Order Number 9022113

Bile acid-induced DNA damage and repair in bacterial and mammalian cells

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The University of Arizona, 1990

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Ann Arbor, MI 48106

BILE ACID-INDUCED DNA DAMAGE AND REPAIR IN BACTERIAL AND MAMMALIAN CELLS

by

Risa Lynne Kandell

A Dissertation Submitted to the Faculty of the

COMMITTEE ON GENETICS (GRADUATE)

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1990

THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Risa Lynne Kandell

entitled Bile Acid-Induced DNA Damage and Repair In Bacterial and Mammalian Cells

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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Harris Bernstein	12/12/89 Date
	Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Harris Bernstein
Dissertation Director

12/12/89
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DEDICATION

The writing of this manuscript and the preceding years of work could not have been accomplished without the love and support of two people, my mother and sister.

To Isabel Kandell - Your confidence in me is boundless. You encourage me to pursue my dreams and catch me when I fall. And you always make me laugh. From you I have learned the greatest of all lessons, to enjoy each day.

To Mona Kandell - Your pride in me makes me shine and your ability to give me center stage with love makes me humble. Thank you for all the laughter and joy we share (and for listening to all my stories, again and again).

My undying gratitude and love go to you both.

My sincere thanks to Esther Ribler for her love and support. This manuscript is dedicated (in memory) to Manuel L. Ribler.

ACKNOWLEDGEMENTS

My sincere gratitude to Harris Bernstein, a true mentor. Your wisdom, patience and guidance are priceless gifts you subtly give your students, while always allowing them freedom to explore. Your gentle way of teaching is best described by Pope - "Men must be taught as if you taught them not and things unknown proposed as things forgot." I thank you for graciously imparting so much knowledge and for giving me the opportunity to learn.

My thankful appreciation to my committee members Dr. Sue Denise, Dr. Tom McCoy, Dr. Dennis Ray and Dr. Oscar Ward for their time and guidance throughout this endeavor.

This project was accomplished with the assistance of many people. I thank my friends and colleagues who gladly shared their knowledge and helped me answer so many questions along the way.

And finally, I thank the following people for their contributions: Carol Bernstein, for her enthusiasm; Steve Abedon, for his unique outlook on life; Paul Hyman, for his kindness and gifted listening ability; Susan Hanson for her optimism; Peh Yean Cheah, for her tireless and cheerful ability to teach; Pat McCreary for forcing me to face my fears (I would still be waiting to take Prelims); and John Obringer for making me realize "Everybody is smart, it's how hard you work that counts." To my dearest friend John Delaney thank you for filling those long dark winter mornings in lab with knowledge, encouragement, and above all joyful laughter.

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ABSTRACT

Colon cancer is the second most common type of cancer in the United States. Its incidence is linked epidemiologically to high levels of bile acids in the feces. Bile acids have been implicated as promoters and cocarcinogens in the etiology of colon cancer and as comutagens and mutagens in bacteria. These observations suggest the hypothesis that bile acids may damage DNA. By using the DNA-damage inducible SOS system in Escherchia coli, this study shows that when bacteria are exposed to bile acids there is induction of the SOS repair system and preferential survival of cells undergoing repair. Additionally, differential killing assays using repair defective bacteria show strains defective in recombinational repair or excision repair have lower survival when treated with bile acids than their parental wild-type conterparts. Human fibroblasts were treated with bile acids and unscheduled DNA synthesis (UDS) was measured. UDS is considered to represent the DNA synthesis step in excision repair. UDS, measured by autoradiography, was found to significantly increase in human fibroblasts upon treatment with bile acids. In addition,

differential cytotoxicity assays with Chinese Hamster Ovary cells showed that different DNA-repair pathway defective cells were sensitive to different bile acids. Introduction of DNA damage and induction of DNA-repair by bile acids implicates them as possible direct carcinogens in the etiology of colon cancer.

CHAPTER I

INTRODUCTION

In the general population the probability of developing colorectal cancer from birth to age 70 years is about 4% (Li,1986). Colon cancer is the second most frequent cause of cancer in the U.S., lung cancer being the first. Approximately 100,000 new cases of colon cancer occur each year this country (Lipkin et al., 1980) Carcinogenicity involves a complex interplay between genetic and environmental factors.

Genetic Factors in Colon Cancer

Certain patients have an inborn or acquired predisposition to development of cancer of the large bowel, often at an early age. The life time risk of developing colorectal cancer among individuals with familial adenomatosis polyposis (FAP) approaches 100% among those surviving to old age (Bussey, 1975). The median age of onset is 40 years. This is approximately two decades earlier than the median age of occurrence of colon cancer in the general population. Familial

adenomatosis polyposis is transmitted as an autosomal dominant. The genetic change in FAP, was first shown by Herrera et al. (1986) to be a deletion of the long arm of chromosome 5. Bodmer et al. (1987) located the FAP gene to the specific region 5q21-q22. Interestingly, Solomon et al. (1987) showed that in 20% of 45 cases of non-familial colorectal cancer there has been a loss of genes located on 5q.

Harnden (1989) argued that a loss of one gene of a homologous pair leaves a hemizygous condition, so that an abnormal allele located on the other chromosome would express. By this view the trait is recessive at the cellular level despite the dominant inheritance patterns found in families. Since the majority of cells of the colonic mucosa are not abnormal in patients who carry FAP, one normal copy of the gene is sufficient for normal function. However, if the one remaining normal copy acquires a somatic mutation, the abnormality is expressed. If the probability of a mutation in the remaining normal copy of the FAP gene is sufficiently high, the likelihood of at least one colon epithelial cell per individual becoming cancerous would approach unity and this would lead to the appearance of a dominant inheritance pattern. Since

the median age of onset is 40 years, it is likely that in that time span a second mutation occurs. The frequency of FAP has been estimated to be 1 in 7000 to 1 in 10,000 live births (Mulvhill, 1983).

Gardner's syndrome and Turcot syndrome are variants of FAP (Bess et al., 1980). Gardner's syndrome like FAP is transmitted in an autosomal dominant fashion. However, Turcot syndrome is believed to be an autosomal recessive condition. Variants are distinguished by additional cancer in areas other than the colon. Other autosomal dominant diseases that predispose individuals to colorectal cancer are Cowden's syndrome, nail-patella syndrome and Torre's syndrome. All are characterized by an early age of colon cancer onset. Additionally, patients with ulcerative colitis are at a high risk for development of colon cancer (Devroede et al., 1971). Two decades after the onset of colitis the risk of colon cancer is 20% and it increases another 20% with each passing decade. Crohn's disease is associated with a greater chance of developing colon cancer between ages 21-55 years, when colon cancer is less common in the general population (Gyde et al., 1980).

Knudson et al. (1973) proposed a two stage model for

carcinogenesis. This model of malignant transformation in hereditary cancer syndromes presupposes that the two stages of malignant transformation in hereditary forms of cancer are germinal mutation and somatic mutation. Knudson hypothesized that a germinal mutation predisposes individual cells to malignant transformation by a single mutagenic event. A germinal mutation should affect all cells in a cell line, resulting in multiple opportunities for primary tumor development. A germinal mutation is present at birth, and the time span required for a single somatic mutation is markedly less than that required for two somatic mutations needed in a cancer patient lacking predisposition. Purtilo et al. (1978) hypothesized that in autosomal dominant diseases the first "hit" represents the primary defect and somatic mutations are then necessary for the development of polyps and adenocarcinomas. Table 1-1 defines some necessary terms in cancer progression. Supportive evidence was found by Kopelovich et al. (1979) for induced neoplastic transformation in skin fibroblasts from patients with hereditary adenomatosis of the colon and rectum by treating cells with tumor promoting agents. Fibroblasts from patients without hereditary

Table 1-1 Terms Used in Cancer Progression

TERMS	DEFINITION
Tumor	abnormal tissue swelling or enlargement often equated with neoplasms
Neoplasm	abnormal growth of tissue, may be benign or malignant
Benign	abnormal growth that does not spread and infiltrate other tissues
Malignant	abnormal growth that does spread and infiltrate other tissues
Polyp	benign tumor of epithelial origin
Adenoma	benign glandular tumor
Adenocarcinoma	malignant glandular tumor

predisposition to colon cancer were not transformed by this treatment.

Pero et al. (1983) have shown that leukocytes from individuals selected because they previously had colorectal cancer or a genetic predisposition to the disease were deficient in their capacity to remove chemically induced lesions from their DNA. Theoretically, such a deficiency in DNA repair would result in accumulation of DNA damage and a potential increase in mutations which might lead, or contribute to, neoplastic transformations. They believed that although leukocytes are not the primary targets for colon cancer, they might reflect a systemic repair abnormality either inherited or environmentally induced that results in susceptibility to colon cancer.

Fearson et al. (1987) suggested a genetic hypothesis for the development of colorectal neoplasia based on clonal analysis of human colorectal tumors from 50 women with either familial or spontaneous type colon cancer. First, it has been shown that benign neoplasms have undergone a generalized DNA methylation in their genomes (Goelz et al., 1985). This change has been shown to inhibit chromosome condensation and may lead to mitotic nondisjunction (Schmid et al., 1983)

resulting in loss or gain of chromosomes. During the growth of adenomatous cells, a mutation in the ras gene (usually codon 12 of the c-Ki-ras gene) often occurs (Bos et al., 1987). Finally, a loss of tumor suppressor genes on chromosome 17p or less frequently on other chromosomes, may be associated with progression from adenoma to adenocarcinoma.

The genomes of colon cancer cells have been found to contain oncogenes (Spandidos and Kerr, 1984). Monnat and coworkers (1987) studied 39 tumor tissue samples from 17 colon cancer patients to determine messenger RNA levels of the c-fos, c-myc, c-Ha-ras, and c-Ki-ras genes. Fifty percent of the cases studied showed an increase in expression in one or more of the oncogenes studied. Moreover, it has been shown by James and Sikora (1989) that microinjections of a mutant protein synthesized by c-ras or overproduction of the normal ras protein induces transformation in a mouse fibroblast cell line.

Environmental Factors in Colon Cancer

Genetic predisposition is not the only explanation for increased risk of colon cancer. The inherited

forms of colon cancer are so rare that they account for a very small portion of colon cancer cases. Doll and Peto (1981a and 1981b) estimated that nongenetic factors play a role in 90% of colon cancer cases in the United States and they suggested that these cases might be prevented by appropriate diet. Of course the interaction of genetic and environmental factors must also be considered. Any genetic predisposition would increase the risk of an environmental factor resulting in development of cancer.

As summarized by Haenszel and Correa (1971), in some high risk populations of 65-75 year-olds, 150 to 300 new cases are reported per 100,000 population per year. These high risk populations include those of Connecticut (in the U.S.), Canada and New Zealand. Some low risk populations have a risk for 65-75 year-olds of only 10 to 40 new cases per 100,000 population per year. This is approximately a 10-fold lower risk. These low risk populations include residents of Poland and Japan. When Polish or Japanese individuals migrate to the United States their colon cancer rates rise to about the U.S. values (Staszewski and Haenszel, 1965; Haenszel and Kurihara, 1968). This implies that the population differences in incidence of colon cancer are

due more to lifestyle differences than genetic differences. Researchers have hypothesized that a number of different agents may play a role in the development of colon cancer.

(a) Fecapentanes

Fecal mutagens found by Bruce (1987) were thought to be implicated as chemicals responsible for the initiation of colon cancer. Fecapentanes are a group of related ethers of glycerol and unsaturated alcohols containing five conjugated double bonds (Hirai and Kingston, 1982; Gupta et al., 1983; Baptista et al., 1984). They are found in the organic solvent extract of the lipid fraction of many feces. Using the Ames' Salmonella Test (Ames et al., 1975) fecapentanes were found to be mutagenic. Fecapentanes are produced from unknown precursors by certain strains of Bacteriodes in the gut (van Tassell et al., 1986). However, further studies revealed that suppositories of fecapentanes did not produce evidence of toxicity to the colon over a prolonged period of exposure (Bruce, 1987). Moreover, in case studies (Bruce and Dion, 1980 and Correa et al., 1981) no association was found between fecal mutagenicity and colonic polyps, hypothesized to be

precursors of colon cancer. Additionally, Dion et al. (1982) has shown that when the diet of individuals who had undergone colon polypectomy was supplemented with ascorbic acid, which has been shown to decrease levels of fecapentanes found in the feces, polyp reoccurrence was not reduced. In animal studies, Vaughan et al. (1987) found that fecapentanes, at concentrations a thousand-fold higher than that found in feces, failed to increase nuclear aberrations in mouse colonic epithelial cells.

(b) Cholesterol and Neutral Sterols

When cholesterol enters the intestine about 50% to 96% of it is broken down by intestinal flora into coprostanol, coprostanone and other neutral sterols. A correlation between fecal concentration of cholesterol and the concentration of secondary breakdown products of cholesterol with colon cancer has been shown (Reddy and Wynder, 1973; Crowther et al., 1976, Reddy and Wynder, 1977; Hill et al., 1982). Using mouse colon instilled with aqueous extracts of feces, Bruce (1987) assayed for chromosomal aberrations, specifically micronuclei. Chromosomal fragments that are formed are often not attached to the spindle fibers and may not be

included in the nucleus of either daughter cell, giving rise to micronuclei which can be scored. An increase in micronuclei was found in the cells treated with the fraction of feces isolated with dichloromethane. Purification of the active fraction led to isolation of two ketosteroids, 4-cholesten-3-one and 5-alpha-cholesten-3-one, which are both derivatives of cholesterol (MacDonald, 1983). 4-cholesten-3-one has also been found to induce sister chromatid exchanges in the colonic epithelium, a genetic effect associated with carcinogenic compounds (Kaul et al., 1987). The cytotoxicity of these 3-ketosteroids implies they may be initiators or promoters of colon cancer. Also, people with ulcerative colitis have greater concentrations of neutral sterols in the feces (Reddy et al., 1977b). Patients with colon cancer (Reddy and Wynder, 1973) and those with predisposing diseases, namely familial adenomatous polyposis, ulcerative colitis, and Gardner's syndrome all excrete more fecal cholesterol and secondary metabolites than the appropriate controls (Core and Watne 1974). Non-degradation of fecal cholesterol however, has also been found in healthy North American individuals (Wilkins and Hackman, 1974). Additionally, Nomura et al. (1983)

showed that Akita Japanese, who show lower mortality and incidence rates for colon cancer than Hawaiian Japanese, have a higher concentration of undegraded cholesterol in their feces.

(c) Dietary Fat

Epidemiological data suggest that the incidence of colon cancer is associated with the consumption of diets high in fat and in protein (Armstrong and Doll, 1975; Doll and Peto, 1981a; Knox, 1977), but the results are conflicting. In a review of the evidence Kolonel (1987) cites six epidemiological studies showing a positive correlation between dietary fat and colon cancer, and eight failing to show this correlation. He further cites six analytical studies (case control and cohort studies) with a positive correlation between fat and colon cancer, but also cites seven failing to show this, and two giving an inverse correlation.

There have been approximately 30 animal studies published (Newberne and Sahaphong, 1989) which have examined the influence of dietary fat on chemically induced colon cancer in rodents. Animal studies have shown a high-fat diet may act to promote colon

tumorigenesis (Bansal et al., 1978; Broitman et al., 1977; Nigro et al., 1973, 1975; Reddy, 1986). When rats were treated with known carcinogens and then fed a high fat diet, there is an increase in intestinal tumors (Nigro et al., 1973). Newberne and Sahaphong (1989) point out the high fat diets were also lower in nutrients and the control diets were also lower in caloric density. Nutritional imbalances and high calories may in themselves contribute to tumorigenesis.

Consumption of fat stimulates bile acid release into the intestine (Reddy, 1981). Bile acids have been implicated in the etiology of colon cancer and will be discussed next.

(d) Bile Acids

1. Structure and Function

There are four main bile acids that have been isolated from human bile, these are cholic acid, chenodeoxycholic acid, lithocholic acid and deoxycholic acid (Hylemon and Glass, 1983). The chemical structure of these bile acids as well as cholesterol are illustrated in Figure 1-1. The two primary bile acids formed in the liver through a series of biochemical reactions starting with the 27-carbon sterol,

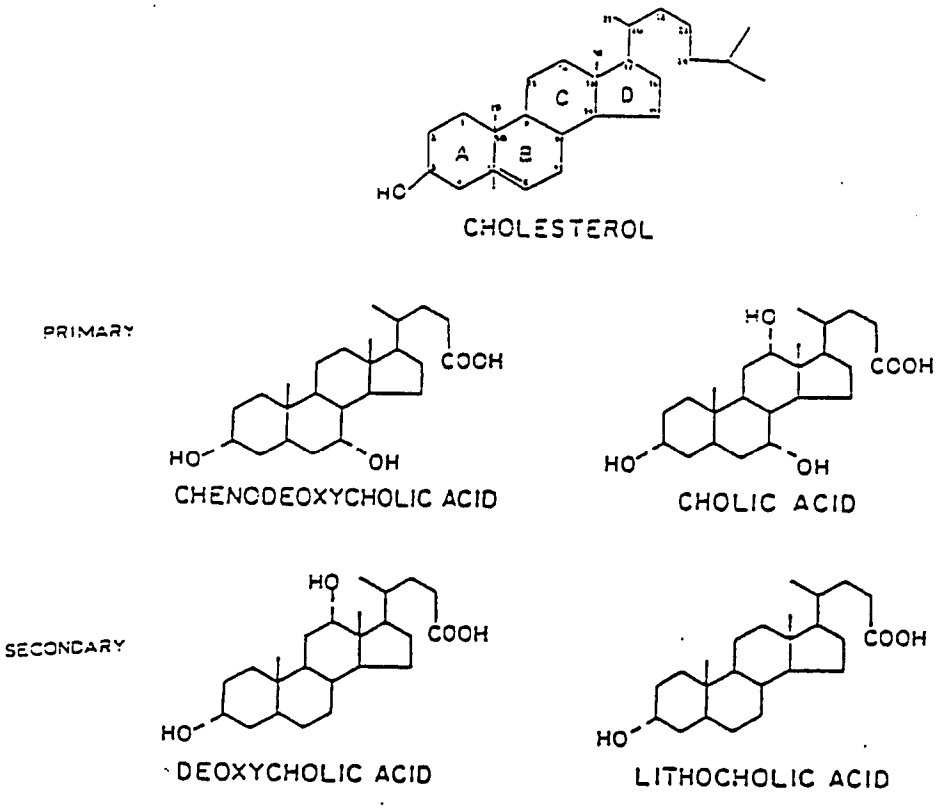

Figure 1-1. The structures of cholesterol and the major bile acids in man.

cholesterol, are cholic acid and chenodeoxycholic acid. Conjugation of the acids occurs in the liver and the conjugates, eg. glycholic acid and taurocholic acid, form sodium and potassium salts. Primary bile acid conjugates compose 60-90% of biliary bile acids. The primary bile acid conjugates are secreted by the liver into the alkaline hepatic bile. In the cecum the conjugated bile acids are deconjugated by the intestinal microflora through hydrolysis to produce free primary bile acids. In man the intestinal microflora can generate at least 15-20 different bile acids from the primary bile acids (Hylemon and Glass, 1983). Bacterial conversion of cholic acid to deoxycholic acid and chenodeoxycholic acid to lithocholic acid through 7-dehydroxylation are the main secondary bile acids produced. Some chenodeoxycholic acid is transformed into the tertiary bile acid ursodeoxycholic acid via 7-ketolithocolic acid. Additionally, bile acids can undergo other chemical modifications including dehydrogenation, oxidation, and isomerization (Macdonald et al., 1983).

The bile acids have numerous important functions. They act as regulators of lipid solubility and bile secretion (Armstrong and Carey 1982, Hofmann et al.,

1983). Bile salts combine with lipids to form water soluble complexes called micelles. Micelles reduce surface tension and in conjunction with phospholipids and monoglycerides are responsible for the emulsification of fat preparatory to its digestion.

Seventy-five to 95% of the bile acids are absorbed from the terminal ileum by active transport. The remainder (130-650 mg/day) enter the colon and are converted to the secondary bile acids (Hylemon and Glass, 1983). In man, bile acids are concentrated and stored in the gallbladder. Bile acids are released into the small intestine in response to a meal. The arrival of food in the duodenum triggers the secretion of cholecystokinin and this hormone triggers the release of bile from the gallbladder into the small intestine. Lithocholate is relatively insoluble and is mostly excreted in the stool, but deoxycholate is partially absorbed from the colon. The absorbed bile acids are transported back to the liver via portal venous and hepatic arterial blood. The normal rate of bile acid synthesis is 0.2 g - 0.4 g per day. The total bile salt pool of approximately 3.5 g recycles 8 times per day via the enterohepatic system (Hoffman, 1977).

2. Role in Colon Cancer

Bile acids are believed to be implicated in the etiology of colon cancer (Reddy, 1981; Reddy, 1983; and Nair, 1988) in part because of their high correlation with dietary fat. Dietary fat is known to increase bile acid secretion into the gut, increase metabolic activity of the gut bacteria, and increase secondary bile acids in the colon. Feces of people that consume large amounts of dietary fat contain greater quantities of bile acids than feces from people whose diet consists primarily of vegetables, grains and fish (Reddy and Wynder, 1973). It has been suggested that dietary fat and cholesterol influence the risk of colorectal cancer via the excretion of bile acids and cholesterol to the lumen, where these compounds may be transformed to secondary bile acids and cholesterol metabolites under the influence of bacterial enzyme systems (Hill, 1985). As reviewed by Hill (1986), there is a high correlation between the presence of bacteria that can change primary bile acids into secondary bile acids and the incidence of colon cancer. He suggested that the secondary bile acids may be of particular importance in the etiology of colon cancer.

In addition, Hill (1986) summarized the many studies relating total fecal bile acid concentrations in populations to the risk of colon cancer. These studies have been highly consistent and this correlation between high fecal bile acid concentration and populations at high risk for colon cancer is now regarded as clearly established. As reviewed by Bruce (1987) and by Nagengast et al. (1988), when fecal pH is reduced by consumption of fiber or lactose, more of the bile acids are in their protonated form and become insoluble. In addition, the ability of bacteria to convert primary bile acids into secondary bile acids is reduced. The epidemiological and animal summaries indicate that there is a lowered risk of developing colon cancer when there is lower fecal pH. This evidence points to total soluble bile acid concentration, and the ratio of secondary to primary bile acids as being important in colon cancer.

Using the Salmonella-mammalian microsome assay, which detects revertant colonies on plates, Silverman and Andrews (1977) found lithocholic acid and one of its conjugates to be comutagenic when tested with activated 2-aminoanthrene, a potent carcinogen. Mutagenicity of bile acids was detected by the

fluctuation test (Greene and Muriel 1976) using Salmonella typhimurium TA100 and TA98 as tester strains (Watabe and Bernstein, 1985). This test is very sensitive for lower concentrations of mutagens (Kuhnlein et al., 1981). Cholic acid, chenodeoxycholic acid, deoxycholic acid and ursodeoxycholic acid were mutagenic in this test while lithocholic acid was not (Watabe and Bernstein 1985). Cheah and Bernstein (1989) showed that chenodeoxycholic acid and deoxycholic acid converted covalently closed circular plasmid DNA to the open circular form, indicating strand breakage. Additionally, they treated the single stranded circular DNA of phage M13 with bile acids and found that the transfection efficiency of this DNA declined up to a thousand-fold.

Nariwawa et al. (1974), Reddy et al. (1977a) and Reddy and Wantanabe (1979) have shown that bile acids can act as promoters by demonstrating that intrarectal instillation of bile acids increases the yield of colon adenocarcinomas in rats receiving the initiator N-methyl-N'-nitro-N-nitrosoguanidine. No tumors were found in rats given bile acid alone. Additionally, cholic acid and lithocholic acid have been shown to be cocarcinogens (Kawasumi et al., 1988). Cocarcinogens

are agents which enhance tumor development by a carcinogen.

In addition to the above genotoxic effects bile acids also increase proliferation of epithelial colon crypt cells (Bull et al.., 1983; Jacobs, 1988). Induction of cellular proliferation supports the finding that bile acids may act as promoters.

Genetic and environmental interactions must also be taken into consideration. Increased bile acid production in the liver depends on a number of host factors which influence the conversion of cholesterol to bile acid (Cannon-Albright et al., 1988). In Western societies, female rates of colon cancer at premenopausal ages exceed the corresponding male rates, while at older ages, male rates predominate (McMichael and Potter, 1980). Both human and animal evidence suggest that this may reflect an effect of sex hormones or reproductive history upon bile synthesis and secretion (McMichael and Potter, 1980). Thus, increased dietary fat and certain host factors would interplay to increase the risk of colon carcinogenesis. Additionally, there is some indication that predisposition to colon cancer could be associated with super-efficient starch absorption (Thornton et al.,

1987). And as pointed out earlier, reduced capacity to repair DNA (Pero et al., 1983) would lead to increased susceptibility to any of the environmental factors believed to be initiators.

Molecular Genetics of Cancer

Human cancers are diseases in which one of the many cells of the body is altered in such a way that it inappropriately and repeatedly replicates itself. These cells propagate to such an extent as to overwhelm other organs and tissues. It is important to understand the sequence of events in carcinogenesis so that we may learn what factors aid or inhibit its progression.

Bishop (1987) explained the conviction that genetic damage might be responsible for cancer grew from diverse roots: the recognition of hereditary predisposition to cancer (Lynch, 1976; Schimke, 1978), the detection of damaged chromosomes in cancer cells (Rowley, 1983), the apparent connection between susceptibility to cancer and impaired ability of cells to repair damaged DNA (Lehmann, 1982) and evidence that relates the mutagenic potential of substances to their

carcinogenicity. Additionally , Bishop adds to the list the discovery that cellular genes, proto-oncogenes, when mutated may become oncogenes capable of neoplastic formation. Figure 1-2 shows the relationship of DNA damage to carcinogenesis. As shown, the cells ability to successfully repair DNA damage plays an integral role in the development of cancer. Inaccurate DNA-repair and error prone repair, may lead to viral induction, gene derepression, mutagenesis and ultimately carcinogenesis.

Carcinogenesis is believed to be a multistep process. The basic concept of the changes cells undergo in carcinogenesis is presented in Figure 1-3. The multistep model of carcinogenesis begins with initiation. Initiators are agents which induce DNA damage and/or mutation. Initiators cause a irreversible stable genetic change (Miller and Miller 1981). Once a cell or group of cells has undergone an initiation event, cancer may develop further by means of promotion. Promoters are agents which induce selective proliferation of initiated cells. Promotion, unlike initiation, is reversible at early stages in the process of carcinogenesis. Because initiation is an heritable event, tumors will develop

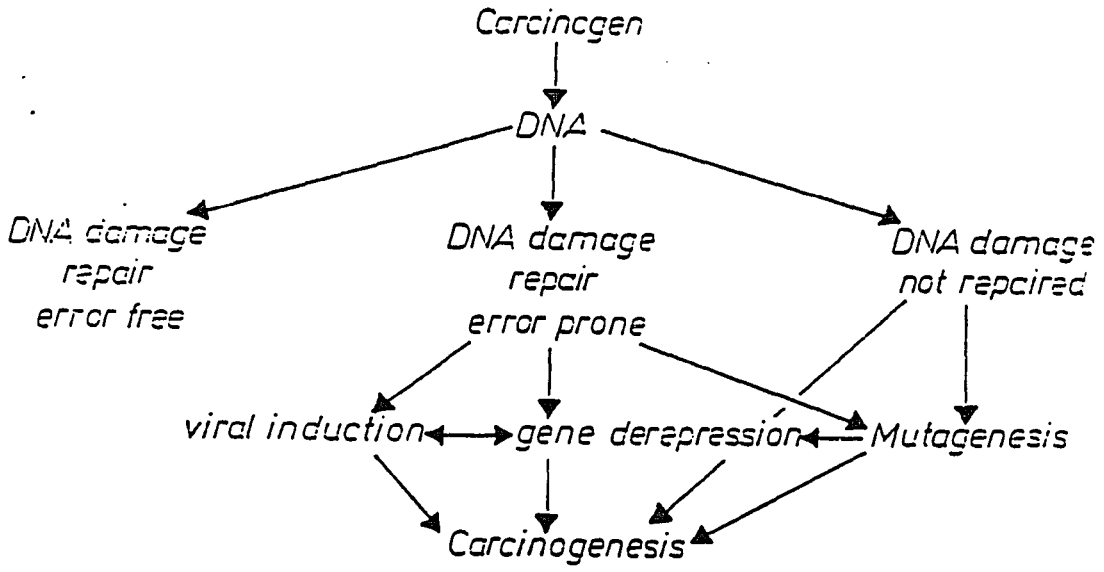

Figure 1-2. DNA damage and carcinogenesis. A carcinogen damages DNA and when the damage is either not repaired (right side) or the repair is error-prone, allowing for mutagenesis (center), the ultimate outcome (bottom) is carcinogenesis (taken from Graffe et al., 1979).

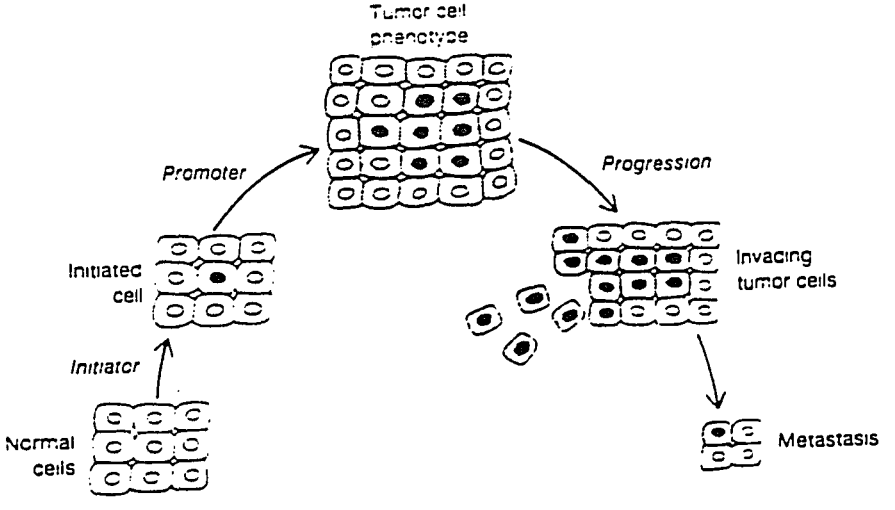

Figure 1-3. Multistep model of carcinogenesis. The bottom left square shows normal cells, which upon subjection to a DNA damaging agent become initiated, next square. Once initiated, a promoting agent causes the tumor cell phenotype to develop. Progression refers to further phenotypic changes resulting in the development of a malignant tumor from a benign tumor (taken from DeVita et al., 1985).

even though the time span between initiation and promotion may be great. Evidence suggests a tumor promoter's primary molecular target is the cell membrane (Weinstein et al., 1980; Trosko et al., 1982). In the absence of a promoting stimulus, no tumors need result following initiation only. Additionally, the effect of promoters is only effective after a certain threshold dose, whereas the effect of initiators may be additive (Boutwell, 1985). Carcinogens often possess both initiating and promoting properties. A few incomplete carcinogens possess only initiating activity (Boutwell, 1985). Progression refers to further phenotypic changes in the benign tumor resulting in malignancy.

Bile Acids versus Bile Salts

Although the distinction between bile acids and bile salts should be made, organic chemists have traditionally employed the term "bile acid" as a generic name for this cholanic class of biological compounds (Carey, 1984). Bile acids are conjugated to glycine or taurine, a derivative of cystine. Amidation increases the solubility of bile acids, thereby

promoting a high intraluminal concentration, which is essential for fat digestion and absorption (Hofman, 1988). All such derivatives of bile acids are fully ionized at the pH present in the intestinal content, and Haselwood (1978) has suggested that the generic term "bile salts" be used. Physiologically, upon conjugation of the bile acids in the liver, it is the salts of these acids that are found in bile and secreted into the small intestine. Bile salts are responsible for the hydrotropic effect (high solublilty) of these acids and the salts more readily form micelles (Carey and Small, 1981). Micelles function to solubilize the nonpolar lipids. The present study employs the traditional terminology in general discussions by using the generic term "bile acid". However, when referring to each particular experiment the specific chemical name, be it salt or acid, is used.

Aim of Study

The purpose of this study is to determine whether bacteria and mammalian cells undergo DNA-repair in response to treatment with chenodeoxycholate and

deoxycholate and whether cells defective in DNA-repair are more sensitive to these agents than their efficient DNA-repair counterparts. A positive result would support the hypothesis that these bile acids are DNA damaging agents and therefore complete carcinogens.

CHAPTER II

MATERIALS AND METHODS

Media for SOS Induction Test

E. coli strain JL1705 was grown overnight in Luria broth (Arber et al., 1983). Luria broth consists of 10 g Bacto-tryptone (Difco), 5 g yeast extract (Difco), 10 g NaCl in 1 liter distilled water adjusted to a pH of 7.2. The strain was maintained on BBL-trypticase plates (Arber et al.,1983). These plates consist of 10 g trypticase, 5 g sodium chloride, 10 g Bacto-agar (Difco) in 1 liter of water adjusted to a pH of 7.2. The test for SOS induction was conducted on BBL-trypticase plates with the addition of 80 ug/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactoside (Xgal), and 50 ug/ml ampicillin. The soft top agar (Steinberg and Edgar, 1962) used for overlay consisted of 6.5 g Bacto-agar (Difco), 13 g Bacto-tryptone (Difco), 8 g sodium chloride, 2g sodium citrate, 3 g dextrose in 1 liter of water with the addition of 100 ug/ml ampicillin. Plates and top agar were autoclaved for 40 minutes and then cooled to aproximately 42°C before the addition of

Xgal and ampicillin. Ampicillin sodium salt was dissolved in sterile deionized water at a concentration of 25 mg/ml, filter sterilized and stored at 4°C. Xgal was diluted in N, N-dimethylformamide at a concentration of 20 mg/ml and stored at -20°C.

Media for Repair Deficient Mutant Sensitivity Assay

E. coli strains AB1157, AB2463, and AB2480 were bubbled in Hershey's media overnight in a 37°C waterbath. Hershey broth (Steinberg and Edgar,1962) consists of 8 g Bacto nutrient broth, 5 g Bacto-peptone, 5 g NaCl, and 1 g Dextrose in 1 liter distilled water adjusted to a pH of 7.2. The strains were maintained on Hershey plates (Steinberg and Edgar, 1962) consisting of 10 g Bacto-agar, 13 g Bacto-tryptone, 8 g NaCl, 2 g sodium citrate and 1.3 g dextrose in 1 liter distilled water. All bacterial strains were stored by taking an alliquot of a fresh overnight culture and diluting 1:1 with 40% sterile glycerol (Lech and Brent, 1987). This solution was placed in a 2.0 ml vial and stored in a -70°C freezer.

Media for Growth of Newborn Human Foreskin Fibroblasts

Newborn human foreskin fibroblasts (NBFF) were grown as a monolayer culture in McCoy's 5A Medium (Modified). The media was purchased from Sigma as a powder and reconstituted with deionized water to which 2.2 g sodium bicarbonate was added. The pH of the media was then adjusted to 7.2 with 1N NaOH and filter sterilized in a 500 ml 22μm Falcon filter unit. Filter sterilizing raised the pH 0.3 units to give a final reading of 7.5. The growth media was supplemented with a 0.5% solution of 10,000 U/ml penicillin plus 10,000 ug/ml streptomycin (Sigma) and 10% fetal bovine serum (FBS) before use. FBS was purchased from Gibco. Media was stored in a dark location at 4°C.

Maintenance of NBFF cells

2.8×10^5 NBFF cells were seeded into a 250 ml tissue culture flask containing 10.0 ml growth medium and placed into a 37°C, 5% CO_2 incubator. Flasks were loosely capped. When flasks were seeded directly from a frozen culture the growth medium was removed and replaced after 24 hours with fresh growth medium. This

is done to remove the dimethyl sulfoxide (DMSO) present in the freezing medium. Cells were fed every 3 days by replacement of old growth medium with fresh medium.

When a confluent monolayer (1.4×10^6) covers the bottom of the flask (every 7-9 days) cultures were split. This procedure entails removing the growth medium and rinsing the flask with phospate buffered saline (PBS). PBS consists of 8.0 g NaCl, 0.2 g KCl, 0.12 g KH_2PO4, 0.91 g Na_2HPO4 in 1 liter distilled water. The pH was adjusted to pH 7.5 with NaOH. The flask was rinsed with PBS to remove magnesium and calcium ions left by the growth medium. Next 2.5 ml of a 0.25% trypsin, 0.2 g EDTA in a 0.9 % sodium chloride balanced salt solution (calcium and magnesium free) was added to the monolayer of cells. When cells lose their ability to adhere to the flask they round up. After 5-10 minutes the flasks were placed under an inverted microscope and loss of cellular adhesion was determined. The side of the flask was then thwacked with the palm of my hand to complete the dislodgement of the cells from the flask bottom. Immediately 5.0 ml of growth medium was added to the flask. This stops the enzymatic action of the trypsin.

The cells, which were then in suspension, were

pipetted into a 15.0 ml conical centrifuge tube and securely capped. Next the tubes were placed in a Sorvall GLC-1 centrifuge and spun at 1,100 RPM for 10 minutes to pellet the cells. Then the medium was removed from the pellet and the cells were resuspended in 5.0 ml fresh growth medium. Cell viability and concentration was determined by placing a drop of the cell suspension into a drop of 0.2% trypan blue solution. Then using a hemocytometer the cells were counted and the percent of cells excluding trypan blue was determined to be the fraction of viable cells. For each culture transfer viability ranged from 94 to 98%. One ml of this suspension was added to a 250 ml tissue culture flask to which 10.0 ml of prewarmed fresh growth medium had been previously added. The flasks were then loosely capped and placed in a 37°C, 5% CO_2 incubator.

Media and Maintenance of Chinese Hamster Ovary Cells

Chinese Hamster Ovary cells (CHO) were grown in Alpha medium (Gibco). This growth medium was supplemented with 10% FBS, and a 0.5% solution of 10,000 U/ml penicillin plus 10,000 ug/ml streptomycin.

CHO cells were grown as monolayer cultures in 250 ml tissue culture flasks. The flasks were seeded with approximately 5 x 10^4 cells. The growth medium was changed every 3 days. Every 5-7 days, when the monolayer became confluent (3 x 10^6 cells/ml) the culture was split. The procedure for splitting the confluent culture was the same as that used for the NBFF cells.

Materials

Sodium deoxycholate (deoxycholate) was purchased from ICN Biochemicals and dissolved in sterile deionized water. Sodium chenodeoxycholate (chenodeoxycholate) was purchased form Sigma Co., St. Louis, Mo and dissolved in sterile deionized water. Chenodeoxycholic acid was purchased from Sigma and dissolved in DMSO. ^{3}H-thymidine (S.A. 60-68 uC/mMol) was purchased from ICN Biochemicals. NTB-3 emulsion, D-19 developer and General fixer were purchased from Kodak.

Bacterial and Mammalian Strains

The bacterial and mammalian strains used in this study are listed in Table 2-1. E. coli strain JL1705 was used for the SOS induction test. E. coli strains AB1157, AB2463 and AB2480 were used in the recA uvrA sensitivity assay. Strain AB1157 is the parental wild-type of the other two strains and it is efficient in repair of DNA damage. Strain AB2463 is recombinational repair defective. Strain AB2480 is defective in excision repair and recombinational repair.

Methodology to Check the Genotype of Bacterial Strains

In strain JL1705 the stuctural gene for beta-galactosidase (lac) is placed under the control of sulA, a DNA damage inducible promoter. Another copy of the lac gene is also present and under normal regulation, but it is mutationally defective. The differential action of Bacto MacConkey Agar (DIFCO, 1985) is based on fermentation of lactose. Bacteria capable of fermenting lactose produce a localized pH drop which, followed by absorption of the neutral red in the agar, imparts a deep red color to the colony.

Table 2-1 Bacterial and Mammalian Strains Used in This Study

Bacteria		
	Type	Reference
JL1705	sulA::lacZ	Lin and Little, 1988
AB1157	Wild-type	Howard-Flanders and Theroit, 1962
AB2463	recA	Howard-Flanders et al., 1966
AB2480	recA uvrA	Howard-Flanders et al., 1969
Human Cells		
NBFF	Newborn foreskin fibroblasts	Dr. Hausler University of Arizona (source)
Chinese Hamster Ovary Cells		
AA8	Wild-type	Hoy et al., 1983
UV4	Excision repair defective; hypersensitive to crosslinks, UV and chemicals that form large monoadducts	Hoy et al., 1983
EM9	Defective in strand-break-rejoining, hypersensitive to X-rays, EMS and MMS	Hoy et al., 1983

Colonies which do not ferment lactose remain colorless. When strain JL1705 cells are grown on MacConkey indicator plates they appear as white to light pink colonies.

The presence of the sulA::lacZ fusion was verified by another test which yields a blue color. First placing 0.2 ml of an overnight culture of JL1705 into top agar to which 100 ug/ml ampicillin and 50 ug/ml Xgal has been added. This mixture was spread on the surface of a Hershey plate. Next, the overlay was allowed to dry and 50 ul of a 200 ug/ml solution of mitomycin-C was placed on the center of the plate. The plates were incubated overnight. The next day a blue ring appeared where the mitomycin-C was placed. In the center of the ring cells are killed due to the high concentration of mitomycin-C. Further away from the center the concentration of the agent has lowered due to diffusion. This area is referred to as the zone of induction (Quillardet and Hofnung, 1985). Because the DNA damaging agent induces sulA, beta-galactosidase is produced which hydolyzes Xgal to give a blue ring. The MaConkey test, performed with the absence of mitomycin-C and the Xgal spot test with mitomycin-C were done with each SOS induction experiment.

The repair defective E. coli strains used in the recA uvrA sensitivity assay were tested for their inabililty to grow after treatment with ultraviolet light. This procedure entailed streaking overnight cultures of AB1157, AB2463 and AB2480 horizontally across a Hershey plate, covering half of the plate with cardboard and placing the plate 20 cm from a hand held ultraviolet lamp (output primarily 254 nm) for 15 to 20 seconds. The procedure was carried out in a darkened room to prevent photoreactivation. The plates were placed in a darkened 37°C incubator overnight. Due to repair defective pathways, differential growth can be observed among these strains.

Growth of Human Foreskin Cells

Freshly isolated human foreskin cells grown in culture for 2 weeks were separated into aliquots and kept frozen at -80°C. For each experiment, an aliquot was defrosted and allowed to grow through one to five generations. Growth rate was found to be consistent (16 hour doubling time) as was cellular adhesion (i.e. no foci indicating transformed cells were witnessed).

Procedure for Checking UV Sensitivity of CHO Strains

CHO cells defective in DNA repair were tested for their inability to survive after exposure to ultraviolet light. A CHO cytotoxicity assay, using UV-light as the DNA damaging agent, was performed. This test is described under CHO cytotoxicity assay. An ultraviolet lamp (output primarily 254 nm) was placed 20 cm from the 24-well cluster tray. The open cluster tray had been covered with cardboard so that only 4 wells, vertically placed, were exposed to the light. Every 10 seconds, 4 additional wells are exposed to irradiation, leaving the last 4 wells covered. In this way the first 4 wells irradiated had a total exposure time of 50 sec and the next columns had 40, 30, 20, 10 and 0 sec exposure times, respectively. The ultraviolet irradiation was performed with each CHO cytotoxicity experiment and the results were found to be consistent. Strain AA8 was sensitive to 50 sec ultraviolet irradiation. Strains EM9 and UV4 were sensitive to 40 and 10 sec of treatment, respectively.

Protocol for SOS Induction Test

The SOS induction test is a modified version of the SOS Chromotest. The standard SOS Chromotest (Quillardet and Hofnung, 1985) is a qualitative bacterial colorimetric assay for genotoxins. In E. coli, DNA damaging agents induce expression of genes referred to as damage-inducible (din) genes (Walker, 1984). This response, which helps the cell survive in the face of DNA damage, is called the SOS response. The SOS Chromotest employs a strain of E. coli (PQ37) in which there is an operon fusion placing lacZ, the structural gene for beta-galactosidase, under the control of the SOS gene called sfiA or sulA. SulA functions to delay cell division subsequent to DNA replication (Huisman and Kondo, 1981). This delay gives the cell time to repair its DNA. In this strain, DNA damage results in turn-on of sulA and also, since it is now in the same operon, lacZ. When lacZ is expressed, it produces beta-galactosidase. This enzyme cleaves a colorless galactoside, Xgal (5-Bromo-4-chloro-3-indolyl-beta-D-galactoside) to form a blue product. I first tried the two parts of the standard Chromotest. One part is carried out in liquid media

(Miller, 1972) and one part is a solid agar spot test. The deoxycholate proved to be too toxic for use during liquid growth of the E. coli. The spot test gave a positive result; a blue induction ring was observed. However, E. coli PQ37 grew very slowly presumably due to an rfa mutation causing membrane sensitivity to sodium deoxycholate. The blue induction ring was observed only after 3 days of incubation with the bile salt. Moreover, the spot test does not lend itself easily to quantification. Thus, I developed a colony assay on solid agar in Petri dishes using another strain.

E. coli JL1705 contains the operon fusion sulA::lacZ, and another copy of the lac structural gene is present. This is under normal regulation and is mutationally defective. Luria broth was inoculated with E. coli JL1705 and the culture was shaken overnight in a 37°C waterbath. A sample of this culture was serially diluted into M9 salts solution. This was done so that a measurable number of colonies would be obtained on the agar plate when the sample is plated. Next, an alliquot (20 to 150 ul) of either deoxycholate, chenodeoxycholate or mitomycin-C was added to molten top agar kept at 42°C in a heating

block. Deoxycholate and chenodeoxycholate were each dissolved in sterile deionized water at a concentration of 100 mg/ml. Mitomycin-C was dissolved in sterile deionized water at a concentration of 200 ug/ml. Ampicillin was added to the top agar tube to give a final concentration of 100 ug/ml. The tube was then vortexed for 10 seconds with a hand touch mixer (Touch Mixer Variable MT-51 American Scientific Products). Following this 0.5 ml of JL1705 at a 10^8 dilution in M9 salts solution was pipetted into the tube and bubbled slightly to mix the suspension. When no test agent was added to the top agar 0.1 ml of JL1705 cells at a 10^8 dilution in M9 salts solution was pipetted into the tube. Since the test agents caused a decrease in survival of the bacteria, when such agents were used five times more cells were added to the top agar to obtain plates with a countable number of colonies. The mixture was then poured onto BBL trypticase plates containing 80 ug/ml Xgal and 50 ug/ml ampicillin. The plates were then incubated for 19 hours in a 37°C incubator. I selected 19 hours as the cut-off point for incubation because a longer time (24 hours) allowed the chromogenic substrate to diffuse into neighboring colonies giving rise to false positive results.

Significantly less than 19 hours was insufficient time for countable size colonies to form. After incubation colonies were counted and scored as either blue or white using a colony counter (New Brunswick Scientific model C-110). The percentage of the initial population of cells that survived was determined by dividing the titer of bacteria treated with the test agent which were able to form colonies by the titer of untreated cells able to form colonies. For each treatment dose three plates were counted and the number of colonies was averaged. The experiment was repeated three times. The average titer of surviving cells and the percentage of these that formed blue colonies was calculated for each dose of test agent. The standard error was calculated based on the data from the three experiments. A control test with 533 ng/ml of mitomycin-C was carried out with each bile acid experiment to provide a positive control. The percentage of blue colonies stayed within 75-80% at this dose of mitomycin-C. The final dose of test agent (x) was calculated from the following relationship:

([test agent])(initial volume)=(x)(total volume)

[test agent] = bile acid or mitomycin-c concentration
initial volume = ul of test agent pipeted into top agar

total volume = no. of ml plate agar + no. ml top agar
+ no. ml bacteria

Protocol for Repair Deficient Mutant Sensitivity Assay

The procedure for the differential killing test was similar to that of the SOS induction test. Three strains of E. coli were used in these experiments. The strains used were AB1157, AB2463, and AB2480. AB1157 is the parental wild-type of the other two strains and has normal DNA repair. When DNA damage occurs in this strain, the bacteria are able to cope efficiently with the damage and survive. AB2463 is a recA mutant with impaired ability to carry out general recombination. Strain AB2480 is defective in both the recA and uvrA genes. The uvrA gene product along with the gene products of uvrB and uvrC is required for excision repair (Kuschner, 1987). Strains defective in uvrA are

more sensitive to ultraviolet light than wild-type. Strain AB2480 is extremely sensitive to DNA-damage because of its inability to undergo both recombinational repair and excision repair.

The bacteria were aerated overnight in 25 ml bubbler tubes in a 37°C waterbath. The next day the cells were serially diluted in M9 salts solution. Aliqots of bile acid were added with an eppindorf pipet (40-180 ul) to molten top agar kept at 42°C in a heating block. The mixture was vortexed with a touch hand mixer for 10 seconds. Next, 0.5 ml of cells at various dilutions were pipetted into the top agar and bubbled slightly to thoroughly distribute the cells. This mixture was then poured evenly onto Hershey plates. The plates were incubated for 19 hours at 37°C. Colonies are then counted. For each dose of bile acid three plates were scored. In the zero dose control when no bile acid was added, four plates were scored. The average titer for each dose was calculated. The experiment was repeated three times and the average titer at each dose and its standard error was calculated. The final dose of the bile acid was calculated following the equation used in the SOS induction test.

Additionally, with each differential killing assay a

ultraviolet sensitivity control was performed. 0.1 ml of an overnight culture of each strain was streaked across a Hershey plate and allowed to dry. Next, half of the open dish was covered with cardboard. The dish was placed 20cm under a hand held ultraviolet lamp (output primarily 254 nm) for 15 or 20 seconds. This procedure was carried out in a darkened room to prevent photoreactivation. The plates were placed in a 37°C darkened incubator overnight to allow growth of surviving cells.

Protocol for Unscheduled DNA Synthesis Assay

To measure unscheduled DNA synthesis (UDS) monolayer cultures of NBFF cells were grown on coverslips. Two coverslips were first flame sterilized and placed into 45 cm petri dishes. All work was then done under a laminar flow hood. Five ml McCoy's growth medium was added to each dish. Next, 0.5 ml of a 10.0 ml NBFF suspension from a T75 monolayer culture split was added to each dish. This was an approximate seeding of 3 $x10^5$ cells/ml. The dishes were placed in a 37°C, 5% CO_2 incubator for 4-5 days. In this time a confluent monolayer was obtained on the coverslips.

(a) Treatment

After 4-5 days the dishes containing the coverslips were rinsed with PBS. Then 5.0 ml PBS containing 10 mM hydroxyurea was added to each dish. The dishes were then allowed to stand at room temperature for 15 minutes. This was done to let hydroxyurea inhibit S phase synthesis. Hydroxyurea inhibits the mammalian ribonucleotide reductase and thus blocks the production of deoxyribonucleotides and hence DNA synthesis (Kornberg, 1980). Although hydroxyurea inhibits semiconservative replication, it has little effect on repair assessed by unscheduled synthesis when used at millimolar concentrations (Cleaver and Thomas, 1981).

Meanwhile, various amounts of bile acid were pipeted into 15.0 tubes containing 5.0 ml PBS and 10 mM hydroxyurea. A stock solution of deoxlycholate was prepared at a concentration of 50 mg/ml in sterile deionized water. A stock solution of chenodeoxycholic acid was prepared at a concentration of 50 mg/ml in DMSO. The final percent of DMSO was 1% in all treatments including the negative control. The tubes were vortexed for 20 seconds to ensure a uniform mixture. After 15 minutes the PBS containing 10mM

hydroxyurea was removed from the coverslips and the PBS bile acid mixture was added. The coverslips were left in the treatment medium for 20 minutes at room temperature.

UV light is a well known DNA damaging agent. The most frequent photoproducts caused by UV light include pyrimidine dimers, an alkali-labile PydC lesion, dihydrothymine, pyrimidine adducts, DNA cross-links, cytosine hydrate, single-strand breaks and DNA-protein cross-links (Bernstein and Wallace, 1983). These diverse types of damages are repaired by a number of pathways, including photoreactivation, excision repair, post-replication repair. Unscheduled DNA synthesis (UDS) indicates excision repair (Cleaver and Thomas, 1981). To ensure that the cells had the ability to repair DNA damage that is measurable by UDS, I exposed the cells to UV light for 30 seconds. Since cells are able to reverse thymidine dimers through enzymatic photoreactivation activated by white light, it was necessary to do this experiment in yellow light. Since it is unknown how bile acids effect DNA, I chose to do the bile acid experiments in yellow light also. This also allowed for consistency among variables in the UV control and the bile acid experiment. White light

remained off from the time of addition of labelled media until its removal. First, the coverslips were placed 20 cm directly beneath a 1 cm^2 aperture from a 254 nm ultraviolet bulb. The dose rate was measured with a dosimeter and found to be 1 $J/M^2/sec$. The coverslips were exposed at this dose rate for 30 seconds.

After exposure to bile acid the coverslips were rinsed twice with fresh PBS to remove the test agent. Next the coverslips that had been exposed to bile acid or UV were placed in petri dishes containing McCoy's medium supplemented with 1% FBS, 10mM hydroxyurea and 1.0 uC/ml ^3H-thymidine (S.A. 60-68 Ci/mmol). The dishes were placed in light tight baskets in a 37°C, 5% CO_2 incubator for 21 hours.

(b) Fixing and Mounting

After 21 hours the labeling media was removed. The dishes were rinsed 3 times with PBS. Then 1% sodium citrate was added to each dish and left on the coverslips for 10 minutes (Lü et al., 1988). This is done to swell the nuclei so that upon staining they are more easily visualized under the microscope. The sodium citrate was then removed and three 10 minute

changes of fixer were added to each dish. The fixer consists of ethanol:acetic acid (3:1). Finally, dishes were allowed to dry overnight.

When the coverslips were completely dry they were marked with a magic marker on the side with cells with a small 7. Seven was chosen because this number is directional, unlike 8 for example which looks the same from the back of the coverslip. This was done so that the side of the coverslip with the cells is distinguishable. Next, the coverslips were carefully removed from the petri dishes by turning the dishes over and slightly bending the dishes so the coverslips fell free. The coverslips were mounted with approximately 50 ul of Permount onto precleaned glass slides. Each slide was labelled according to bile acid or UV treatment and left to dry over night.

(c) Autoradiography

The mounted coverslips were prepared for autoradiography. This process entails covering the slides with a light sensitive emulsion which, upon development, detects the decay of ^{3}H-thymidine particles encorporated into the DNA of the cell. NTB-3 Emulsion is extremely sensitive to light. All

procedures using this emulsion were conducted in a photography darkroom with no safety light on.

The procedure for covering the slides in emulsion is described next. First, approximately 3 tablespoons of gelled NTB-3, kept at 4°C were taken from a light-tight container and placed in a Coplin jar. The Coplin jar had previously been wrapped repeatedly with electrical tape to make it light-tight. Next, the Coplin jar containing the emulsion was wrapped in two layers of heavy duty aluminum foil. The jar was placed in a 45°C incubator and not disturbed for 1 hour. After this time the emulsion was in a liquid state. Next, in the darkroom, slides, placed back to back, were dipped for 3 seconds into the jar, removed, separated and placed vertically into a test tube rack. Forty slides were dipped per experiment. The rack was then placed in a light tight tank. The slides remained there to dry for 4 hours.

Afterwards the slides were placed in a smaller light tight black box containing a capsule of Drierite. The boxes were sealed with electrical tape. I found that 3M tape worked better than other electrical tapes because it had the least static electricity when removed from the box. Static electricity gives off

light which is detected by the emulsion and ruins areas on the slides. The boxes were then covered with aluminum foil and placed in a carton so that the slides layed horizontally. The carton was placed at 4°C for 7 days.

(d) Development and Staining

The box containing the slides was placed at room temperature for 1 hour. Fresh developer and fixer were made for each experiment. D-19 developer was dissolved in deionized water as directed by the manufacturers instructions and cooled to 14°C. Kodak General fixer was also dissolved in deionized water and cooled to 14°C. The cells were stained with hematoxylin.

(e) Quantitation and Statistical Analysis

Grains over nuclei were counted. An area adjacent to the nucleus of similar size was also counted and this number was subtracted from the nuclear count to give a mean grain count. Nuclei with mean grain counts equal to or greater than 50 were considered to be in S-phase (replicative synthesis) and not used in the study (Cleaver and Thomas, 1981).

The z-test was used to determine whether there was a

significant difference between the mean grain count per population per dose of test agent versus the mean grain count when no test agent was used (Duncan et al., 1977). The z-test determines the significance of the difference between two means and may be used with large sample size (n > 30). This test is referred to as a t-test when a smaller sample size (n < 30) is used. The sample size in this assay was large (n ≥ 450). The following equation defines z:

$$z = \frac{(\bar{Y}_1 - \bar{Y}_2)}{s_p \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \qquad s_p = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2}}$$

n = size of sample

Y = mean of sample

s_1 = standard deviation of sample 1

s_2 = standard deviation of sample 2

A z value of greater than +1.96 implies statistical significance at the .05 level and a z value of greater than +2.58 implies significance at the .01 level.

CHO Cytoxicity Assay

Screening for differential genotoxicity of mammalian cells defective in DNA repair as compared to wild-type cells, can be accomplished by using the method developed by Hoy et al. (1984). Upon exposure of Chinese Hamster Ovary (CHO) cells to a DNA damaging agent, DNA repair mutants are less able to survive than wild-type.

In 0.5 ml of Alpha growth medium, 3 X 10^4 cells of wild-type (AA8) or 4 x10^4 cells of repair mutant EM9 or repair mutant UV4 were added to each well of 24-well cluster tray (Costar). The tray was placed in a 37°C, 5% CO_2 incubator and kept immobile for approximately 2 hours while attachment of the cells took place. The conditions of temperature, medium volume, and absence of tray movement during cell attachment are critical for ensuring uniform spreading of the cells.

After 2 hours the growth medium was removed from each well and replaced with chenodeoxycholate or deoxycholate dissolved in sterile deionized water and added at various dilutions to growth medium without serum. The wells were treated for 1 hour with the exposure media and left at room temperature. Cells in the control wells were treated with the exposure media

without the test compound. At the end of one hour 2.5 ml growth medium was added to each well to dilute the compound, and then the medium was removed by aspiration. 2 ml fresh growth medium was added to each well and the cultures were incubated at 37°C with 5% CO_2. After 72 hours the cells were fixed with 95% ethanol, and stained with crystal violet.

Cytotoxicity was measured by comparing the staining patterns of the wild-type cells to those of the mutants. In general, reduced staining results from a combination of cell killing, growth inhibition and detachment of cells. To quantitate the differential cytotoxicity, an integer between 0 and 5 is assigned to the staining intensity of each well with 0 designating no cell growth and 5 designating confluent cell growth. The lowest concentration of test compound that produces a two-unit difference from the no test agent control (e.g. 5 to 3 or 4 to 2) is defined as the lowest effective concentration of the compound (LEC). The LEC of the wild-type strain is divided by the LEC of the repair defective strain to give the differential cytotoxicity ratio (DC). The staining intensity was scored by myself and 3 other people in the lab who were unaware as to which agent was being tested.

CHAPTER III

RESULTS

The SOS response is induced by agents which damage DNA or inhibit replication (Little and Mount, 1982). Ultraviolet radiation, alkylating agents and crosslinking agents are all capable of turning on genes controlling a variety of responses in E. coli. These responses include an enhanced capacity for DNA repair, mutagenesis, inhibition of cell division and prophage induction (Witkin, 1976).

Two proteins regulate the SOS response. They are the LexA and RecA proteins (Little and Mount, 1982). The LexA protein represses a set of genes situated throughout the genome. An inducing agent, e.g. DNA damage, activates the RecA protein, which in turn cleaves the LexA repressor inactivating it, and the SOS genes are derepressed. Roberts and Devoret (1983) have suggested that the RecA protein is activated when it binds to gaps caused by the replication fork encountering a lesion. As the pool of LexA diminishes, various SOS genes, including recA, begin to be expressed at greater levels (Walker, 1984) and the

SOS responses are observed.

E. coli JL1705 carries a sulA::lacZ fusion. SulA is an SOS gene responsible for delayed cell division (Walker, 1984) and is normally repressed. When an SOS-inducing agent is present, the sulA promoter is derepressed and in this strain due to the sulA::lacZ fusion, the product of lacZ, beta- galactosidase, is produced. By measuring beta-galactosidase production, I was able to quantify the SOS response in the presence of a DNA damaging agent.

Effect of Mitomycin-C on SOS Induction

Mitomycin-C, a known DNA crosslinking agent has been used previously to induce the SOS response in strains carrying the gene-lacZ fusion as assayed by beta-galactosidase production (Keyon and Walker, 1980). Production of this enzyme was determined by its ability to hydrolyze Xgal, a colorless substrate, to form a blue product which can be measured. Colonies of E. coli JL1705 in which the SOS-response is induced turn blue due to the production of beta-galactosidase and the consequent hydrolysis of Xgal. Since the lacZ operon, which is also present, is not functional due to

a mutation in the structural lacZ gene, JL1705 colonies are typically white, unless induced by a DNA damaging agent.

E. coli JL1705 was incubated for 19 hours with mitomycin-C on nutrient plates (Lin and Little, 1988) containing 80 ug/ml Xgal. As evident in Figure 3-1, with increasing concentration of mitomycin-C, there was a corresponding increase in the percent of blue colonies among total colonies. This implies that with increasing concentrations of this DNA-damaging agent, there was an increase in the percent of bacteria that have an induced SOS-response and/or that bacteria with an SOS-response are more likely to survive than uninduced bacteria. Additionally, Figure 3-1 shows that the percent of the initially added bacteria which survived decreases as the concentration of mitomycin-C increased. As the amount of DNA-damage increased, a smaller proportion of the total damage can be repaired, resulting in survival of a smaller fraction of the cells.

There are three possible fates for a cell: survival without SOS-induction, survival with SOS-induction or death. These destinies are represented in Figure 3-2. The abscissa represents the increasing concentrations

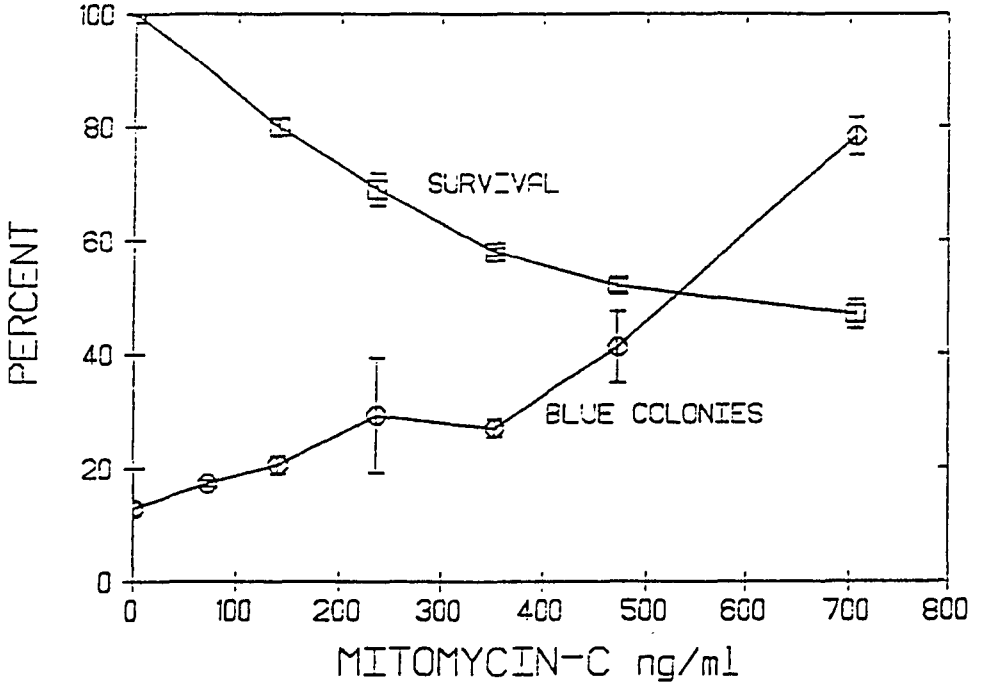

Figure 3-1. Effect of mitomycin-C on E. coli JL1705 on survival and SOS-induction. Blue colonies represent percent of blues among survivors. Error bars indicate standard error of three repeat experiments.

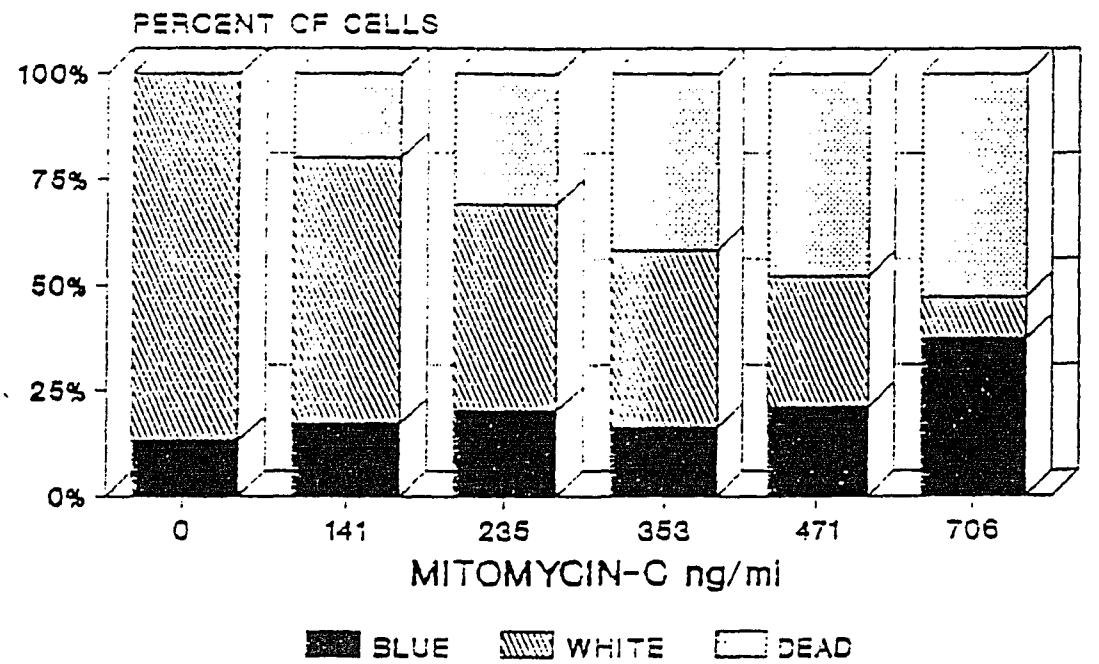

Figure 3-2. The three fates of the bacteria (JL1705) after treatment with mitomycin-C. Blue are those colonies which were SOS-induced. White represents those colonies which were not SOS-induced. Percentages are based on the mean of three experiments.

of mitomycin-C (0 to 706 ng/ml) with which the bacteria are incubated. The ordinate represents the percent of total bacteria found in each category. As shown, with increasing concentrations of mitomycin-C the percent of cells in which the SOS-response is induced (blue colonies) increased. After treatment with 706 ng/ml mitomycin-C, approximately 50% of the cells have died and of those surviving 90% were induced (blue). The remaining 10% were not SOS-induced (white). Some blue colonies may be expected, even without mitomycin-C present due to spontaneous DNA-damage.

Effect of Chenodeoxycholate on SOS-Induction

As shown in Figure 3-3 when the concentration of chenodeoxycholate in which the cell were incubated for 19 hours was increased from 0.0 ug/ml to 450 ug/ml there was an accompanying increase in the percent of blue colonies among surviving colonies, indicating increasing SOS induction and/or preferential survival of induced bacteria compared to uninduced bacteria. The increase in blue colonies among survivors was approximately 11-fold. Once again there was a low level, approximately 10%, of colonies which were SOS-

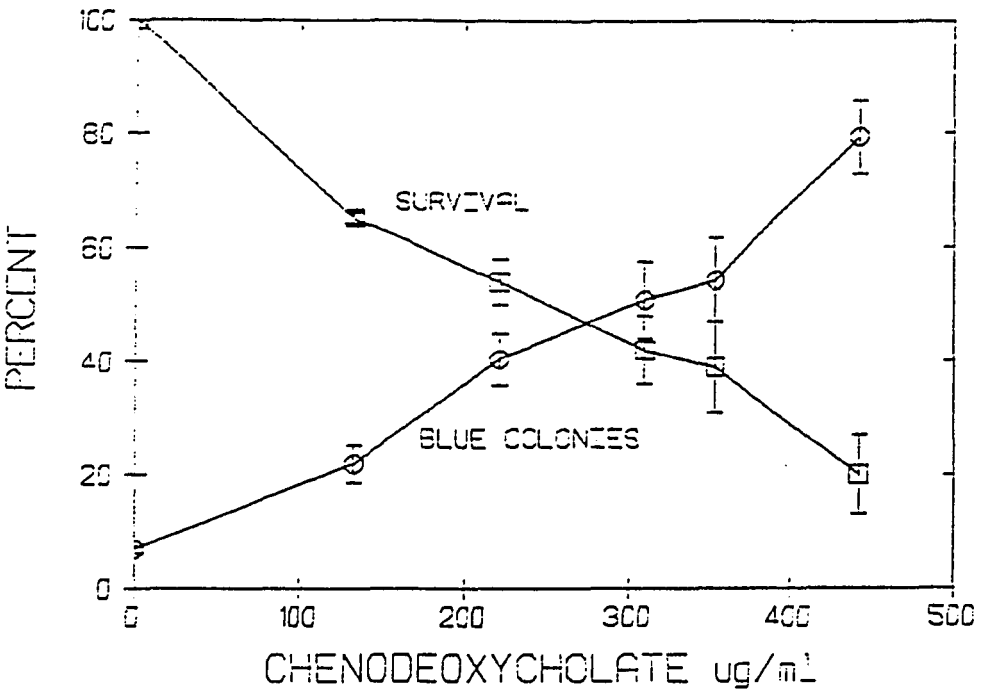

Figure 3-3. Effect of chenodeoxycholate on survival and SOS-induction. Blue colonies represent SOS-induced cells among survivors. Error bars indicate the standard error of the mean of three repeat experiments.

induced with no addition of testing agent. Additionally, as seen in Figure 3-3 the percent of total surviving colonies upon incubation decreases upon incubation with increasing concentrations of chenodeoxycholate. At the highest concentration of chenonodeoxylcholate (441 ug/ml), less than 25% of the cells survive. As shown in Figure 3-4, SOS-induction increased about 3-fold from 0 concentration of chenodeoxcholate, to 221 ug/ml, 309 ug/ml, and 353 ug/ml of chenodeoxycholate. At the highest dose of chenodeoxycholate, 441 ug/ml, the fraction of SOS-induced cells among the total population declined somewhat, and this may reflect death of induced cells at this dose.

These results show clearly that SOS-induced cells have a much higher probability of surviving than uninduced cells. That chenodeoxycholate induced the SOS response, and that induced cells were more likely to survive in the presence of chenodeoxycholate implies that chenodeoxycholate is a DNA damaging agent.

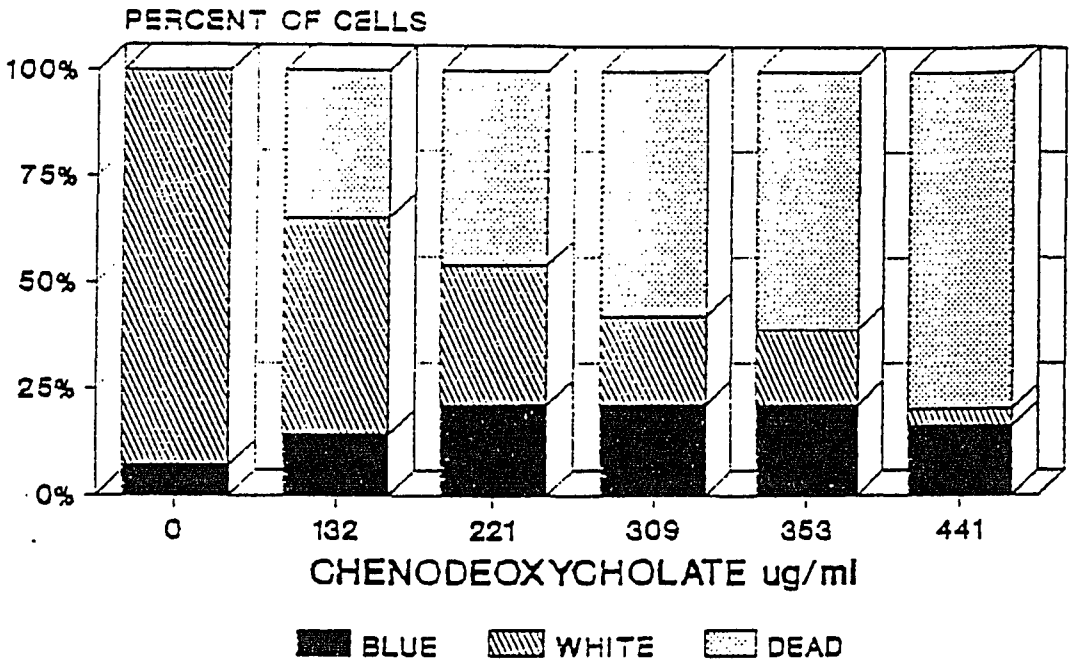

Figure 3-4. The three fates of E. coli JL1705 incubated with chenodeoxycholate. Blue represents repair initiated colonies and white represents those colonies that survived but were not SOS-induced. Percentages are based on the mean of three experiments.

Effect of Deoxycholate on SOS-Induction

The same protocol as previously described for mitomycin-C and chenodeoxycholate was used for testing deoxycholate. Briefly, increasing doses of deoxycholate were added to top agar overlay to which E. coli JL1705 was added. This was spread on nutrient plates (Lin and Little, 1988) containing 80 ug/ml Xgal and the plates were incubated for 19 hours. In Figure 3-5 it can be seen that as the dose of deoxycholate increased from 0.0 ug/ml to 441 ug/ml the percent of blue colonies among surviving colonies also increased, indicating a rise in SOS-induction and/or preferential survival of induced bacteria compared to uninduced bacteria. The percent of blue colonies is 8.6 fold higher at 441 ug/ml deoxycholate then at 0.0 ug/ml. Additionally, survival decreased, to 20% at higher doses of deoxycholate. Figure 3-6, shows that the maximum increase in SOS-induction is 1.9 fold greater at 397 ug/ml deoxycholate than the zero dose control. There was also preferential survival of those cells undergoing SOS-induction. It can be seen in Figure 3-6 that cell survival decreased as doses of deoxycholate increased. There was less than 5% white cells (not

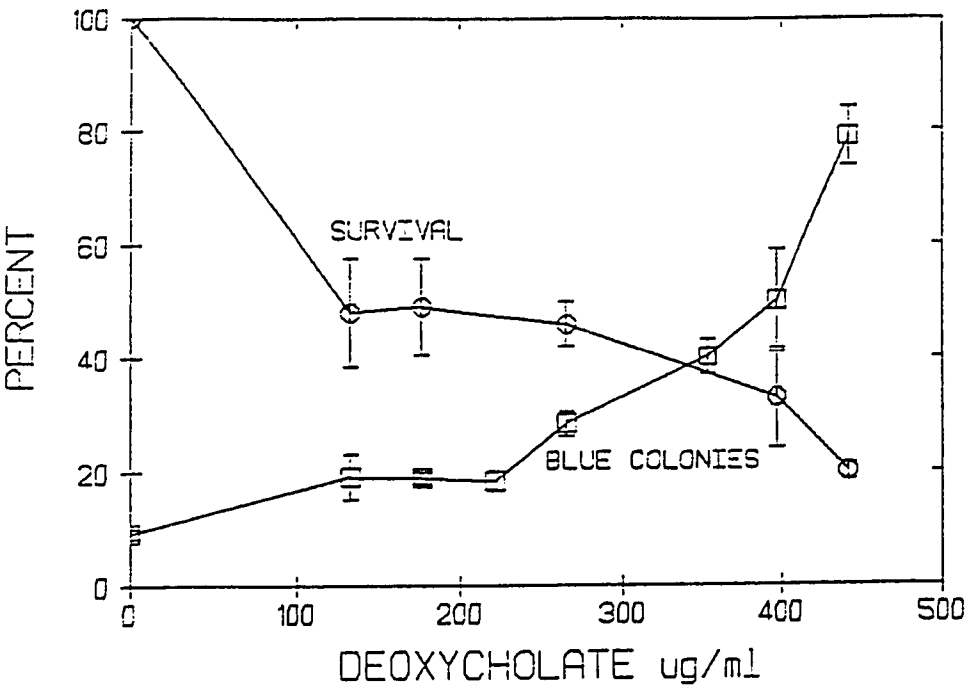

Figure 3-5. Effect of deoxycholate on SOS-induction and survival in E. coli JL1705. Blue colonies represent percent blues among survivors. Error bars indicate standard error of the mean of three repeat experiments.

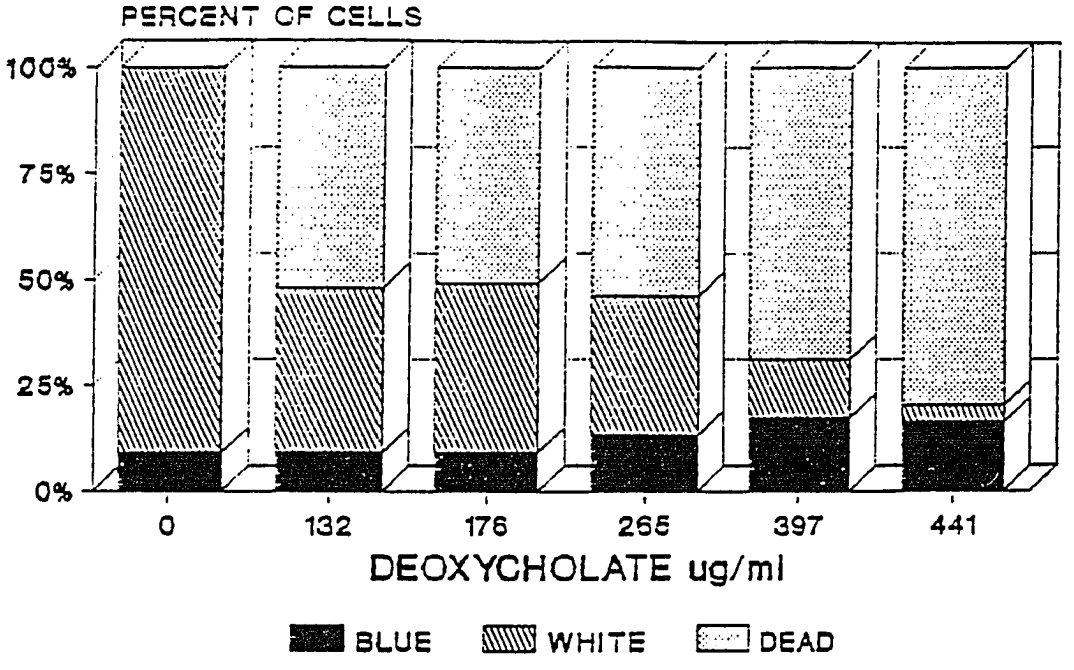

Figure 3-6. The three fates of E. coli after incubation with deoxycholate. Blue represents SOS-induced colonies and white represents those colonies that survived but were not SOS-induced. Percentages are based on the mean of three experiments.

SOS-induced) surviving at the maximum dose. However, there was not a proportional decrease in SOS-induced cells. The percent of SOS-induced cells remained constant and then increased at the three highest doses of deoxycholate while the percent of survival of uninduced cells steadily declined. This indicates that cells undergoing enhanced DNA-repair have a higher proportion of survival than uninduced cells when incubated with this bile acid.

Repair Deficient Mutant Sensitivity Assay

This test is based on the relative sensitivities of repair deficient and wild-type strains of E. coli to inactivation by DNA-damaging agents. Bacterial differential survival assays provide one of the simplest means of detecting chemically-induced DNA damage. These tests work on the principle that the failure to repair DNA damage can result in cell death (Tweats et al., 1981). Strains deficient in DNA repair have reduced survival compared to repair-proficient strains upon treatment with DNA damaging agents. E. coli mutants defective in the recA gene have reduced ability to repair damage caused by a wide spectrum of

agents. E. coli mutants defective in the uvrA gene are unable to repair a different spectrum of DNA damage.

Effect of UV Light on Repair Defective Strains

In this test, 0.1 ml of each strain was streaked across a Hershey plate and allowed to dry. Next, a piece of cardboard was placed over half of the open dish. Strains were irradiated with a hand held ultraviolet lamp (output primarily 254 nm) from a distance of 20 cm for 15 sec. This procedure was carried out in a darkened room to prevent photoreactivation (Kushner,1987). The plates were placed in a darkened incubator at 37°C and left overnight. As shown in Figure 3-7A and B the repair defective strains were unable to grow on the half of the dish which was irradiated. The top row shows the wild-type E. coli. This wild-type was able to repair ultraviolet-induced DNA damage and grew across the entire plate. RecA is defective in recombinational repair, but to some extent is still able to undergo excision repair. This strain grew weakly across the plate as shown in the middle rows of Figure 3-7. As the time of ultraviolet irradiation increased from 15

A B

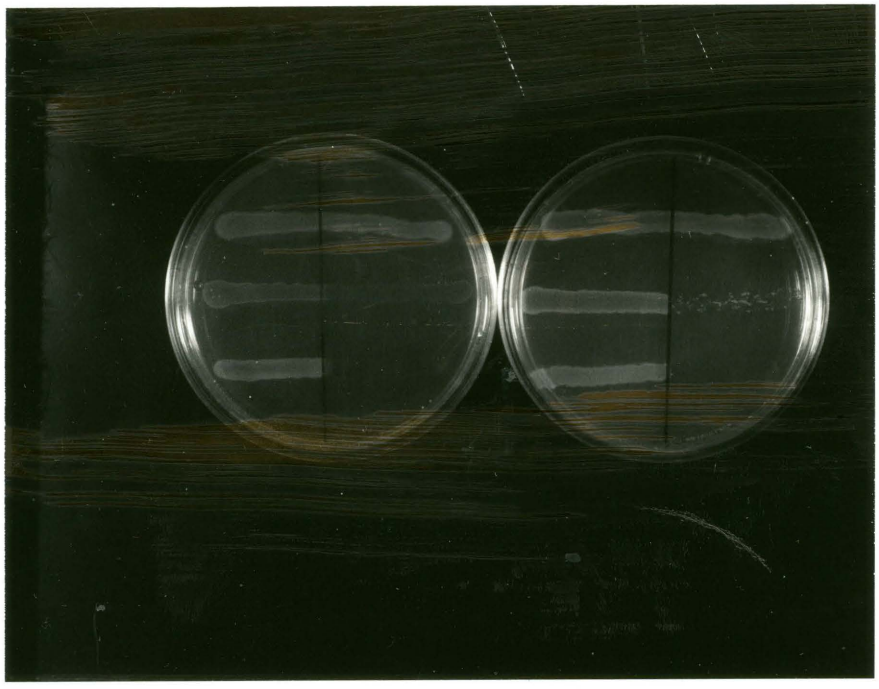

Figure 3-7. Effect of UV irradiation on wild-type and repair defective strains. Right half of each dish received UV irradiation. Left dish (A) was exposed to 15 sec UV. Right dish (B) received 20 sec of exposure. The top row is the wild-type, the middle row is recA and the bottom row is recAuvrA.

to 20 sec, the strain was less able to survive the damage as seen by its weaker growth in plate B. The recA uvrA strain, (bottom row Figure 3-7 A and B) was unable to grow in the presence of either a 15 sec or a 20 sec exposure to ultraviolet light as seen by its absence on both plates on the half of the dish that received treatment. Whenever the bile acids were tested for ability to inactivate these strains the above UV sensitivity test was done to check the strains for their respective DNA-repair defects.

Effect of Deoxycholate on Repair Defective Strains

This test is procedurally similar to the SOS-induction test. Increasing doses of deoxycholate were premixed with the molten top agar. Then bacteria, diluted in M9 salts solution, were added. Next, the solution was poured on to Hershey plates and incubated overnight at 37°C. The next day colonies were counted and the surviving fraction determined.

Figure 3-8 shows the results of this test. The error bars indicate the standard error of the mean of three repeat experiments. The abscissa represents the increasing concentrations of deoxycholate used, 0.0 to

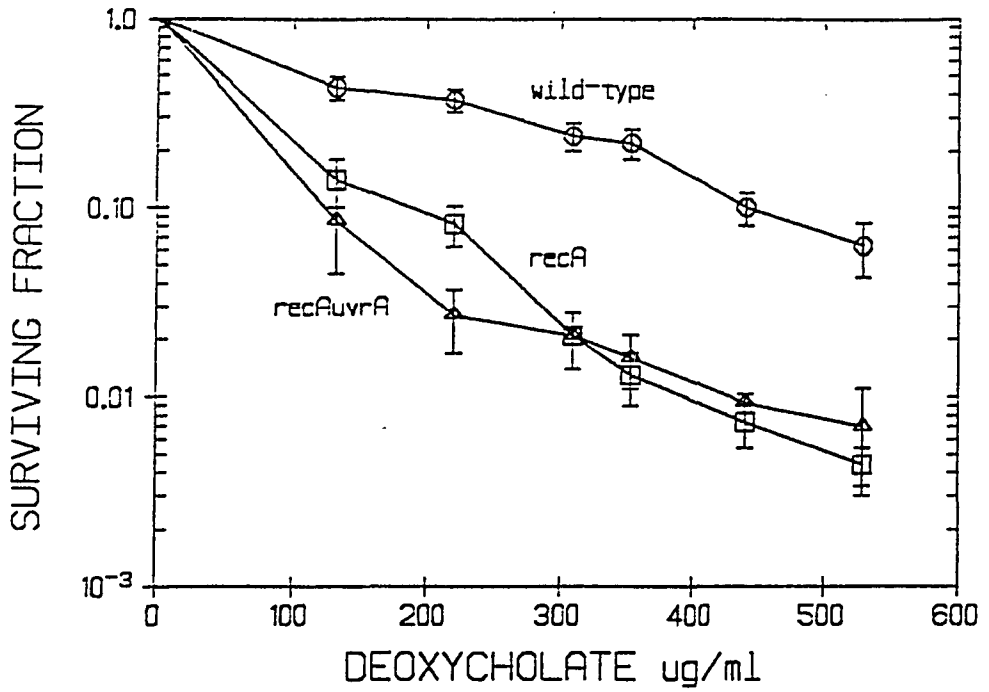

Figure 3-8. Survival of wild-type and repair defective E. coli strains upon treatment with deoxycholate. Error bars indicate standard error of the mean of repeat experiments.

540 ug/ml. The ordinate represents the surviving fraction. This is determined as the percent survival at a particular dose compared to the number present at 0.0 dose. Each point on the graph represents data from three plates per dose per experiment or a total of nine plates per dose. For the 0.0 dose four plates were counted in each experiment or a total of 12 plates for all threee experiments. To obtain accurate and reliable colony counts, bacterial dilutions were chosen so that plate counts ranged between 100 and 500 colonies.

At the highest dose of deoxycholate, 540 ug/ml, there was a 10-fold decrease in survival of both repair defective strains compared to wild-type. Interestingly, there was no significant difference in survival between the recA strain and the recA uvrA strain. The recA mutant is defective in recombinational repair and the double mutant has an additional defect in excision repair. However, the double mutant was not more sensitive to deoxycholate. These results indicate that lethal DNA damages caused by deoxycholate are within the spectrum that is repaired by the recA pathway. There do not appear to be any further lethal damages that require the uvrA

pathway of repair.

Effect of Chenodeoxycholate on Repair Defective Strains

The same protocol as that used with deoxycholate was followed with chenodeoxycholate. Figure 3-9 shows the results of the overnight incubation of wild-type and the two DNA-repair defective strains of E. coli with increasing doses of chenodeoxycholate. At the highest dose of chenodeoxycholate there was approximately a 100-fold reduction in survival of the recA mutant strain compared to the wild-type. Interestingly, the recAuvrA double mutant is more sensitive than the recA single mutant. The surviving fractional of wild-type cells decreased from 1.0 to 0.15. However, the surviving fraction of recAuvrA cells decreased from 1.0 to 0.0001. These results indicate that the DNA damages caused by chenodeoxycholate fall both within the spectrum repaired by the recA pathway and within the spectrum repaired by the uvrA pathway.

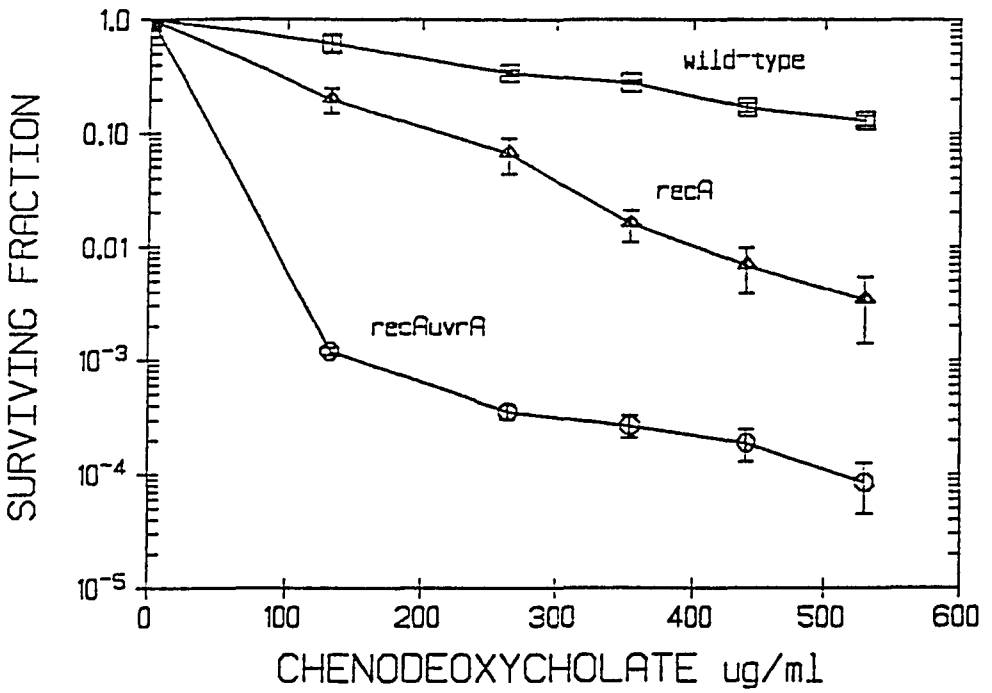

Figure 3-9. Survival of wild-type and repair defective E. coli strains upon treatment with chenodeoxycholate. Error bars indicate standard error of the mean of repeat experiments.

Effect of Deoxycholate on Unscheduled DNA Synthesis

Unscheduled DNA synthesis (UDS) is a measure of the repair synthesis mammalian cells undergo in response to DNA damage. Cells of human origin exhibit this phenomena to a greater extent, however, than rodent cells (Painter and Cleaver, 1969). UDS was first reported in Hela cells by Rasmussen and Painter, (1964) in response to ultraviolet radiation. Human cells defective in excision repair from individuals with the inherited condition xeroderma pigmentosum, show reduced incorporation of tritiated thymidine into DNA in response to UV irradiation (Painter and Cleaver 1969).

I used this procedure to measure the incorporation of tritiated thymidine into human foreskin fibroblast cells in response to treatment with bile acids. Autoradiography was used to detect the emission of beta-particles from the ^{3}H-thymidine incorporated into the DNA during the 21 hours following a 20 min treatment with each bile acid. The emitted particles are detected, upon development of the slides, as silver grains over the nucleus. Most of scheduled replicative synthesis is inhibited by the addition of a solution of 10 mM hydroxyurea before and during treatment with the

bile acid and during labelling. The few cells (< 2%) with 50 grains or more are distinctive. These were considered to be in replicative synthesis and were not counted (Cleaver and Thomas, 1981). Background grains were subtracted from nuclear grains to give a mean grain count.

As seen in Table 3-1, with increasing concentrations of deoxycholate, there was an increase in the mean grain count per cell in two separate experiments. The mean grain count increased when treatment increased from 0.0 to 25 ug/ml deoxycholate and leveled off at 50 ug/ml. The induced level of UDS shown in Table 3-1 is not as high as in the positive control in which the cells were exposed to 30 sec of ultraviolet light, but it was substantial and consistent. Additionally, in the comparison of grain counts between each of the treated samples and the untreated control the differences are significant ($p \leq 0.05$) or highly significant ($p \leq 0.01$).

Table 3-1 UNSCHEDULED DNA SYNTHESIS
SODIUM DEOXYCHOLATE

EXPT	DOSE ug/ml	NUMBER OF SLIDES	NUMBER OF CELLS	MEAN GRAIN COUNT PER CELL AND STANDARD ERROR	FOLD INCREASE
1	0.0	3	450	3.93±0.43	1.00
	12.5	3	450	5.17±0.52*	1.31
	25.0	5	750	8.36±0.47**	2.13
	50.0	4	600	7.36±0.51**	1.87
	100.0	4	600	7.81±0.49**	1.99
	250.0	4	600	6.92±0.48**	1.76
	UV 30sec	3	450	12.64±0.40**	3.22
2	0.0	6	900	4.38±0.32	1.00
	12.5	6	900	6.63±0.40**	1.51
	25.0	6	900	6.74±0.40**	1.53
	50.0	5	750	6.94±0.46**	1.58
	75.0	5	750	5.51±0.36**	1.26
	100.0	5	750	7.31±0.42**	1.67
	UV 30sec	3	450	21.87±0.42**	4.99

* p=significant ≤ 0.05
** p=highly significant ≤ 0.01

Effect of Chenodeoxycholic acid on UDS

As seen in Table 3-2, in two separate experiments there was an increase in mean grains per cell when fibroblasts were treated with increasing doses of chenodeoxycholic acid. This increase was statistically highly significant at concentrations of ≥ 100 ug/ml chenodeoxycholic acid. Additionally, in all the UDS experiments, exposure to UV light resulted in a highly significant increase in mean grains per cell.

Effect of Deoxycholate and Chenodeoxycholate on Repair Defective CHO cells

The sensitivity of DNA-repair defective Chinese Hamster Ovary cells (CHO) to these bile acids was tested using the assay developed by Hoy et al. (1984). Increased cytotoxicity, as measured by loss of staining intensity, of the mutant cells compared to the wild-type cells was interpreted as a measure of unrepairable, lethal DNA damage. The lowest dose of the compound which caused a two-unit difference in staining intensity from the zero dose treatment was determined to be the lowest effective concentration of

Table 3-2 UNSCHEDULED DNA SYNTHESIS
CHENODEOXYCHOLIC ACID

EXPT	DOSE ug/ml	NUMBER OF SLIDES	NUMBER OF CELLS	MEAN GRAIN COUNT PER CELL AND STANDARD ERROR	FOLD INCREASE
1	0.0	3	450	4.25±0.43	1.00
	25.0	3	450	3.63±0.30	.85
	50.0	3	450	4.72±0.42	1.10
	100.0	3	450	7.58±0.57 **	1.78
	250.0	3	450	6.00±0.45 **	1.30
	UV 30sec	3	450	16.57±0.49 **	3.22
2	0.0	4	600	3.72±0.34	1.00
	50.0	3	450	3.36±0.32	.90
	75.0	3	450	4.89±0.40 *	1.31
	100.0	3	450	5.14±0.44 **	1.38
	150.0	3	450	6.19±0.45 **	1.66
	00.0	3	450	5.45±0.38 **	1.47
	UV 30sec	3	450	16.15±0.27 **	4.34

* p=significant ≤ 0.05
** p=highly significant ≤ 0.01

the compound (LEC). AA8 is the wild-type strain. The differential cytotoxicity ratio (DC) is obtained by dividing the LEC of wild-type by the LEC of each mutant. Table 3-3 shows that the DC ratio of deoxycholate, but not chenodeoxycholate, is significant in strain EM9. This strain is hypersensitive to X-rays and is defective in strand-break rejoining. In contrast, chenodeoxycholate, but not deoxycholate, causes a significant DC ratio in strain UV4. This strain is defective in excision repair and crosslink removal. This study showed that both bile acids are DNA-damaging agents. Also, the results imply that different intact repair pathways are needed to enable the cell to recover from the bile acids.

A summary of results from each assay used in this study is given in Table 3-4.

Table 3-3 Results of CHO Cytotoxicity Assay

STRAIN	COMPOUND	LEC	DC RATIO
AA8 (wild-type)	Chenodeoxycholate	1000 ug/ml	1
	Deoxycholate	1000 ug/ml	1
UV4 (excision repair mutant)	Chenodeoxycholate	500 ug/ml	2*
	Deoxycholate	1000 ug/ml	1
EM9 (defective in strand-rejoining)	Chenodeoxycholate	1000 ug/ml	1
	Deoxycholate	500 ug/ml	2*

* A DC ratio of 2 or greater means there is significant differential cytotoxicity.

Table 3-4 Summary of Results

Assay	Bile Acid	Result
SOS Induction	Chenodeoxycholate	SOS induction (3-fold) and preferential survival of induced cells
	Deoxycholate	SOS induction (1.9-fold) and preferential survival of induced cells
Repair Deficient Mutant Sensitivity	Chenodeoxycholate	uvrA recA sensitive ++ recA sensitive +
	Deoxycholate	uvrA recA sensitive + recA sensitive +
UDS	Chenodeoxycholic acid	increased repair synthesis
	Deoxycholate	increased repair synthesis
CHO Repair Mutants	Chenodeoxycholate	UV4 had increased sensitivity
	Deoxycholate	EM9 had increased sensitivity

\+ 10 fold less survival than wild-type
++ greater than 10 fold less survival than wild-type

CHAPTER IV

DISCUSSION

Each assay in this study relied on the cell's ability to respond to DNA damage. Various DNA repair pathways have evolved in bacterial and mammalian cells to correct or tolerate DNA damage. These DNA repair pathways are discussed next.

DNA Repair Pathways

In both prokaryotes and eukaryotes there are enzymatic systems which the cell employs to ensure that the hereditary information contained in the nucleotide sequence of DNA remains intact. Fidelity of replication is one method used to make certain the perpetuation of the DNA sequence is correct. The mechanisms by which high fidelity is accomplished by various DNA polymerases include: (1) accurate base pairing during polymerization and (2) proofreading of replication errors (Kornberg, 1980). Additionally, damage to DNA must be repaired to preserve the information encoded in the base sequence. Cells have

established several enzymatic systems to accomplish this task.

(a) Photoreactivation

Ultraviolet irradiation induces a variety of DNA damages, the most common of which are pyrimidine dimers (Kuschner, 1987). These dimers arise from the formation of a cyclobutane ring across the 5-6 double bond of adjacent pyrimidines. Photoreactivation involves the enzymatic cleavage of cyclobutane pyrimidine dimers in the presence of visible light (300 to 600 nm) (Rupert et al., 1958). Photoreactivating enzymes have been found in prokaryotes, lower eukaryotes and human cells (Sutherland, 1974).

(b) SOS Response

The E. coli SOS system was the first regulatory network to be characterized that is induced by DNA damage (Walker, 1985). It is the largest, most complex, and best understood DNA-damage inducible network. Little and Mount (1982) summarized the system of gene regulation which controls the SOS response. This system involves the actions of two proteins: LexA and RecA. The LexA protein represses a set of unlinked

genes located throughout the genome. The RecA protein, when activated by an inducing signal, cleaves LexA thereby inactivating it (Slilaty and Little, 1987). The SOS genes are thus derepressed and the SOS response ensues.

Exposure of E. coli to agents or conditions that either damage DNA or interfere with DNA replication results in increased expression of genes that are members of the SOS network (Walker,1984). The SOS responses include (1) inhibition of cell division, (2) Weigle reactivation, (3) induction of prophage, (4) and increased capacity to carry out inducible excision repair and (5) an increased capacity to repair double strand breaks (Witkin, 1976). Despite concentrated efforts, the existence of an inducible repair and mutagenesis process in mammalian cells that is analogous to the bacterial RecA-dependent SOS system is still in doubt (Rossman and Klein, 1985).

(c) Excision Repair

Excision repair is an important process for the accurate removal of DNA damages. The principle of excision repair is as follows: the DNA damage is recognized; a phosphodiester strand scission is made;

the damage, along with adjacent nucleotides, is excised from the strand containing it; and the deleted stretch is resynthesized utilizing the intact complementary strand as a template (Hanawalt et al., 1979). Excision repair is possible only in duplex regions of DNA due to the need of a template. A glycosylase, a specific enzyme which recognizes usually one specific type of damaged base, may begin the excision repair process by removing the altered or incorrect base from the DNA (Lindahl, 1982). This base removal results in an apurinic or apyrimidinic site which may be recognized by a specific endonuclease (Hanawalt et al., 1979).

Studies of excision repair in E. coli have revealed three genes, uvrA, uvrB, and uvrC to be required for this repair process. These were identified through studies of a series of mutants characterized by their inability to excise pyrimidine dimers (Howard-Flanders, et al., 1966). The general reaction consists of the nonspecific binding of the UvrA-ATP protein dimer to damaged DNA. Next, UvrB protein interacts with this complex to form a stable DNA-UvrA-UvrB complex. In the presence of the UvrC protein cleavage of the DNA phosphodiester backbone occurs both at the 5' and 3' sides of the DNA damage. The complex and damage-

containing oligonucleotide is released upon interaction with DNA helicase II and possibly DNA polymerase I. The resulting gap is filled by repair synthesis which is carried out by DNA polymerase I and completed by DNA ligase (Kushner, 1987). Neither uvrA or uvrB mutants perform incision (Shimada et al., 1968). UvrC mutants do perform incision but, at a much slower rate than wildtype (Seeberg and Johansen, 1973).

As long as the damage is confined to one strand (e.g. incorrect bases, damaged bases, single strand breaks, dimers and monoadducts), excision repair is effective in its removal. However, E. coli cells in which damage to both complementary strands occurs, must use the recombinational repair pathway to cope with this damage.

Studies of cells from patients with xeroderma pigmentosum (XP) give much information about excision repair in mammalian cells. Fibroblasts from XP patients are sensitive to UV light and like uvr mutants of E. coli they are also sensitive to a variety of chemicals which form bulky adducts. The excision repair pathway in mammals may act in an analogous way to the exision repair pathway in E. coli (Hanawalt, 1979).

(d) Recombinational Repair

Seven genes of E. coli have been identified whose products play roles in homologous recombination (Walker, 1985). They are recA, recB, recC, recF, recJ, recN and ruv. The expression of recA, recN and ruv is induced by DNA damage and controlled by the SOS regulatory network. Mutations in any one of the above genes results in decreased survival of E. coli when exposed to DNA damaging agents. RecA, RecB and RecC gene products act directly in two repair process, daughter-strand gap repair and double strand break repair (Walker, 1985).

Daughter strand gap repair is a post-replication recombinational repair pathway in which damages are tolerated rather than removed. When a replicating strand encounters a DNA damage, replication is blocked. Presumably replication resumes at a site past the lesion (Walker, 1985). The gap is then filled in through recombination between the daughter strands.

Double-strand breaks in DNA may be created by the action of various agents. One of the most widely studied of these agents is ionizing radiation (Walker, 1985). Double-strand break repair is an inducible system and requires the presence of another DNA duplex

with the same base sequence as that damaged by the lesion. RecA and RecN gene products are necessary for this repair pathway.

It has been shown that the molecular weight of DNA synthesized in mammalian cells at early times after UV irradiation is relatively small compared to that synthesized in unirradiated control cells. At later times the molecular weight of this DNA increases to normal size even though the parental strand still contains UV-damage (Lehmann, 1972; Waters, 1979). Additionally, van Zeeland (1984) investigated how long pyrimidine dimers form a block for the nascent DNA strands in UV-irradiated human cells. He found that in normal human fibroblasts 15 minutes after UV irradiation the length of the nascent strands are equal to the inter-dimer distance found in parental DNA. However, after longer periods of time the newly synthesized DNA becomes rapidly larger. These results suggest that DNA synthesis in UV irradiated mammalian cells, although initially blocked at sites of UV damage, eventually continues past these damages. Most data suggest that DNA damages encountered by the leading strand are capable of blocking fork progression, unlike damages encountered by the lagging

strand. It is believed that the lagging strand leaves a gap directly across from the dimer which eventually is filled in by some mechanism (Griffiths and Ling, 1989). Problems to be solved in mammalian DNA repair include the extent to which nucleosomes and higher orders of chromatin structure influence sites and rates of repair, the role played by degrees of supercoiling and the influence of transcriptional and replication processes on repair (van Zeeland, 1984).

Short Term Carcinogenicity Testing

Bacteria and mammalian cells are widely used as test systems to detect DNA damage. DNA damage is recognized as a major factor in the initiation of carcinogenesis (Bishop, 1987). Some of the bacterial assays measure revertant colonies resulting from mutations produced upon treatment with a genotoxic agent (Ames et al., 1975; Greene and Muriel, 1976). Other tests, as described below, measure additional manifestations of DNA damage.

(a) SOS Chromotest

Quillaret and Hofnung (1985) developed the SOS Chromotest which measures the production of beta-galactosidase from a sulA::lacZ fusion. In this strain beta-galactosidase production is under control of a DNA damage inducible SOS gene. A uvrA mutation was introduced into this strain by Quillardet and Hofnung (1985) so that it would be deficient in excision repair. This increases the induction response to numerous DNA damaging agents. An rfa mutation renders the strain lipopolysaccharide deficient. This allows better diffusion of certain molecules into the strain, but also renders the strain highly sensitive to bile salts as seen by growth inhibition on McConkey plates which contain bile salts. Quillardet et al. (1985) tested 83 compounds using this assay, and found that 90% of the compounds previously shown to be mutagenic in the Ames test were SOS inducers.

The SOS-induction test used in this study makes use of the same sulA::lacZ fusion. However, strain JL1705 is uvrA+ and rfa+ which may decrease its sensitivity to some DNA damaging agents.

(b) Reduced Survival of *E. coli* Mutants

Reduced survival of repair defective *E. coli* has been shown by Tweats (1981) to be effective in detecting DNA damaging agents. DeFlora *et al*. (1984) tested 135 genotoxic agents in three different versions of a bacterial DNA-repair sensitivity assay: (1) liquid micromethod procedure, (2) 2 h preincubation test on agar plates measuring colony formation and (3) diameter size on an agar spot test. Each version of the assay measured survival of repair defective bacteria compared to wild-type after exposure to a test agent. DeFlora *et al*. (1984) found the combined overall accuracy of the tests to be 72.4% in detecting agents previously reported as carcinogenic in the literature. Additionally, the liquid micromethod procedure correlated well to the Ames reversion test. The DNA repair tests were superior to the Ames Test in detecting direct-acting agents (ie. those which did not need metabolic activation). Knamüler *et al* (1988) found that repair defective *E. coli* (uvrBrecA) injected intragastrically into the colon and small intestine of mice had a lower survival than wild-type cells. Additionally, this decrease in survival was only obtained in the intestine and not other organs. They

hypothesized that genotoxic factors were present in the intestine of mice.

(c) UDS in Mammalian Cells

To test the reliability of the UDS assay as a tool for detecting chemical carcinogens, San and Stich (1975) examined 64 different compounds. These compounds were tested with human skin fibroblasts and included precarcinogens that required metabolic activation, proximate and ultimate carcinogens, man-made and naturally-occurring compounds, non-carcinogenic but mutagenic chemicals as well as non-mutagenic chemicals. The results revealed the following pattern: (1) cells exposed to proximate and ultimate carcinogens responded with DNA repair synthesis; (2) precarcinogens either elicited no detectable levels of unscheduled ^{3}HTdr incorporation, or triggered DNA repair at high concentrations or at longer exposure times (2-5 hours); (3) non-carcinogenic compounds failed to initiate DNA repair synthesis. This results indicate that the UDS assay on cultured human fibroblasts is a sensitive system for the detection of chemical carcinogens.

(d) CHO Repair Mutant Cytoxoicity

The Chinese hamster cytotoxicity test assay introduced by Hoy et al. (1984) to detect and classify DNA-damaging agents using DNA-repair deficient strains is becoming widely used. Hoy and coworkers tested 15 direct-acting and seven metabolism-dependent agents that were expected to produce bulky, covalent DNA adducts. All produced a differential cytotoxicity response in at least two of the DNA-repair defective mutants. Thirteen non-DNA damaging agents were also tested and none produced a differential cytotoxicity response, suggesting that this outcome is specific for DNA damage. This assay also provides reliable information about the type of damage. The UV4 strain has shown dramatic hypersensitivity to bifunctional alkylating agents in this assay (Hoy et al., 1983) and the EM9 strain has shown sensitivity to chemicals which produce strand breaks.

Similarities and Differences of Chenodeoxycholate and Deoxycholate

Throughout this study it was consistently found that both chenodeoxycholate and deoxycholate damage DNA as

evidenced by: (1) the increase in measurable DNA repair synthesis in human fibroblasts found after treatment with either agent, (2) the increased sensitivity of DNA repair defective bacteria and mammalian cells to treatment with either agent and (3) the preferential survival of DNA repair induced cells in a bacterial population exposed to these bile acids.

Additionally, there were certain dissimilarities found between the two bile acids in these assays which are helpful in classifying each with respect to the type of DNA damage it may cause. These are discussed below.

(a) SOS Induction Test

As shown in Figures 3-1 and 3-2, bacteria which have an increased capacity for DNA repair, as evidenced by the presence of SOS induction, preferentially survive treatment with either chenodeoxycholate or deoxycholate. However, upon treatment with chenodeoxycholate there is a 3-fold increase in SOS induction compared to a 1.9-fold increase with deoxycholate. This difference in induction of the SOS response may be due to the type of DNA damage produced by each bile acid. It is believed that single stranded

regions of DNA may act as an inducing signal for the SOS response (Walker, 1984). Moreover, Roberts and Devoret (1983) have suggested that RecA is activated when it binds to single strand gaps produced by the replication apparatus when it encounters DNA damage. If the SOS inducing signal is a specific type of DNA damage as hypothesized (Barbe et al., 1983), deoxycholate may not cause this type of damage as efficiently as chenodeoxycholate.

However, an alternate argument for the lower SOS induction upon treatment with deoxycholate is that the concentration needed to induce the SOS response is toxic and the cells are unable to survive. Since bile acids have been shown to damage mammalian cell membranes (Vahoung et al., 1981), the dose needed for SOS induction by deoxycholate in E. coli may cause membrane damage from which the cells are unable to recover.

(b) Repair Deficient Mutant Sensitivity Assay

E. coli cells defective in DNA repair responded quite differently to chenodeoxycholate and deoxycholate. Cells defective in excision repair were 100-fold more sensitive to chenodeoxycholate than

deoxycholate. Since a uvrA mutation defective in excision repair (Walker, 1984) in the presence of a recA mutation caused increased sensitivity to chenodeoxycholate, E. coli must be able to use excision repair to remove at least part of the potentially lethal DNA damages caused by chenodeoxycholate. RecA uvrA double mutant cells show no significant further decline in survival than the recA single mutant when treated with deoxycholate as shown in Figure 3-8. This indicates that the excision repair pathway is unable to repair a significant fraction of lethal DNA damages caused by deoxycholate.

(c) Unscheduled DNA Synthesis

To determine whether bile acids cause DNA damage in mammalian cells, unscheduled DNA synthesis was measured. As discussed above (UDS in Mammalin Cells, section c) UDS is a measure of repair synthesis that cells undergo in response to a DNA-damaging agent and it is believed to specifically reflect excision repair (Rasmusson and Painter, 1966). After exposure to a genotoxin, incorporation of ^{3}H-thymidine into DNA is measured by autoradiography. Repair synthesis is associated with an increase in mean autoradiogrphic

grains per cell. Treatment of human fibroblasts with increasing doses of either chenodeoxycholic acid or deoxycholate caused an increase in mean grain counts (Tables 3-1 and 3-2). These results indicate that the bile compounds tested cause DNA damage in human cells. It can be seen that treatment with deoxycholate, a secondary bile acid causes a significant increase in the mean grain count at lower doses than chenodeoxycholic, a primary bile acid (25 ug/ml as opposed to 100 ug/ml). Previously, it has been shown that factors which increase bacteria present in the gut responsible for the conversion of primary bile acids into secondary ones, increase the risk of colon cancer (Bruce, 1987; Nagengast et al., 1988). The finding that deoxycholate is more potent in the UDS assay is in accord with the above results.

It is also important to note that the level of repair synthesis triggered is indicative of the chemical's potency only when comparing similar molecular compounds (ie. isomers and derivatives) (San and Stich, 1975). In this respect it is valid to compare chenodeoxycholic acid to its derivative deoxycholate. However, no such correlation is true when comparing carcinogens of different molecular

structure. San and Stich (1975) showed that the two compounds 4-nitro-quinoline-oxide and monochloroacetic acid, similar in their in vivo carcinogenic effects produced quite different levels of UDS.

(d) CHO Cytotoxicity Assay

The study of the effect of bile acid cytotoxicity on CHO cells shows each bile acid caused DNA-damage. Also, the results imply that different intact repair pathways are needed to enable the cell to recover from each of the bile acids since different repair mutants are sensitive to each one. The mutant defective in strand break rejoining (EM9) was sensitive to deoxycholate, while the excision defective mutant (UV4) was sensitive to chenodeoxycholate. These results are similar to those obtained with the E. coli sensitivity tests. Mutants sensitive to UV-type damage were more sensitive to chenodeoxycholate, whereas mutants sensitive to X-ray mimetic agents were more sensitive to deoxycholate. This implies an excision repair type pathway is needed for recovery from damage caused by chenodeoxycholate and that a recombinational type repair pathway is needed for recovery of damage caused by deoxycholate.

Dosage of Bile Acid

Between 130 to 650 mg of bile acids enter the colon per day and are converted to secondary bile acids (Hylemon and Glass, 1983). Reddy (1981) found that healthy subjects excrete 0.2 mg chenodeoxycholic acid per g dry feces and 3.4 mg deoxycholic acid per g dry feces. Subjects on diets with 25% more fat than control subjects, excreted the same amount of chenodeoyxhcholic acid as those without the excess fat in their diet, but the excreted amount of deoxycholic acid significantly increased to 5.3 mg per g dry feces. Additionally, in a study by Thompson (1983) it was shown that 7 to 8 mg of total bile acids per g dry feces were excreted by healthy subjects as compared to 10 mg per g in subjects with colorectal cancer. The amount of deoxycholic acid excreted by subjects from the U.S, an area with high colon cancer incidence, was approximately 2.2 mg per g dry feces, whereas Japanese subjects, whose incidence of colon cancer is lower, excreted only 0.5 mg per g dry feces (Thompson, 1983). The dose range in all assays used in this study was between 12.5 ug/ml to 500 ug/ml. This is considerably lower than that found excreted in the feces and the

dose used by Cheah and Bernstein (1989) to obtain damage in naked DNA (10 to 120 mg/ml). The dosages used in this study are consistant with those used in another study (Kulkarni and Yielding, 1985). In this study, single strand breaks in DNA occurred after whole cells were exposed to 100 ug/ml lithocholic acid for 30 minutes.

In conclusion, these studies indicate that the bile acids chenodeoxycholate and deoxycholate cause DNA damages, and that these damages are at least partially repairable. This conclusion supports the hypothesis that bile acids are primary carcinogens and are implicated in the etiology of colon cancer.

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