Richards, 1981).

Changes which were seen in epithelial cell proliferation sometimes were consistent with changes seen in colon carcinogenesis. The increase in cell proliferation caused by DMH is consistent with its carcinogenic effect in the colon. The increases in the number of cells per colon crypt seen after intrarectal bile acid injections are consistent with the enhancement of MNNG-induced carcinogenesis seen after intrarectal bile acid injections (Reddy et al., 1978). And the increase in colon cell number seen after feeding dietary agar corresponds to its effect on colon carcinogenesis.

For the other diets fed, however, their effects on colon cell proliferation were inconsistent with their

effects on colon carcinogenesis. Dietary fat did not alter epithelial cell kinetics, even though in most studies it has been shown to increase DMH-induced colon carcinogenesis. The lack of a change in bile acid concentration or excretion when the dietary fat level is increased may explain the absence of a change in colon cell number or proliferation. When wheat bran was fed, a slight increase was seen in cell number even though 1) fecal bile acid concentrations were lowered and 2) wheat bran is known to decrease chemically-induced colon carcinogenesis. Apparently the physiological change in bile acid concentration produced by wheat bran was not as important as other changes produced. The increase in cell number seen in this study and in the study by Jacobs and Schneeman (1981) are not consistent with the decrease seen in colon carcinogenesis when wheat bran is fed. Finally, when carrageenan is fed, no change in cell proliferation was seen even though carrageenan enhances chemically-induced colon carcinogenesis.

Several conclusions can be drawn from this study. First, dietary agar and lithocholic acid intrarectal injections slightly enhanced colon epithelial cell proliferation. Both treatments increased the number of cells per crypt, although they did not affect the number of cells synthesizing DNA or the DNA synthesis rate. In addition, dietary wheat bran and deoxycholic acid

intrarectal injections both slightly increased the number of cells per crypt. Second, dietary agar's (and dietary wheat bran's) effect was not mediated through bile acids: their concentration in feces was lower in rats fed these diets. Finally, DMH injections increased the total number of cells per crypt and the number of labeled cells per crypt.

CHAPTER 5 . . . . METABOLISM OF 1,2-DIMETHYLHYDRAZINE BY CULTURED RAT COLON EPITHELIAL CELLS

INTRODUCTION

1,2-Dimethylhydrazine (DMH) is a chemical which specifically induces colon tumors in several rodent species (Fiala, 1977). The tumors which it induces are histologically similar to those seen in humans. Therefore, DMH has been used as a model carcinogen in animals to study colon carcinogenesis.

The mechanism by which DMH induces colon tumors has been the subject of much research. DMH is metabolized through a series of reactions to the methyldiazonium ion, which then methylates nucleophilic sites in the cell - DNA, RNA, and protein (Fiala, 1977; Miller, 1978). After the injection of labeled DMH in vivo, labeled azomethane (AM) is exhaled, labeled azoxymethane (AOM) and methylazoxymethanol (MAM) are excreted into the urine, and labeled methyl groups bind to DNA in the colon and other organs (Fiala et al., 1976a; Fiala et al., 1976b; Fiala et al., 1977; Rogers and Pegg, 1977; Likhachev et al., 1977; Cooper et al., 1978; Herron and Shank, 1981). From these experiments, Fiala (1977) has proposed that DMH is metabolized to AM, AOM, MAM, and finally to the methyldiazonium ion, which then methylates DNA.

Although DMH induces colon tumors, it has not been established whether DMH can be completely metabolized by colon epithelial cells. Weisburger (1971) hypothesized that DMH is partially metabolized in the liver and the gut

before reaching the colon. Autrup et al. (1977, 1978) found that colon organ cultures metabolize DMH to products which bind to DNA; however, DMH could have been partially metabolized by other cell types in the culture or by bacteria in the culture.

In this study, I sought to determine if DMH can be metabolized by isolated colon epithelial cells. Cells were cultured with labeled DMH. The metabolism of DMH was quantitated in three ways: gaseous metabolites leaving the culture were trapped, metabolites in media were separated and quantitated by high performance liquid chromatography (HPLC), and DNA from the cells was isolated to measure the binding of DMH metabolites to DNA. Cells from both conventional and germfree rats were used to insure that DMH was not being metabolized by intestinal bacteria contaminating the culture. DMH was also incubated in media without cells to see if it degraded spontaneously. I found that cells from both conventional and germfree rats metabolize DMH to gaseous metabolites, metabolites in media, and metabolites which bind to DNA.

MATERIALS AND METHODS

Chemicals. ^{14}C-1,2-dimethylhydrazine (DMH) dihydrochloride (8.4 mCi/mmole) was from New England Nuclear, Boston, MA; unlabeled DMH dihydrochloride, bovine pancreatic ribonuclease (Type 1-A), T_1 ribonuclease (Grade V), DNA (Type 1), porcine liver esterase (Type 1),

azoxymethane (AOM), and methylazoxymethanol (MAM) acetate were from Sigma Chemical Co., St. Louis, MO; proteinase K was from Boehringer Mannheim Biochemicals, Indianapolis, IN; fetal bovine serum, CMRL-1066 media without glutamine, Penicillin-Streptomycin solution, amphotericin B, and a solution of non-essential amino acids were from Grand Island Biological Company, Grand Island, NY.

Animals. Germfree 250 gm. male Sprague-Dawley rats were from Harlan Industries, Cumberland, IN. Colon epithelial cells were isolated from the rats the morning after they were delivered by the supplier. Conventional male Sprague-Dawley rats (250 gm.) were from Spartan Research Animals, Haslett, MI. These rats were housed in groups of 2 or 3 until sacrifice and were fed an open-formula, cereal-based diet. The animal room was temperature- and humidity-controlled and was on a twelve-hour light-dark cycle.

Isolation and Cultivation of Colon Epithelial Cells. Colon epithelial cells were isolated using a method similar to that of Weiser (1973). Two rats were used per experiment. Rats were sacrificed by decapitation and the colons were excised at the anus and the ileocecal valve. Next, the colons were rinsed with a 0.154 M NaCl, 1mM dithiothreitol solution to remove the colon contents. Next, one end of the colon was tied with thread, the colon was filled with solution A (1.5mM KCl, 96mM NaCl, 27mM

sodium citrate, 8mM KH_2PO_4, 5.6mM Na_2HPO_4, pH 7.3) and the other end of the colon was tied. The colon was then placed into a sterile beaker containing sterile saline heated to 37°; this was placed in a humidified incubator heated to 37° and incubated for 15 minutes. This step removes residual fecal material and mucus from the colon. After the incubation, solution A and the residual contents were drained from the colon and discarded. Next, the colon was filled with solution B (70mM NaCl, 5.6mM KH_2PO_4, 3.9mM Na_2HPO_4, 1.1mM KCl, 1.5mM EDTA, and 0.5mM dithiothreitol) and reincubated for 20 minutes as described above. This step causes dissociation of the epithelial cells from the intestinal mucosa. After incubation, the solution was drained into a sterile centrifuge tube. The colon was then rinsed four times with a phosphate-buffered solution (70mM NaCl, 5.6mM KH_2PO_4, 3.9mM Na_2HPO_4 and 1.1mM KCl) and agitated by hand to obtain maximum dissociation of the epithelial cells. These rinses were added to the centrifuge tube and the cells were obtained by centrifugation. Krebs-Ringer bicarbonate buffer (0.154 M NaCl, 0.154 M KCl, 0.11 M $CaCl_2$, 0.154 M KH_2PO_4, 0.154 M $MgSO_4$, 0.154 M $NaHCO_3$, 0.1 M Na_2HPO_4, pH 7.4) containing 2 mM glucose was then added to the cell pellet. Cells were mixed with the buffer by drawing the mixture into a sterile 10-ml serological pipet 3 or 4 times. The cells

were recentrifuged and the pellet was mixed similarly with 10 ml of the culture media. Two 5-ml aliquots of the cell suspension were transferred to 150 mm plastic petri dishes. 15 ml of media was then added to the cell suspension. The culture media used was CMRL-1066 without glutamine and was supplemented with glucose (5 mg/ml), fetal bovine serum (5%), hydrocortisone (0.67 ug/ml), penicillin (125 Units/ml), streptomycin (125 ug/ml), amphotericin B (2 ug/ml), minimum essential media non-essential amino acids (10 mM for each amino acid). All isolation and culture procedures were done in a clean air hood using conventional aseptic technique.

Cell Viability. Cell membrane integrity was assessed by the trypan blue exclusion method. Cell suspension in media was made 0.07% with trypan blue and stained and unstained cells were counted in a hemocytometer after 5 minutes exposure. A cell containing any amount of blue stain was considered non-viable. The viability of the cells was also assessed by examining the oxygen consumption of the cells. 0.5 ml of cell suspension in media was mixed with oxygenated Krebs Ringer bicarbonate buffer solution containing 2 mM glucose. The disappearance of oxygen from the solution was measured with a Biological Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, OH).

Incubation of Colon Epithelial Cells with DMH. ^{14}C-DMH

dihydrochloride was diluted 50% with unlabeled DMH dihydrochloride before it was added to the culture. DMH dihydrochloride was added to the media at a concentration of 8 ug/ml (0.25 uCi/ml or 10 uCi total in 2 petri dishes). This concentration was chosen because it did not affect cell viability (measured by either trypan blue or O_2 consumption), and because it was found in preliminary experiments that this dose produced a high level of binding to total cellular macromolecules, which were isolated as described by Munro and Fleck (1968). The two petri dishes were placed in a humidified, controlled atmosphere culture chamber (Bellco Glass, Inc., Vineland, NJ) which was located inside a larger incubator (National Appliance Co., Model 3331-2, Portland, OR). The cells were incubated at 37^{o} in an atmosphere consisting of 95% air and 5% CO_2. This mixture was constantly drawn through the culture chamber. All gases leaving the incubator were passed through a series of three flasks: two containing 100 ml of ethanol cooled to -72^{o} by a surrounding ethanol-dry ice bath and a third containing 500 ml of H_2SO_4. These solutions will trap radioactive AM leaving the incubator (Fiala, Kulakis, Bobotas, and Weisburger, 1976). One ml aliquots of the fluids were withdrawn every hour. 15 ml of triton-toluene (1:2) containing 2,5-diphenyloxazole (4 grams/l) and 1,4 bis[2-(4-methyl-5-phenylazolyl)]-benzene (0.2 grams/liter)

was added to each aliquot and the amount of ^{14}C present was determined by liquid scintillation spectrometry. The cells were incubated for 6 hours. At this time the plates were removed from the incubator, and the cell suspensions were transferred to a single 50 ml centrifuge tube. After centrifugation for 6 minutes at 2500 x g, the culture media was transferred to another centrifuge tube, frozen, and stored at -20°. The media was later analyzed by HPLC to determine the metabolism of DMH to other metabolites. The cell pellet was used to isolate DNA.

This experiment was conducted four times using 8 conventional rats, twice using 4 germfree rats, and twice using media alone.

Isolation of DNA. DNA was isolated from the cells by a phenol extraction method. Cells were vortexed for 5 minutes in a lysing solution (2% Triton X-100, 2mM $CaCl_2$, 2mM $MgSO_4$, 30mM KCl, and 100mM NaCl). After centrifugation at 2500 x g for 5 minutes, nuclei were resuspended in 4 ml of 0.1M Tris buffer (pH 7.0) containing 1% sodium lauroyl sarcosine and Proteinase K (0.1 mg/ml) and were then vortexed until a clear, viscous solution was formed. The solution was then extracted with equal volumes of chloroform:phenol (1:1) until the protein interface had disappeared. Between extractions, the solutions were centrifuged at 6500 x g for 20 minutes. Next, the supernatant layer was incubated with bovine

pancreatic ribonuclease (125 ug/ml) and T_1 ribonuclease (100 Units/ml) for 30 minutes at 37°. The solution was then re-extracted with chloroform-phenol until the protein interface had disappeared. DNA was precipitated by the addition of an equal volume of ethanol. After centrifugation at 12,000 x g for 20 minutes at 4°, the DNA was washed once with ethanol, 3 times with ether, and then dried. The DNA was redissolved in water and its UV absorption at 260 nm was determined with a Beckman ACTA III UV-visible double beam Spectrophotometer. DNA standards ranging from 10 ug/ml to 100 ug/ml were prepared in water; the DNA concentration of the cell isolates was estimated from the DNA standard curve. The DNA solutions were then mixed with liquid scintillation fluid and the amount of ^{14}C bound to DNA was determined by liquid scintillation spectrometry.

Separation of DMH and Its Metabolites by HPLC. DMH and its metabolites AOM and MAM were separated similar to a method described by Fiala, Bobotas, Kulakis, and Weisburger (1976). Media from the cell cultures was thawed, centrifuged, and filtered through a 0.45 um filter. 100 ul of media was then injected into the HPLC. The HPLC consisted of a Tracor 980A solvent programmer, a Milton Roy Co. mini Pump (model 396), a Valco injector (model SVOV-6-1), a Whatman Partisil PXS10/25 ODS-2 column, and an LDC Spectro Monitor III flow cell

spectrophotometer (The eluant was monitored at 217 nm). Fractions were collected in an ISCO fraction collector, model 272, equipped with an ISCO volumeter, model 400. The solvent programmer was programmed as follows: 0-4 minutes, 100% water; 4-14 minutes, a linear water to methanol gradient (10%/min.); 14-35 min., 100% methanol; and 35-60 minutes, 0.5 M phosphoric acid. Ten minutes were required for the solvent to flow from the solvent programmer to the detector. The flow rate was 1 ml/min. Fractions were collected every 20 seconds for the first 20 min., and every minute from 20-60 min. Scintillation fluid (6 ml) was added to the fractions, which were then counted using liquid scintillation spectrometry. DMH, AOM, and MAM were used as standards. AOM and MAM were monitored at 217 nm. Before injection into the HPLC, MAM acetate was reacted with porcine liver esterase for 30 minutes at 37^{o} to release free MAM (Fiala, Bobotas, Kulakis, and Weisburger, 1976). Labeled DMH was injected into the HPLC and fractions were collected to determine its elution time. All standards were diluted in media before use.

RESULTS

Cell Viability. The concentration of cells in the media averaged $6.44 \pm 0.69 \times 10^6$ cells/ml from conventional rats and $8.62 \pm 2.74 \times 10^6$ cells/ml from germfree rats. In freshly isolated cells, 44.6 ± 4.1% of the cells from

conventional rats and 45.4±0.4% of the cells from germfree rats excluded the trypan blue dye. The ability to exclude dye decreased during the culture period; by 10 hours, all cells permitted some dye to pass through the cell membrane and respiration had greatly decreased. The addition of DMH up to 8 ug/ml did not affect cell viability or respiration. Because of decreased viability with incubation time, cells were incubated for 6 hours in all subsequent experiments.

Metabolism of DMH to Gaseous Metabolites. Gaseous metabolites were captured by passing gases leaving the incubator through ethanol at -72° and 1 M H_2SO_4. AM, which has a boiling point of 2°, was effectively trapped in cold ethanol: very little radioactivity was found in the H_2SO_4 bath. CO_2 is also released as a metabolic product of DMH; it could not be trapped, however, because of the large amount of CO_2 in the gas mixture which passed through the incubator.

The metabolism of DMH to gaseous metabolites is shown in Figure 7. Results are shown as total DPM captured rather than as DPM/cell so that these results could be compared with those when media alone was used. Metabolism by cells from germfree rats was greater than metabolism by cells from conventional rats; in both cases, the amount of gaseous metabolites released increased throughout the 6-hour incubation period. Metabolism of DMH was seen when

Figure 7. Metabolism of 1,2-Dimethylhydrazine to Gaseous Metabolites by Media or Media Plus Colon Epithelial Cells from Either Conventional or Germfree Rats. All gases leaving the incubator were trapped as described in the Materials and Methods. Results are expressed as the total DPM that were captured. Cultures from conventional rats had 6.4±0.7 million cells/ml of media; cultures from germfree rats had 8.6±2.7 million cells/ml of media. The DMH concentration in the media was 8 ug/ml (0.25 uCi/ml).

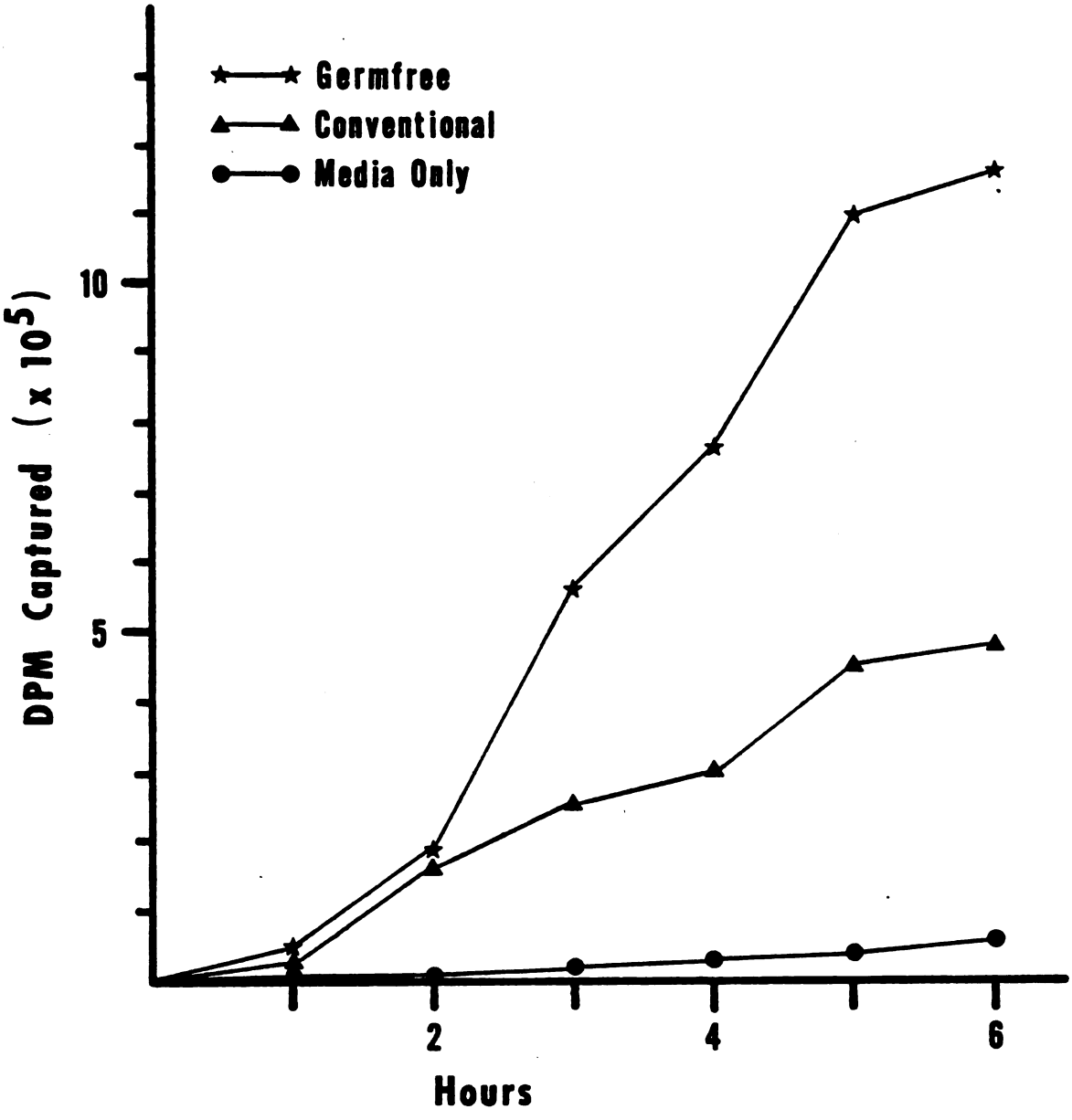

Germfree
Conventional
Media Only
DPM Captured (x 10^5)
10
5
2
4
6
Hours

Figure 8. High Performance Liquid Chromatography of Methylazoxymethanol, Azoxymethane, and 1,2-Dimethylhydrazine.

a. High Performance Liquid Chromatography of MAM, AOM, and DMH Standards. AOM and MAM were monitored at 217 nm. DMH was monitored by determining the radioactivity in 1 ml fractions. Four elution periods are noted on the graph: a) 0-4 min., 100% water; b) 4-14 min., a linear water to methanol gradient (10% per min.); c) 14-35 min., 100% methanol; and d) 35-60 min., 0.5 M phosphoric acid.

b. High Performance Liquid Chromatography of Media from a Culture Derived from Conventional Rats. Fractions were collected every 20 sec. for the first 20 min., and every min. from 20-60 min., and the amount of radioactivity in each fraction was determined.

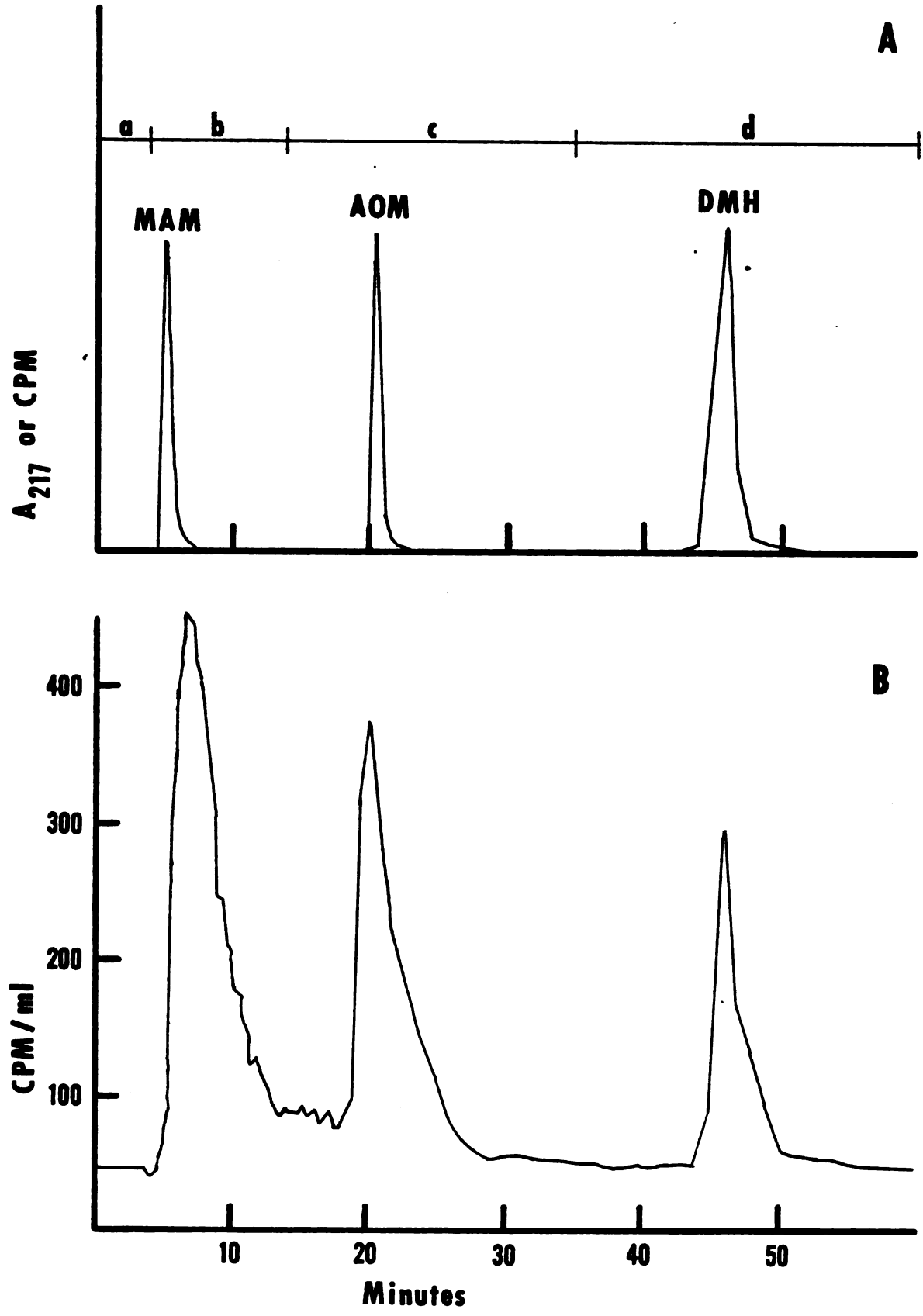

A
a
b
c
d
MAM
AOM
DMH
A_{217} or CPM
B
400
300
200
100
CPM/ml
10
20
30
40
50
Minutes

Table 10. Metabolism of 1,2-Dimethylhydrazine to Methylazoxymethanol and Azoxymethane in Media

	% of Total Radioactivity			% Degradation of DMH
	DMH	AOM	MAM	
Standard	80.2±3.1[1]	13.0±2.0	6.8±1.2	---
Media Only	67.5±3.5	15.5±3.8	17.3±0.3	15.8
Conventional	15.9±0.8	32.6±1.4	51.5±1.1	80.2
Germ-free	17.7±5.8	31.7±3.4	50.6±9.2	77.9

[1]Results are mean ±SEM

[2]% Degradation of DMH = 100 - $\frac{\text{\% DMH in sample}}{\text{\% DMH in standard}}$

DMH was incubated in media without cells; however, metabolism was much less than when cells were present. When DMH was incubated with cells from germfree rats, 2.6% of the total label was trapped in cold ethanol and H_2SO_4; with cells from conventional rats, 1.1% was trapped; and with media alone 0.1% was trapped.

Metabolism of DMH to Metabolites Found in Media. DMH and its metabolites AOM and MAM were separated by reverse-phase HPLC. MAM eluted at 7 minutes (immediately after the void volume), AOM eluted at 20 minutes (during the MeOH/H_2O gradient), and DMH eluted at 47 minutes (with phosphoric acid) (Fig. 8). When the labeled DMH standard was injected, 80% of the radioactivity was found in the DMH peak, with the rest in the AOM or MAM peaks (Table 10). When DMH was incubated in media containing no cells, some degradation to AOM and MAM was seen (15.8%). When DMH was incubated with colon epithelial cells - from either conventional or germfree rats - DMH was extensively degraded to MAM and AOM.

Binding of DMH Metabolites to Colon Epithelial Cell DNA. Colon epithelial cells from both conventional and germfree rats metabolized DMH to products which bound to DNA (Table 11).

DISCUSSION

These results clearly demonstrate that colon epithelial cells have the ability to metabolize DMH. When colon

Table 11. Binding of 1,2-Dimethylhydrazine Metabolites to DNA in Cultured Colon Epithelial Cells

Source of Cells	DPM Bound to DNA (DPM/ng DNA)
Conventional Rats	382±44[1]
Germ-free Rats	550±160

[1]Results are mean ± SEM.

epithelial cells were incubated with DMH, they metabolized it to gaseous metabolites (AM), to MAM and AOM in media, and to products bound to DNA.

Cells from germfree rats metabolized DMH at a higher or at a comparable rate for the criteria examined. Therefore, it is doubtful that bacteria, which contaminate the cultures derived from conventional rats, were responsible for the metabolism of DMH.

DMH was metabolized to products in the media and to gaseous metabolites when it was incubated with media alone. The metabolism of DMH in media could be caused by 1) spontaneous degradation of DMH or 2) metabolism by an enzyme from the fetal calf serum in the media. When cells were added to the media, the metabolism of DMH to metabolites in the media and to gaseous metabolites increased dramatically. Therefore, metabolism of DMH by the media plays only a minor role.

DMH metabolites bound to DNA in cells from both conventional and germfree rats. The adducts that formed with DNA could not be examined because of the low specific activity of the DMH and because of the short incubation period. The radioactivity in DNA was insufficient to enable the determination of specific adducts. When labeled DMH is injected in vivo, labeled methyl groups bind to DNA in the colon and in other organs (Rogers and Pegg, 1977; Likhachev et al. 1977; Cooper et al., 1978;

Herron and Shank, 1981). Methyl groups bind at both the N^7 and O^6 positions of guanine. When DMH is added to rat or human colon organ cultures, methyl groups also bind to DNA at the N^7 and O^6 positions of guanine (Autrup et al., 1977).

These data clearly show that metabolism by the liver or colon bacteria is not necessary for methylation of colon epithelial cell DNA. If extracolonic metabolism of DMH exists, its extent is unclear; the contribution of the liver and colon bacteria to colon DNA methylation and colon carcinogenesis is unknown. It is likely, though, that the complete metabolism of DMH by colon epithelial cells plays a major role in colon carcinogenesis.

CHAPTER 6 . . . 1,2-DIMETHYLHYDRAZINE METABOLISM IN THE RAT: ITS DIURNAL VARIATION AND ITS ALTERATION BY DIETARY FAT AND FIBER

INTRODUCTION

Diet has been shown to be a factor in the development of colon cancer in both epidemiological and experimental studies. Populations which consume high levels of dietary fat and low levels of dietary fiber have a high incidence of colon cancer (Correa and Haenszel, 1978). In most studies in experimental animals, dietary fat enhances the development of colon tumors (Reddy et al., 1974a; Reddy et al., 1976a; Reddy et al., 1977c; Nigro et al., 1975; Bull et al., 1979). Some dietary fibers, such as wheat bran, inhibit chemically-induced colon carcinogenesis; others, such as agar or carrageenan, enhance it (Barbolt and Abraham, 1978; Barbolt and Abraham, 1980; Watanabe et al., 1978; Watanabe et al., 1979; Wilson et al., 1977).

The mechanisms by which these dietary changes alter the development of chemically-induced colon tumors are still unclear. Several studies have suggested that diet influences colon carcinogenesis by altering the levels of bile acids in the colon: bile acids have been shown to be promoters in colon carcinogenesis (Narisawa et al., 1974; Reddy et al., 1976b; Reddy et al., 1977d). But diet might also influence the metabolism of the carcinogen which induces colon tumors.

In this study, I examined the effect of dietary fat, wheat bran, and agar on the metabolism of the colon

carcinogen 1,2-dimethylhydrazine (DMH). The binding of labeled DMH metabolites to the DNA of isolated rat colon epithelial cells was determined at several time intervals after the injection of labeled DMH. I isolated colon epithelial cells, rather than using the whole colon, because colon tumors originate from epithelial cells. In addition, the clearance of DMH and its metabolites from blood was determined, since DMH is thought to reach the colon primarily through the blood (Hawks and Magee, 1974). I found that diet did not alter the amount of DMH metabolites bound to DNA even though the level of DMH plus its metabolites in serum was altered by diet. A marked diurnal variation in DMH metabolism in colon epithelial cells was also seen.

MATERIALS AND METHODS

Chemicals. ^{14}C-1,2-dimethylhydrazine dihydrochloride (8.4 mCi/mmole) was from New England Nuclear, Boston, MA; unlabeled DMH dihydrochloride, bovine pancreatic ribonuclease (Type 1-A), T_1 ribonuclease (Grade V), and DNA (Type 1) were from Sigma Chemical Co., St. Louis, MO; proteinase K was from Boehringer Mannheim Biochemicals, Indianapolis, IN; TS-1 tissue solubilizer was from Research Products International, Elk Grove Village, IL; agar was from Difco Laboratories, Detroit, MI; standard wheat bran was from the American Association of Cereal Chemists, St. Paul, MN.

Experimental Protocol. Weanling male Sprague-Dawley rats (Spartan Research Animals, Haslett, MI) were housed individually in hanging-wire, stainless-steel cages. The animal room was temperature- and humidity-controlled and was on a 12-hour light-dark cycle. The rats were fed one of four purified diets: a low-fat, low-fiber control diet; a high-fat diet; a wheat bran diet; and an agar diet (Table 12). The diets had a constant energy to protein ratio. I assumed that no energy was derived from agar. Wheat bran (which was found to contain 36% neutral detergent fiber) was added to provide the same level of fiber as the agar diet. The amount of corn oil, casein, and glucose in the diet were adjusted to account for the amount of fat, protein, and carbohydrate in the wheat bran. Food and water were provided ad libitum. Diets were fed to the rats for 4 weeks.

At the end of this time period, rats were injected with DMH. Before injection, labeled and unlabeled DMH were dissolved in saline and adjusted to pH 6.8 with NaOH. The rats were injected s.c. with 10mg of DMH dihydrochloride per kg body weight and 25uCi of ^{14}C-DMH per kg body weight. All rats were injected between 9 A.M. and 10 A.M. At several time periods (12, 24, 36, 48, 60, 72, and 96 hours) after injection, rats were sacrificed by decapitation, blood was collected, and serum was obtained. Three or four rats from each diet

TABLE 12

COMPOSITION OF DIETS
(Percent of Diet)

	Control	High-fat	Wheat bran	Agar
Casein	20.0	27.5	15.9	18.5
Methionine	0.3	0.4	0.3	0.3
Choline	0.2	0.3	0.2	0.2
Vitamin mix[1]	1.0	1.4	0.9	0.9
Mineral mix[2]	4.0	5.5	3.7	3.7
Corn oil	5.0	37.0	4.0	4.6
Glucose	69.5	27.9	54.4	64.4
Wheat bran	---	---	20.6	---
Agar	---	---	---	7.4

[1]American Institute of Nutrition, 1976.

[2]Draper et al., 1964.

were sacrificed at each time point. After the colon was removed, colon epithelial cells were isolated using the method of Weiser (1973). The colon was incubated with the EDTA-dithiothreitol solution for 50 minutes. During the incubations, the colons were placed individually in a 250 ml beaker, covered with saline, and placed in a Dubnoff metabolic shaking incubator at 37°. After the incubation, the colon was manually agitated to obtain the maximum number of cells.

DNA was isolated from the cells by a phenol extraction method. Cells were vortexed for 5 minutes in a lysing solution (2% Triton X-100, 2mM $CaCl_2$, 2mM $MgSO_4$, 30mM KCl, and 100mM NaCl). After centrifugation at 2500 x g for 5 minutes, nuclei were resuspended in 4 ml of 0.1M Tris buffer (pH 7.0) containing 1% sodium lauroyl sarcosine and Proteinase K (0.1 mg/ml) and were then vortexed until a clear, viscous solution was formed. The solution was then extracted with equal volumes of chloroform:phenol (1:1) until the protein interface had disappeared. Between extractions, the solutions were centrifuged at 6500 x g for 20 minutes. Next, the supernatant layer was incubated with bovine pancreatic ribonuclease (125 ug/ml) and T_1 ribonuclease (100 Units/ml) for 30 minutes at 37°. The solution was then re-extracted with chloroform-phenol until the protein interface had disappeared. DNA was precipitated by the

addition of an equal volume of ethanol. After centrifugation at 12,000 x g for 20 minutes at 4^{o}, the DNA was washed once with ethanol, 3 times with ether, and then dried. The DNA was redissolved in water and its UV absorption at 260 nm was determined with a Beckman ACTA III UV-visible double beam Spectrophotometer. DNA standards ranging from 10 ug/ml to 100 ug/ml were prepared in water; the DNA concentration of the cell isolates was estimated from the DNA standard curve. The DNA solutions were then mixed with 15 ml of triton-toluene (1:2) containing 2,5-diphenyloxazole (4 grams/liter) and 1,4-bis[2-(4-methyl-5-phenylazolyl)]-benzene (0.2 grams/liter) and the amount of ^{14}C bound to DNA was determined by liquid scintillation spectrometry.

To determine radioactivity in serum, I added TS-1 tissue solubilizer to an aliquot of serum and digested the sample for 6 hours at room temperature. After the sample had been completely digested, the pH was adjusted to 7 with glacial acetic acid, liquid scintillation fluid was added, and the amount of radioactivity was determined by liquid scintillation spectrometry.

Statistics. Data was analyzed using analysis of variance. When significant f values were detected, treatment means were compared to the control mean using Bonferroni t statistics.

RESULTS

The weight gains and final body weights of the rats were not affected by diet.

Figure 9 shows the effect of diet on the binding of radioactive DMH metabolites to DNA from colon epithelial cells. Diet did not alter binding to DNA for any of the times examined. A diurnal variation, however, was evident. Rats which were killed at 9 P.M. (12, 36, and 60 hours) tended to have higher binding than those killed at at 9 A.M. (24, 48, 72, and 96 hours). After the rats were injected at 9 A.M., a large amount of binding occurred by 12 hours. At 24 hours, there was a slight increase in binding but not nearly as large as in the preceding 12 hour period. At 36 hours a large increase in binding was seen, followed by a decrease at 48 hours. By 60 hours, another increase in binding was seen except in rats fed the high-fat diet. At 72 and 96 hours, binding appeared to level off. The maximum value at 9 P.M. in rats fed the control diet was 47% higher than the maximum value at 9 A.M. The corresponding figures for the other diets are as follows: high-fat diet, 10%; wheat bran diet, 108%; agar diet, 78%.

Figure 10 shows the effect of diet on serum radioactivity levels after the injection of labeled DMH. In rats fed the high-fat diet, the serum radioactivity level was significantly higher ($P<0.05$) than in control rats at

Figure 9. The Effect of Dietary Fat, Wheat Bran, and Agar on the Binding of Radioactive 1,2-Dimethylhydrazine Metabolites to DNA from Colon Epithelial Cells.

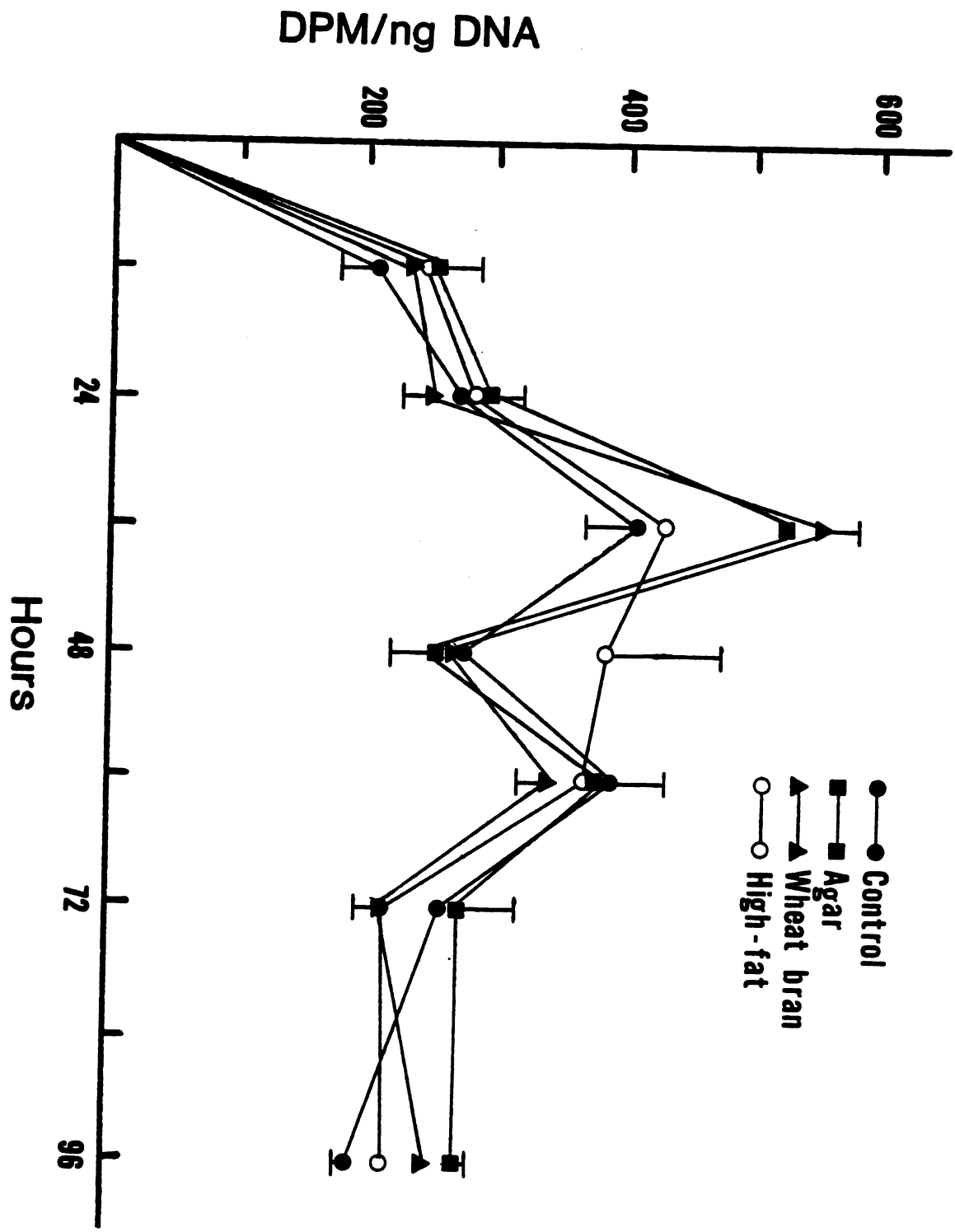
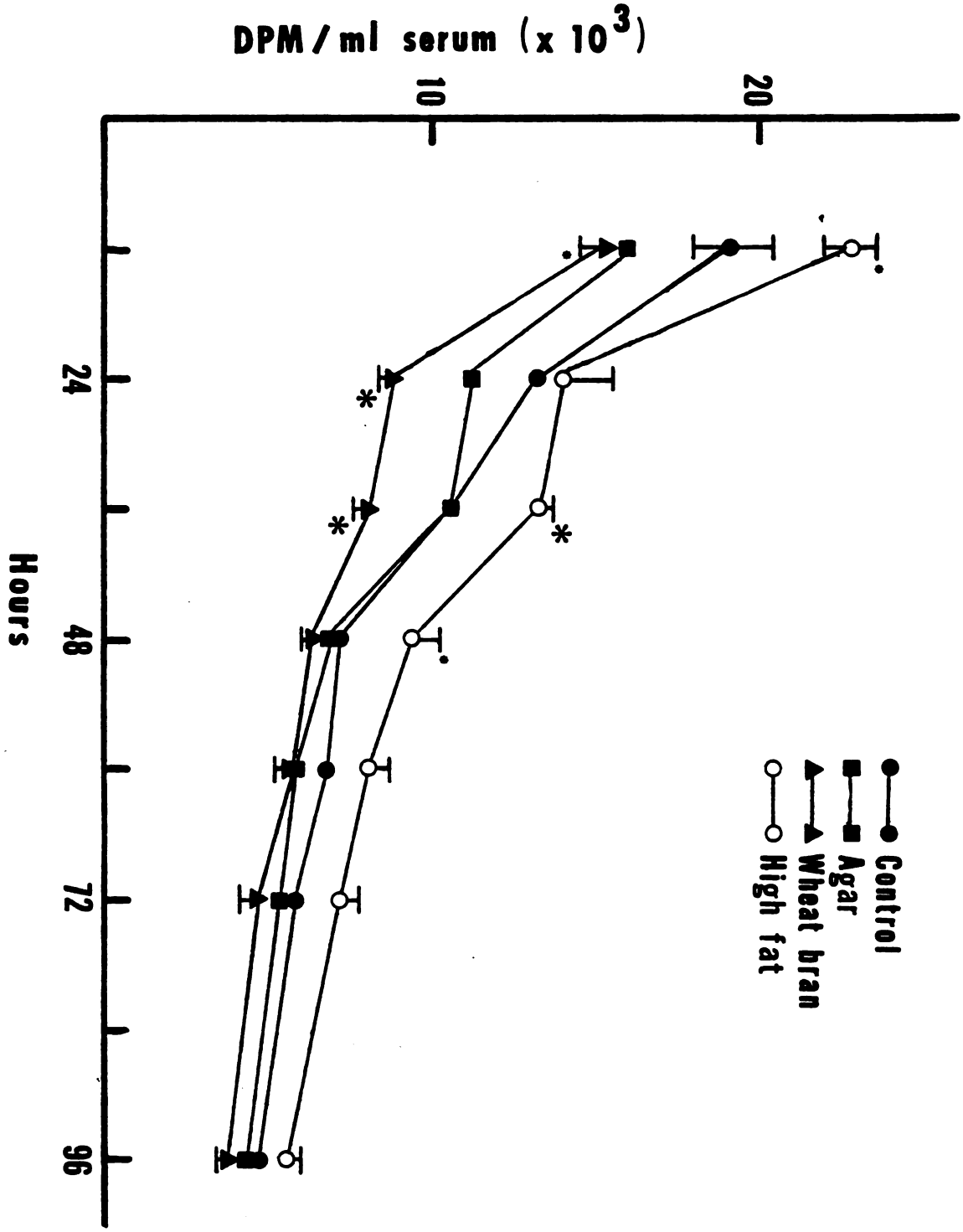

DPM/ng DNA
200
400
600
24
48
72
96
Hours
Control
Agar
Wheat bran
High-fat

Figure 10. The Effect of Dietary Fat, Wheat Bran, and Agar on Serum Radioactivity Levels after the Injection of Labeled 1,2-Dimethylhydrazine. Values from rats on experimental diets were compared to control values using the Bonferroni t-test. Statistical differences are indicated at the $P<0.05$ level (*) and at the $P<0.1$ level (•).


DPM / ml serum (x 10^3)
10
20
24
48
72
96
Hours
Control
Agar
Wheat bran
High fat

36 hours, and was slightly higher ($P<0.1$) at 12 and 48 hours. In rats fed the wheat bran diet, serum radioactivity was lower ($P<0.05$) at 24 and 36 hours and slightly lower ($P<0.1$) at 12 hours. Dietary agar did not affect serum radioactivity. The disappearance of ^{14}C from serum fits a two-pool model (Table 13), with the first pool measured with the 12-36 hour-period and the second with the 48-96 hour-period. The half-lives ranged from 8.6 to 23.3 hours for the first pool and from 62.1 to 74.5 for the second. Diet did not alter the half-life for either pool ($P>0.05$).

DISCUSSION

These results show that dietary fat and fiber do not affect DMH-induced carcinogenesis by altering the binding of DMH metabolites to DNA. At all time periods examined, none of the dietary treatments affected the binding of DMH metabolites to DNA after the s.c. injection of labeled DMH. These results suggest that the combined effect of DMH metabolism and the removal of methyl groups from DNA is not altered by dietary fat or by either of the fibers tested. The experimental design did not allow the testing of these effects separately. These diets most likely modify tumor promotion, probably by changing the levels of certain metabolites in the colon. For a diet to alter DMH metabolism, it probably must produce an intracellular change; selenium, for example, likely inhibits the action

TABLE 13

Effect of Diet on the Half-Lives of DMH Metabolites in Serum

	Half-life of radioactivity (hours)	
	Pool 1	Pool 2
Control	12.0 ± 1.2[2]	74.5 ± 11.2
High-fat	13.3 ± 12.0	62.1 ± 32.2
Wheat bran	8.6 ± 7.1	63.5 ± 7.6
Agar	23.3 ± 17.7	69.3 ± 6.2

[1]The half-life was estimated with the 12-36 hour period for the first pool, and with the 48-96 hour period for the second pool.

[2]Results are means ± SEM.

[3]Half-life for neither pool was affected by diet ($P > 0.1$).

of DMH by altering its intracellular metabolism (Harbach and Swenberg, 1981).

In contrast, the binding of DMH metabolites to DNA was markedly altered by the time of day that the rats were sacrificed. The increase in binding seen at the 9 P.M. time points could be caused by 1) increased metabolism of DMH between 9 A.M. and 9 P.M. or 2) decreased DNA repair during this time period.

DMH is metabolized through a series of intermediates before being converted to the methyldiazonium ion, which is the ultimate carcinogen (Fiala, 1977). DMH is first metabolized to azomethane, which is exhaled through the lungs or further metabolized to azoxymethane (AOM) and then to methylazoxymethanol (MAM) (Fiala et al., 1976b). Both AOM and MAM are excreted in the urine (Fiala et al., 1976a). MAM can also be converted to the methyldiazonium ion. MAM degrades spontaneously, but is metabolized much faster when it is incubated with alcohol dehydrogenase (Feinberg and Zedeck, 1980). DMH is metabolized in colon epithelial cells, but partial (or complete) metabolism of DMH in other organs is likely. It is probable that changes in the activities of one or more of the enzymes of DMH metabolism were responsible for the diurnal variation which was seen.

These results confirm that DMH metabolites bind to DNA in the colon. When DMH is injected in vivo, it is

metabolized to methyl groups which bind to the O^6 and N^7 positions of guanine in colon DNA (Likhachev et al, 1977; Rogers and Pegg, 1977; Cooper et al., 1978; Herron and Shank, 1981). Binding at the O^6 position is important because it can lead to miscoding and mutation (Miller, 1978). None of the above studies examined diurnal variation in DMH metabolism.

Serum radioactivity after the injection of labeled DMH was, in general, increased by feeding high-fat diets and decreased by feeding wheat bran. The mechanism of action is not known: extra-colonic metabolism or excretion may have been involved. The absorption of DMH from the injection site could also have been affected by diet. The dietary fat intake could possibly have altered the composition of lipids in cellular membranes. No diurnal variation in serum radioactivity was apparent. Thus, the diurnal variation in DNA binding in colon epithelial cells (and possibly in other tissues) was not reflected in the levels of radioactivity which were seen in serum. The maximum serum radioactivity concentration was seen at 12 hours - the first time point. Pozharriski et al. (1975) found that serum radioactivity was maximal 3 hours after the subcutaneous injection of labeled DMH. The radioactivity in serum could be from DMH or any of its metabolites - azomethane, AOM, or MAM. The two pools in serum may represent DMH and one or more of its metabolites.

The differences in serum radioactivity levels imply that different levels of DMH were reaching the colon epithelial cells in rats fed the different diets. These changes, however, were not reflected in the binding of DMH metabolites to DNA. This discrepancy could be caused by several factors: the activity of the enzymes of DMH metabolism may change so much because of their diurnal variation that the levels of DMH or its metabolites entering the cell are of minor importance; the total radioactivity levels in serum may mask changes in metabolite profiles; metabolites of DMH may enter the cell or be metabolized at a different rate than DMH; or diurnal variation in the cell cycle could change the concentration of methyl groups in DNA.

Several conclusions can be drawn from this study. First, the binding of DMH metabolites to DNA in colon epithelial cells undergoes a marked diurnal variation. Rats killed at 9 P.M. had higher levels of DMH metabolites bound to their DNA than those killed at 9 A.M. Second, diet does not alter the amount of DMH metabolites bound to DNA in colon epithelial cells even though diet does change the level of DMH plus its metabolites in serum.

This experiment shows that dietary fat and fiber do not alter DMH-induced colon carcinogenesis by an effect on DMH metabolism or DNA repair. More likely, these dietary components alter tumor promotion. The discovery of a

diurnal variation in DMH metabolism has implications for experiments using DMH. The time of day at which DMH is injected will alter the rate and possibly the mechanism of its metabolism.

SUMMARY AND CONCLUSIONS

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These experiments were conducted to study the effects of nutrition on colon carcinogenesis. I first sought to determine the effect of specific dietary factors (dietary fat and agar) on the development of chemically-induced colon tumors. The rest of my research focused on the mechanisms by which these and other dietary components alter colon carcinogenesis. I examined whether diet alters colon carcinogenesis by affecting tumor initiation, tumor promotion, or the colon mucosa directly.

In the first experiment, the effect of dietary fat and agar on DMH-induced colon carcinogenesis was examined using a factorial design. Feeding agar significantly increased the number of colon tumors per animal and slightly increased the malignant tumor incidence. Increasing the dietary fat level did not affect the tumor number and only slightly increased the incidence of malignant tumors. Dietary agar decreased fecal neutral sterol and bile acid concentrations; agar's effect, therefore, was not mediated by these metabolites.

One way agar could enhance colon carcinogenesis is by directly altering the colon mucosa. To test this hypothesis, I fed agar and control diets to rats for 6 weeks and then

examined the colon topography using scanning electron microscopy. Feeding agar did change the mucosa in some animals: the surface contained numerous folds and clefts which appeared to be interruptions in the epithelial lining at higher magnification. These results showed that agar may enhance colon carcinogenesis by damaging the colon mucosa.

Another way in which diet may alter colon carcinogenesis is by an effect on tumor promotion. Because the colon mucosa is constantly exposed to fecal material and the chemicals in it, and because tumor promotion also requires constant exposure of a tissue to a chemical over a long period of time, materials in the feces are good candidates for tumor promoters. The bile acids are one such group: their levels in the colon are altered by diet and they have been shown to be tumor promoters in rat colon (Mitchell and Eastwood, 1976; Reddy et al., 1977; Reddy et al., 1978).

Since tumor promoters act by increasing cell proliferation (Diamond et al., 1980), I examined the effect of varying colon bile acid concentrations on colon epithelial cell proliferation. Bile acid concentrations were altered by intrarectally injecting bile acids or by varying the fat and fiber content of the diet. DMH was injected into half of the rats to serve as an initiator.

In the first study, deoxycholic acid, lithocholic acid or saline were intrarectally injected for 4 weeks after an

earlier subcutaneous injection of DMH. Although the bile acid injections did not alter the DNA synthesis rate, lithocholic acid significantly and deoxycholic acid slightly increased the number of cells per crypt. DMH injections increased both the DNA synthesis rate and the number of cells per crypt.

In the second study, the levels of dietary fat or fiber were varied, again after an earlier subcutaneous DMH injection. Three fibers were fed: wheat bran, which inhibits chemically-induced colon carcinogenesis, and agar and carrageenan, which enhance it (Wilson et al., 1977; Barbolt and Abraham, 1978; Watanabe et al., 1978; Watanabe et al., 1979; Barbolt and Abraham, 1980). All three fibers significantly lowered fecal bile acid concentrations, but dietary fat did not affect fecal bile acid concentrations or excretion. None of the dietary treatments changed the DNA synthesis rate, but agar significantly and wheat bran slightly increased the number of cells per crypt. DMH also slightly increased the number of cells in this experiment.

These results indicate that the bile acid injections and dietary agar and wheat bran may induce a slight hyperplasia in the colon. This is consistent with the promoting abilities of the bile acids and with the increase in colon carcinogenesis caused by dietary agar; wheat bran, however, increased cell number even though it inhibits colon carcinogenesis. DMH strongly enhanced cell proliferation in the

first experiment and slightly enhanced it in the second; this is consistent with its strong carcinogenicity in the colon.

Finally, diet may influence colon carcinogenesis by an effect on tumor initiation. In my research I studied the carcinogen DMH, which induces tumors predominately in the epithelial cells of the colon. I first examined whether DMH can be metabolized by isolated rat colon epithelial cells. The cells were isolated from the colon and were then cultured with labeled DMH for six hours. I found that the cells metabolized DMH to gaseous metabolites, to the metabolites AOM and MAM in the media, and to products which bind to DNA.

Having determined that DMH is in fact metabolized by colon epithelial cells, I next examined the effects of diet on DMH metabolism. Because the effects of fiber cannot be examined in cell culture, the in vivo metabolism of DMH was studied. I examined the effect of dietary fat, wheat bran, and agar on 1) the metabolism of DMH to products which bind to DNA of rat colon epithelial cells and 2) the clearance of DMH from serum. None of the diets altered the amount of ^{14}C bound to DNA for any of the time periods examined. A diurnal variation, however, was seen: rats killed at 9 p.m. had higher binding than those killed at 9 a.m. Serum radioactivity was generally increased by high-fat diets and decreased by dietary wheat bran; agar did not affect serum

radioactivity.

Several conclusions can be drawn from these studies. Dietary agar enhances DMH-induced colon carcinogenesis. Agar's effect is not mediated by bile acids, but it may act by directly damaging the colon mucosa. These changes may cause the increase in crypt cell number which is seen when agar is fed. The DNA synthesis rate, however, is not changed by dietary agar, any of the other dietary treatments, or intrarectal bile acid injections. Intrarectal injections of lithocholic acid and deoxycholic acid increase crypt cell number, which is consistent with their promoting effect in the colon. And DMH enhances colon epithelial cell proliferation, which is consistent with its carcinogenicity in the colon. Finally, although DMH is metabolized by colon epithelial cells, its in vivo metabolism to products which bind to colon epithelial cell DNA is not affected by diet even though diet does alter the level of DMH plus its metabolites in serum. It appears, therefore, that dietary fat and fiber act by influencing the promotional phase of carcinogenesis.

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