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RAT COLON EPITHELIAL CELLS: ISOLATION, CULTIVATION, AND BENZO(a)PYRENE METABOLISM.

presented by

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has been accepted towards fulfillment
of the requirements for

MASTER degree in SCIENCE

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Date 5-21-81

RAT COLON EPITHELIAL CELLS: ISOLATION, CULTIVATION, AND BENZO(a)PYRENE METABOLISM.

By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Food Science and Human Nutrition

1981

G11565 6

ABSTRACT

RAT COLON EIPITHELIAL CELLS: ISOLATION, CULTIVATION

AND BENZO(a)PYRENE METABOLISM

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The purpose of this study was two-fold: 1) to isolate and culture colon epithelial cells and 2) once culture conditions were established to study the metabolism of the environmental procarcinogen, benzo(a)pyrene, (BP). Colon epithelial cells collectively referred to as colonocytes were isolated from germ-free rats. These colonocytes were maintained for 12 weeks. Colonocytes underwent morphological changes in differentiaing from cuboidal-type to columnar type epithelial cells with microvilli which characterizes epithelial cell differentiation in the colon.

Colonocytes from conventional rats metabolize BP when cultured for 24 hours. Metabolites of BP produced by colonocytes caused mutation in the Ames test. Bile acids and tryptophan metabolites, compounds affected by diet and implicated in colon carcinogenesis, caused both increased and decreased BP metabolism by the colonocytes.

This study demonstrates that colonocytes can be cultured to study cell biology and xenobiotic metabolism.

ACKNOWLEDGEMENTS

I would like to sincerely thank Dr. Maurice Bennink for his never-ending patience, guidance and fostering of professional ethics during the past several years.

I would also like to thank my committee members Drs. I. Gray, C. Chang, and R. Young for their helpful suggestions.

Most of all I would like to express my sincere thanks to my parents, who have been very supportive in any endeavor I have undertaken.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER THREE

LIST OF FIGURES

INTRODUCTION

Approximately 114,000 new cases of colorectal cancer were diagnosed in the United States and approximately 53,000 people died from this disease in 1980. Although colorectral cancer incidence has plateaued in recent years it still remains relatively high in Western societies.

There is considerable evidence suggesting that most colon cancer is caused by environmental agents, specifically diet. Research involving dietary effects on the etiology of this disease is therefore desirable. Methods which utilize mammalian cells may increase our understanding on how carcinogenesis occurs at the cellular level. Moreover, methods which utilize cells of the target tissue (colon) could help us elucidate how dietary factors influence colorectal carcinogenesis.

In vitro systems have been developed to study a wide variety of diseases. Unfortunately, cell types other than the types found in the target tissue are frequently used. My goal was to develop an in vitro system in which colon epithelial cells, the target cell for colon tumorigenesis, are cultured and used to examine the metabolism of carcinogens. A specific goal was to determine how carcinogen metabolism is influenced by compounds which can be increased in the gut due to dietary modifications.

CHAPTER ONE . . . . . LITERATURE REVIEW

Literature Review

EPIDEMIOLOGY

Epidemiological studies indicate that many cancers are caused, mediated, or modified by a multitude of environmental factors. A major emphasis of cancer research is to study and identify environmental factors thought to be related to carcinogenesis. Regarding cancer of the colon, epidemiological data indicate that the incidence of this disease is associated primarily with environmental factors rather than genetic or racial factors (1).

The incidence of colonic cancer not only seems to vary with geographic area but also with socioeconomic status of the population (2). Burkitt (3) reported that the incidence of colon cancer is high in North America and Northwest Europe and low in South America, Asia and Africa. The importance of environmental influences on colon cancer is also documented by a higher incidence of colon cancer in first and second generation Japanese immigrants to California and Hawaii compared to Japanese living in Japan (4). Environmental rather than genetic dependence of colon cancer risk is supported by the differences in incidence between American and African blacks (5).

DIET AND CANCER

Based on colon cancer distribution patterns in the world and data dealing with migrant populations, dietary factors, particularly dietary fat, animal protein, and dietary fiber, should merit primary consideration. Wynder et al. (2) reported that populations in high risk areas consume diets high in animal fat and protein whereas people in low risk areas eat foods low in these components. Data also revealed that within most populations, animal protein and fat consumption increases with socioeconomic status. Reddy (6) reported that there is a strong association between dietary fat intake, concentration of fecal bacteria, fecal bile acid and neutral sterol concentration, and the risk of colon cancer among different populations. He also postulated that bile acids and cholesterol metabolites in the colon play a modifying role in colon carcinogenesis, and that concentrations of these compounds in the colon are modulated by dietary factors.

The effect of bile acids on colon carcinogensis has received substantial support from animal studies. Chomchai et al. (7) showed that diverting bile from the proximal half of the small intestine to the distal half increases azoxymethane-induced intestinal tumors. Tumors increased in both the small intestine and colon and was thought to be due to an increase in bile acid concentration in these anatomical areas. Reddy (8) reported that lithocholic acid and deoxycholic acid, two secondary bile acids present in high concentrations in the large bowel of man, acted as promoters of colon carcinogenesis. Similarly, both the incidence of chemically-induced colon tumors and concentration of bile acids present in feces increased when rats were fed high-fat

diets. Cohen et al. (9) observed that dietary supplementation of the primary bile acid, cholic acid, significantly enhanced colorectal tumorigenesis. Fecal analysis revealed there was a significant increase in deoxycholic acid concentration also. Tumor enhancement was attributed to this secondary bile acid. Sarwal et al. (10) added another primary bile acid, chenodeoxycholic acid, to diets of rats and similarly observed an increase in tumor incidence. Interpretation of these results add support to the concept that some bile acids act as promoters in colon carcinogenesis.

Regarding other dietary factors, a high intake of animal protein may also play a causal role in colonic cancer (11). Haenzel et al. (4) found that colon cancer incidence increased with an increased consumption of beef. This undoubtedly could also be due to fat consumption since almost half of our dietary fat is derived from meat. Chung et al. (12) reported that certain tryptophan metabolites could be responsible for the increased risk of this disease. He demonstrated that tryptophan concentration and bacterial tryptophanase activity, the enzyme which can metabolize tryptophan, were significantly higher in feces from rats on all meat diets compared to feces from rats fed a normal diet. Oyasu et al. (13) observed that indole, a bacterial metabolite of tryptophan enhanced urinary bladder tumorigenesis when administered with the known carcinogen, 2-acetylaminofluorene. Thus the formation of indole and other tryptophan derivatives in the gastrointestinal tract could be important in the etiology of cancer, especially in populations with a high protein intake that could elevate concentrations of tryptophan in the colon where it can be metabolized by the microflora (12).

A lack of dietary fiber has received considerable attention regarding colon cancer incidence. Populations eating primarily unrefined diets rich in fiber have a low incidence of colon cancer while populations eating primarily refined diets containing little fiber have a relatively high incidence of this disease (2). The proposed benefits of dietary fiber are as follows: 1) a more rapid transit of indigestible material through the gut 2) a rapid transit reduces the opportunity for gut bacteria to produce a carcinogen and 3) a rapid transit reduces the opportunity for a carcinogen, ingested or produced in the gut, to act upon the intestinal mucosa (14). However, much experimental evidence is needed to evaluate the above hypotheses concerning fiber and colon carcinogenesis.

The ingestion of xenobiotics and their role in gastrointestinal tract carcinogenesis has just recently received attention. Many theories regarding diet and cancer deal primarily with metabolic and/or hormonal processes. Nutrient excesses and deficiencies which may promote or inhibit cancer have also received much consideration. However, many suspected carcinogenic and/or procarcinogenic compounds are present in foods as well. Only a few studies have examined tne safety of endogenous but exotic chemical constituents of foods. Procarcinogens can be present in the diet. produced during cooking, or formed from dietary constituents in the gastrointestinal tract.

Nitrite and nitrate present as food additives or naturally occuring compounds may form carcinogenic N-nitrosamines when ingested (15). This occurs when nitrates are converted to nitrites and combine with secondary or tertiary amines in the stomach. Aflatoxin B_1, a natural

carcinogenic mycotoxin produced by the mold Aspergillus flavus, has also been detected in foods such as cereal grains and nuts.

The presence of mutagens or carcinogens in cooked foods has also gained considerable attention in recent years. Mutagenic compounds have been isolated from cooked meat and fish (16). Several groups have reported that mutagenic compounds were present in broiled or fried hamburger (17,18) and also in the charred parts of fish and beef (19). Data indicate that these mutagenic compounds may be the pyrolysis products of amino acids (17), polycyclic aromatic hydrocarbons (PAH) such as benzo(a)pyrene (BP) (19), or products resulting from non-enzymatic browning (20,21). Present studies indicate that many of these compounds are mutagenic in in vitro assays and carcinogenic in some animal models; however, their role as carcinogens in man remains to be established.

Food contamination with PAH's is a seemingly uncontrollable phenomenon in our environment due to pollution of the atmosphere, water and soil. PAH contamination can also occur through food processing and preparation (19). Charcoal-broiled meats, smoked meats and cheeses, and consumable foods grown in heavily polluted environments all contain detectable levels of PAH's (22). Suitable animal experiments to indicate whether or not there might be a hazard in the ingestion of PAH contaminated foods are obviously desirable.

Investigations to study the possible relationship between diet and colon tumorigenesis are needed to understand the etiology of colonic cancer. Composition of diet determines the intraluminal components of exogenous and endogenous origin, the composition of intestinal

microflora and indigestible material which in turn may produce possible cocarcinogenic and/or carcinogenic compounds from the intraluminal contents (6). These suspected carcinogenic compounds may in turn act upon the intestinal mucosa to cause precancerous changes and lead to possible tumorigenesis.

Many procarcinogens and promutagens require metabolic activation before they become ultimate carcinogens or ultimate mutagens. In order to study carcinogen/mutagen activation at the cellular level one must understand this activation process. PAH activation will be described to illustrate a general metabolic activation process. The initial step in PAH activation involves a group of enzymes identified as the microsomal mixed function oxidases (MFO) (23). This group of non-specific MFO's belong to the cytochrome P-450 (cyt P-450) family. The metabolic interface between environmental PAH's and the biological organism is this cyt-P450 containing enzyme system. This enzyme system associated with PAH metabolism is located on the endoplasmic reticulum or the microsomal fraction of the cell. This enzyme system is induced by a variety of compounds although large variations among different tissues, different species, and different strains of the same species has been observed (24).

Knowledge of the cyt P-450 containing enzyme system has been gained from studies involving the metabolism of a model PAH, BP. In this enzyme system BP is initially oxygenated by the MFO. aryl hydrocarbon mono-oxygenase (AHH), and an epoxide is produced. The epoxide produced can have several metabolic fates: 1) non-enzymatic conversion to phenols 2) hydration of the epoxide intermediates to dihydrodiols via

epoxide hydrase 3) enzymatic or non-enzymatic conjugation of oxygenated intermediates to water-soluble glutathione 4) enzymatic and non-enzymatic conjugation to form glucuronide or sulfate conjugates and 5) non-enzymatic covalent binding to cellular macromolecules. Further action by AHH can convert the dihydrodiols to the highly reactive diol-epoxides. These diol-epoxides can also covalently bind to cellular macromolecules such as DNA, RNA. and protein. It is believed that this covalent binding to DNA is a major cause of mutagenesis and possible carcinogenesis (25). The conjugation of the oxygenated BP intermediates to sulfate, glucuronic acid, or glutathione produces a water-soluble detoxified metabolite. This detoxification process is thought to be a protective mechanism fundamental to the cell's survival.

Whether or not a compound is a mutagen and/or carcinogen in vivo fundamentally lies in the interface between the activation and detoxification pathways. The detoxification pathways produce a readily excretable, non-reactive, water-soluble conjugated metabolite whereas activation pathways result in a highly reactive intermediate which can bind to DNA. The carcinogenic susceptibility of mammalian cells may be determined through an increase or a decrease in either of these metabolic pathways

Carcinogenesis is believed to be a two-stage process. It consists of an initiation and promotion stage (26). It is generally thought that the initiation step is a mutation involving DNA alteration or damage. Promotion involves the expression of this DNA damage and ultimately tumorigenesis. Compounds can be classified two ways. Mutagens cause the initial DNA damage and promoters which enhance tumor expression.

Bile acids are believed to be tumor promoters (6).

IN VITRO STUDIES

One way to study the interaction between carcinogenic compounds and the intestinal mucosa is through in vitro cultivation of intestinal tissue. Autrup et al. (27) developed a colon explant system in order to study the metabolism of suspected environmental carcinogens directly in the target organ. They found that colon explants from rats (28) and humans (29) could be maintained in culture for up to 20 days and these explants could metabolize the procarcinogen, BP, into metabolites which bind to cellular DNA. It is generally believed that such covalent binding between the carcinogen metabolite and cellular macromolecules, such as DNA, RNA, and proteins. leads to initiation (25). In a later study, Autrup et al. (30) compared BP metabolism in human and rat colon explants and found qualitative differences but not quantitative differences in the amount of BP metabolized. This suggests a high correlation between human colon and rat colon metabolic properties in the quantitative sense even though they differed in metabolic profiles. This difference in metabolism may be due to differences in the detoxification pathways. One must note that even though Autrup et al. (29) reported the binding of BP to DNA. BP has never been shown to cause colon cancer.

Previously, rat colon explants have been used to study the metabolic capabilities of the colonic mucosa (27,28,29,35,38). However these colonic explants contain not only epithelial cells. which are the target cells for colon tumorigenesis, but fibroblasts and smooth muscle cells. These other cell types may influence epithelial cell metabolism and/or

have some metabolic properties of their own. Investigations in our laboratory have indicated that fibroblasts obtained from rat fetus colonic tissue can metabolize BP (37). A cell culture system which utilizes only colon epithelial cells was needed to elucidate the mechanisms by which carcinogens act at the target cell level. Since human colonic tissue is difficult to obtain, we investigated the in vitro cultivation of rat colon.

Fang and Strobel (31) scraped rat colon mucosal cells from the intestinal wall and used these cells to study the activation of carcinogens. They reported that the 9000xg supernatant, which contains the microsomal fraction, of rat colon mucosal cells activated procarcinogens to metabolites that are mutagenic in the Ames test. The Ames test, a standard test for mutagenicity, involves plating the suspected compound with different strains of histidine-dependent Salmonella typhimurium on histidine deficient agar plates. If mutations occur in these bacteria, a colony of mutated bacteria (histidine auxotrophs) will appear on the plates. An increase in the number of colonies or revertants indicates an increase in mutagenicity.

Stohs et al. (32) isolated metabolically active small intestinal cells using the enzymes collagenase and hyaluronidase. He postulated that the use of isolated whole cells facilitated the study of the linked cyt P-450 mediated and conjugated phases of drug metabolism. With microsomes or the 9000xg subcellular fraction it is more difficult to reproduce the correct intracellular concentrations and circumstances of all the necessary enzymes and cofactors. However it is quite possible that isolating cells with collagenase and hyaluronidase may

indeed alter cellular metabolism by affecting the cell's membrane.

The use of isolated colon epithelial cells to study xenobiotic metabolism has never been investigated. In the following chapter I will describe the adaptation of a dissociation method by Weiser (33) which produces viable colon epithelial cells without the use of enzymes. The rat colon epithelial cells are dissociated using a chelating (EDTA) solution. As described by Weiser (33) this dissociation procedure is time dependent, whereby 52 minutes will dissociate the mitotically active cells found in the crypts of the intestine. Cells isolated by this method represent the normal population of cells found lining the colon; i.e. ranging from differentiated columnar-type epithelial cells to undifferentiated cuboidal-type epithelial cells in the crypts. Weiser (33) reported that epithelial cells obtained from intestinal scrapings showed only one third the capacity for incorporation of D (1-^{14}C) glucosamine compared to intestinal cells isolated by his less traumatic isolation procedures. Harrison and Webster (34) demonstrated that a complexing reagent, such as EDTA, can be useful in the dissociation of intestinal cells, while having a significant effect in maintaining the cell membrane.

The development of a cell culture technique which utilizes viable colonic epithelial cells would have tremendous potential in studying parameters surrounding cellular metabolism of carcinogens. Since most carcinogens have to be activated before they act as ultimate carcinogens, a cell culture system may be invaluable in testing compounds which may enhance this activation process, especially those compounds which may be increased in the gastro-intestinal tract due to

dietary modifications. Moreover, the balance of activation and detoxification of xenobiotics could be studied with cell culture systems.

CHAPTER TWO . . . . . .ISOLATION AND CULTIVATION OF COLONOCYTES.

Introduction.

Isolation and cultivation of mammalian cells are important approaches in studying cellular metabolism and biology. Some types of cells are relatively easy to cultivate for prolonged periods as primary cultures, however, cultivation of epithelial cells has met with little success. Epithelial cells of the colon are involved in inflammatory diseases and in colon cancer, two serious health problems in the United States. Research in these areas could conceivably benefit from cell biology studies using epithelial cells. This report describes procedures for isolation and cultivation of epithelial cells from colons of rats; the long term goal is to use colon epithelial cells to study cellular events which may be important in inflammatory diseases and neoplasia in the colon.

Colon epithelial cells include columnar, goblet, crypt and endocrine cells which will be collectively referred to as "colonocytes" as suggested by Roediger and Truelove (36). Many variations of the methods to be described in cell isolation and cultivation were used: the following describes procedures that produced the greatest increase in cell number and is used routinely to cultivate colonocytes in this laboratory. An increase in cell number and changes in morphology representing cell differentiation will be collectively referred to as cell growth.

Materials and Methods.

Animals. Germ-free female Sprague-Dawley rats, 250g. were from Harlan Industries, Cumberland, Indiana 46229. Colonocytes were isolated from the rats the day they arrived from the supplier.

Chemicals. Fetal bovine serum (FBS), CMRL-1066 medium with glutamine, Eagle's Minimum Essential medium (EMEM), Waymouths MB 752/1 medium, Penicillin-Streptomycin solution, Amphotericin B and a solution of non-essential amino acids were purchased from Grand Island Biological Company, Grand Island, New York 14072. Rat tail collagen, Type IV, and hydrocortisone were purchased from Sigma Chemical Company, St. Louis, Missouri 63178. All other chemicals and supplies used were from local suppliers.

Collagen coating of cover slips and petri dishes.

To coat the bottom of petri dishes with collagen a sterile collagen solution (0.2 mg/ml in 1% acetic acid) was transferred to 10 cm petri dishes (Falcon). The collagen solution was poured off after 5 minutes and the petri dishes were allowed to dry under sterile conditions at room temperature. To coat cover slips with collagen for scanning electron microscopy (SEM) of the colonocytes, droplets of the collagen solution were placed on cover slips (Corning). The cover slips were allowed to dry and then sterilized by autoclaving.

Isolation and cultivation of colonocytes.

A procedure used to isolate epithelial cells from the small intestine (Weiser et al. 33) was adapted for isolation of colonocytes. Rats were sacrificed by exsanguination and colons were excised at the cecum and rectum. Excised colons were rinsed with a 0.154 M NaCl, 1 mM dithiothreitol solution to remove intestinal contents. After rinsing, one end of the colon was tied with thread and the colon was filled with solution A (1.5 mM KCl, 96 mM NaCl, 27 mM sodium citrate, 8 mM KH_2PO_4, 5.6 mM Na_2HPO_4, pH 7.3) to reduce the mucous coating of

the epithelial cells. The other end was then tied and the colon was placed in a petri dish in a humidified incubator (5% CO_2, 95% air) maintained at 37^{o}. After 15 minutes, solution A and residual contents were discarded. The colon was then filled with solution B (70 mM NaCl, 5.6 mM KH_2PO_4, 3.9 mM Na_2HPO_4, 1.1 mM KCl, 1.5 mM EDTA, 0.5 mM dithiothreitol) and placed in the incubator for 52 minutes. By 52 minutes this time-dependent dissociation method will dissociate most cells including the mitotically active cells found in the crypts of Leiberkuhn of the colon. Solution B, containing the dissociated cells, was drained into a sterile centrifuge tube. The colon was rinsed twice with phosphate-buffered solution (70 mM NaCl, 5.6 mM KH_2PO_4, 3.9 mM Na_2HPO_4 and 1.1 mM KCl) and shaken vigorously to obtain the maximal number of cells. The rinses were added to solution B and the cells were collected by centrifugation at 300 x g for 5 minutes. The loose cell pellet was resuspended in 2 ml of culture medium and transferred to 15 cm culture dishes that had been previously coated with rat tail collagen and rinsed once with culture medium. The cells were spread over the plate with a glass rod and incubated for 30 min. After this attachment period, culture medium was carefully added to immerse the cells, and the culture dishes were incubated for designated periods. The culture medium consisted of CMRL-1066 with glutamine, supplemented with glucose (5 mg/ml), FBS (5%), penicillin (125 U/ml), streptomycin (125 ug/ml), amphotericin B (2 ug/ml), minimum essential media non-essential amino acids (final concentration was 10 mM for each amino acid), and hydrocortisone (0.67 ug/ml). All isolation and culture procedures were performed in a laminar flow hood using conventional aseptic techniques.

All solutions and equipment were autoclaved and kept at room temperature, except for the media which were either purchased sterile or filter sterilized through 0.22 uM Millipore filters. After two days of incubation at 37^{o}, the medium was carefully removed so that the attached cells were not disturbed and fresh media was added. If cultures became contaminated with bacteria or yeast, the dishes were rinsed with a sterile balanced salt solution (BSS - 137.0 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 $Na_2HPO_4 \cdot 7H_2O$, 6 mM $NaHCO_3$, 5.5 mM glucose, 0.2% phenol red) before addition of fresh medium.

Cell viability.

Cell membrane integrity was assessed by the trypan blue exclusion method. Cell suspension in BSS were made 0.07% with trypan blue and stained and unstained cells were counted in a hemacytometer. Viability was expressed as the percentage of total cells that were unstained after five minutes exposure to trypan blue.

Scanning electron microphotographs.

Isolated colonocytes were spread on collagen coated cover slips and incubated in petri dishes similar to the procedure described above. At designated times colonocytes attached to the glass cover slips were fixed in a 4% glutaraldehyde solution. After a series of ethanol dehydration steps, the cells were critical-point dryed and gold plated. The cover slips were scanned using a JBL-100 scanning electron microscope.

Results and Discussion.

Inappropriate cellular environment, lack of cell attachment to culture dishes, prolonged cell doubling time and bacterial contamination are some of the major obstacles impairing successful long-term cultivation of colonocytes. Colonic tissue taken aseptically from germ-free rats provided a source of bacteria-free epithelial cells to overcome one of the major difficulties. We have been unsuccessful in cultivating colonocytes from conventional rats because of bacterial contamination. Washing the cells repeatedly before plating and incubation in the presence of penicillin-streptomycin with or without rifampicin (up to 100 units or mg/ml of media) reduced bacterial contamination. However,some bacteria became resistant to the antibiotics within three to five days and caused the colonocytes to cease growing and to degenerate.

Initially, freshly isolated colonocytes were plated in EMEM with 10% FBS, since this medium supported rapid proliferation of intestinal fibroblasts isolated from fetal rats (37). Culturing colonocytes in EMEM resulted in minimal growth (increase in cell number) accompanied by cellular destruction as assessed by light microscopy. Waymouths MB 752/1, a medium used to maintain rat tracheal explants (40), was tested in a similar manner. Minimum growth and extensive cellular destruction occurred with this medium also. Viable cultures of rat colonocytes beyond one week were not feasible using either EMEM or Waymouths MB 752/1 medium.

CMRL-1066, a medium used to maintain rat colon explants (29,39) was compared to EMEM and Waymouths media. CMRL-1066 medium supplemented

with glucose, non-essential amino acids, FBS and hydrocortisone promoted in vitro growth with minimal cellular destruction. Morphologically, the epithelial cells were more uniform (cuboidal) and had less cell membrane deterioration compared to cells grown in the two media previously described. Colonocyte growth was greater if the supplemented CMRL-1066 medium contained 5% FBS than if the medium contained 10% FBS. Additions of insulin, putrescine, arginine, pyruvate or essential fatty acids to the supplemented CMRL-1066 medium did not improve cell viability or growth and thereafter were omitted from the supplemented CMRL-1066 medium. With the use of CMRL-1066 supplemented media cultures of viable colonocytes have been maintained for as long as 12 weeks. Doubling times remain several-fold longer than doubling times for fibroblasts or myogenic cells; the prolonged doubling time for colonocytes excludes the use of colonocytes for mutagenicity studies. However, with the use of CMRL-1066 medium we have been able to investigate how compounds which are altered by dietary composition influences colonocytes metabolism. The studies will be reported elsewhere.

Attachment of freshly isolated colonocytes to the plastic surface of dishes was very poor. Adding medium to dishes immediately after the colonocytes were spread on the surface of the dish resulted in virtually no cell attachment. But rinsing the culture dish with media, spreading the colonocytes on the rinsed surface and incubating the cells for 30 minutes before adding medium allowed some of the colonocytes to attach. If colonocytes are incubated for periods longer than 30 minutes before addition of medium, cell survival is decreased. Coating the surface of the culture dishes with rat tail collagen significantly increased cell

attachment. Our current practice is to spread isolated colonocytes on culture dishes coated with rat tail collagen and previously rinsed with medium; medium is carefully added after the colonocytes have been incubated for 30 minutes.

Freshly isolated colonocytes were a mixed population of epithelial cells. Figure 1 shows a typical culture 24-hrs after isolation. Clumps of cells consisting of whole or partial crypts, individual cells and some cellular fragments attached to the collagen coated dishes. The viability of freshly isolated cells was 52% as determined by trypan-blue exclusion. Presumably some non-viable cells attached to the dish also. Within one week only cuboidal and columnar-type epithelial cells remained attached (Figure 2); cellular fragments, debris and dead cells degenerated or washed away during periodic medium changes. Within two weeks of culture, isolated islands of cuboidal cells in monolayers were prevalent (Figure 3). These islands of cells grew larger with time and at the borders of these monolayers a progression of cuboidal cells to columnar-type cells was observed (Figure 4). This appears to represent in vitro differentiation from the cuboidal-type epithelial cells found near the bottom of the crypt columns to the columnar- type epithelial cells that are found near the top of the crypt column or lining the intestinal lumen. SEM of freshly isolated cells showed three types of cells - cuboidal or spherical cells which probably represent endocrine and undifferentiated cells, columnar or absorptive type cells and mucous-secreting goblet cells which have the typical "wine glass" appearance (Figures 5 & 6). Figure 7 shows a SEM of mixed colonocytes (mostly cuboidal-type cells) that have been in culture for eight weeks.

Microvilli form on absorptive cells in vivo as the differentiated cell progresses from the bottom of the crypt column to the luminal surface. Freshly isolated colonocytes and cells that had been cultured for eight weeks were compared. Microvilli on eight week old cells were morphologically similar to those found on freshly isolated cells: this may indicate in vitro differentiation. Figure 8 shows two freshly isolated columnar-type colonocytes with microvilli-like projections on their upper surface. Similarly, cells grown in culture for eight weeks also showed microvilli-like projections (Figure 9). A greater magnification of these projections (Figure 10) clearly shows that these projections were morphologically similar to microvilli. Columnar-type colonocytes that attach after isolation degenerated by three weeks of culture. Therefore, the columnar-type cell in Figure 9 must have differentiated from the cuboidal-type cells shown in Figure 7.

In summary, rat colonocytes isolated from germ-free rats can be cultured in supplemented CMRL-1066 medium for 12 weeks or longer on collagen-coated plastic dishes. After three weeks of culture, cuboidal-type cells are the only viable cells in culture. Cuboidal cells appear to differentiate to columnar-type cells with microvilli-like projections by eight weeks of culture.

Figure 1. Phase micrograph of freshly isolated colonocytes. Original magnification (400 X).

Figure 2. Phase micrograph of colonocytes cultured for one week. Original magnification (400 X).

Figure 3. Phase micrograph of colonocytes cultured for two weeks. Original magnification (400 X).

Figure 4. Phase micrograph of colonocytes cultured for six weeks. Original magnification (400 X).

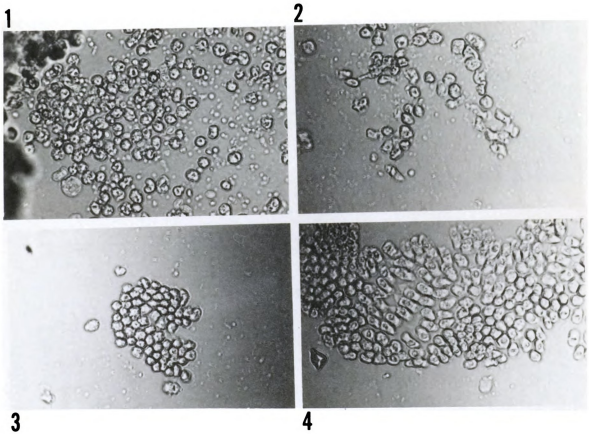
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Figure 5. Scanning electron micrograph of freshly isolated colonocytes. Original magnification (2,600 X).

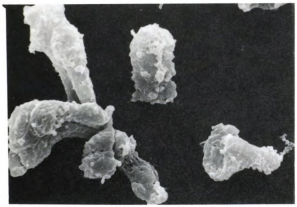
Figure 6. Scanning electron micrograph of freshly isolated colonocytes. Original magnification (6,000 X).

Figure 7. Scanning electron micrograph of colonocytes cultured for 8 weeks. Original magnification (1,200 X).

Figure 8. Scanning electron micrograph of freshly isolated colonocytes. Original magnification (6,000 X).

Figure 9. Scanning electron micrograph of a columnar epithelial cell cultured for 8 weeks. Original magnification (6,000 X).

Figure 10. Scanning electron micrograph of "microvilli" found on columnar epithelial cell cultured for 8 weeks. Original magnification (32,000 X).



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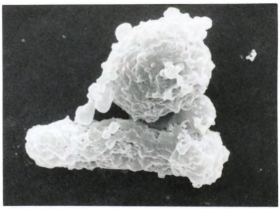

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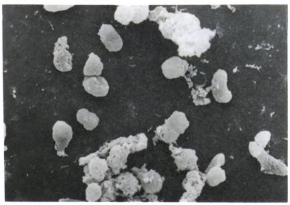

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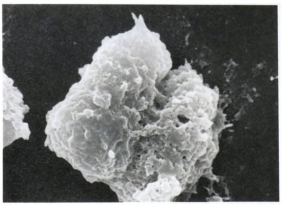

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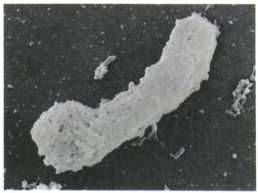

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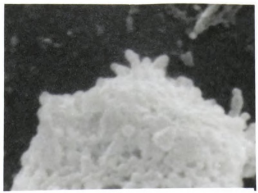


10

CHAPTER THREE . . . . . COLONOCYTE METABOLISM OF BENZO(a)PYRENE AS INFLUENCED BY BILE ACIDS, SKATOLE AND INDOLE.

Introduction

Once cell culture conditions for in vitro cell growth were elucidated the metabolic properties of rat colon epithelial cells could be studied. The cell culture procedures described in chapter 2 were used to study xenobiotic metabolism, specifically polycyclic aromatic hydrocarbons (PAH). Benzo(a)pyrene (BP), a ubiquitous xenobiotic, was used as the model PAH contaminant. Initial metabolic studies (37) demonstrated that not only will these cells grow in culture, they will also metabolize xenobiotics. The potential usefulness of this system to study xenobiotic metabolism seemed immense. This chapter will describe the use of cultured colonoytes to study PAH metabolism, especially BP metabolism when co-cultured with compounds that are increased in the colon when a high-fat, high-protein diet is consumed. The overall objective was to look at how intraluminal contents of the colon, specifically those compounds which can be determined by diet and which are implicated in colon carcinogenesis, affect PAH metabolism.

Materials and Methods

Animals. Male (200-250g) Sprague-Dawley rats were purchased from Spartan Animals, Haslett, Michigan 48840. Conventional rats were used in the studies because of their cost and accessability. They were allowed laboratory chow (Wayne Lab Blox, Chicago, IL 60606) and water ad libitum.

Chemicals. [^{3}H]-Benzo(a)pyrene, specific activity 30.2 Ci/mmole, was obtained from New England Nuclear, Boston MA 02118. Lithocholic acid, deoxycholic acid, skatole, and indole were purchased from Sigma Chemical Company. Fetal calf serum, CMRL-1066 media, penicillin-

streptomycin. amphotericin B and non-essential amino acids were purchased from Grand Island Biological Company, Grand Island, NY 14072.

Cytotoxicity. Cell membrane integrity was assessed by the trypan blue exclusion method. Cell suspension in BSS were made 0.07% with trypan blue and stained and unstained cells were counted in a hemacytometer Viability is expressed as the percentage of total cells which were unstained after 5 minutes exposure to trypan blue. The cytoxic effect of BP and test solutions were determined by viable cell counts after 24 hr incubations.

BP Metabolism. Colonocytes from conventional Sprague-Dawley rats were isolated and cultured similarly to those procedures used for germ-free rats as described in chapter 2. An equal aliquot of the cell suspension was pipeted into 15 cm culture dishes (FALCON). The cultures were incubated with [^{3}H]-benzo(a)pyrene (BP, 1.0 ug/ml of media) for 12 or 24 hrs. After the indicated incubation period, aliquots of culture medium containing the colonocytes and BP metabolites were analyzed by the radioactive assay of van Cantfort et al. (41). This isotopic assay determines all BP metabolites. Where applicable, the DNA content of the cell cultures was determined by the colorimetric assay of Setaro & Morley (42).

Statistics. One way analysis of variance was performed as described by Gill (43). When F values were greater than critical values, treatment differences were determined by the Bonferroni t-test.

Induction of BP Metabolizing Enzymes. Male Sprague-Dawley rats were injected with B-naphthaflavone (s.c., 80 mg/kg) once a day for 3 days prior to exsanguination. Cell isolation, culture conditions and

determination of BP metabolism were the same as described for cells isolated from rats which didn't receive B-naphthaflavone.

Mutagenicity of BP Metabolites. Colonocytes were cultured in the presence of 1.0 ug BP/ml of media and an aliquot of medium was extracted three times with ethyl acetate/acetone (2:1) after a 24 hr incubation period. The extracts were dried with Na_2SO_4 and evaporated under nitrogen. The extracts were dissolved in DMSO and tested for mutagenicity.

Ames Test. The Ames test was performed according to the standard procedure described by Ames et al (46). Ethyl acetate/acetone (2:1) extractable metabolites dissolved in DMSO were tested using the Salmonella typhimurium strains TA 100 and TA 1535. The S-9 fraction from rat liver was not used in these mutagenicity tests.

BP Binding to Cellular Macromolecules

Similar 24 hr incubations were performed and 5 ml aliquots from the colonocyte cultures were analyzed for BP bound to cellular macromolecules. Cellular macromolecules were precipitated twice with ice cold 10% trichloroacetic acid. The precipitate was washed twice with ice cold 1% potassium acetate in ethanol to remove lecithans, twice with ethanol: chloroform (3:1, v:v) and once each with ethanol:diethyl ether (3:1, v:v) and diethyl ether to remove the remaining lipids (44). The resulting precipitate was solubilized by addition of NCS tissue solubilizer (Amersham, Arlington, Heights, IL). The solubilized sample was neutralized with HCl, added to 15 ml Tritontoluene scintillation cocktail [1:2; 4 g 2.5-diphenyloxazole, 0.6g 1,2-bis 2(4-methyl-5-phenylazoxy)] and counted by liquid scintillation spectometry.

Results and Discussion

Cultured colon epithelial cells isolated from conventional rats were able to metabolize the procarcinogen, BP. Initial studies were performed using cells from two different rats and results are shown in Table 1. The inherent high variation of in vitro investigation is consistent with these data. Colonocytes cultured for 72 hrs had a 1 to 2-fold increase in BP metabolism compared to 24 hr cultures. However, beyond 24 hrs bacterial contamination increases dramatically due to the endogenous microflora present in conventional rat intestines. Culture medium heavily contaminated with bacteria can adversely affect in vitro metabolism. Also, it has been reported (45) that enteric bacteria can hydrolyze BP into carbon fragments, which could possibly show up as BP metabolites. For these reasons only 12 and 24 hour cultures were used in our investigations.

To determine the cytotoxicity of BP. cells were incubated with varying levels of BP for 24 hours. Cell viability was determined prior to and after the 24 hr incubation period. The results are shown in Figure 1. Increasing the concentration of BP decreased the amount of viable cells from 46% at 24 hrs with only solvent to 36% when the culture medium contained 1.0 ug BP/ml. Increasing the BP concentration up to 10.0 ug/ml caused further decreases in viability. A low level of BP is therefore desirable to eliminate or reduce cytotoxicity. However. a lower level of BP may not be sufficient to cause significant metabolism by isolated colonocytes. The amount of BP metabolized per million viable cells (Figure 2) increases as BP concentration increases, but BP metabolism did not increase in proportion to BP concentration

Table 1

Benzo(a)pyrene metabolism in rat colonocyte cultures[a]

	Benzo(a)pyrene metabolism[b]
Rat #1	28.14
Rat #2	38.56

[a]All cultures were treated with 1.0 ug BP/ml of culture media and incubated for 24 hours.

[b]ng BP metabolites per mg DNA minus zero time control.

Figure 1. The effect of Benzo(a)pyrene concentration on colonocyte viability.

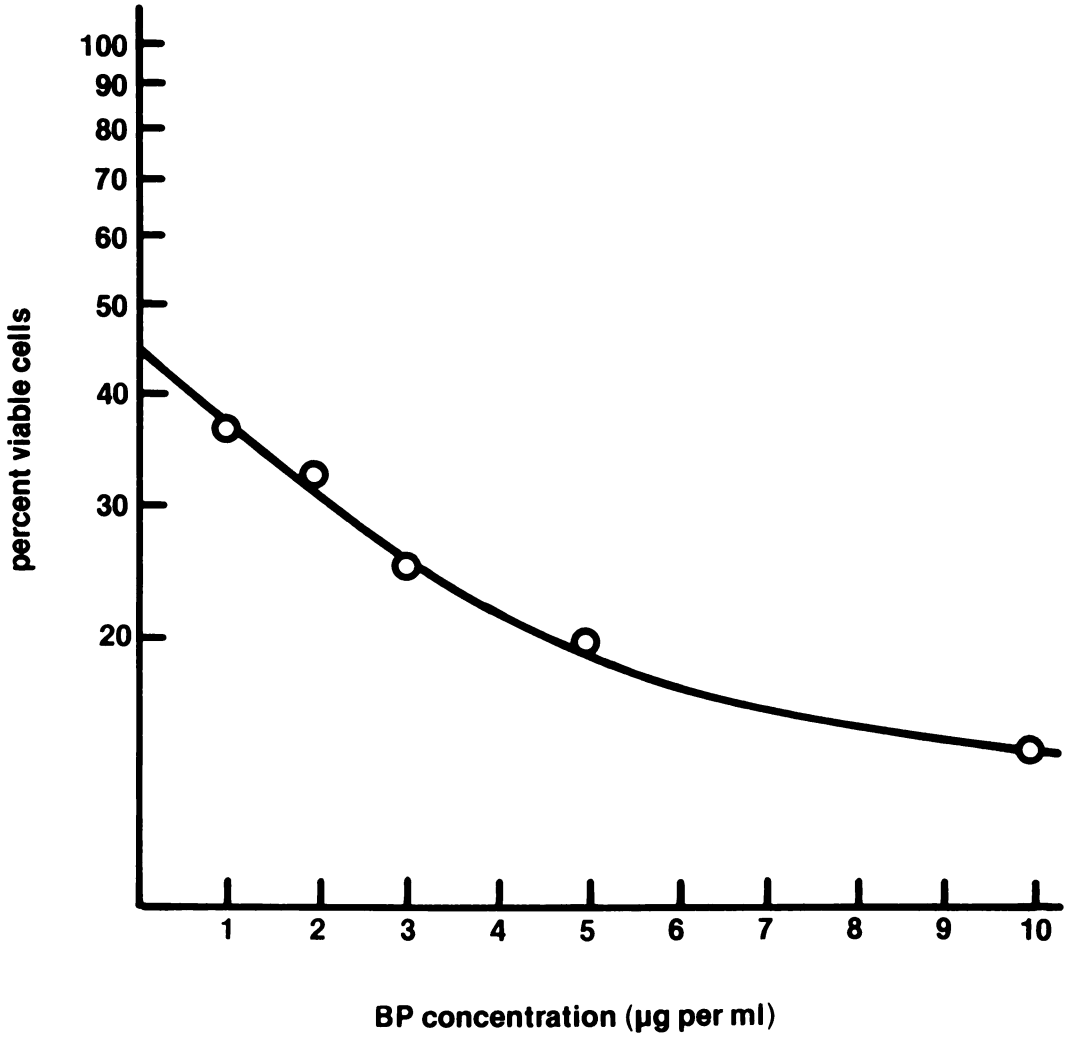
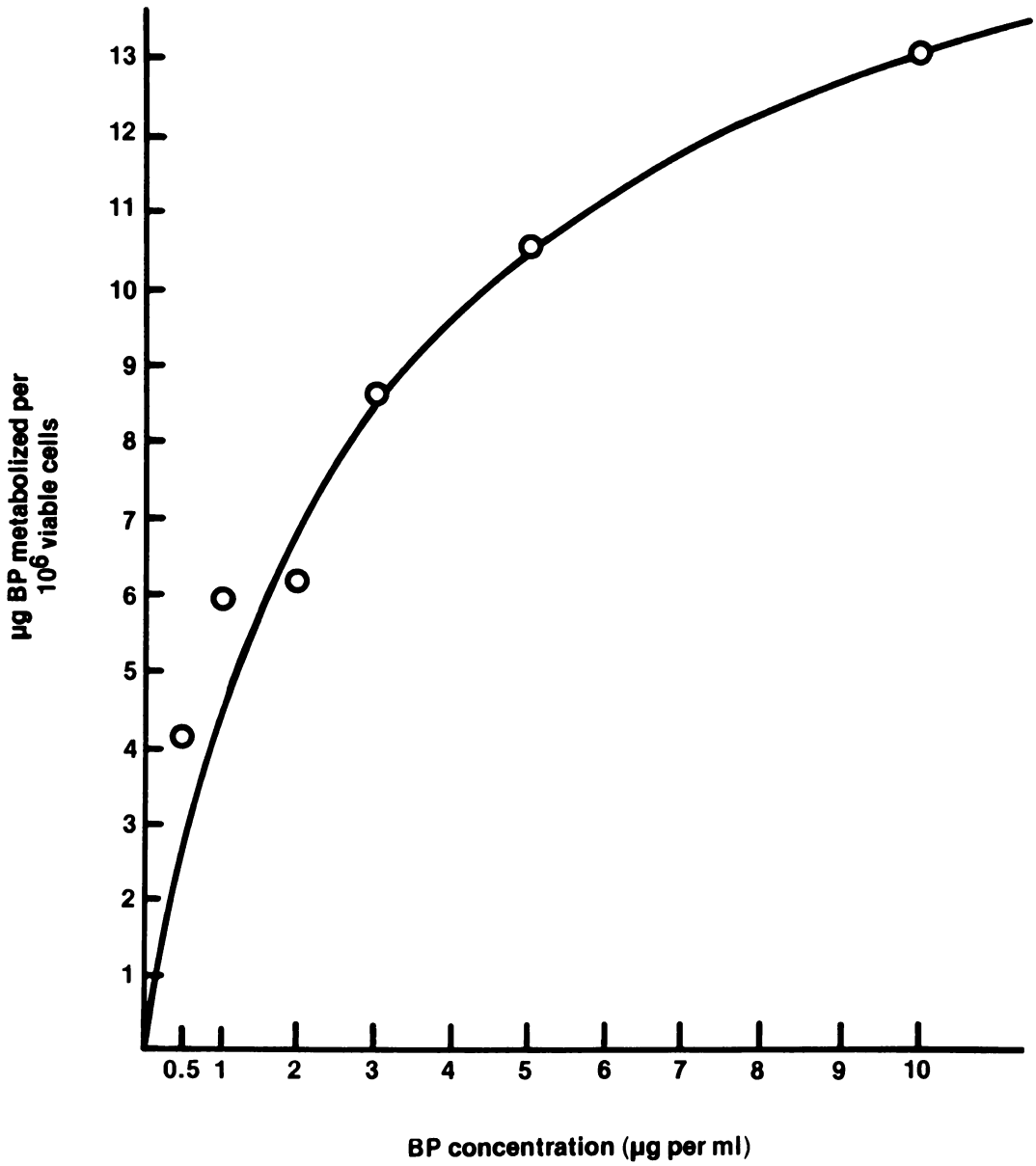

100
90
80
70
60
50
40
30
20
percent viable cells
1
2
3
4
5
6
7
8
9
10
BP concentration (µg per ml)

Figure 2. The effect of Benzo(a)pyrene concentration on colonocyte metabolism.


µg BP metabolized per 10^6 viable cells
13
12
11
10
9
8
7
6
5
4
3
2
1
0.5 1 2 3 4 5 6 7 8 9 10
BP concentration (µg per ml)

even when BP concentrations were 1.0 ug/ml or less. Most likely the non-linear response was due to decreased cellular metabolism without a concomittant decrease in cell viability as BP concentrations increased. Since the gut metabolites which were to be tested for their effect on PAH metabolism are also cytotoxic, BP concentrations in later experiments were limited to 1.0 ug/ml of media which was the greatest BP concentration that showed nearly linear BP metabolism in response to increased BP concentrations.

The next experiments were performed with rats that had been injected with B-naphthaflavone, an inducer of mixed function oxidases (MFO) which are involved in cellular metabolism of PAH's such as BP in rat colon (24). This increase in the mixed function oxidases, especially aryl hydrocarbon monooxygenase, has been postulated to play a role in the carcinogenic effect of PAH's. Reports of either an enhancement or inhibition of carcinogenesis are presently found in the literature. It has been reported that various xenobiotics and dietary treatments may also induce these microsomal enzymes (47). It was hypothesized that colonocytes isolated from pretreated rats would metabolize significantly more BP compared to colonocytes isolated from control rats injected with vehicle. The hypothesis was true since colonocytes from B-naphthaflavone treated rats metabolize 20-30 times more BP (362 ng BP metabolized per unstimulated cell culture vs 5.22 ug BP metabolized per stimulated cell culture). These results demonstrate that colonocytes are reacting the same as microsomes from pretreated rats compared to control rats.

An important next step was to achieve an understanding of how

colonocytes metabolize and possibly activate potential carcinogens; therefore, the mutagenicity of BP metabolites produced in vitro was investigated. The mutagenicity of a compound may be an indication of its carcinogenic potential. Organic extractable metabolites were tested in the Ames test (46), a standard test for mutagenicity. The Ames test involves plating the metabolites with different strains of Salmonella typhimurium on histidine-deficient agar plates. If mutations occur, a colony will appear on the plates. An increase in the number of colonies or revertants per plate indicates an increase in mutagenicity. The organic extract tested contains both metabolized and unmetabolized BP. BP added without in vitro metabolism/activation showed no increase in revertants per plate over background level. Any increases in reverants is therefore due to the presence of mutagenic metabolites. The results are presented in Table 2. An increase in the level of organic extract from 24 hr intestinal cell cultures resulted in an increase in revertants per plate. The Salmonella typhimurium strain TA 100 is more sensitive and has a higher spontaneous mutation rate, and can therefore more readily revert compared to the TA 1538 strain. The trend for increased mutagenicity with increased levels of the organic extract is apparent with strain TA 1538, however the number of revertants per plate is less for TA 1538 than for TA 100. These studies indicate that colonocytes isolated from conventional rats will activate BP into mutagenic metabolites during a 24 hr incubation period.

Deoxycholic acid and lithocholic acid, two secondary bile acids, are considered to be promoters in colon carcinogenesis (48). Therefore, these bile acids may affect xenobiotic metabolism. This hypothesis was tested by incubating colonocytes with either lithocholic acid or

Table 2.

Mutagenicity of organic-extractable benzo(a)-pyrene metabolites from rat colonocyte cultures[a]

	Revertants/plate[b]	
ug/plate added	TA 100	TA1535
0.00	80	10
1.25	93	21
2.50	109	20
5.00	120	28
10.00	152	28

[a]All cultures were treated with 1.0 ug BP/ml of culture media and incubated for 24 hours.
[b]10 plates/level/*Salmonella typhimurium* strain.

deoxycholic acid along with BP. The two bile acids were initially tested at the 100 uM concentrations because previous investigations in this laboratory indicated that these compounds were slightly cytotoxic at 100 uM concentrations. The results are shown in Table 3. In cell cultures incubated for 12 hrs, deoxycholic acid inhibited BP metabolism significantly ($p < 0.05$). Lithocholic acid tended to inhibit BP metabolism although not significantly. Cells cultured for 24 hrs in the presence of bile acids tended to overcome the inhibitory effect on BP metabolism found with 12 hr incubations. This stimulation in BP metabolism by bile acids was significantly different ($p < 0.05$).

The effects of skatole and indole, two bacterial metabolites of tryptophan which have been implicated in colon carcinogenesis (12), on BP metabolism were tested also and results are shown in Table 3. With 12 hr incubations, skatole and indole exerted opposite effects upon BP metabolism; indole inhibited while skatole enhanced BP metabolism ($p < 0.05$), with skatole showing the greatest increase in amounts metabolized. Since skatole enhanced BP metabolism the most, varying concentrations of skatole were added to colonocyte cultures. All these concentrations of skatole increased BP metabolism ($p < 0.05$, Table 4) with the 250 uM concentration of skatole producing the greatest increase in BP metabolism. The decrease in BP metabolism seen with 500 uM skatole compared to 250 uM skatole could be due to cytotoxicity at the higher concentration.

Table 5 shows the effect that deoxycholic acid and lithocholic acid have upon BP metabolism in colonocyte cultures from rats pretreated with B-naphthaflavone. Both deoxycholic acid and lithocholic acid had an

Table 3.

Treatment effects upon benzo(a)pyrene metabolism in rat colonocyte cultures[a]

	BP Metabolism Incubation Time (hours)	
Treatment	12	24
Solvent	100[b]	100[c]
100 uM Lithocholic acid	94	123
100 uM Deoxycholic acid	83*	114*
100 uM Skatole	635*	353*
100 uM Indole	48*	132*

[a]All cell cultures were treated with 1.0 ug BP/ml of culture media.

[b]100% equals 205 ug BP/15 ml cell culture, normalized for viability and to solvent control. Asterisked values are statistically different ($p < 0.05$).

[c]100% equals 362 ug BP/15 ml cell culture, normalized for viability and to solvent control. Asterisked values are statistically different ($p < 0.05$).

Table 4.

Effects of varying concentrations of skatole upon benzo(a)pyrene metabolism in rat colonocyte cultures[a]

Treatment	BP Metabolism
Solvent	100[b]
100 uM Skatole	353*
250 uM Skatole	638*
500 uM Skatole	529*

[a]All cell cultures were treated with 1.0 ug BP/ml of culture media and incubated for 24 hours.

[b]100% equals 128 ng BP/15 ml cell culture, normalized for viability and to solvent control. Asterisked values are statistically different ($p < 0.05$).

Table 5.

Treatment effects upon benzo(a)pyrene metabolism in B-naphthaflavone treated rat colonocyte cultures [a]

Treatment	BP Metabolism
Solvent	100[b]
100 uM Lithocholic acid	55*
100 uM Deoxycholic acid	81*
100 uM Indole	95*
100 uM Skatole	203*
250 uM Skatole	237*

aAll cell cultures were treated with 10 ug BP/ml of culture media and incubated for 24 hours.

b100% equals 5.22 ug BP/15 ml cell culture, normalized for viability and to solvent control. Asterisked values are statistically different ($p < 0.05$).

inhibitory effect upon BP metabolism compared to the control cultures ($p<0.05$). Lithocholic acid inhibited BP metabolism to the greatest extent. The inhibitory effect of bile acids on BP metabolism by colonocytes from induced rats is in contrast to the results found when these bile acids were incubated with colonocytes from control rats (Table 3 compared to table 5). It should be noted that even though the bile acids inhibited BP metabolism by coloncytes from induced rats compared to the solvent control, the amount of BP metabolized by colonocytes from B-naphthaflavone treated rats and incubated in the presence of either bile acid is still significantly greater than BP metabolism by colonocytes from control rats incubated in the presence of bile acids.

The effect of tryptophan metabolites upon BP metabolism by colonocyte cultures from induced rats was also investigated. Skatole had a quite different effect upon BP metabolism compared to indole and two bile acids (Table 5). At the 100 uM level, skatole enhanced BP metabolism ($p<0.05$). At the 250 uM level of skatole, BP metabolism increased to the greatest degree.

The differences in BP metabolism exhibited between colonocytes from uninduced and induced rats are quite dramatic. Investigations to study these differences could lead to a better understanding of enzyme induction and carcinogenesis. Recent studies (49) have implicated an associated increase in detoxification pathways with enzyme induction. This increase in detoxification of xenobiotics should result in an increase in the detoxified conjugated metabolites such as glucuronides, sulfates, etc. which are water soluble thereby allowing easier exit from the body.

Reactive intermediates of PAH metabolism are believed to bind to cellular macromolecules (CM) such as DNA, RNA and protein and initiate the carcinogenic process. These reactive intermediates are formed when the PAH's are metabolized by the cell. They are the intermediary products formed when the initial compound is sequentially metabolized to its detoxified metabolite. One might find an associated increase in binding to CM upon an increase in metabolite conjugation and/or cellular metabolism. To check this hypothesis aliquots of cells were taken from 24 hr cell cultures and the CM were extracted and counted for bound metabolites. The cell cultures from stimulated rats had a two-fold increase in binding of BP to CM compared to cultures fom non-stimulated rats. In the cell cultures from stimulated rats both of the bile acids, lithocholic and deoxycholic, slightly increased the binding of BP to CM (Table 6). Increasing the concentration of skatole in cell cultures from stimulated rats caused a similarly greater increase in binding of BP to CM. However, in the cell cultures from non-stimulated rats the two bile acids decreased BP binding to CM compared to the control level. Skatole similarly increased the level of binding to CM in unstimulated cell cultures when compared to the binding in stimulated cell cultures. Indole slightly increased BP binding to CM. The effects of tryptophan metabolites on BP metabolite binding to CM followed similar patterns to their effects upon BP metabolism by cultures from both stimulated and unstimulated rats. It should be pointed out that although cell cultures from stimulated rats increased BP metabolism approximately 20-fold compared to cell cultures from unstimulated rats, there was only a 2-fold increase in BP binding to CM. An increase in

Table 6.

Treatment effects upon benzo(a)pyrene binding to cellular macromolecules[a]

Treatment	BP Bound to CM	
	B-naphthaflavone induced cell cultures	Uninduced cell cultures
Solvent	100[c]	100[b]
100 uM Lithocholic acid	120*	62*
100 uM Deoxycholic acid	128*	71*
100 uM Indole	NT[d]	113*
100 uM Skatole	239*	180*
250 uM Skatole	331*	244*

[a]All cell cultures were treated with 1.0 ug BP/ml of culture media and incubated for 24 hours.

[b]100% equals 452 ng BP/15 ml cell culture, normalized for viability and to solvent control. Asterisked values are statistically different ($p < 0.05$).

[c]100% equals 661 ng BP/15 ml cell culture, normalized for viability and to solvent control. Asterisked values are statistically different ($p < 0.05$).

[d]NT=no treatment.

cellular detoxification pathways may serve as a possible explanation for the observed differences between cultures from stimulated and unstimulated rats.

In all except the initial studies, the amount of BP metabolized or the amount of BP bound to CM was normalized regarding cell viability. Presenting the data this way diminishes the cytotoxic effects of the compounds tested. Metabolism per viable cell better explains the effects test compounds exert. Bile acids and tryptophan metabolites were cytotoxic to a greater degree in the cells from stimulated rats compared to the cells from non-stimulated rats. In both type of cells, the bile acids and indole were much less cytotoxic than skatole. This indicates that skatole exerts a more profound effect upon colonocytes and thus may affect colonocyte metabolism to a greater degree. The data substantiates this conclusion. In cells from non-stimulated rats it is quite possible that bile acids, although being cytotoxic, allowed more of BP to enter into the remaining viable cells. This may cause the observed increase in BP metabolism. A greater interaction of BP with cellular membranes may have resulted also since bile acids are powerful solubilizing agents. This could enhance BP metabolism also.

In the cell cultures from stimulated rats all compounds tested showed a greater decrease in cell viability compared to cell cultures from non-stimulated rats in comparison to their control cultures where only BP was added. This indicates there are differences between cells from stimulated and unstimulated rats other than their quantitative differences in BP metabolism. It appears that cells from stimulated rats are much more sensitive to bile acids and tryptophan metabolites.

The significance of this increased in vitro cytotoxicity is unknown. The difference in viability between cells from stimulated and non-stimulated rats when no compounds were added suggests that cells from stimulated rats were not affected by the isolation procedures to the extent of cells from unstimulated rats. Possible differences in cell membranes and/or growth characteristics in cells from stimulated rats should not be overlooked.

Conclusion

Lithocholic and deoxycholic acids tend to enhance BP metabolism by colonocytes from non-stimulated rats. One hypothesis suggests that since PAH's are lipid soluble they would be carried along with the lipid portion of the intestinal contents and would therefore not come in contact with the intestinal mucosa. Bile acids, however, are powerful solibulizing agents and would tend to maintain the carcinogens in solution and aid their interaction with cellular surfaces. Increased interaction with cellular surfaces or membranes probably allows greater entry of carcinogens into the cell and therefore enhanced metabolism. This increased interaction may explain the increased cytotoxicity as well. Reddy et al. (48) suggests that the tumor promoting effect of bile acids is probably due to the alteration of cellular membranes to allow greater entry of carcinogens.

The effects of the tryptophan metabolites upon BP metabolism are also probably due to the metabolite's effect upon cell membranes. Skatole has been shown to exert structural alterations in biological membranes (50). This could explain their effect on BP metabolism in the cell cultures from non-stimulated rats.

The effects that both bile acids and tryptophan metabolites have upon BP metabolism in cell cultures from stimulated rats could be due to a difference in cellular mechanisms. Induction of the microsomal enzymes cause increased metabolism of PAH's, however, it is unknown how this induction affects the metabolism of other compounds. Further investigations concerning the metabolic properties of cells from stimulated rats are needed.

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