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DEVELOPMENT OF AN INTESTINAL CELL CULTURE METHOD
TO STUDY DIETARY EFFECTS ON INTESTINAL MUTAGENS

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DEVELOPMENT OF AN INTESTINAL CELL CULTURE METHOD TO STUDY DIETARY EFFECTS ON INTESTINAL MUTAGENS

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ABSTRACT

DEVELOPMENT OF AN INTESTINAL CELL CULTURE METHOD TO STUDY DIETARY EFFECTS ON INTESTINAL MUTAGENS

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Intestinal fibroblasts were cultured to develop a method to screen potential mutagenic and carcinogenic agents found in the gastrointestinal tract. Cells were isolated from fetal rat intestines and cultured in Eagle's Minimum Essential Medium with 10% fetal calf serum and antibiotics. Cell doubling time was 20 hours and the cells were subcultured 2-5 times before experimental treatment. Optimum conditions for determining mutation frequency were: exposure time to mutagenic agents, 3 hours; expression time, 3 days; cloning in selective medium, 2 weeks; and 1.0 mM ouabain in culture medium to select mutant cells.

Bile acids, often implicated as carcinogens in colon cancer, were tested for mutagenesis in this system. Deoxycholic, lithocholic and chenodeoxycholic acids were more cytotoxic than cholic, hyodeoxycholic and 3,12-dione-5β-cholanic acids. Deoxycholic acid produced the highest mutation frequency at 0.5 mM. Lithocholic and chenodeoxycholic acids at 0.5 mM also were mutagenic. Cholic, hyodeoxycholic

and 3,12-dione-5β-cholanic acids at concentrations $\leq$ 1.0 mM were not mutagenic. Cholic acid at 2.0 mM was mutagenic. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was used to initiate cell mutation and bile acids were tested as promoting agents in this system. When MNNG treated cells were incubated in 1.0 mM ouabain medium containing cholic acid at 0.1 mM or 0.2 mM or chenodeoxycholic acid at 0.1 mM, the mutation frequencies increased above the mutation frequency induced by MNNG alone. When deoxycholic and lithocholic acids at 0.1 mM were used in ouabain medium, mutation frequencies decreased. This decrease in mutation frequency is probably due to the greater cytotoxicity of deoxycholic and lithocholic acids rather than the inability of these acids to promote MNNG induced mutation.

Organic and water extracts of feces and bile collected from rats fed high-fat, low-fiber or low-fat, high-fiber diets also induced higher mutation frequencies.

The effects of dietary fat and fiber on steroid metabolism, bowel function, and anaerobic cecal bacteria were studied in rats. Both wet and dry fecal mass were increased when bran and agar were added to the diet. Intestinal transit time decreased as the level of bran in the diet increased. There was no significant difference in daily bile acid excretion either when bran was added to the diet or when the fat and agar content of the diets were varied. However, the greater fecal mass caused the fecal concentration of bile acids to be decreased when dietary fiber was added to the diet.

More extensive bile acid degradation was found as the level of bran in the diet increased. More cholic acid was degraded when cecal contents from rats fed bran diets were incubated anaerobically than when cecal contents from rats fed a bran-free diet were incubated anaerobically. The number of anaerobic cecal bacteria was not altered by the bran content of the diet. Therefore, bacterial type or activity rather than the number of viable anaerobic bacteria in the large bowel appears to be more important in bile acid degradation.

Neutral steroid excretion and concentration were higher in rats fed a high-fat, low-fiber diet compared to rats fed a low-fat, high-fiber diet. Neutral steroids were more extensively degraded in the low-fat, high-fiber fed rats. Rats fed the high-fat, low-fiber diet excreted more coprostanol in the feces than coprostanone, whereas more coprostanone was found in the feces of rats fed the low-fat, high-fiber diet. This different ratio of coprostanol/coprostanone in the feces of rats fed the two different diets implies that microbial activity in the large bowels of the two groups of rats was different.

Results of this study indicate that 1) the intraluminal environment can be changed due to variations in the diet, 2) it is feasible to use intestinal fibroblasts isolated from rat fetuses to screen potential mutagens found in the gastrointestinal tract. The second conclusion is based on the observations that compounds which caused cell mutations are either a known carcinogen or strongly implicated in colon cancer.

To my mother

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INTRODUCTION

Epidemiologic studies show that the incidence of colon cancer varies with geographic area and socioeconomic level. It is high in Western countries and low in African and Asian countries. Based upon studies with migrant populations, many investigators agree that environmental factors play a major role in the tumorigenesis of colon cancer, but genetic factors play a minor role. It has been shown that the risk rate of developing colon cancer among the immigrants from the low risk areas into the United States is high,while the risk rate of their counterparts in the native country remains relatively low.

Among the environmental causes, dietary factors have been suggested in the etiology of colon cancer. Several hypotheses have been proposed how diet may play a role in colon carcinogenesis. Dietary factors include fat, protein, and fiber levels in diet. Diet influences intraluminal components and intestinal microflora. Intestinal microflora may produce carcinogens or cocarcinogens from the intraluminal metabolites. Also, the diet can change gut function and the mass of the material in the colon. Bowel function controls the length of time that colonic cells are exposed to carcinogenic agents, and the mass of indigestible material in the

colon affects the concentration of carcinogenic agents that come in contact with colonic cells. Bile acids, cholesterol and protein have been suggested as substrates which microflora metabolize to produce carcinogens.

The relationship between diet, intraluminal components, microflora, and gut function needs to be elucidated. Through studying the foregoing interrelationships one might be able to identify agents responsible for the etiology of colon cancer. A great deal of research has been conducted in an effort to elucidate the relationship between diet and colon carcinogenesis in animal models. However, there is not general agreement among investigators which component(s) of the diet is (are) etiologically important in colon carcinogenesis.

The existing animal bioassays are time consuming and expensive. There is an urgent need to develop a relatively rapid and reliable in vitro method to screen suspected carcinogenic agents which are found in the gut. Positive compounds can then be tested by the existing bioassays. Compounds which are positive in the bioassays then can be related to diet.

The objectives of this research are: 1) to study the effect of diet on steroid metabolism, gut function and cecal bacteria, and 2) to develop an in vitro method to screen suspected carcinogens identified in the literature and from objective 1.

LITERATURE REVIEW

Nutritional and dietary factors have been thought to play a role in the development of cancer in man and animals for many years. However, experimental evidence to support this concept and to explain the mechanisms involved is very limited at the present time.

First I will review the epidemiologic findings which implicates a dietary relationship to cancer. The relationships of specific dietary components and site of cancer will be discussed also. Secondly, I will review experimental evidence which implicates dietary components and cancer. Finally, I will review currently employed methods to evaluate carcinogenicity and/or mutagenicity of compounds which can be found in the gastrointestinal tract.

Food, food additives and contaminants may be involved in the cause and/or the development of some of the common cancers. Epidemiologic data show that there is an uneven distribution of many major types of cancer in the world. Race, culture, environmental factors and diet are factors which could influence the incidence rate of cancer. The question of which component(s) account for the difference in cancer incidence remains unanswered.

Some observations have been reported. Cancer of colon and breast are most prevalent in North America and Western European countries (1,2). The incidence of gastric cancer is high in Japan, Chile, South Africa and Finland. These epidemiologic data provide circumstantial evidence that diet may be important in the cause of cancer, but nothing about its etiology and pathogenesis.

Results of studies with immigrant population do not support genetic and racial differences as a major cause of cancer (3-6). Rather, environmental factors appear to account for the geographic differences in cancer distribution. Generally the areas with low incidence of colon and breast cancer have a low standard of living with limited amount of industrialization, whereas the high incidence areas are economically developed and industrialized countries. Air and water pollutants common to the industrialized countries, food additives, customs and stress of Western society and dietary pattern have been suggested as possible environmental factors which cause the cancer (1).

Most investigators agree that diet is a major environmental factor which could cause cancer, especially cancer of the gastrointestinal tract. Populations in high risk areas consume diets containing large quantities of animal fats and protein, refined carbohydrate (7-9) and small quantities of dietary fiber (10), whereas people in low risk areas eat foods low in meat and meat products but high in fish and vegetables, unrefined cereals and fiber rich foods such as legumes and tubers (1).

In addition to food consumed, nutritional status may affect the incidence of tumors. High energy intake, nutritional excesses or deficits, and consumption of alcohol increase the risk of certain cancers. Specific deficiencies of essential nutrients have been shown to enhance experimental chemical carcinogenesis. These include the deficiencies of vitamin A (12,13), methionine and choline (14), selenium (15), zinc (16), copper (16), vitamin C (17) and protein (18).

Table 1, cited from Lowenfels and Anderson (11), summarizes possible relationships between diet and cancer.

Cancer of the colon. Cancer of the large bowel is a leading cause of cancer related deaths in United States. Mortality due to cancer of the large bowel is second only to lung cancer in males, and third only to breast and lung cancer in females. The incidence of colon cancer is high in North America, Western Europe and Australia, moderate in Eastern Europe, Israel, and Ibeiran Peninsula and low in Asia, Africa, Central and South America (Figure 1). This difference in the distribution could be attributed to genetic or environmental factors. There is some evidence that genetic factors may play a role in colon carcinogenesis (19). Relatives of colon cancer patients have a greater risk of developing this cancer. Also, persons with familial polyposis coli, Gardner's syndrome, adematous polyps, and Pentz-Jegher's syndrome, all of which are hereditary diseases, have an increased risk of colon cancer.

TABLE 1. Diet and Cancer: Possible Relationships

Factor	Organ or organ system at risk
Energy Content	Overall incidence of cancer may be affected by total caloric intake
Type of Diet	
Abrasive, irritant foods	Stomach
High fat	Colon, breast
Low residue	Colon
Toxic Plants	
Cycad palm	Small, Large bowel
Bracken fern	Small, Large bowel
Safrole	Liver
Vitamins	
A	Epithelial tissues
Riboflavin	Epithelial tissues
Carcinogens	
Aflatoxins	Liver
Nitrosamines	Upper gastrointestinal tract
Benzopyrene	Stomach
Miscellaneous	
Alcohol	Oropharynx, larynx, esophagus, liver
Artificial sweeteners	Bladder

Adapted from Lowenfels and Anderson (11).

Figure 1. Geographical distribution of colonic cancer 1968-1969. Adapted from Kassira et al. (1).

Geographic Variance in Colonic Cancer Mortality Rates
(Age-Adjusted-per 100,000) 1968-1969

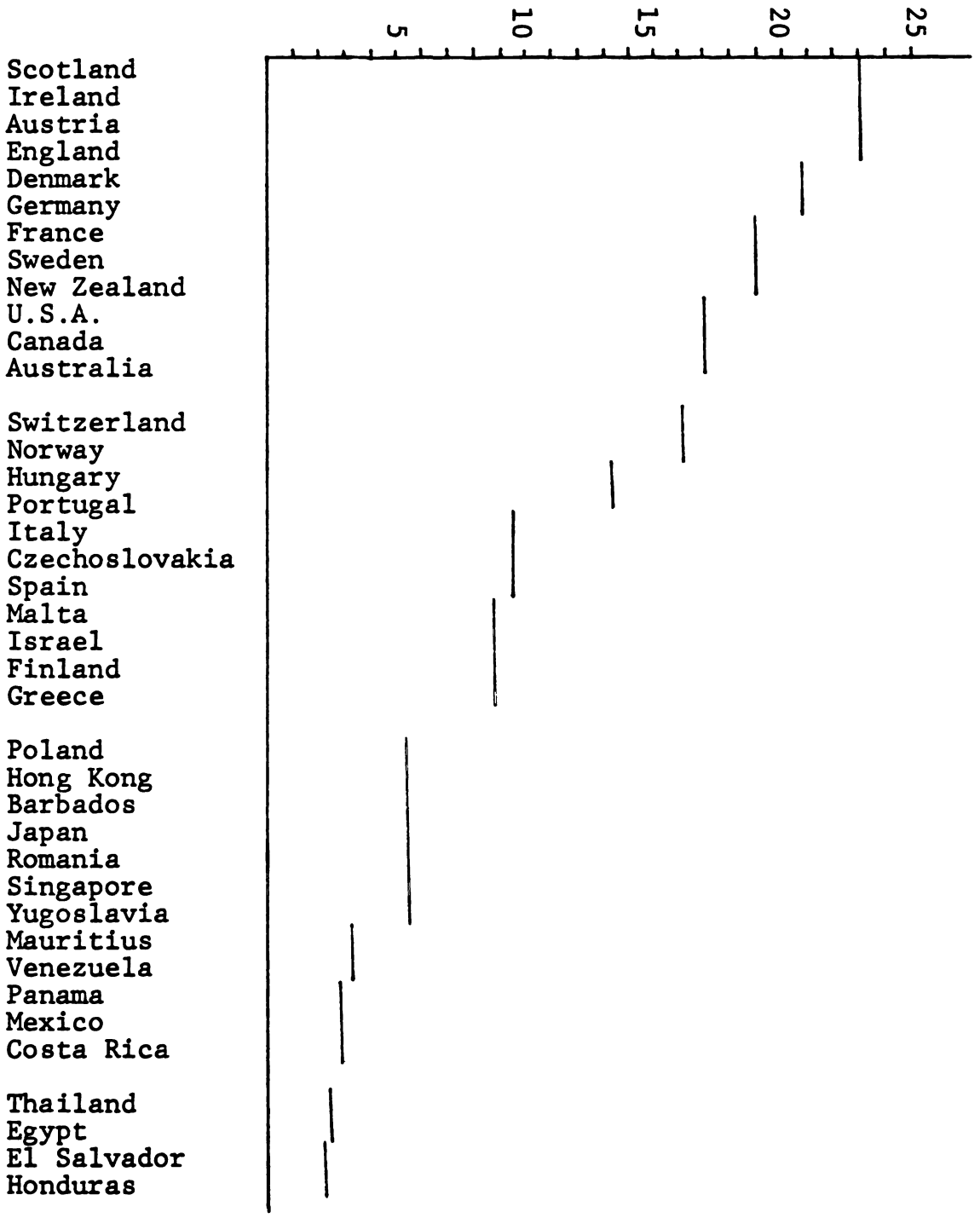

FIGURE 1.

Studies with migrant populations, however, suggest that the incidence of colon cancer is more associated with environmental causes than with genetic factors. While the incidence of colon cancer in Japan is low compared to the United States, the risk rate increases sharply among the immigrated Japanese in California (3) and Hawaii (4). The incidence of colon cancer in the first generation of Japanese immigrants is similar to the incidence of colon cancer in Japan, but the incidence in the second generation is essentially the same as that found in native American whites. This is especially true for males. This implies that prolonged exposures to the American environment may be primarily responsible for the higher incidence of colon cancer in the second generation. Data from other immigrant populations show similar trends. The colon cancer mortality rate among Polish immigrants into the United States is rapidly approaching the mortality rate for American whites, while the mortality rate in Poland remains low (5). The importance of environmental effects is also supported by the work of Mass and Modan (6) who compared the incidence of colorectal tumors among Jews who immigrated into Israel from different parts of the world. The incidence was high among Europe-American born Jews, moderate among Israel born Jews, and low among Asia-Africa born Jews. It is also reported that American blacks have much higher incidence of colon cancer than African blacks (10,20).

In view of the above epidemiological findings it seems unlikely that the difference in the incidence of large bowel cancer is due mainly to genetics. There is good correlation between incidence of colon cancer and economic development. The more affluent societies have the higher incidence of the colon cancer.

Among the environmental causes, diet has been proposed as a factor in the development of colon cancer. Diets in countries with a high risk of colon cancer differ from diets of countries with a low incidence of colon cancer. The high risk countries consume more animal fat and protein, processed sugar, and refined carbohydrate (7-9) and less fiber (10) than countries with a low risk of developing colon cancer. Haenszel et al. (21) found that Hawaiian Japanese, who ate "Western-style" meals more frequently than typical Japanese meals, had a high incidence of colon cancers. Seventh-Day Adventists, who are vegetarians, are reported to have 20% less large bowel cancer than control American whites (22).

From these observations, many investigators have developed a hypothesis relating diet and tumorigenesis in the colon. Composition of the diet can alter the intraluminal components and microflora of the gut. Also, the metabolism of intestinal microflora can be altered by the diets. It is hypothesized that carcinogenic or cocarcinogenic compounds are produced from normal intraluminal components by

altered microfloral populations or altered microfloral metabolism. Cocarcinogenic compounds refer to compounds which are not carcinogenic by themselves but which may stimulate carcinogenesis in the presence of a carcinogen. Thus, the diet may have its effect both by altering the supply of substrates for carcinogen or cocarcinogen production, and by changing the type and number of the intestinal bacteria available to act on such substrates. A diet high in animal fat, animal protein, and refined carbohydrate may lead to different populations of intestinal microflora than diets low in animal products and high in dietary fiber (23). Also, differences in diets can change endogenous secretions into the gut which influences intraluminal environment.

The key question is what diet-dependent components, modified by intestinal bacteria, could possibly account for the development of colon cancer. Due to structural similarities between steroids and polycyclic aromatic carcinogens (23,24), bacterial metabolites of bile acid and cholesterol have been suspected to have a role in carcinogenesis. As the fat content of the diet increases, the amount of bile secreted into the gut increases. (25) Also, the concentration of fecal bile acids and neutral steroids increase as the fat level in diet increases. Moreover, the high fat diet may change the population of intestinal flora which could result in more extensive bacterial degradation of steroids into products which may have carcinogenic or cocarcinogenic activity.

A number of bacterial metabolites of amino acids are also suspected as carcinogens or cocarcinogens. Bacterial production of ammonia, indole and other tryptophan metabolites in the large bowel from unabsorbed protein are also mentioned in relation to colon carcinogenesis (7, 26).

Dietary fiber may be another component capable of altering colon carcinogenesis through its effect on gut function and microflora population and metabolism. It is well agreed that as fiber in the diet decreased, fecal mass becomes smaller and intestinal transit time gets longer (27-29). The small stool mass and the longer transit time might be very important factors in causing colon cancer. Any carcinogen ingested or formed in the gut would not only be present in a more concentrated form in small indigestible masses, but would also be held in contact with cells for a prolonged period. Transit time also has been proposed as one of the factors determining the extent of microbial degradation of compounds. (30) However, human studies indicate that bowel transit time is not a crucial factor in determining the extent of bacterial metabolism in the gut (31).

There is some epidemiological evidence which shows that high and low risk groups of people consuming different dietary regimes have differencies in fecal constituents and in gut bacteria. Hill and Aries (23,32) studied the excretion and degradation of bile acids and neutral steroids in

feces among four populations with different risk rates. English and Scots who consumed mixed "Western-type" diets excreted more bile acids and neutral steroids in their feces than Indians on a rice diet or Ugandans on a matoke diet. Additionally, bile acids and neutral steroids were more concentrated and more degraded in the stools of the English and Scottish subjects. A higher ratio of total anaerobic bacteria to total aerobic bacteria also was found in high risk populations. Anaerobic bacteria metabolize steroids more actively than the aerobic bacteria (23). Various studies show that vegetarians have a lower fecal concentration of bile acids and neutral steroids than omnivorous people living in the same city (Table 2).

Reddy et al. (33) compared fecal constituents among Americans on a typical mixed "Western" diet, American vegetarians, American 7th-Day Adventists, Japanese Americans on a Japanese diet, and Chinese Americans on a Chinese diet. They found a higher fecal β-glucuronidase activity for Americans eating a mixed "Western" diet than for the other populations. Activity of β-glucuronidase, an inducible enzyme, is associated with many components of microflora present in the feces and therefore used as an indicator of metabolic activity of gut bacteria (34). Glucuronide formation is a major detoxification mechanism in animals. Many endogenous and exogenous compounds that are excreted in bile as glucuronide conjugates are deconjugated by bacterial

TABLE 2. Effect of diet on the fecal steroid concentration

Population	No. of Subjects	Duration of Diet	Fecal steroid concentration		Source
			Bile acids	Neutral steroids	
High meat to no meat	8	4 weeks	86[a]	68[a]	Reddy (37)
Vegans[b]	15	Long-term	58	82	Aries (38)
Vegetarians[b]	12	Long-term	52	39	Reddy (33)
7th-Day Adventists[b]	11	Long-term	21	33	Reddy (33)

[a]Results expressed as percentage of control values.

[b]Compared with normal omnivorous people in the same city.

Adapted from Hill (8).

β-glucuronidase and further modified by intestinal bacteria. Therefore the difference in β-glucuronidase activity indicates that the intestinal microflora of Americans eating "Western diet" are more able to hydrolyze glucuronide conjugates than microflora of other groups. Americans eating a "Western diet" excreted more fecal bile acid and neutral steroid and also these steroids were more extensively degraded.

Fecal steroid excretion for cancer and other disease patients have been compared. Reddy and Wynder (35,36) reported that there was a greater concentration of total bile acid and neutral steroids in the feces of colon cancer and adenomatous polyps patients when compared to patients with other digestive diseases or to healthy controls. Moreover, the steroids were more extensively degraded in the feces from cancer or adenomatous polyps patients. Feces from colon cancer patients had a higher activity of fecal bacterial 7α-dehydroxylase, the enzyme which converts cholic and chenodeoxycholic acids to deoxycholic and lithocholic acids, respectively. Hill et al. (39) demonstrated that there were higher levels of both fecal bile acid metabolites and nuclear dehydrogenating clostridia (NDC) in large bowel cancer patients than in patients with other diseases. NDC are able to perform the nuclear dehydrogenation reaction. It is hypothesized that nuclear dehydrogenation of bile acid can result in the production of 20-methyl cholanthrene, a potent carcinogen.(23)

It appears that the fat and protein content of diet can influence the incidence of naturally occurring or chemically induced colon cancer.

Effect of diet on experimentally induced cancer. Reddy and coworkers (40) investigated the effect of type and quantity of dietary fat on colon tumor incidence in rats treated with 1,2-dimethylhydrazine (DMH), an organospecific carcinogen. Rats fed either a 20% lard or a 20% corn oil diet were more susceptible to colon tumor induction by DMH than rats fed 5% of either fats. There was no difference in tumorigenesis between the lard and the corn oil fed groups at the same level of fat. They also studied the biliary and fecal bile acid and neutral steroid excretion in these rats (25). Rats fed corn oil or lard at the 20% level showed markedly increased concentrations of biliary and fecal bile acids and neutral steroids compared to rats fed diets containing 5% fat. Animals treated with DMH excreted more biliary and fecal bile acids and neutral steroids than the rats on same level of dietary fat without DMH treatment. Since DMH treatment increased bile acid excretion, this result may indicate that animals subject to tumor development may have greater excretion of bile acids and neutral steroids.

When beef fat was added to a normal chow diet to 35% by weight, rats developed more azoxymethane (AOM) induced intestinal tumors than rats fed the normal chow diet (41). However, the results of this report should be viewed with caution. Since weight gain for animals fed the high fat

diet was greater, the observed difference in tumor induction may have resulted from variation in calorie intake as well as fat content of the diet. In addition, adding fat to the chow diet would have resulted in dilution of all nutrients, therefore the different tumor production may be due to dietary deficiencies.

There is some evidence that bile acids may act as carcinogens. As early as 1940, Cook et al. (42) reported that deoxycholic acid dissolved in sesame oil and injected under the skin induced tumors in mice at the site of injection. However, Shear (43) could not find any tumors in mice injected with dry crystals of deoxycholic acid. Since Cook et al. did not include a proper control group receiving only solvent (sesame oil), questions can be posed regarding a possible role of sesame oil in the carcinogenesis. Later Lacassagne et al. (44) showed that apocholic acid produced tumors in mice at the site of injection. They also found that 7-dehydrocholesterol and 3β-acetoxy-bisnor-Δ^5-cholenic acid had carcinogenic activity (45). Their control groups received solvent (olive oil) and did not develop any tumors.

Nigra and others (46) found an increased incidence of tumor in the large intestine of rats treated with chemical carcinogens when the animals were fed a 2% cholestyramine-containing diet compared to rats fed a regular chow diet. Since cholestyramine binds bile acids and causes more bile acids to enter into the large intestine, it seems that bile acids in the large bowel may have a tumor promoting effect.

In another experiment, these investigators transplanted the bile duct in rats from the proximal half to the distal half of the small intestine of rats to divert more bile acids to the colon (47). They found more tumors in rats with diverted bile ducts than in the control rats when all were given the same amount of AOM.

Reddy, Wynder and coworkers demonstrated that deoxycholic acid enhances the carcinogenic effect of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in the large intestine of germ-free rats (48). When rats were given repeated intrarectal doses of sodium deoxycholic acid followed by intrarectal instillation of MNNG, they developed more tumors in the large intestine than rats receiving MNNG alone. They also demonstrated that lithocholic and taurodeoxycholic acids promoted cancer in conventional rats given intrarectal instillation of MNNG. (49) Sodium cholate and sodium chenodeoxycholate also showed tumor promoting effects in MNNG induced colon cancer in both germ-free and conventional rats, however, these salts of bile acids had greater tumor promoting effect in conventional rats than germ-free rats. These results indicate that secondary bile acids produced by gut microflora have greater tumor promoting effects than primary bile acids (50). None of these bile acids, however, showed carcinogenic activity when administered without MNNG.

In a recent report (51), cholecystectomized mice showed an increased rate of colon carcinoma induced by DMH compared to intact control mice. It was reported that

cholecystectomized animals increase bile acid flow from the liver to the large intestine without the gallbladder to store the bile (52). Thus, there would be an increased concentration of secondary bile acids, produced by bacteria, in the colon leading to increased carcinogenic or cocarcinogenic activity.

In a review of the distribution of small bowel tumors, it was found that most tumors occur in the duodenum or proximal jejunum near the entrance of bile duct where the concentration of bile is the highest. This result also suggests that bile acids may have a promoting role in tumor development (53).

Relationship of gut microflora to colon cancer. Some attempts have been made to show a correlation between gut bacteria and the risks of colon cancer. Reddy et al (54) demonstrated that gut flora have a role in chemical carcinogenesis by comparing germ-free and conventional rats given DMH. Conventional rats treated with DMH developed more tumors in the large bowel than germ-free rats treated with DMH. They concluded that gut flora had a role in metabolizing DMH to an active carcinogenic compound.

An association between the level of anaerobic bacteria, the extent of steroid degradation and the risk of colon cancer among different populations has been suggested (23). However, not all investigators agree. Hill (23) examined the fecal flora of individuals living in areas with a high and low risk for colon cancer in an effort to identify

species related to the development of cancer. The same broad group of bacteria were found in the feces of all the populations studied. However, fecal samples from British and American subjects yielded more anaerobes than feces of Ugandans, Indians, and Japanese. The latter low risk populations had many more facultative and aerobic bacteria in their feces than did British and Americans. Thus, the ratio of anaerobes to aerobes was much higher in people eating a "Western-mixed" diet than in those on a vegetarian diet.

A greater proportion of colon cancer patients had NDC present in their feces than did control patients with other diseases (39). Control patients had gastrointestinal disease and diseases which were not related to gastrointestinal tract. Higher numbers of bacteroides and clostridial species were found in feces of people from high risk populations (8, 26). Eubacterium was found in greater numbers in feces from people from low risk populations. However, Finegold et al. (55,56) could not find any significant difference in the anaerobic species of bacteria between the high risk groups (Japanese-American on mixed Western diet and polyps patients) and low risk populations (Japanese-American on a Japanese diet and patients with other disease). Moore and Holdeman (57) also failed to find a specific species of bacteria which is characteristic of high risk populations. Instead, they reported that individuals in low risk areas tend to maintain higher concentration of a few species, whereas the high risk group had a more heterogenous flora in fecal specimens.

However, the flora of each person is so complex and the variation among individuals within a population is so great, that it is very difficult to point out differences between groups, even if differences do exist.

Several studies have been conducted in an attempt to change the composition of fecal flora by dietary modification. The inclusion of wheat bran (58), pectin, guar gum, bananas, plantain bananas, medium chain triglyceride, and olive oil (59) had no effect on changing the gut flora.

Reddy et al. (37) studied the composition of fecal flora from volunteers who ate a high meat "mixed-Western" diet or a non-meat diet. Both diets had the same protein content but different fat levels. Total number of anaerobic fecal bacteria were higher in specimens from the volunteers while they were consuming the high meat diet than during the period when the volunteers consumed the non-meat diet. Henteges et al. (61) conducted a similar experiment except their high meat and non-meat diet had the same fat level but different protein levels. No differences were found in the total number of anaerobes in the feces of the subjects during the studies of either diet. The discrepancies between these two studie may indicate that the total fat content of the diet, not protein, has an effect on changing the fecal microflora. Differences in methodology may also have produced the discrepancy.

Even though number and type of microflora may not differ between the high and low risk populations, the

metabolic activities of flora are reported to differ. A high proportion of the anaerobes isolated from stools from people from high incidence areas were able to dehydroxylate bile acids. The proportions were very small when the organisms were isolated from stools from the low incidence population (8). Also a high proportion of the clostridia isolated from stools of people from high risk populations were able to dehydrogenate the steroid nucleus.

The metabolic activities of gut microflora can be changed by diet. The activity of β-glucuronidase produced by bacteria can be increased by increasing the level of fat and meat in diet (35,62). Changes in the diet have caused changes in the ability of flora to metabolize various chemicals in experimental animals (63).

Methods for studying carcinogenesis and mutagenesis. At this point in colon cancer research, there is an urgent need to identify compounds which are capable of causing cancer. Only then can a cause and effect relationship between carcinogenic agents and diet be made, which in turn will lead to a better understanding of the role of diet in the prevention or development of cancer.

Even though epidemiological data indicate that dietary differences are associated with the development of cancer, it has not been demonstrated that tumor formation can be caused by dietary manipulation in animals or man. Moreover, it is not likely that it will be feasible to produce colon cancer by dietary manipulation in the future. One of the plausible methods to study the effects of diet on

carcinogenesis is to study chemical carcinogenesis in animals fed various diets. However, the interaction between carcinogen and the gut components are hard to predict. Even if a difference was found in carcinogenesis between groups of animals fed different diets, it still would be subject to argument what component of the diet or what metabolite induced by diet was responsible for the difference. The intrarectal instillation of test compounds into rats as used by Wynder and others (48-50) does permit identification of carcinogens and cocarcinogens. However, all these methods of animal bioassays are too time consuming (6 months to 2 years) and expensive to use as screening methods to test all suspected carcinogenic compounds. There is a desperate need for a rapid and reliable in vitro method for screening purposes.

In the past several years, many attempts have been made to develop reliable in vitro methods to test viral and chemical agents responsible for tumor induction. Cell culture techniques have been used in many laboratories for screening carcinogens and for elucidating fundamental cellular and molecular mechanisms of carcinogenesis. The majority of the cell culture work has been conducted with fibroblasts which produce sarcomas upon inoculation into host animals. Most human cancers originate from epithelial tissues, and it would obviously be desirable to study epithelial cells in culture. However, researchers have not yet

successfully grown epithelial cells in culture for subsequent demonstration that they can be transformed.

There are two common cell types used for the demonstration of transformation. One is primary and secondary cultures from Syrian hamster embryo cells (64). The other is permanent mouse cell lines derived from C_3H mouse prostate. The hamster embryo cell system has the advantage that the cells are diploid, whereas the prostate cells are aneuploid. However, the hamster cells have a short life time and low cloning efficiency, while the mouse prostate cells have an unlimited life span and very high cloning efficiency. There are other cell types used by various laboratories. C_3H/10T1/2 cells derived from C_3H mouse embryos, Balb/3Ts cell lines from mouse embryo cells, Chinese hamster lung cells, and rat embryo fibroblasts are some of the commonly used cell types.

The biggest limitation in using cell culture techniques to demonstrate cell transformation is that no universal marker exists to differentiate transformed cells from normal cells. The most frequently used markers for the recognition of transformed cells are: 1) changes in morphology of cells and colonies, 2) changes in growth properties, 3) chromosomal aberrations, 4) immortality in culture, and 5) ability to produce tumors when injected into syngenetic animals. In the first method, transformed cells are characterized by criss-cross random cell orientation, increased number of nucleoli per cell, and changes in nuclear-cytoplasmic

ratio. In the second method, neoplastic cells have the ability to grow in fluid suspension, whereas most normal cells grow only if they are attached to a surface. The transformed cells also have the capacity to produce three dimensional colonies in soft agar and to grow with reduced concentrations of serum. Tumor cells lose the density-dependent inhibition of cell division and form piled up foci after confluency is reached. On the other hand normal cells stop growing after confluency has been reached and form only monolayers of cells. Scoring these piled-up foci has been most widely used to quantitate transformation in cell culture. In the third method, there is a correlation between chromosomal abnormalities and development of cancer (65). By chromosome stains, the chromosomal changes in the cell can be detected. In the fourth method, cell longevity is the criteria. Transformed cells demonstrate immortality in culture, while normal cells have a finite life span. The fifth method is the ultimate criterion for demonstration of malignant transformation. If cells suspected as being transformed can produce tumors in a host animal, then one can safely state that the cells were transformed.

All the above criteria, except the ability to produce tumors in animals, have been observed to some extent in normal cells. Therefore artifacts and the bias of investigators are greatly involved (66).

One of the more promising methods for screening chemical agents for potential carcinogenic activity is to

determine the mutation frequencies caused by suspected agents in microbial or in cell culture systems. It is frequently observed that many known carcinogens also induce mutation in cell cultures and in microorganisms (67, 68).

It is generally thought that carcinogenesis is a two or multi-step process (69-71), consisting of initiation and promotion stages. Many feel that the initiation step is a mutation involving DNA damage and is an irreversible event. The following promotion stage is a reversible process involving gene modulation. There are two classes of carcinogens: one class contains mutagens which will cause the initial irreversible DNA damage, and the other class contains promotors which will enhance tumor expression. The initial mutation may occur in germ cells, in which case tumors may be inherited; or it may occur in somatic cells, in which case the tumor is not inherited. (69)

One of the most widely accepted methods to score mutation is the method developed by Bruce Ames and coworkers (72). They developed several series of histidine-requiring auxotrophs of Salmonella typhimurium (TA-1535, TA-1537, TA-1538, and TA-100) by specific mutation. These strains are easily reverted in response to a wide range of chemical mutagens. By omitting histidine in culture medium, the mutagen-induced revertant colonies, which do not require histidine for growth, can be scored easily. Initially, only a limited number of known carcinogens appeared to be mutagenic in this system. However, with the understanding that

a carcinogen may have to be activated by an *in vivo* system before it can act as an ultimate carcinogen, the bacterial system was coupled with mammalian microsomal enzyme preparations to metabolize the procarcinogen to an ultimate carcinogen. With this coupled system, more and more known carcinogens are able to cause mutation in bacteria. The biggest limitation in this system is that the carcinogens which are not initiators (i.e. promoters) will not be detected. Some strains of *E. coli*, yeast, fungi, and animal cells also have been used for mutation assays using different types of selective systems.

It seems more reasonable to use a mammalian cell system rather than a microbial cell system to study mutagenesis and/or carcinogenesis, if a specific and selective method could be developed. There are three major types of mutants isolated in mammalian cell culture, namely, temperature-sensitive conditional lethal mutants, nutritional auxotrophs and drug-resistant phenotypes. Temperature-sensitive mutants have a smaller range of temperatures in which they can grow compared to wild-type cells. In other words, the mutants cannot grow either at the lower end of the normal temperature range or at the higher end of the normal temperature range. This defect probably arises from a change in the thermal kinetics of denaturation or assembly of a particular protein. Nutritional auxotrophs need certain nutrients for growth which is not necessary for the wild-type cells. This requirement is due to mutation which causes the

inactivation of an enzyme in the biosynthetic pathway of the certain nutrients, such as amino acids or vitamins.

Drug-resistant mutants can grow in the presence of a drug which is cytotoxic to normal cells. Drug-resistant mutants have been isolated from the mammalian cells. Mutation in the gene for hypoxanthine-guanine phosphoribosyl transferase (HGPRTase) leads to cells which are resistant to such purine analogues as 8-azaguanine and 6-thioguanine. Mutation in the gene for the plasma membrane Na/K ATPase leads to ouabain resistant cells and mutation in the gene for protein kinase leads to dibutyryl cyclic AMP (Bt_2AMP) resistant cells (73). Mutants which are resistant to 5-bromodeoxyuridine (BrdU) and to colchicine have also been isolated (14). BrdU, a thymidine analogue, resistant cells have decreased thymidine kinase activity. Colchicine binds to a protein subunit of cellular microtubles and therefore interferes with the formation of microtubles. A wide variety of other drugs have also been used to select mutants in culture (74). Wild-type cells die when they are incubated with these drugs, therefore, drug-resistant mutants can be selected by adding the drug in the culture medium.

One of the major limitations in mutagenic studies is that only a relatively small number of the mutant forms are identified. The nutrition auxotrophs and drug-resistant mutants require the hit of a mutagen on a restricted number of target genes. If the hit occurs at another site, the event will not be scored because of the lack of a suitable

detection system. Another drawback is that there are mutagens which do not have tumor producing effects and there are carcinogens which do not show mutagenic activity. Many more classes of compounds appear to be mutagenic than carcinogenic (65).

Until recently, cancer promoters were not detected in mutation frequency assay system. Trosko et al. (75) very nicely demonstrated the effect of promotors in the V-79 Chinese hamster cell culture by using the ouabian and 8-azaguanine systems.

I chose to use cells isolated from the intestine of rat fetuses to develop a model system to screen agents that are possibly responsible for colon cancer. Ouabain resistance was the criteria chosen to determine mutation frequency. Cells resistant to ouabain have been isolated from mouse and Chinese hamster cells (76), from human lymphoblasts (77, 78), Ehrlich ascites cell (79), and Hela cells (80).

Ouabain, a steroid glycoside, is a specific inhibitor of the plasma membrane Na/K ATPase. Inhibition of this enzyme decreases the active transport of potassium and cells exposed to ouabain die due to potassium starvation. Ouabain resistance results from a mutation in the structural gene for ATPase due to a point mutation. The point mutation results in a single amino acid substitution, which allows the enzyme to be active in the presence of ouabain. Mutant cells could: 1) have another K^+-transport site with an altered response to ouabain, 2) lose all or part of the ouabain

receptors in membrane, or 3) have a decreased affinity for ouabain (74, 77).

Point mutation is due to nucleotide base-pair substitution at a speific site in the wild-type gene, such as GC in the wild-type is replaced by AT in the mutant. Point mutation results in a functional protein with altered specificity due to a single amino acid substitution. By treating cells with base-pair substitution mutagens (ethyl methane sulphonate, MNNG, UV light), the frequency of ouabain-resistant cells increase markedly (76, 78).

Unlike the 8-azaguanine system, selection of ouabain-resistant mutants is not dependent upon the cell density seeded (74) and the expression time is shorter (75). However ouabain-resistant cells are not observed when cells are treated with a frameshift mutagen, such as ICR-191. In frameshift mutations, one or a few nucleotide bases are inserted into, or deleted from, a specific site within the gene. Therefore they shift the "reading frame" of the translation process, leading to a non-functional protein. Frame shift mutation can be detected easily by using 8-azaguanine as a selective marker.

The inducibility of mutation frequency by mutagens has been used by many investigators to score mutation frequency induced by environmental mutagens/carcinogens for the purpose of screening mutagenic/carcinogenic agents and for better understanding of the mechanisms. Most of these mutation studies have been done with an established cell line

such as 3T3 cells and V-79 Chinese hamster cells.

I have used freshly isolated fibroblasts from the intestine of rat fetuses since using these cells has the advantage of utilizing cells from the target tissue. It would be more appropriate to use intestinal epithelial cells, but intestinal epithelial cells have not been successfully grown for transformation studies. Some workers have successfully isolated intestinal epithelial cells for metabolic studies, but it has not yet been possible to grow them in vitro for a prolonged period of time.

MATERIALS AND METHODS

PART I

Part I was designed to study the effects of dietary fat and fiber on steroid metabolism, intestinal transit time and intestinal microflora in rats. In experiment 2, organic and water soluble extracts were prepared to use for cell culture experiments in Part II.

Experiment 1

In experiment 1, the effects of wheat bran, the most frequently studied dietary fiber, was evaluated.

Animals and housing. Male Sprague-Dawley rats weighing approximately 230 g were divided into 4 groups of 11 rats each. The rats were housed individually in stainless steel cages with raised wire floors. The animal room was maintained at $20\pm1^{o}C$ with controlled humidity and a 12-hour light-dark cycle.

Diets. The diet composition is shown in Table 3. Three levels of wheat bran, 2 g (Diet 2), 4 g (Diet 3), and 8 g (Diet 4), were isocalorically substituted for cornstarch in the control diet (Diet 1). The caloric values of wheat bran (2.13 kcal/g) and of other dietary nutrients were taken from Composition of Foods, Agricultural Handbook No. 8, USDA,

TABLE 3. Composition of diets for experiment I (g/100 kcal)

	Diet 1	Diet 2	Diet 3	Diet 4
Na-caseinate	4.7	4.2	4.2	3.3
Vitamin mix[a]	0.2	0.2	0.2	0.2
Mineral mix[b]	0.9	0.9	0.9	0.9
Lard[c]	3.3	3.3	3.3	3.3
Corn Oil[d]	1.3	1.1	1.1	0.9
Cholesterol	0.2	0.2	0.2	0.2
L-methionine	0.07	0.07	0.07	0.07
Choline·Cl	0.04	0.04	0.04	0.04
Cornstarch	11.6	10.9	10.0	8.4
Wheat Bran[e]	-	2.0	4.0	8.0

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[a]The vitamin mix was composed of thiamin·HCl 11 g; pyridoxine 11g; riboflavin 11g; CaPantothenate 33 g; p-aminobenzoic acid 55 g; menadione 25 g; inositol 50 g; ascorbic acid 100 g; niacin 50 g; vitamin B_{12} 0.015 g; biotin 0.3 g; folic acid 2 g; retinol acetate 1×10^7 IU; α-tocopherol 50,000 IU; vitamin D_3 110,000 IU and cerelose to 5 kg.

[b]The mineral mix (salt mix No. 4164, Teklad, Inc., Madison, WI.) contained g/100 g: calcium acetate·H_2O, 6.293; calcium diphosphate·$2H_2O$, 28.525; dipotassium phosphate, 28.443; ferric citrate·$5H_2O$, 2.44; magnesium sulfate·$7H_2O$, 10.053; potassium iodide, 0.65; sodium diphosphate·$12H_2O$, 14.630; sodium chloride, 9.546.

[c]Farmer Peet's Shortenin'-Pork and beef fat-Peet Packing Company, Chesaning, Michigan.

[d]Mazola oil

[e]Feed grade wheat bran

1963 (82). These three levels of wheat bran were added to provide 1.1, 2.2, and 4.4 g of neutral detergent residue (NDR) per 100 kcal for diets 2,3 and 4, respectively. The neutral detergent residue of wheat bran was analyzed according to Van Soest and Wine (83). These diets were fed for 4 weeks (period I) and then all animals were switched to the bran-free control diet for another 4 weeks (period II).

Analyses. In the beginning of the 4th week of period I, 100 mg of chromic oxide was administered intragastrically to 6 rats in each group to measure intestinal transit time. Fecal collections were made every 8 hours for the first day and then every 12 hours for the next 2 days. The feces were dried to a constant weight at 60°C in a forced air oven and stored for chromic oxide analysis. Chromic oxide was determined according to the method of Bolin et al. (84), and transit time was designated as the time required for 95% of ingested chromic oxide to be excreted.

During the 4th week of periods I and II, feces were collected for 3 successive 24 hour periods and dried as above. Wet and dry weights were determined gravimetrically. The samples were pooled by group for bile acid analysis.

Bile acids were extracted from aliquots of pooled feces as described by Grundy et al. (85). Cholic acid (24-^{14}C) was added to each sample at the beginning of the analysis as an internal standard to correct for incomplete recoveries during extraction. Separation of bile acids from fatty acids

was as described by Makita and Wells (86). Methyl esters of bile acids were prepared with excess of ethereal-diazomethane. The methylated bile acids were dried, silylated (85) and trimethylsilyl ethers (TMS ethers) were quantitated on a GLC equipped with a flame ionization detector. The column temperature was 210°C for 20 minutes after sample injection and it was programmed at 8°C/min to 270°C and held at 270°C until the last bile acid was eluted. Holding the temperature at 210°C for 20 minutes allowed remaining fatty acid methyl esters to be separated from the TMS ethers of bile acids. The injector temperature was 280°C; detector temperature, 290°C; H_2 flow, 30 ml/min and N_2 carrier gas flow, 35 ml/min. The column was 6 ft long, 1/8 in O.D. and packed with 3% SP2401 on Supelcoport 100/120. Cholic, lithocholic, deoxycholic, chenodeoxycholic, 12-keto-deoxycholic and 3,12-diketo-deoxycholic acid standards were methylated and silylated as above with 5-α-cholestane as an internal standard. The area under the recorder tracing for each standard was cut out and weighed. Standard curves were prepared by plotting the ratio of bile acids to 5-α-cholestane vs. the quantity of bile acids injected. The quantity of bile acids excreted in the feces was determined from the standard curves. An average standard curve was used to quantitate all bile acids. Bile acids are reported as the sum of all bile acids. Bile acids which did not chromatograph with cholic and chenodeoxycholic acids are designated as degraded bile acids.

At the end of the 4th week of each experimental periods I and II, cecums from 3 animals from each group were removed for anaerobic bacterial count and *in vitro* bile acid fermentation. A portion of cecal contents from each rat was serially diluted to 10^{-9} concentration and incubated in anaerobic roll tubes at 37°C for 5 days using M98-5 medium without hemin as described by Salanitro et al. (87). Colonies were counted after a 5-day incubation period. This procedure was conducted as soon as the rats were killed and all steps were performed aseptically and anaerobically. Cholic acid fermentation was determined by incubating 1 ml of the 10^{-1} dilution of cecal contents with 0.1 mmole of cholic acid, 10 ml of dilution solution and 1 g of diet which the animal had been eating. The fermentations were conducted anaerobically in 50 ml serum bottles at 37°C for 24 hours. Fermentation was stopped by injecting 2 ml of concentrated HCl into the serum bottles. Blank fermentations were carried out by injecting sterile dilution solution instead of cecal dilutions into the serum bottles. Microbial degradation of cholic acid was designated as the quantity of cholic acid fermented to other bile acids minus blank fermentations. Bile acids were extracted from the fermentation reaction and analyzed as described for the fecal samples.

Experiment 2

In experiment 2, male Sprague-Dawley rats (average 260 g) were allotted into 2 groups of 10 rats each. The conditions of animal care were same as experiment 1.

Diets. Composition of the diets is shown in Table 4. Fat provided 45% of the energy in the high-fat, low-fiber diet and there was no added fiber. Fat provided 7% of the energy in the low-fat, high-fiber group and 2 g of agar per 100 kcal was added as fiber source. Agar provided 7% fiber by weight and the caloric value of agar was excluded in the energy calculation. The rats were fed the diets for 4 weeks.

Analyses. After the diets were fed for 3 weeks, feces were collected for one week at 9 a.m. each morning. The fecal samples were kept frozen at -20°C until all the collections were made and then the daily collections were pooled for each animal. The feces were dried as in experiment 1. Wet and dry weights were determined gravimetrically.

Bile acids and neutral steroids were extracted from aliquots of pooled feces after saponification with 20% KOH in ethylene glycol as described by Evrad and Janssen (88). ^{3}H-cholesterol and cholic acid (24-^{14}C) were added to each sample at the beginning of the analysis as internal standards to correct for incomplete recoveries during extraction.

5-α-cholestane was added to the neutral steroids as an internal standard for GLC analysis and the dried steroids were silylated as described for the bile acids in experiment 1. Cholesterol, coprostanol and coprostanone standards were silylated and chromatographed with 5-α-cholestane as an internal standard. Neutral steroid separation was on 6 ft. x 1/8 in. column packed with 3% OV 17 on 100/120 Gas Chrome Q.

TABLE 4. Composition of diets in experiment 2 (g/100 kcal)

	Diets	
	High-fat, low-fiber	Low-fat, high-fiber
Na-caseinate	4.0	4.0
Vitamin Mix[a]	0.2	0.2
Mineral Mix[b]	0.8	0.8
Tallow[c]	4.2	-
Corn Oil[d]	0.8	0.8
Cholesterol	0.1	-
L-methionine	0.06	0.06
Choline·Cl	0.06	0.06
Cornstarch	9.8	19
Agar[e]	-	2

[a]See footnote a, Table 3

[b]See footnote b, Table 3

[c]Beef fat was melted and filtered through cheese cloth.

[d]Mazola oil

[e]Difco Bacto-Agar, Difco Laboratories, Detroit, Michigan.

The injector temperature, detector temperature, and column temperature were 270°C, 290°C, and 250°C, respectively. All other conditions were the same as described for bile acid separation in experiment 1. A Varian CDS 111 electrical integrator was used to quantitate bile acid and neutral steroid content of the samples. Neutral steroid excretion is expressed as the sum of cholesterol, coprostanol and coprostanone excretion.

The separation of bile acids from fatty acids, methylation and silylation of bile acids were done as in experiment 1. The bile acids were separated on a 6 ft.long x 1/8 in. O.D. column packed with 3% SP 2100 on Supelcoport 100/120.

The column temperature was 210°C for 10 minutes after sample injection and then the temperature was programmed 4°C/min to 270°C and held at 270°C until all bile acids were eluted. Other GLC conditions were same as described for experiment 1.

After steroid analyses, all feces were pooled by group and the feces were extracted with chloroform and water for use in the second part of experiment. The feces were acidified with HCl in an aqueous slurry and excess chloroform was added. The mixture was mixed well in a separatory funnel, and the chloroform layer was collected. This procedure was repeated until no apparent color was extracted with chloroform. The chloroform extract was dried in a flash evaporator. The extract was transferred to a screw cap tube

with chloroform and dried under a stream of nitrogen. Aliquots of the chloroform extracts were analyzed for bile acid and neutral steroid contents as above.

To prepare the water extract of the feces excess water was added to the feces and heated at 60°C for 1 hour. Heating appeared to extract more compounds. The resultant slurry was filtered through Whatman #1 filter paper and the collected filtrate was dried in a flash evaporator. Both dried fecal and water extracts were kept at 4°C until used.

At the end of the 4 week feeding period, the bile duct was cannulated with polyethylene tubing (PE 10, Intramedic®, Parsippany, N.J., I.D. 0.011", O.D. 0.024"). The rats were anaesthetized by ether. One end of the tubing was tied in place in the bile duct and the other end of the tubing was routed under the skin to the midpoint of the back and pulled out through a small puncture in the skin. The tubing was taped in a small test tube and the test tube was placed on back of the rat with tape in such a way that the collected bile in tube would not be spilled by the movement of rat. Bile was emptied at necessary intervals from the tube. Bile was collected for 24 hours and food and water were available to the rats during the bile collection. Bile was kept frozen at -20°C for steroid analysis. Bile acids and neutral steroids were extracted from aliquots of bile from each rat and analyzed by GLC as described for fecal samples. After steroid analysis, the bile was pooled by group and dried in a vacuum oven at 60°C with a nitrogen atmosphere and stored at 4°C for the experiment in part II.

PART II

Part II was designed to develop a rapid, in vitro method to screen agents which could be responsible for the cause and development of colon cancer. Several cell culture techniques were evaluated to screen potential intestinal mutagens.

1. Culture Media and Growth Conditions

Unless stated otherwise, the growth medium was made with Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal calf serum, antibiotics and fungicide (125 units/ml of penicillin, 125 µg/ml of streptomycin, 3.44 µg/ml of fungizone). All of the above were purchased from Grand Island Biological Co., Grand Island, N.Y.

Buffered Saline Solution (BSS) contained 137 mM NaCl, 2.7 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2 \cdot 6H_2O$, 0.15 mM $NaH_2PO_4 \cdot H_2O$, 1.36 mM $Na_2HPO_4 \cdot 7H_2O$, 6mM $NaHCO_3$, 5.5 mM glucose and phenol red as a pH indicator. The pH was adjusted to 7.4 with CO_2 gas. $CaCl_2$ and $MgCl_2 \cdot 6H_2O$ were omitted from the BSS to prepare Ca^{++}, Mg++ -free BSS which was used to prepare a solution for trypsinizing cells.

Ouabain (2mM, Sigma Chemical Co., St. Louis, Mo.) containing EMEM was prepared in batch quantities, sterilized by filtering through a 0.22 micron filter and stored at $4^{\circ}C$. This was diluted and reconstituted with 10% fetal calf serum and antibiotics as needed. Ouabain has been reported to be stable in solution for periods of many months (76).

Cultures were incubated in growth medium at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

To stain and count colonies, cultures were rinsed 3 times with BSS, fixed in absolute methanol for 5 minutes, and stained with Giemsa stain for 20 minutes at room temperature. After staining, the colonies were counted with a stereo microscope (10-30X).

2. Preparation of Reagents Used.

N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was dissolved in sterile distilled water (20 µg MNNG/50 ml water). The MNNG solution was added to culture dishes containing growth medium to provide indicated concentrations. No further attempt was made to sterilize the MNNG solution.

3α, 12α-dihydroxy-5β-cholanic acid (deoxycholic acid), 3α-hydroxy-5β-cholanic acid (lithocholic acid), 3α, 6α-dihydroxy-5β-cholanic acid (hyodeoxycholic acid) and 3, 12-dione-5β-cholanic acid were dissolved in dimethyl sulfoxide (DMSO). The solutions were filter sterilized with a 0.2 micron FGLP solvent resistant filter (Millipore Corporation, Bedford, Ma.). These sterile solutions were added to culture dishes to provide indicated concentrations. The final DMSO concentration was less than 0.5%.

3α, 7α, 12α-trihydroxy-5β cholanic acid (cholic acid) and 3α, 7α-dihydroxy-5β-cholanic acid (chenodeoxycholic acid) were neutralized with 1 N NaOH, made 5mM by dissolving in growth medium and filter sterilized. These solutions were diluted with growth medium to the indicated concentrations before use. If cholic and chenodeoxycholic

acids were dissolved in DMSO and added to the culture dishes containing growth medium, the DMSO solutions would not go into solution or suspensions. Instead, DMSO solutions of cholic and chenodeoxycholic acids caused protein coagulation and pH alterations of the growth medium.

Deoxycholic and 3,12-dione-5β-cholanic acids were purchased from Steraloids, Inc., Wilton, N.H. Cholic and hyodeoxycholic acids were purchased from Sigma Chemical Co., St. Louis, Mo., and lithocholic and chenodeoxycholic acids were purchased from Aldrich Chemical Co., Milwaukee, Wis.

The chloroform extract of feces from experiment 2 was weighed, dissolved in DMSO and sterilized by filtration. Also, the fecal water extract was weighed, dissolved in water and filter sterilized.

Dried bile was reconstituted to the original volume with growth medium and filter sterilized. This bile containing medium was diluted with growth medium to provide various concentrations before use.

3. Isolation of Intestinal Epithelial Cells

First Approach: Sprague-Dawley rats were killed and the large intestine was removed. The large intestine was cut longitudinally and the inside was rinsed with BSS supplemented with antibiotics and fungicide (125 units/ml of penicillin, 125 μg/ml of streptomycin, 50 μg/ml of gentamycin, 3.44 μg/ml of fungizone).

The mucosal surface of the intestine was removed from the muscular layers by scraping with a scalpel. The

resultant tissue was trypsinized with 0.1% trypsin for 3 minutes to dissociate cells. This was centrifuged 1500 x g for 3 minutes and the precipitate was suspended in culture medium (EMEM supplemented with 10% horse serum and same concentrations of antibiotics as in BSS) and plated in a culture dish (Falcon and Corning). After 24 hours, and then daily, the culture medium was discarded, the cells were rinsed with BSS and fresh culture medium was added.

Second Approach: Tissue culture/cell culture technique.

Small pieces (approximately 2x2 mm) were cut from the large intestine of a rat. These pieces were laid on a plastic culture dish mucosal side down without medium. The dish was rinsed with growth medium (EMEM supplemented with 10% fetal calf serum and same concentrations of antibiotics as above) before use. The dish which contained several pieces of intestinal explants was placed in the incubator for 1 hour during which time the explants were allowed to attach to the plastic surface. After 1 hour of incubation, the growth medium was very carefully added to the dish so that the attached pieces were not disturbed. The dish was incubated for 4 days without medium change or disturbance. After 4 days of incubation, the culture dish was rinsed and fresh culture medium was added daily thereafter. Most of the tissue was washed away with the daily rinsing. Epithelial cells grew out from where the explants had attached to the dish.

Third Approach: Epithelial cells were isolated from the large intestine of Sprague-Dawley rats according to the procedure presented by Weiser (89). Citrate, not enzyme, was used to dissociate the epithelial cells. A piece of sterile tubing (Bardic Feeding Tube, size 8, C.R. Bard Ind., Murray Hill, N.J.) was inserted in each end of the large intestine and tied in place to fill and remove solutions with a syringe. All the solutions were made as described (89), filter sterilized and antibiotics and fungicide were added (150 units/ml of penicillin, 150 µg/ml of streptomycin, 50 µg/ml of gentamycin, 3.51 µg/ml of fungizone). The crypt and villus cells were isolated together by incubating the intestine for 52 minutes with solution B.

4. Isolation of Intestinal Fibroblasts.

Pregnant Sprague-Dawley rats in the 18-19 day of gestation were purchased. Both the small and large intestine was taken from the rat fetus. The intestine was chopped into small pieces and put into 0.2% trypsin in Ca^{++}·Mg^{++} -free BSS in a sterile culture tube for 5 minutes. The tube was mixed vigorously during this time with a vortex mixer to dissociate the cells. After centrifugation for 3 minutes, the precipitate was resuspended in growth medium and plated. After 6 hours, the medium was changed since there was a yellow secretion in the intestine which greatly decreased cell viability. Thereafter, the cells were washed and culture medium was changed daily. The attached cells were trypsinized with 0.2% trypsin whenever necessary to prevent

confluency. The trypsinized cells were collected by centrifugation and replated until there were a sufficient number of fibroblasts to conduct an experiment.

5. Protocols of Experiments.

Fibroblasts from the intestine of a rat fetus were subcultured 2-5 times before the experimental treatment. The fibroblasts were plated in 15 cm plates and grown to about 20% confluency.

On the day of treatment, the compounds at designated concentrations were added to the plates and incubated for 3 hours. After the 3 hour incubation, the cultures were washed with BSS twice and growth medium was added to the plate. The medium was changed daily for 3 days (expression time). After 3 days cells were trypsinized and counted with a hemocytometer. Plating efficiency was determined by seeding 200 cells in 6 cm plates with growth medium. Triplicate dishes were used for measuring plating efficiency. Culture medium was changed every 3 days. After 8 days, cells were fixed, stained and counted. Plating efficiency was calculated as follows:

$$\text{Plating efficiency} = \frac{\text{Average number of colonies in plate (A)}}{200}$$

Ouabain resistant mutants (Oua-R mutants) were determined by plating 3×10^4 cells in 6 cm plates with 1.0 mM ouabain containing medium. Four to 8 replicate dishes were used. Medium was changed every 3 days. After 14 days the cells were fixed and stained, and colonies were counted.

Oua-R mutants (mutation frequency) was calculated per 10^5 survivors, taking into account the plating efficiency of each plate as follows:

$$\text{Oua-R mutants}/10^5 \text{ survivors} = \frac{10^5}{(\text{plating efficiency}) \times (3 \times 10^4)} \times \text{average number of ouabain resistant colonies in mutagenesis plates (B)}$$

To determine optimal ouabain concentration and expression time to select Oua-R mutants, different concentrations of ouabain in medium and different times for the expression period were used as indicated. Protocols for the determination of plating efficiency (A) and mutagenesis (B) are summarized in Figure 2.

Most mutagenic compounds are cytotoxic so that percent cell survival needed to be determined. Percent survival for the test compounds was determined by plating 200 cells in 6 cm plates. 16-18 hours later, the cells were incubated with various concentrations of the test compounds for 3 hours. The culture dishes were then washed twice with BSS and fresh growth medium was added to the dish. Medium was changed every 3 days and the cells were fixed and stained after 8 days. Percent survival was calculated as follows:

$$\% \text{ survival} = \frac{\text{Average number of colonies in dishes (C)}}{200} \times 100\%$$

Figure 2 also shows the protocol for determination of cytotoxicity for the various test compounds.

Figure 2. Protocols for (A) plating efficiency, (B) mutagenesis, and (C) cytotoxicity.

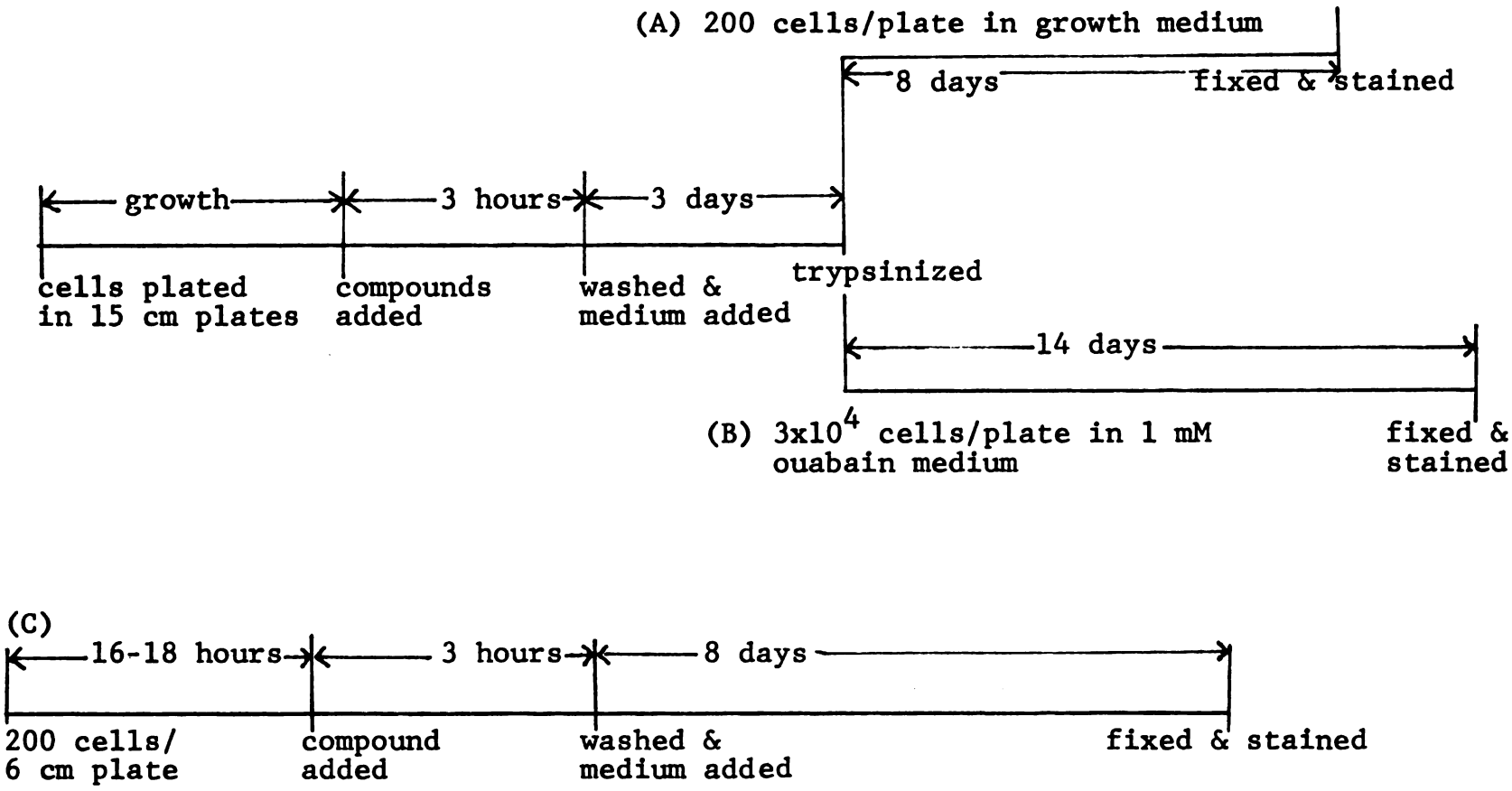

(A) 200 cells/plate in growth medium
8 days
fixed & stained
growth
3 hours
3 days
cells plated in 15 cm plates
compounds added
washed & medium added
trypsinized
14 days
(B) 3x10^4 cells/plate in 1 mM ouabain medium
fixed & stained
(C)
16-18 hours
3 hours
8 days
200 cells/ 6 cm plate
compound added
washed & medium added
fixed & stained

FIGURE 2.

The tumor promoting effect of bile acids were tested as described by Trosko et al. (75) with some modifications. Cultures were treated with 1.5 μg MNNG/ml of medium for 3 hours, washed with BSS and growth medium was added. After the expression period, cells were trypsinized and plated for the determination of plating efficiency and Oua-R mutant selection as described above. At this time various bile acids, at indicated concentrations, were added to the plates used for both plating efficiency and mutagenesis determination. Medium was changed every 3 days and each time bile acids were added. After 2 weeks, cultures were fixed, stained and colonies were counted.

6. Doubling Time Determination.

Fibroblasts in 15 microscopic fields per plate were counted every 24 hours for 3 consecutive days. The average number of cells were plotted against time to determine doubling time. Doubling times were determined on two different occasions with a different source of cells on each occasion. Triplicate plates were utilized on each ocassion.

RESULTS

PART I

Experiment 1

Energy consumption and weight gain were similar among groups throughout the experimental periods (Table 5). Both wet and dry fecal mass were increased as the level of bran in the diet increased. When the rats were switched to the bran-free diet (period II), fecal mass decreased to levels similar to those in controls in period I. Intestinal transit time was inversely proportional to the level of bran in the diet. No significant differences in total bile acid excretion were found among rats fed different levels of bran containing diets. However, fecal concentration of bile acid (mg of bile acids per g of wet feces) decreased as the level of bran in the diet increased since fecal weights were greater when bran was added in the diet. The fecal bile acid concentration decreased by 25, 50 and 65% of control group for diets 2 (2 g of bran per 100 kcal), 3 (4g of bran), and 4 (8 g of bran), respectively. The percent of fecal bile acids that were degraded increased with level of bran in the diet. When rats were fed the control diet during period II this difference in bile acid degradation disappeared. Cholic acid degradation also increased in the

TABLE 5. Bowel function, steroid metabolism and bacterial content of the cecum in rats fed graded levels of wheat bran.

	Diet 1[a]	Diet 2	Diet 3	Diet 4
Energy consumption, kcal/day/rat				
Period I[b]	86	83	84	84
Period II[c]	86	86	80	72
Weight gain, g/day/rat				
Period I	5.1	4.9	5.0	5.3
Period II	2.4	2.5	2.4	2.4
Fecal excretion, Period I				
wet mass, g/day/rat	1.0±0.07[d]	2.1±0.14*	2.5±0.12*	4.6±0.25*
dry mass, g/day/rat	0.6±0.05	1.3±0.05	1.7±0.09*	3.2±0.13*
Fecal excretion, Period II				
wet mass, g/day/rat	0.9±0.12	1.5±0.15*	1.1±0.11	1.1±0.10
dry mass, g/day/rat	0.6±0.05	0.8±0.04	0.7±0.04	0.7±0.04
Transit time, hours	68.0±5.0	62.7±0.7	57.3±1.9*	21.5±1.9*
Fecal bile acids, Period I				
excretion, mg/day/rat	11.0±2.6	15.1±1.4	13.7±1.6	17.4±5.0
concentration, mg/g wet feces	10.9±2.8	8.0±1.5	5.5±1.6	4.0±1.2*
% degradation,[e] Period I				
feces	44±3	57±4	63±2*	73±0*
<u>in vitro</u>	28±12	49±11	37±9	46±8
% degradation,[e] Period II				
feces	54±2	52±2	52±2	52±2
<u>in vitro</u>	31±7	27±7	26±2	27±10

TABLE 5 (Continued . . .)

	Diet 1[a]	Diet 2	Diet 3	Diet 4
Bacterial count[f]				
Period I	7.6	7.6	4.3	4.7
Period II	5.0	6.7	7.2	8.6
Cecal weight, g				
Period I	3.2 ± 0.2	4.1 ± 0.4	4.2 ± 0.2	3.7 ± 0.6
Period II	4.2 ± 0.2	3.6 ± 0.2	3.5 ± 0.4	4.8 ± 0.1

[a]See Table 3 for diet description.

[b]Diets contained graded levels of wheat bran. (0,2,4,8 g of wheat bran per 100 kcal for diets 1,2,3,4, respectively).

[c]All diets in Period II were bran-free diets.

[d]Mean $\pm$ S.E.

[e]% of secondary bile acids.

[f]Number of viable bacteria per g of cecal contents x 10^{-10}.

*Significantly different from control ($p < 0.05$). Treatment differences were tested by Dunnett's test.

in vitro fermentations from cecal contents of bran-fed rats, however, the linear increase in cholic acid degradation with increased levels of bran in the diet was not observed in the *in vitro* fermentation. When rats were fed a bran-free diet, the percent bile acid degradation in feces and *in vitro* fermentation was comparable to that of control rats in period I.

When a low level of bran (2g of bran) was added in the diet, the number of anaerobic bacteria per g of cecal contents stayed the same as in the control group, but the concentrations of anaerobic bacteria in cecal contents decreased with higher levels of bran (4 g or 8 g of bran) in the diet. Since there was no difference in cecal weights between groups, it is likely that the number of anaerobic bacteria per cecum also decreased with increased levels of bran in the diet.

Experiment 2

There was no difference in energy intake and weight gain between the two groups of rats for the 4 week period (Table 6). Both wet and dry fecal weights were significantly higher for the group fed the low-fat, high-fiber diet compared to the group fed the high-fat, low-fiber diet.

Total bile acid excretion was similar between the two groups, however, the concentration of bile acids was lower in the group fed the low-fat, high-fiber diet. Total neutral steroid excretion and neutral steroid concentrations were significantly greater in rats fed the high-fat, low-fiber diet compared to rats fed the low-fat, high-fiber diet.

TABLE 6. Fecal mass and steroid metabolism in feces and in bile from rats fed either a high-fat low-fiber diet or a low-fat, high-fiber diet.

	High fat, low-fiber diet	Low-fat, high-fiber diet
Energy consumption, kcal/day/rat	82	76
Weight gain, g/day/rat	4.8	4.3
Fecal excretion		
wet mass, g/day/rat	2.2±0.2[a]	5.7±0.5*
dry mass, g/day/rat	1.1±0.05	2.0±0.05*
Fecal bile acids		
excretion, mg/day/rat	12.7±1.2	12.1±0.6
concentration, mg/g wet feces	6.2±0.9	2.3±0.3*
Fecal neutral steroids		
excretion, mg/day/rat	58.7±2.6	13.4±1.3*
concentration, mg/g wet feces	29.5±3.5	2.4±0.3*
% degradation[b]	53 ±2.5	81 ±0.5*
coprostanol/coprostanone	2.2±0.2	0.5±0.02*
Bile		
bile acid concentration, mg/ml	5.2±1.4	5.1±1.5
neutral steroid concentration, mg/ml	0.12±0.02	0.08±0.02

[a]Mean ±S.E.

[b]% of coprostanol and coprostanone.

*Difference is significant between the 2 groups ($p < 0.05$). Treatment differences were tested by Student's t test.

However, more extensive degradation of cholesterol to coprostanol and coprostanone was seen in the feces of rats fed the low-fat, high-fiber diet. In the high-fat, low-fiber group, more cholesterol was degraded to coprostanol than to coprostanone; however, rats fed the low-fat, high-fiber diet excreted more coprostanone than coprostanol in the feces.

There was no difference in bile acid or neutral steroid concentration in bile collected from the bile duct of rats fed the two diets.

PART II

1. Intestinal Epithelial Cell Isolation

It was not possible to obtain cells using the scraping method. Cells were dissociated with the use of citrate but they did not attach to the bottom of the plate and did not grow.

Figure 3 shows the epithelial cells isolated by the tissue culture/cell culture technique. The pieces of tissue degenerated with time (approximately 5 days) and the cells grew out from the vicinity of tissue explant attached to the surface of the plates. Many of these cells survived for up to 5 weeks, but there was no sign of increasing cell numbers.

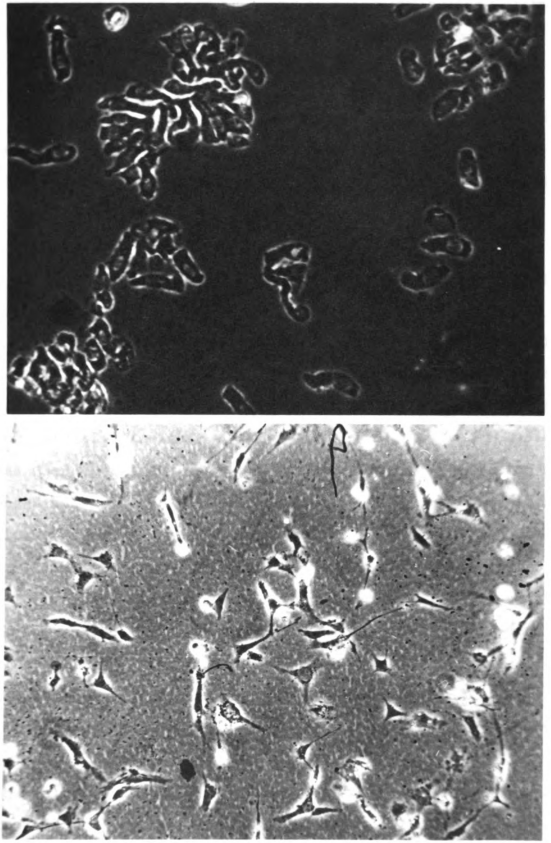
In all of the above trials the biggest problem encountered was contamination. Since the large intestine has

Top

Figure 3. Phase micrographs of epithelial cells isolated from rat intestine by tissue culture/cell culture method (x 655) (see material and method).

Bottom

Figure 4. Phase micrographs of fibroblasts isolated from intestine of rat fetus. (x 464).



a large number and many varieties of microorganisms, it was almost impossible to eliminate the growth of microflora.

2. Isolation of Fibroblasts and Measurement of Doubling Time.

Figure 4 shows the fibroblasts isolated from the intestine of rat fetus. The problem of microbial contamination was greatly reduced when cells were isolated from fetal tissue. Doubling time of these cells were 20 hours.

3. Mutagenesis Study

In order to measure the dose response of fibroblasts to MNNG, cells were treated with different levels of MNNG for 3 hours and then the Oua-R colonies were selected at 1.0 mM ouabain in growth medium. The number of Oua-R colonies increased linearly, whereas cell survival was decreased as MNNG concentration increased (Fig. 5). 1.5 μg MNNG/ml of medium was used thereafter to optimize conditions for selection of Oua-R mutants. This concentration of MNNG gives high mutation frequencies with reasonably high percent survival.

To determine the optimum ouabain concentration to select Oua-R mutants, cells treated with 1.5 μg MNNG/ml of medium and non-treated control cells were grown in medium with different concentrations of ouabain. The MNNG treated cells had more Oua-R colonies than non-treated cells at all concentrations of ouabain (Fig. 6). When cells were grown in medium with ouabain concentrations greater than 1.0 mM, the majority of the cells did not survive in either the control or the MNNG treated groups. Cells forming colonies at

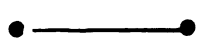


Figure 5. Cytotoxicity and ouabain resistant mutant response to MNNG dose.

●———● ouabain resistant mutants per 10^5 survivors.

o--------o percent survival

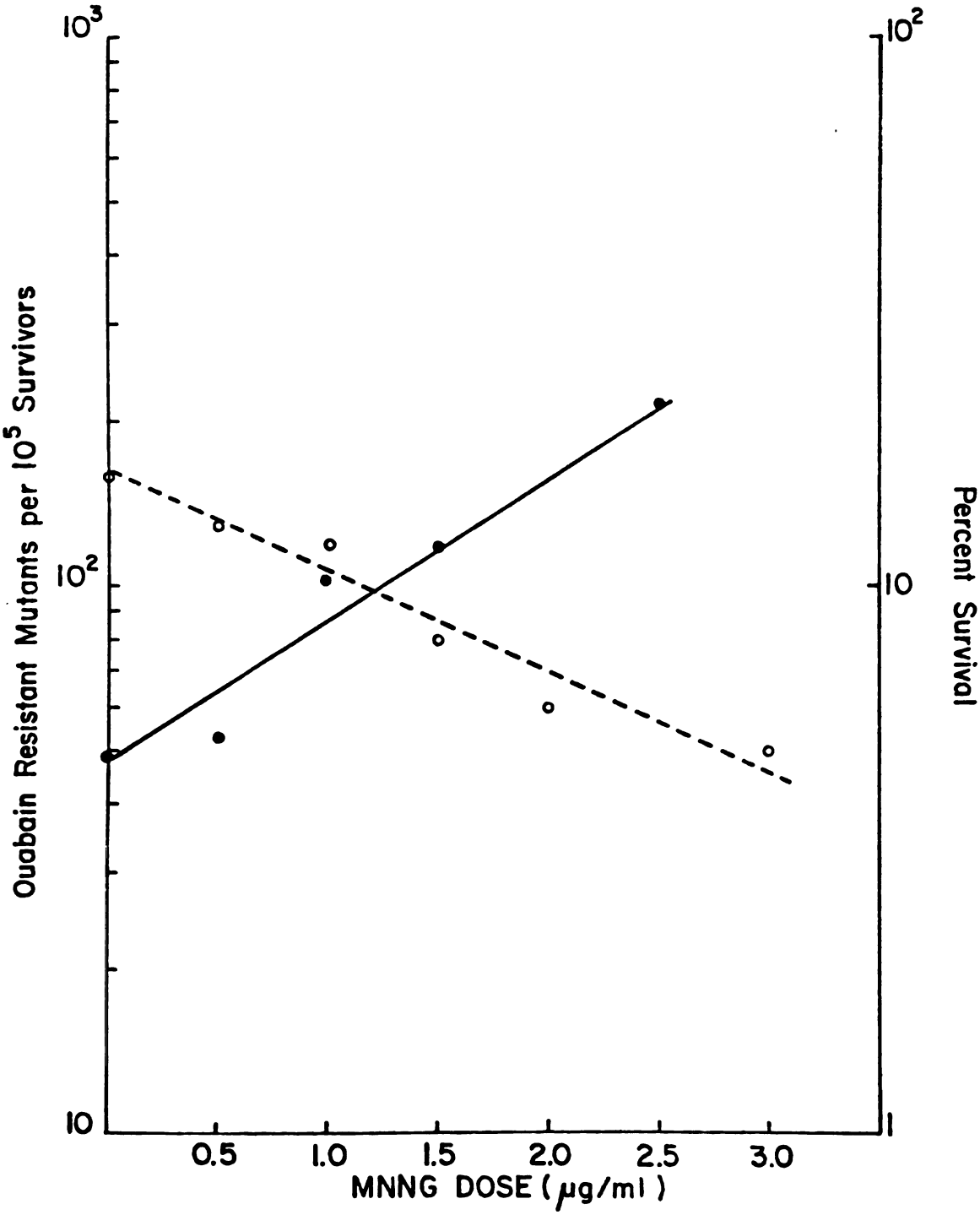

10^3
10^2
10
10^2
10
1
Ouabain Resistant Mutants per 10^5 Survivors
Percent Survival
0.5
1.0
1.5
2.0
2.5
3.0
MNNG DOSE (μg/ml)

FIGURE 5.

FIGURE 6. Effect of ouabain concentration on control and MNNG treated cells.

●————● control cells

o--------o MNNG treated cells (1.5 μg/ml)

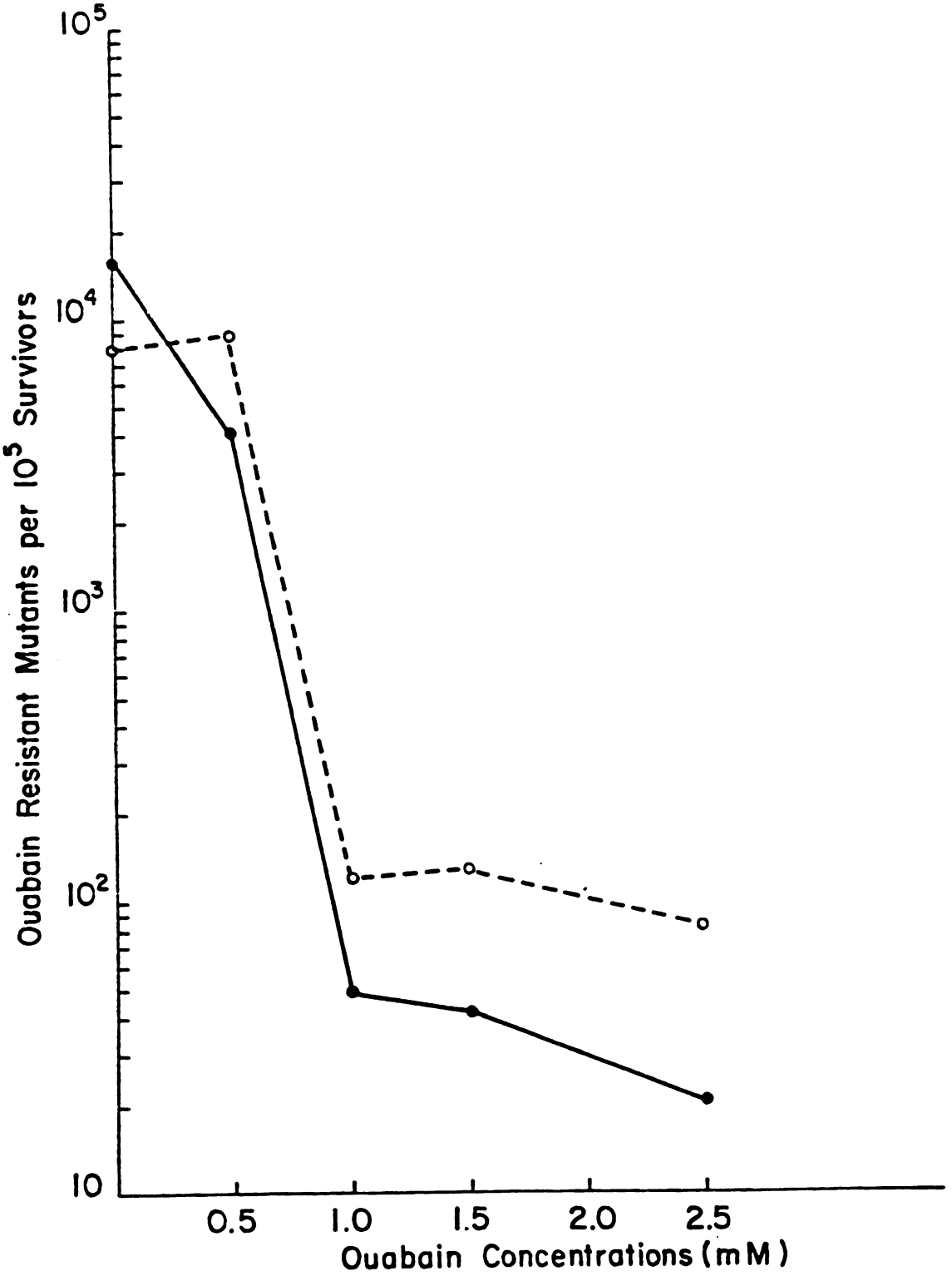

10^5
10^4
10^3
10^2
10
Ouabain Resistant Mutants per 10^5 Survivors
0.5
1.0
1.5
2.0
2.5
Ouabain Concentrations (mM)

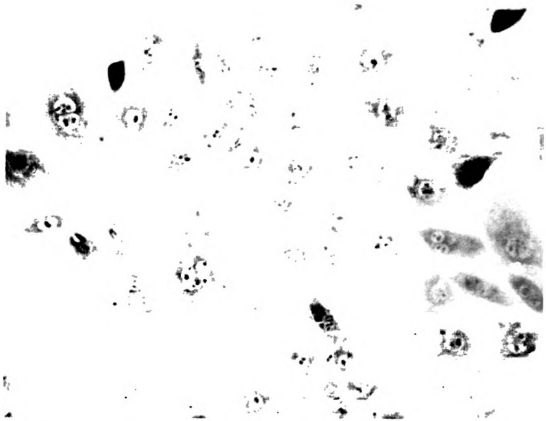
FIGURE 6.

ouabain concentrations greater than 1.0 mM in the medium are considered ouabain resistant or mutant cells. Also, as the concentration of ouabain in the medium increased, the colony size decreased. A concentration of 1.0 mM ouabain in the medium was used for all the subsequent experiments because this concentration of ouabain optimized the difference between MNNG-induced mutation and spontaneous mutation and because 1.0 mM ouabain allowed more satisfactory growth of mutant colonies than higher concentrations. Figure 7 shows colonies grown in either growth medium or in 1.0 mM ouabain-containing medium.

To determine the optimum-expression time, plates were treated with 1.5 μg MNNG/ml for 3 hours. MNNG-treated cells were then trypsinized and replated, and 1.0 mM ouabain was then added on subsequent days to optimize for selection of Oua-R cells. Plates with more than 3 days of expression time were trypsinized and subcultured into 15 cm plates to prevent confluency. Figure 8 show the results. Mutation frequency increased linearly until 3 days of expression time, but decreased with time thereafter.

Bile acids were found to be cytotoxic but the degree of cycotoxicity of the various bile acids was different. Figure 9 shows the cytotoxicity of six different bile acids when cells were treated with graded concentrations of these acids. There appear to be two groups of bile acids in terms of cytotoxicity. Lithocholic, deoxycholic and chenodeoxycholic acids were much more cytotoxic than cholic, hyodeoxycholic and 3,12-dione-5β-cholanic acids.

FIGURE 7. Bright field micrograph of Giemsa stained cells (x 688) (top) colony grown in growth medium for 8 days, (bottom) colony grown in 1.0 mM ouabain-containing medium for 14 days.



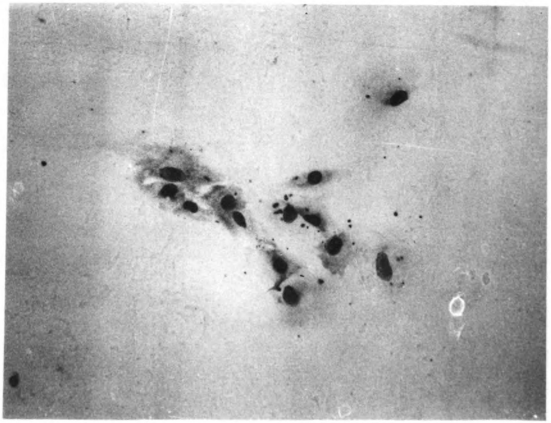


FIGURE 8. Expression time of MNNG (1.5 μg/ml)-induced ouabain resistant mutations. Expression times shown are intervals between MNNG treatment and exposure to ouabain-containing medium (1.0 mM).

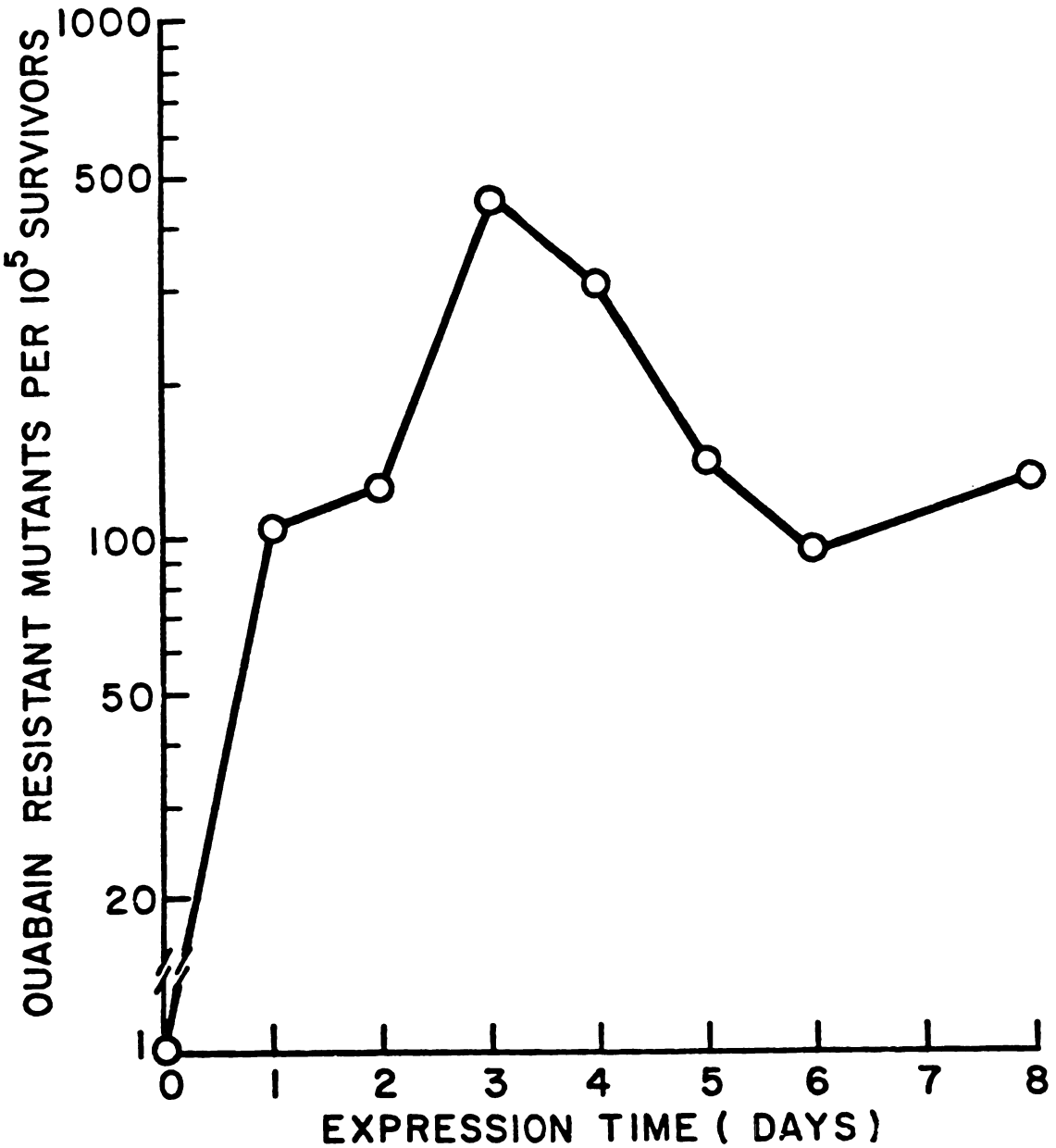
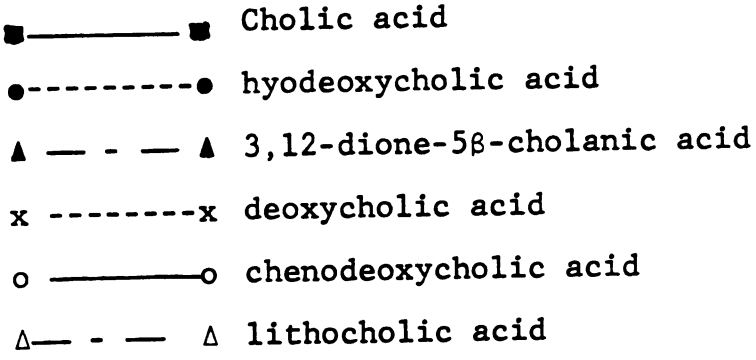

OUABAIN RESISTANT MUTANTS PER 10^5 SURVIVORS
1000
500
100
50
20
1
0
1
2
3
4
5
6
7
8
EXPRESSION TIME (DAYS)

FIGURE 8.

FIGURE 9. Cytotoxicity of selected bile acids



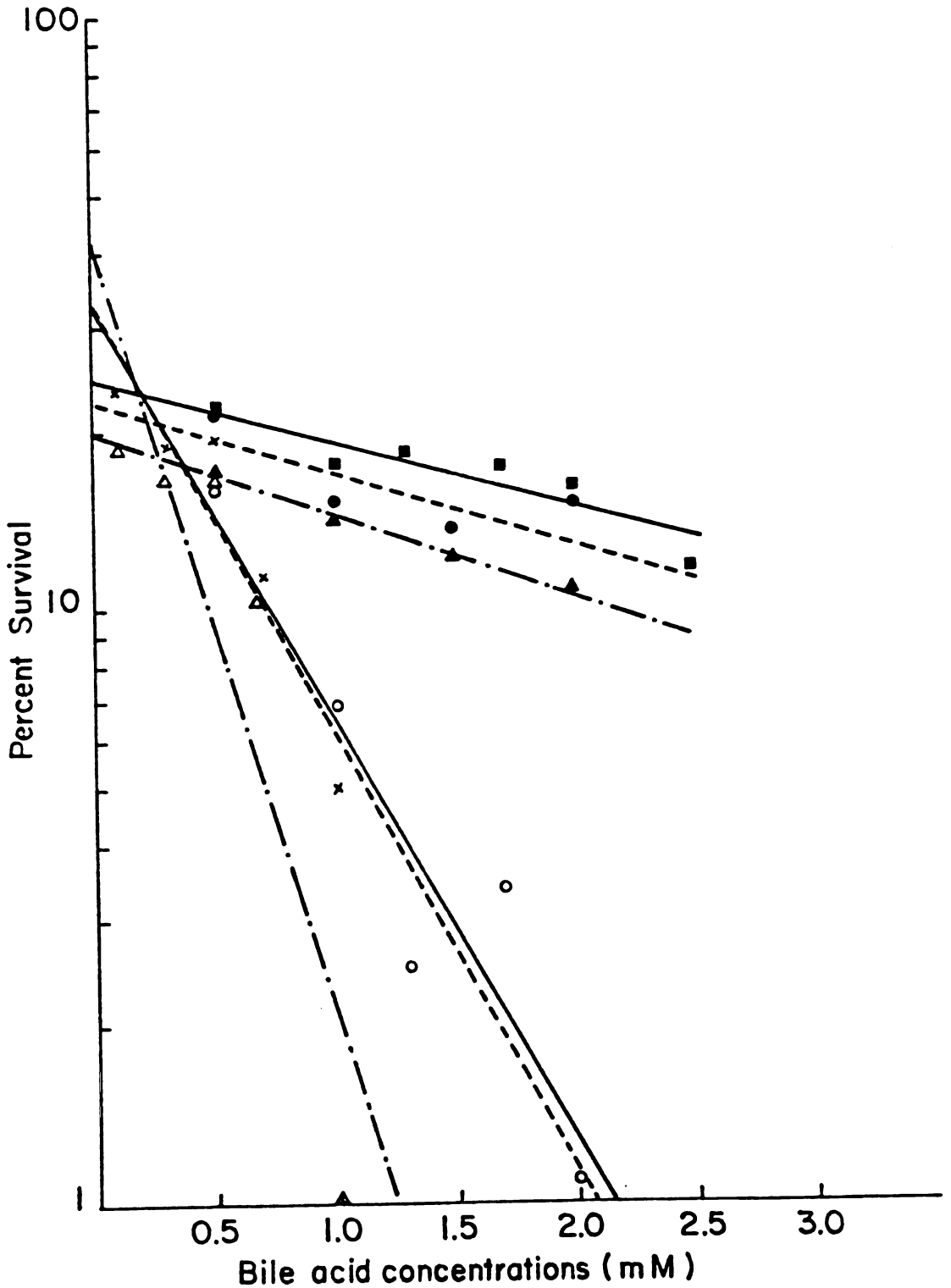

100
10
1
Percent Survival
0.5
1.0
1.5
2.0
2.5
3.0
Bile acid concentrations (mM)

FIGURE 9.

Since a preliminary experiment showed that deoxycholic acid induced a greater mutation frequency than the spontaneous mutation frequency, cells were treated with graded levels of deoxycholic acid (0.1 mM-1.0 mM) to see if there was a dose response. As shown in Table 7, mutation frequency increased until deoxycholic acid concentration was 0.5 mM. Concentrations greater than 0.5 mM, however, decreased the mutation frequency. This decrease is probably due to the high cytotoxicity of deoxycholic acid at these concentrations so that most cells died rather than undergoing mutation. At 1.0 mM there were so few cells living after treatment that a meaningful mutation frequency could not be obtained.

The variations in mutation frequency within each experiment and within a treatment from 3 experiments are shown in Table 8. Standard error between experiments were approximately 20% of the mean values.

Table 9 shows the results of several experiments in which the mutagenic and comutagenic effect of MNNG and various bile acids were examined. Since the spontaneous mutation frequency (control value) was different from one experiment to another, the values are presented as a percent of the control values. Since induced mutation frequencies by deoxycholic acid was highest at 0.5 mM (Table 7), other bile acids were tested for their mutagenicity at this concentration. Chenodeoxycholic and lithocholic acids at 0.5 mM

TABLE 7. Cytotoxicity and ouabain resistant mutants response to graded levels of deoxycholic acid.

Concentration of deoxycholic acid, mM	% survival	Oua-R mutants / 10^5 survivors
0 (control)	21	112[a]
0.1	23	166
0.3	19	155
0.5	19	256
0.7	11	171
1.0	5	-

[a]Mean values were from 8 plates per each concentration. Results were from 2 experiments.

TABLE 8. Variations in mutation frequency within each experiment and within a treatment from three experiments

Experiment	Treatment	
	1.0 μg MNNG / ml of medium	Deoxycholic acid, 0.5 mM
1	203[a]±86	222±128
2	140 ±12	230±12
3	280 ±108	393±332
	$\bar{X}$ = 208[b]± 40	$\bar{X}$ =282± 56

[a]Mean ± standard deviation for 4-8 plates per value. Value is expressed as % of control.

[b]Average mean value in 3 experiments ± standard error.

TABLE 9. Mutagenic and comutagenic effect of selected bile acids.

Treatment	Relative mutation frequency (percent of controls)		
	MNNG concentration, μg/ml		
	0	0.5	1.0
Control[a]	100[b]	124	205
Cholic acid, 1.0 mM	103	-	59
Cholic acid, 2.0 mM	339	208	189
Chenodeoxycholic acid, 0.5 mM	167	177	-
Lithocholic acid, 0.5 mM	147	172	141
Deoxycholic acid, 0.5 mM	393	272	-
3,12-dione-5β-cholanic acid, 1.0 mM	87	-	120
Hyodeoxycholic acid, 1.0mM	85	-	196

[a]Control cells were untreated for cholic and chenodeoxycholic acids comparisons. Control cells were treated with DMSO for other bile acids which were dissolved in DMSO. (see material and methods).

[b]Mean value for 1-3 experiments. Each experiment had 6-8 plates for each bile acid.

produced a higher mutation frequency (Oua-R mutants) than spontaneous mutation frequency. However, cholic, hyodeoxycholic and 3,12-dione-5β-cholanic acids did not show increased mutation frequency at 0.5 mM. Therefore, these bile acids were tested at 1.0 mM concentration. None of these bile acids produced a greater mutation frequency than the spontaneous mutation frequency at this concentration. However, when the concentration of cholic acid was increased to 2.0 mM, a greater mutation frequency than spontaneous mutation frequency was produced. Hyodeoxycholic and 3,12-dione-5β-cholanic acids were not tested at this level.

Mutagenic effects of MNNG and bile acids were not additive at two MNNG dose levels, 0.5 and 1.0 μg MNNG/ml. Instead the mutation frequency was lower when cells were treated with bile acids and MNNG than when treated with bile acids alone.

The chloroform extract from feces of rats fed the low-fat, high-fiber diet was more cytotoxic to cells than the chloroform extract of feces from rats fed the high-fat, low-fiber diet (Table 10). This is probably due to the higher concentration of bile acids in the fecal extract of low-fat, high-fiber fed rats. When cells were treated with a low dose of extract (0.5 mg extract/ml) from rats fed the high-fat, low-fiber diet, the mutation frequency was similar to the mutation frequency of cells treated with DMSO only. However, when cells were treated with 0.75 mg of extract/ml,

TABLE 10. Mutagenic effect of chloroform extracts of feces from rats fed a high-fat, low-fiber diet or a low-fat, high-fiber diet.

Diet	Treatment	Concentration in each treatment (μg/ml medium)		% survival	Oua-R mutants / 10^5 survivors
		Neutral steroids	Bile acids		
	Control (DMSO)	-	-	22	112[a]
High fat, low-fiber	0.5 mg/ml[b]	28.1	20.6	21	102
	0.75 mg/ml	42.1	30.8	17	197
	1.0 mg/ml	56.2	41.1	11	184
Low-fat, high-fiber	0.5 mg/ml	35.7	32.5	14	146
	0.75 mg/ml	53.4	48.6	11	200
	1.0 mg/ml	71.5	65.1	0	-

[a]Mean values from 4-7 plates per each concentration. Results were from 2 experiments.

[b]mg of chloroform extract/ml of medium.

there was a 76% increase in Oua-R mutants compared to the control. A greater extract concentration (1.0 mg of extract/ml) did not cause a further increase in mutation.

Unlike the cells treated with the chloroform extract of feces from the rats fed the high-fat, low-fiber diet, there was 30% increase in Oua-R mutants when cells were exposed to 0.5 mg of extract/ml of medium from rats fed the low-fat, high-fiber diet. When the cells were treated with 0.75 mg of extract/ml, the mutation frequency was comparable for both groups treated with the same concentration of extracts.

When cells were exposed to water extracts of feces from rats fed both diets, cell survival was as high as for control cells at all extract concentrations tested. The water extracts from feces of rats fed both diets produced higher mutation frequencies than the spontaneous mutation frequency at all the levels studied (Table 11). However, there was no dose response and there was no difference in mutation frequency between the extract from rats fed a high-fat, low-fiber diet and a low-fat, high-fiber diet.

Bile was very cytotoxic. When cells were treated with bile that was reconstituted with medium to provide 20% of the original bile concentration, no cells survived. With concentrations of 0.01 to 0.1 of original concentrations of bile, cell survival decreased and Oua-R mutants increased linearly over control level. There was no difference in mutation whether the bile was from rats fed the high-fat,

TABLE 11. Mutagenic effect of water extracts of feces from rats fed a high-fat, low-fiber diet or a low-fat, high-fiber diet.

Diet	Treatment	Oua-R mutants / 10^5 survivors
	Control	7[a]
High-fat, low-fiber	4 mg/ml[b]	14
	10 mg/ml	14
Low-fat, high-fiber	4 mg/ml	13
	10 mg/ml	9
	25 mg/ml	15

[a]Values were from one experiment with 8 plates per concentration.

[b]mg of water extract/ml of medium.

low-fiber diet or from rats fed the low-fat, high-fiber diet (Table 12).

Comutagenic effects of MNNG with fecal extracts and bile from rats fed the high-fat, low-fiber diet were tested. Each compound was tested at one dose level (0.75 mg chloroform extract/ml, 4 mg water extract/ml and 10% of original concentration of bile) which showed a greater mutation frequency than spontaneous mutation frequency (Tables 10-12). Like various bile acids, the mutagenic effects of MNNG and either chloroform extracts of feces or bile were not additive (Table 13). There was a slight increase in comutagenicity with water extract of feces and MNNG, however, the difference should not be considered significant without further verification.

Since bile acids are often proposed as cancer promotors, the promoting effect of bile acids were tested in this system as in the scheme presented by Trosko (75). The results are shown in Table 14. Since deoxycholic and lithocholic acids are very cytotoxic, most cells did not survive and form colonies after 2 weeks of incubation with these bile acids in the medium. Also, colony formation was very poor. Therefore, the decreased mutation frequency when cells were incubated with these bile acids probably is not meaningful. When cells were grown in 0.2 mM deoxycholic acid medium for 2 weeks, no cells survived.

When cells were grown in medium with cholic acid at 0.1 mM and 0.2 mM and chenodeoxycholic acid at 0.1 mM,larger

TABLE 12. Mutagenic effect of bile collected from bile ducts of rats fed a high-fat, low-fiber or low-fat, high-fiber diet.

Diet	Bile Concentration	Cholesterol (μg/ml medium)	Bile acids (μg/ml medium)	% survival	Oua-R mutants 10^5 survivors
	0 (Control)	-	-	22	7[a]
High-fat, low-fiber	0.011[b]	1.3	60.4	21	19
	0.025	3.0	136	18	24
	0.1	12	544	8	18
	0.2	24	1088	0	-
Low-fat, high-fiber	0.011	0.93	57	22	18
	0.025	2.1	128	18	24
	0.1	8.4	511	13	32
	0.2	16.8	1022	0	-

[a]Results were from one experiment with 6 plates per concentration.

[b]ml of bile/ml of medium (see materials and methods).

TABLE 13. Mutagenic and comutagenic effects of fecal extracts and bile from rats fed a high-fat, low-fiber diet.

Treatment	Relative mutation frequency (percent of control)	
	Mutagenicity	comutagenicity with MNNG (0.5 μg/ml)
Control[a]	100[b]	147
Chloroform extract (0.75 mg/ml)	175	147
Water extract (4 mg/ml)	175	196
Bile (0.1 original conc.)	257	142

[a]Control cells were untreated for water extract of feces and bile comparisons. Control cells were treated with DMSO for fecal chloroform extract test.

[b]Results were from one experiment with 5-7 plates per treatment.

TABLE 14. Promotor effect of selected bile acids.[a]

Treatment	Oua-R mutants / 10^5 survivors
Control	297[b]
DMSO, 0.05%	363
Deoxycholic acid, 0.1 mM	174
Lithocholic acid, 0.1 mM	103
Cholic acid, 0.1 mM	684
Cholic acid, 0.2 mM	548
Chenodeoxycholic acid, 0.1 mM	618

[a]All cells were treated with 1.5 μg MNNG/ml and grown in growth medium for 3 days. Cells were replated for plating efficiency and for mutagenesis by adding bile acids in growth medium at the indicated concentrations. Both groups of cells were cultured for 2 weeks.

[b]Results were from one experiment with 7-8 plates per treatment.

colonies were formed compared to the cells incubated with deoxycholic or lithocholic acids. Mutation frequencies were increased in this particular experiment demonstrating that some bile acids can act as mutagenic promotors. It is surprising that chenodeoxycholic acid was less cytotoxic than deoxycholic and lithocholic acids at 0.1 mM since they showed similar cytotoxicities at 0.5 mM (Fig. 9).

DISCUSSION

Many biological metabolites in the colon are thought to be toxic to colonic cells (76). These cell toxins could be important agents which cause colon cancer. A larger stool mass and shorter intestinal transit time might be very significant factors in preventing colon cancer. Heavier stools have a diluting effect on toxic substances, and a shorter transit time reduces the duration of exposure of the tissues to toxic substances.

The greater fecal mass and shorter transit time in bran-fed rats agree with the results of other investigators (27,29). Inclusion of agar, a purified fiber source, in the diet also increased stool weights. Increase in fecal mass is often correlated with bowel regularity (30). Daily bile acid excretion was increased in bran-fed animals, even though the increase in excretion was not statistically significant. However, fecal bile acid concentration was lower and decreased linearly with increasing levels of bran in the diet. Other workers have also reported a greater bile acid excretion in feces when fiber was added to diet. Rats fed Purina chow or cellulose-containing diets had greater fecal excretion of cholic acid and its degradation products than rats fed a purified semi-synthetic diet (90). Jenkins et al.

(27) also reported increased bile acid excretion and reduced fecal bile acid concentration in human subjects fed a diet containing wheat fiber.

The second experiment was designed to determine how dietary fat and fiber content affected steroid metabolism. Diets high in beef fat (9, 91) and low in dietary fiber (10) have been implicated as a cause of the colon cancer through an alteration in steroid metabolism and gut function. For these reasons, high beef-fat (tallow), low-fiber and low-fat, high-fiber (agar) diets were compared. Agar was used as the source of fiber since it has been shown in our laboratory that agar has a great capacity to increase stool weight and decrease transit time (28). High cholesterol ingestion has been associated with an increased excretion of fecal bile acids by humans (92), and high fat diets (either lard or corn oil) also increased fecal bile acid excretion in rats (25). Since the high-fat, low-fiber diet was also a high cholesterol diet, it was expected that the daily fecal bile acid excretion would be greater for the rats fed the high-fat, low-fiber diet than for rats fed the low-fat, high-fiber diet. However, there was no difference in daily fecal bile acid excretion between these two dietary groups, and as shown in the experiment 1, fiber in the diet actually increased the daily fecal bile acid excretion. Therefore, it seems that the high fat content of one diet and the high fiber content of the other diet offset each other as far as daily bile acid excretion was concerned. Perhaps the most important fact was

that the bile acid concentration of feces from rats fed the low-fat, high-fiber diet was several-fold lower due to the dilution effect of the larger fecal mass.

As expected, neutral steroid excretion and concentration was higher for rats fed the high-fat, low-fiber diet than for rats fed the low-fat, high-fiber diet. It is well established that diets rich in fat and cholesterol lead to high excretion of neutral steroids in feces (25).

If bile acids and neutral steroids are important carcinogens or cocarcinogens, then the reduced steroid concentration noted for the high fiber diets in experiment 1 and 2 would decrease the quantity of these steroids to which the colonic cell surface is exposed.

It has been proposed that the inclusion of fiber in the diet may influence the type and number of microflora in the large bowel. A modification in bowel microflora could lead to an alteration in the rate and extent to which cholic or chenodeoxycholic acids are metabolized to secondary bile acids such as deoxycholic, lithocholic, and corresponding ketone derivatives. This would also influence the metabolism of cholesterol to coprostanol and coprostanone.

Results in experiment 1 showed a decrease in concentration of cecal anaerobic bacteria with the inclusion of bran in the diet. Even though the absolute number was fewer, it is doubtful that this decrease in anaerobic bacteria concentration has physiological importance. In this type of work,

a difference of more than one log is considered physiologically important (93).

Inclusion of bran in the diet increased bile acid degradation as bran concentration in the diet increased, and the inclusion of agar in the diet also increased the degradation of neutral steroids. The trend of bile acid degradation was similar for feces and *in vitro* fermentation studies. Therefore, it appears that the total number of anaerobic bacteria may not be directly related to degradation of bile acids.

A change in bacterial species present in the cecum and colon could change the extent of bile acid and neutral steroid degradation. Since bacterial concentrations and cecal weights were similar for rats fed all levels of bran and there was a decrease in transit time for bran-fed rats, the increased bile acid degradation is likely due to a change in types of intestinal microflora. The difference in microbial activity due to diet was demonstrated in experiment 2 when neutral steroid metabolism is examined. It is of interest to note that the ratio of fecal coprostanol to coprostanone is very different for rats fed the two different diets. These data suggest that microorganisms which metabolize cholesterol to coprostanol are predominant in rats fed the high-fat, low-fiber diet, whereas bacteria which convert coprostanol to coprostanone are more prevalent in the large bowel of rats fed the low-fat, high-fiber diet. The probable difference in types of intestinal microflora in this

experiment is likely due to both the fat and the fiber content of the diet. The possibility that gut ecology caused the change in neutral steroid metabolism rather than changes in bacterial types, however, cannot be excluded.

It is generally believed that secondary bile acids and cholesterol metabolites are more active carcinogens or cocarcinogens than primary bile acids and cholesterol (94). Many investigators have postulated that high fiber (95) and low fat (96) diets result in decreased steroid degradation in the colon, and therefore, decreased incidence of colon cancer. The data in this study are contradictory to this hypothesis, at least with respect to the effects of bran fiber, agar, and tallow in rats. If fiber is beneficial in reducing colon cancer, its major effects will be through the dilution of carcinogens or cocarcinogens in colonic contents as Hill suggested (96), and/or through the reduction in time in which the colonic cells are exposed to these agents.

It was unexpected to find similar bile acid and cholesterol concentrations in bile of animals fed either a high-fat, low-fiber or a low-fat, high-fiber diet. With a diet high in fat, it is expected that more bile acids would be secreted into the gut to aid in fat digestion and absorption. However, one needs to know the concentration of bile acids in bile and the bile flow rate before total bile acid secretion can be ascertained. The flow rate of bile was not determined since bile was collected under stressful conditions immediately after the bile duct was cannulated.

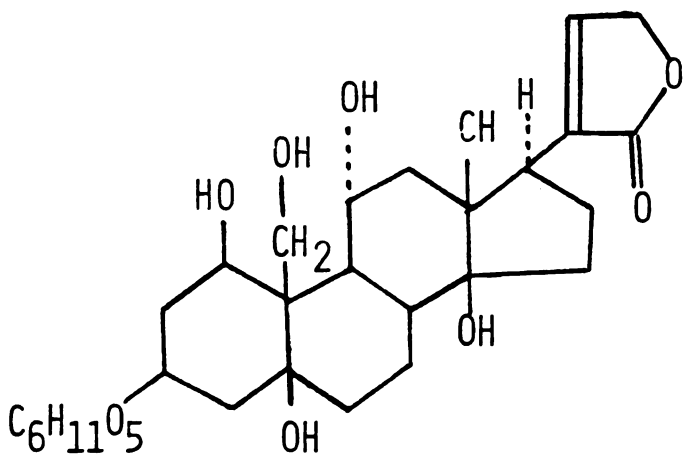
Therefore, bile flow probably did not reflect physiological conditions since the rats secreted widely varying amounts of bile during the same 24 hour period. The difference in bile flow was not related to diet, but rather seemed to be due to a difference in response of each rat to stress and in the success of creating a free-flowing cannula.

Reddy et al. (25) reported a greater biliary concentration of bile acids when rats were fed a high fat diet rather than when the rats were fed a low fat diet. But their rats were fed high and low fat diets for 10 weeks and the bile was collected only for 4 hours. In this study, the rats were fed their respective diets for only 4 weeks and bile was collected for 24 hours. Rats in this study may have secreted more dilute bile with time, since they did not consume much of the available food for the 24 hour period following surgery. If early differences in bile acid concentration existed between groups, this difference may have been obscured with prolonged collection time.

The experiments which were designed to develop an intestinal cell culture method to screen mutagens were conducted on the assumption that mutagenesis plays a role in carcinogenesis and that ouabain resistant cells are true mutants.

Ouabain is a specific inhibitor of the plasma membrane Na/K ATPase. Structure of ouabain is shown below. ATPase is responsible for the active transport of sodium and

potassium. In the presence of ouabain, cells can not survive due to the inhibition of this transport system. Ouabain resistant mutation is due to a genetic alteration in the structural gene for the ATPase system which allows the survival of cells by permitting transport activity in the presence of ouabain (78). A frameshift mutation can not contribute to ouabain resistance, since a frameshift mutagen would cause a mutation which results in a complete loss of enzyme activity. Thus, the potassium transport function would be completely blocked in the presence of ouabain (78, 97). For the same reason, when ouabain resistance is used as a marker for mutagen screening, deletion-type mutagens will go undetected.

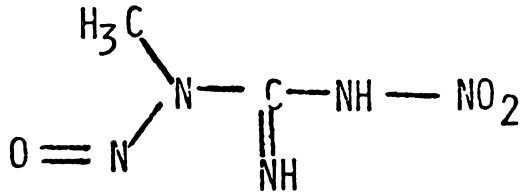


OUABAIN ($C_{29}H_{44}O_{12}$)

The results of this study indicate that mutants can be selected by using ouabain as a selective agent and MNNG is an effective mutagen for inducing ouabain resistant mutation in intestinal fibroblasts isolated from rat fetuses. MNNG induced Oua-R mutation in a dose dependent manner. Structure of MNNG is shown below.

Factors which must be determined in establishing protocols for mutagenesis assays include 1) dosage of the potentially mutagenic agent, 2) expression time for induced mutations, 3) conditions for optimal detection of mutant phenotypes.

Oua-R mutants could be selected when ouabain was present at 1.0 mM, since ouabain inhibits the growth of wild-type cells at concentrations equal to or greater than 1.0 mM. In studies by other investigators, a ouabain concentration of 1.0 mM was needed to select Oua-R mutants from wild-type cells of other rodents such as mouse and hamster cells (76, 97). However, it should be noted that a 1.0 μM concentrations



N-METHYL-N'-NITRO-N-NITROSOGUANIDINE

of ouabain was sufficient to select Oua-R mutants in human cells (77, 78).

As shown in Figure 7, cells grown in ouabain-containing medium exhibited both reduced size of individual cells as well as fewer cells per colony. Baker et al. (76) have also observed a decrease in colony size with increasing ouabain concentration. Colonies surviving 0.5 mM ouabain probably resulted from incomplete inhibition of wild-type cell multiplication by ouabain rather than by the presence of ouabain resistant cells.

The frequency of ouabain resistant cells reached its maximum when they were allowed to grow in growth medium for 3 days after mutagen treatment. The 3 day expression time before the cells were exposed to ouabain allowed approximately 3 doubling times, provided that mutagen-treated cells grow at the same rate as normal cells. If more than 3 days were allowed for expression, the mutation frequency decreased. Chang et al. reported that UV-induced Oua-R mutation frequency was maximum with 2 days of expression time in Chinese hamster cells (97). If more than 2 days for expression was allowed, they also found a decline in mutation frequency. A MNNG concentration of 1.0-1.5 μg/ml was effective in inducing mutation in this study as in other studies (98).

In many mutation frequency studies, cells are not subcultured after treatment with a mutagen. Huberman et al. (99) used 8-azaguanine as marker and Chang et al. (97) used ouabain as a selective agent to determine mutation frequency,

but neither investigator subcultured the cells after mutagen treatment. Instead they seeded a specific number of cells into plates, exposed the cells to a mutagen, allowed growth in medium for the appropriate expression period, and then put drug-containing medium in the plates to select mutants. When this protocol was followed for intestinal fibroblasts from rat fetus, Oua-R cells did not form distinctive colonies and scoring was very difficult. But, when the cells were subcultured after the expression period, the Oua-R cells formed distinctive colonies and were easier to score. The protocol used in this study is more widely practiced with cells grown in suspension (73) than cells attached to surfaces.

The spontaneous mutation frequency in the cell system used in this study was higher than reported for other cell systems, and the spontaneous mutation frequency varied from one experiment to another. The reported frequency of Oua-R colonies in wild-type cell populations (spontaneous mutation frequency) was 2.0×10^{-6} in Chinese hamster (V-79) cells (97), 1.0×10^{-6} in human lymphoblastoid lines (77), and 1.0×10^{-5} in Chinese hamster ovary (CHO) cells (76). Spontaneous mutation frequency could vary 2 or more orders of magnitude not only between different cell lines but also in the same line assayed at different times (74). In the cell system used here, the spontaneous mutation frequency ranged from 7.0×10^{-5} to 112×10^{-5}. In one report by Trosko et al. the spontaneous mutation frequency of Oua-R

cells also varied from 2.1×10^{-5} in one experiment to 3.6×10^{-6} in another experiment (75).

The higher spontaneous mutation frequency found in this study as compared to other cell systems is likely due to the difference in cell types. The intestinal fibroblasts of the rat fetus have a finite life span and must be frequently isolated from intact animals. Most frequently used cell types for cell culture work are established cell lines which have an unlimited life span. It is likely that many of the cell characteristics have changed during the constant subculturing.

It has been reported that the fibroblasts isolated from different organs have different characteristics. Skin and lung fibroblasts derived from the same human fetuses showed several differences (100). Fetal lung cultures had faster cell replication rate, greater ^{3}H-thymidine incorporation into DNA, higher cell numbers at confluency, smaller cell volumes, decreased cellular RNA and protein contents and longer life span in culture compared to fetal skin fibroblasts. Also, these two fibroblast cultures responded differently to the addition of hydrocortisone to culture medium. Examination of cornea, heart, and skin fibroblasts from the same embryonic chicks also revealed differences in fibroblasts isolated from different organs (101). They were different in saturation densities, in sensitivity to treatment with trypsin and EDTA, in sensitivity to EDTA alone and in the mode of cell arrangement at confluency. For these

same reasons fibroblasts from different animals and sources could exhibit differences in ouabain resistance.

There could be many reasons why the spontaneous mutations noted in this study varied from experiment to experiment. One possible explanation for variation in spontaneous mutation frequency is that cells used for each experiment were isolated from different animals. Additionally, the number of subcultures before treatment was different. Consequently trypsinization could conceivably change the characteristic of cells. However, in the experiments where the spontaneous mutation frequency was high, the mutation frequency of treated cells was also high and vice versa. Therefore, for the purpose of screening mutagens, the variation in spontaneous mutation would not affect the comparison of results as long as a control group is included in each experiment.

Cloning efficiency (percent survival) of non-treated fibroblasts was relatively low (approximately 20%). It seems that the cloning efficiency of freshly isolated cells is lower than the cloning efficiency of cells from established cell lines. Maher et al. reported that the cloning efficiency of fibroblasts from human foreskins was 20-35% (102), whereas the cloning efficiency of 90-100% for aneuploid V-79 Chinese hamster cells (75, 99) was reported. Even though the cloning efficiency of cells used in this study was relatively low, the lower cloning efficiency would not affect the results of experiments.

Since bile acids have been postulated as the agents which could cause colon cancer, selected bile acids have been tested in this system. The bile acids tested here are the ones reported to be found in the feces (103) and in bile of rat. 3,12-dione-5β-cholanic acid was included since a bile acid with a GLC relative retention time similar to 3,12-dione-5β-cholanic acid was often noted in rat feces when the bile acids were quantitated in experiments 1 and 2 of part I. Also, the diketo-acid is even more degraded than deoxycholic acid which is reportedly a potential carcinogen.

Interestingly, the various bile acids had different cytotoxicities and different solubilities. Since bile acids are lipophilic rather than hydrophilic, difficulty was encountered in incorporating bile acids into culture medium. When deoxycholic, hyodeoxycholic, and 3,12-dione-5β-cholanic acids were dissolved in DMSO, they went into solution easily in medium without changing the medium pH. However, the lithocholic acid in DMSO did not go into solution in medium, therefore suspensions of lithocholic acid were used. When cholic and chenodeoxycholic acids were dissolved in DMSO, they changed the pH of the medium upon addition and caused protein coagulation. Therefore, these acids were dissolved directly into medium after neutralization by NaOH. Thus two methods were utilized to incorporate bile acids into medium.

There appear to be two groups of bile acids among the bile acids tested as far as cytotoxicity is concerned.

Deoxycholic, lithocholic and chenodeoxycholic acids were more cytotoxic than cholic, hyodeoxycholic and 3,12-dione-5β-cholanic acids. When cells were incubated with deoxycholic, lithocholic and chenodeoxycholic acids at 1.0 mM for 3 hours, most cells did not survive. However, cholic, hyodeoxycholic, and 3,12-dione-5β-cholanic acids had relatively high percent survival when they were incubated at 2.0 mM concentrations for 3 hours.

Even though the bile acids were cytotoxic to cells in a mammalian cell system, Silverman and Andrews did not find any noticeable cytotoxic effect of bile acids in Salmonella tester strains used for the Ames test (104). It appears that there is a direct relationship between bile acid cytotoxicity and mutagenicity. It has been generally thought that mutation frequency increases with decreasing cell survival (74). More cytotoxic bile acids (deoxycholic, lithocholic and chenodeoxycholic acids) produced a greater mutation frequency at a lower concentration (0.5 mM), while less cytotoxic bile acids (cholic, hyodeoxycholic and 3,12-dione-5β-cholanic acids) did not show an increased mutation frequency even at 1.0 mM. However cholic acid at 2.0 mM was mutagenic. Even though hyodeoxycholic and 3,12-dione-5β-cholanic acids did not show mutagenic activity at 1.0 mM, it is possible that these steroids would induce mutation at higher concentrations, since a dose dependent response was noted for deoxycholic acid and cholic acid.

The dosage difference among various bile acids that was required to produce mutagenesis agrees with the animal studies done by Wynder and coworkers (49,50). They found tumor promoting effects of deoxycholic, lithocholic, cholic, and chenodeoxycholic acids after intrarectal instillation of MNNG in rats. 1.0 mg sodium taurodeoxycholic and lithocholic acids were given at each time whereas 20 mg of sodium cholate and sodium chenodeoxycholate were used to produce approximately the same tumor promoting effect.

Deoxycholic acid has been found to be mutagenic in bacteria (105), but not in the Salmonella-mammalian-microsome test (104). The different results with different assay systems emphasizes the importance of using a system which is more closely related to the human.

Even though a comutagenic effect of bile acids and MNNG was not observed in this study with rat intestinal fibroblasts, bile acids could be comutagenic in this system with other known carcinogens, such as 2-aminoanthracene (2-AA). Lithocholic acid and its conjugates showed comutagenic activity with suboptimal amounts of 2-AA in TA-1538, even though bile acids themselves were not mutagenic (104). However, comutagenesis was not observed when lithocholic acid was tested with MNNG, 2-acetyl-aminofluorene (AAF), 3-methylcholanthrene (MCA), and benzo(a)pyrene (BP). The decrease in mutation frequency when cells were treated with bile acids and MNNG agrees with the results of Silverman and Andrews (104). When Salmonella tester strains

were treated with lithocholic acid and MNNG, the number of revertants decreased by 25% compared to the cells treated with solvent and MNNG only. The reason for the decreased mutation frequency when cells were treated with bile acids and MNNG, as compared to bile acids or MNNG only, is not known. Since bile acids are surface-active agents, they may change membrane permeability and inhibit MNNG transport across the membrane. Untransported MNNG could then react with bile acids in such a way as to inhibit bile acid transport or activity. This is purely speculation, but appropriate experiments could be designed to prove or disprove the hypothesis. Also, the levels of bile acids tested here for comutagenicity was the optimal concentration needed to induce maximum mutation frequencies. Suboptimal concentrations of bile acids may show synergistic effects with chemical mutagens which would demonstrate comutagenicity.

The extracts of rat feces fed with either high-fat, low-fiber or low-fat, high-fiber diets, were tested for mutagenic and comutagenic activity in this system. Since the extracts were added to the medium on a weight basis, extract concentration in the medium does not represent the physiological concentration of these extracts in feces of either groups.

Both chloroform and water extracts of feces from rats fed either of the two diets produced higher mutation frequencies than the spontaneous mutation frequency. Since extracts from both diets increased the mutation

frequency at different concentrations, it seems that the concentration of fecal metabolites in the colon could play an important role in colon carcinogenesis. The finding that a water extract did not exhibit cytotoxicity but did exhibit mutagenicity is contrary to the general belief that there is a direct relationship between cytotoxicity and mutagenicity.

It was also interesting to find the mutagenic effects of bile. It has been postulated that conjugated bile acids are less carcinogenic or non-carcinogenic compared to unconjugated bile acids. Since most bile acids in bile are present in the conjugated form, conjugated bile acids may also enhance mutation. It is possible that other components of bile, but not the conjugated bile acids, induced the mutation.

In the study of Silverman and Andrews (104), not only lithocholic acid but also the conjugates of lithocholic acid were found to be comutagenic with 2-AA. One can still argue that the conjugated bile acid may have a lower mutagenic potency than free bile acids. This possibility is suggested by the work of Silverman and Andrews (104). The number of revertants decreased by 40% and 20% when bacteria were treated with taurolithocholic acid + 2-AA and with glycolithocholic acid + 2-AA compared to when bacteria were treated with lithocholic acid + 2-AA, respectively.

It was not surprising to find that there was no difference in mutagenic activity of the bile from the two

dietary groups, since the steroid concentrations of the bile were not different. The mutation inducibility of bile is consistent with work of Chomchai et al. (47). They found more tumors in the colon of DMH treated rats when the bile duct was diverted from the proximal half to the distal half of the small intestine. Chloroform and water extracts of feces and bile did not show comutagenic effects with MNNG as in the case of bile acids.

Since the chloroform and water extracts of feces and bile were shown to elicit mutagenic activity in the rat intestinal fibroblast assay system, the next step would be to separate these extracts into more definitive groups of compounds. These groups could then be tested for the mutagenic effect until the compounds responsible for the mutagenesis are identified.

Trosko et al. (75) nicely demonstrated the tumor promoting effect of 12-0-Tetradecanoyl-phorbol-13-acetate (TPA) in UV-irradiated Chinese hamster cells, using ouabain and 6-thioguanine resistance as selective markers. Whereas TPA did not show any cytotoxic effect, bile acids were cytotoxic and incubation of cells with bile acids for 2 weeks killed or markedly inhibited the growth of cells, especially in ouabain medium. The promotor study was carried out only once. Results of the present experiment only suggest the possibility of developing an experimental protocol to test promotors in this cell system. However, to adapt this system for tumor promotor studies, all the conditions should be

optimized with a known promotor, like TPA. The dose of test compounds would have to be tested, especially for cytotoxic compounds like bile acids. It should not be concluded that some bile acids have tumor promoting effects from the result of this experiment only.

There still are many limitations in using this system as a screening technique. One problem is that colonies are small and are difficult to detect. Another problem is that calculation of mutation frequency depends entirely on the number of cells seeded. Therefore, the accuracy of calculating mutation frequency is dependent upon the accuracy of counting cells by a hemocytometer. Variation of the cell counts by a hemocytometer was large and it is likely that a bias was involved. Most experiments presented here were repeated at least twice. One way to reduce variation and bias is to repeat each experiment two or three times. Also, a large number of replicate dishes perhaps 20-30 plates or more should be used for each datum point.

Even though many of the compounds tested showed mutagenic activity in intestinal fibroblasts cultures, it would be presumptious to speculate that these compounds would also cause mutation or tumorigenesis in an intact animal. Even if any of the compounds are proven to be mutagenic or carcinogenic in animals, the extrapolation of such results to man should be exercised with great caution.

I pursued this study purely to develop a method for screening purposes. If a compound is identified as a

mutagen by an *in vitro* system, an *in vivo* assay system should follow. At the same time, efforts should be made to relate the presence of the compound as a function of diet, microflora metabolism and gut function. Only then can meaningful recommendations be made for the general public in an attempt to reduce the risk of developing colon cancer.

CONCLUSIONS AND RECOMMENDATIONS

The most notable change associated with dietary fiber in the diet was an increased fecal mass and reduced intestinal transit time. With the experimental designs employed here, the variations in dietary fat and fiber content did not affect the total daily bile acid excretion in rats. However, the fecal bile acid concentration was lower when dietary fiber is incorporated in the diet due to the larger fecal mass. Since bile acids are suspected carcinogens or cocarcinogens, dietary fiber in the diet may play a role in decreasing the incidence of colon cancer by diluting bile acids in the large bowel. Also, dietary fiber might have a protective role in colon carcinogenesis by reducing the time which colonic cells are exposed to carcinogenic or cocarcinogenic compounds.

The difference in bile acid degradation in rats fed graded levels of bran and the difference in neutral steroid degradation in rats fed diets with different levels of fat and agar indicate that the metabolic activity of gut microflora can be modified by diet.

Fibroblasts were isolated from the intestine of rat fetuses to screen potential mutagens and/or carcinogens which can be found in the gastrointestinal tract. Increased

ouabain resistant mutation frequencies were produced when cell cultures were treated with MNNG, bile acids, organic and water extracts of rat feces and rat bile. Since a dose response was seen in most of the experiments, the concentration of bile acids and/or intraluminal components is likely to be an important factor in development of colon cancer.

Results of this study indicate that intestinal cell culture techniques are feasible for screening mutagens and/or carcinogens which can be found in the gut. However, additional work should be done in an attempt to improve this cell culture technique before it is recommended for routine use. Such factors as the optimal cell age, optimum condition to increase colony size and the number of replicate plates required for statistical validity should be evaluated. Also, many known carcinogens and mutagens should be tested to ascertain mutagenic activity in this system.

Since frameshift mutation is not detected by employing ouabain as a selective agent, a selective agent which can detect frameshift mutation, such as 8-azaguanine, should be developed. The basic parameters to select mutants in this cell culture system have been determined and adaptation of the present protocol for 8-azaguanine use should not be too difficult. Unless a compound shows negative results in several systems, it should not be concluded that the compound in question does not have mutagenic activity.

Some of the compounds which show positive results in this mutation assay should be tested in in vivo systems. The ultimate criterion will be to inject mutated cells into an immunologically incompetent animal to test for tumorigenesis.

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