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Title: Antimutagenic Potency of Wheat Grain and Berry Extracts *in vitro* and Anticarcinogenicity of Wheat Grain *in vivo*

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Abstract approved _____

David E. Williams

The antimutagenic potency of wheat grain and berry extracts was studied *in vitro* against several heterocyclic amines (HCAs) using the Salmonella mutagenicity assay and the anticarcinogencity of wheat grain was studied *in vivo* using the rat colonic aberrant crypt focus assay.

Wheat bran, which binds HCAs *in vitro*, as well as refined wheat and unrefined whole wheat, inhibited the mutagenic activities of 2-amino-3-methylimidazo [4, 5-*f*] quinoline (IQ), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) when they were co-incubated and the supernatant (minus grain) was added to the Salmonella mutagenicity assay. The water-soluble fraction alone from refined and unrefined wheat, but not bran, also inhibited these mutagens *in vitro*. *In vivo*, AIN-93G diets containing refined wheat or unrefined wheat were examined for their ability to inhibit IQ-induced colonic aberrant crypt foci (ACF) in the F344 rat. A slight increase in the number of aberrant crypts /ACF (AC/ACF) was seen after 16 weeks in rats treated post-initiation with refined wheat ($p<0.05$), and fewer foci with 2 or 3 aberrant crypts (ACF-2) were found in rats given unrefined whole wheat *post*-initiation compared with animals treated with the same diet during the

initiation phase ($p< 0.05$). There was no significant difference in the profile of IQ urinary metabolites or excretion of promutagens 0-48 hours after carcinogen dosing, and grains had no effect on hepatic cytochrome P450 (CYP) 1A1, CYP1A2, aryl sulfotransferase, or *N*-acetyltransferase activities; however, a slightly higher UDP-glucuronosyl transferase activity was observed in rats fed unrefined wheat compared with refined wheat diets ($p<0.05$). Thus, despite their antimutagenic activities *in vitro*, only marginal effects were seen with refined and unrefined wheat *in vivo* with respect to induction of hepatic enzyme activities, carcinogen metabolism, or IQ-induced ACF in the rat colon.

The fresh juice and extract of crandall black currant (*Ribes aureum*) were not mutagens in the Salmonella mutagenicity assay. Berry extract or fresh juice at levels to 50 μl (22 mg berry) in a 500 μl pre-incubation system significantly inhibited the mutagenicity of IQ, a mutagen from cooked meat, by 32% when rat liver S9 bioactivation system was present. One hundred μl of crandall black currant extract gave 89% inhibition of IQ mutagenicity ($p<0.05$). However, the mutagenicity of 2- hydroxyamino-3-methylimidazo[4,5-*f*] quinoline (*N*-hydroxy-IQ), a direct-acting metabolite of IQ, was not affected. An *in vitro* fluorometric assay showed the activity of cytochrome P 450 (CYP) 1A1 and CYP 1A2 was decreased. Inhibition of CYP 1A2 activity may be an important mechanism of antimutagenicity of crandall black currant extract. Similar results were also observed with other berry samples.

Key word: cereal grains, black currant, berry, aberrant crypt foci, heterocyclic amines, CYP1A1, CYP1A2, Salmonella mutagenicity assay.

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Antimutagenic Potency of Wheat Grain and Berry Extracts *in vitro* and Anticarcinogenicity of Wheat Grain *in vivo*

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CHAPTER 1

INTRODUCTION

DIET AND CANCER

Cancer is the second leading cause of death in the United States. Over one million people are diagnosed with cancer each year. Approximately one out of every two American men and one out of every three American women will have some type of cancer at some point during their lifetime (American Cancer Society, 2002). The association between diet and cancer has been established through epidemiological studies (review by Doll, 1990). Consumption of high levels of fat or salted food and well –done cooked meat are all thought to increase the risk of cancer, whereas, vegetables, fruits, dietary fiber, certain micronutrients and some fatty acids protect against some kinds of cancers (Doll, 1990; Block, *et al.*, 1992; Willett, 1994). Since cancer is mostly a preventable disease, more and more research is concentrated on seeking chemoprevention agents. Among these agents, fruits and dietary fibers are most promising.

MUTAGENESIS AND CARCINOGENESIS

Carcinogenesis is a multistage process that involves a complex series of cellular and molecular events leading to the development of neoplasia. There are three stages in carcinogenesis: initiation, promotion and progression. Initiation is a series of early events in the carcinogenetic process whereby an exogenous or endogenous carcinogen induces alterations of the genetic make-up of the cell. Genotoxic carcinogens are usually activated by metabolism, forming electrophilic

compounds capable of binding to DNA forming adducts. As a consequence, normal growth control genes are mutated or their expression is altered to produce active oncogenes and /or there is loss or inactivation of tumor suppressor genes. The next stage is promotion, which is characterized by alteration of genetic expression and reversible clonal growth of the initiated cell population. Progression is the last stage and involves the increased growth and expression of promoted cancer cells from a focal lesion to an invasive tumor mass, often accompanied by an increasingly abnormal complement of gene expression (Butterworth *et al.*, 1992; WCRF/AICR, 1997).

Gene mutation is an important step in the initiation of carcinogenesis by genotoxic carcinogens, providing an association between mutagenic and carcinogenic activity. Mutagenicity testing is an important mechanism for screening mutagens and predicting carcinogenesis. More than 200 assays for mutagens have been proposed, among them, the Salmonella mutagenicity assay is one of the most widely utilized. The Salmonella mutagenicity assay is a good assay for predicting rodent carcinogens, especially genotoxic carcinogens with structure alerts (Ashby and Tenant, 1991; Tennant and Ashby, 1991; Gold *et al.*, 1993; 1999).

The Salmonella mutagenicity assay was originally developed by Bruce Ames and associates (Ames *et al.*, 1973). In this assay, bacteria cells that are deficient in DNA repair and lack the ability to grow in the absence of histidine are treated with test compounds, with or without a rat liver metabolic system, after which reversion to the histidine- positive phenotype is ascertained.

COLORECTAL CANCER AND ABERRANT CRYPT FOCI

Colorectal cancer is the third most common cancer diagnosed in Americans (American Cancer Society, 2002). The cause of colorectal cancer is still not clear. Besides genetic factors (Vogelstein *et al.*, 1988; Fearon, 1992), evidence from epidemiological and animal studies also supports a relationship between diet and

the risk of colorectal cancer (Giovanucci *et al.*, 1994; Potter, 1996; Slattery *et al.*, 1997). Since the process of carcinogenesis usually takes many years, early-detection and prevention may save many lives and millions of dollars in health care costs.

Aberrant crypt foci (ACF) are good intermediate biomarkers for colon cancer. ACF can be easily recognized by their dilated irregular lumenal opening, thicker epithelial lining and pericrystal zone on the colonic mucosal surface of rodents treated with colon carcinogen (Bird, 1987). ACF has also been described in human colon (Roncucci *et al.*, 1991; Pretlow *et al.*, 1991). ACF are considered putative precursors of colon cancer (Bruce *et al.*, 1993) (Figure 1.1).

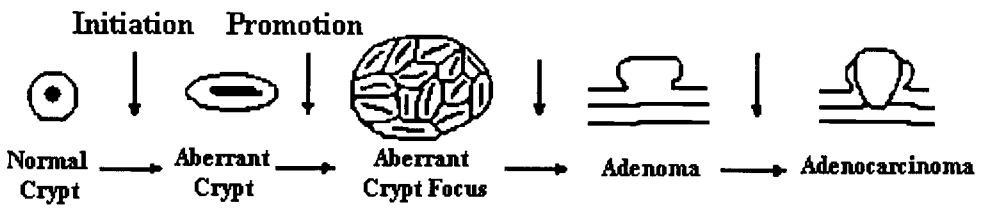

Figure 1.1 A scheme showing the relation between normal crypts, aberrant crypts, aberrant crypt foci, polys and colon cancer. (from Bruce *et al.*, 1993)

The ACF assay provides a short –term test for colon carcinogenesis, and affords a quick and simple way to screen colon carcinogens and anticarcinogens in the animal model. The ACF assay can also be used to identify the mechanisms of carcinogenesis (Bruce, *et al.*, 1993). Anti-carcinogen can also be given to animals before or after carcinogen treatment to test whether the effect is on the initiation or promotion stage of ACF.

Although the induction or promotion of ACF cannot be considered synonymous with the initiation and promotion of colon cancer, the ACF assay is

still a useful assay to screen carcinogenic and chemoprevention agents in the diet (Review by Bruce *et al.*, 1993 and Dashwood, 1999).

DIETARY HETEROCYCLIC AMINES

Heterocyclic amines (HCAs) are group of mutagens and carcinogens formed in cooked meat and fish. They were first found in 1977(Nagao *et al.*, 1977). Currently, over 20 HCAs have been isolated and identified and most of them are highly mutagenic to *Salmonella typhimurim* TA98 when a metabolic system is applied (Sugimura, 1997). Most HCAs are carcinogenic in rodents when given in the diet at 0.01-0.08% and induce cancer in the liver and in other organs (Sugimura, 1997). The amounts of individuals HCAs ingested from food varies (Wakabayashi *et al*, 1992; Augustsson *et al.*, 1997) and might not be sufficient to cause cancer development in man based on a simple calculation of the doses of HCAs required to induced cancer in long term animal experiments at a 50% rate (TD_{50} value) (Sugimura, 1997). However, carcinogenesis is affected by many factors, some HCAs have still been judged by IARC as possible or probable human carcinogens (IARC, 1993).

The mutagenesis or carcinogenesis of HCAs requires metabolic activation. They can be activated by phase I enzyme (hepatic cytochrome P450 (CYP)1A2) to *N*-hydroxylamines, and subsequently *O*-esterified to *N*-acetoxyarylamines, finally forming a nitrenium ion that interacts with DNA to generate covalent adducts, which causes genotoxic damage (Snyderwine *et al.*, 1992). There are also some other phase I and phase II enzymes which can function in the detoxification pathways of HCAs, forming more water soluble and more easily excreted metabolites (Davis *et al.*,1993; Windmill *et al.* 1997; Nowell *et al.*, 1999). Anti-carcinogens that operate as blocking agents during the initiation phase may change the activities of phase I and phase II enzymes in favor of detoxification of HCAs, or bind directly to the procarcinogen or ultimate carcinogen(s). Post-initiation

mechanisms would include all steps that favor increased apoptosis *versus* cell proliferation (Hayashi *et al.*, 1996).

WHEAT GRAINS AND CANCER PREVENTION

Cereals are major staples of the human diet. There is growing evidence that cereals, especially whole grains, play an important role in prevention of cancer (review by Kushi *et al.*, 1999).

As for the mechanism of the protective effect of whole grains, most studies have concentrated on the isolated components in whole grains. Dietary fiber, which is abundant in bran, is inversely associated with colorectal cancer risk in many studies (reviewed by Kushi *et al.*,1999). However, there have been other studies that are more equivocal with respect to this association (Fuchs *et al.*, 1999; Alberts *et al.* 2000).

Besides dietary fiber, whole grains are also rich sources of a wide range of phytochemicals with probable anticarcinogenic properties. Antioxidants, including vitamins, trace minerals, phenolic acids, are also found in the grains (Thompson, 1994). Antioxidants protect cells against oxidative damage, which plays an important role in carcinogenesis. Phenolic acids are also involved in the induction of detoxification systems, specifically phase II conjugation reactions. Ferulic acid, the major phenolic acid of wheat grain, is found in the bran and blocks azoxymethane (AOM)-induced colon carcinogenesis by elevating glutathione S-transferase (GST) activity (Kawabata *et al.*, 2000).

In 1998, Jacobs *et al.* analyzed 40 case-control studies and concluded consumption of whole grain protected against various cancers (Jacobs *et al.*, 1998). Several case-control studies of cancer have also reported on risk associated with intake of refined grains. In contrast to the generally inverse associations seen with whole-grain intake, refined grains tend to be associated with increased risk of colorectal cancer (Bidoli *et al.*, 1992; La Vecchia *et al.*, 1988; Slattery *et al.*, 1997).

But in one prospective study conducted in Japan, consumption of rice and wheat, both of which are usually consumed as refined grains, were inversely associated with risk of colorectal cancer (Hirayama, 1981).

Further research on the health benefits of whole grains, especially focus on the protection mechanism(s) using animal model, is warranted.

In the present study, the effects of unrefined whole wheat, refined wheat, and wheat bran on the mutagenicity of HCAs were assessed using the Salmonella assay, and *in vivo* anti-tumor experiments were conducted by dietary supplement of whole wheat and refined wheat in male F344 rats treated with IQ.

BERRIES AND CANCER PREVENTION

Berries are groups of small fruits that grow in many part of world. Besides their good taste and beautiful color, the health benefit of berries has also been recognized. Blackberries were used medicinally up to the 16th century, the juice being recommended for infections of the mouth and eye (Jennings, 1988). Dietary supplementation with blueberry and strawberry for 8 weeks to 19-month-old Fischer 344 rats was effective in reversing age-related deficits in several neuronal and behavioral parameters. This protection is thought to be due to the high antioxidant activity of berries (Joseph *et al.*, 1999). Supplementation of diets with blueberry also improves eyesight (Kajimoto, 1999) and prevents urinary tract infections (Howell *et al.*, 1998).

Black currant grows wild in northern Europe and North America. Black currant is used primarily in the juice processing industry. The raw juice is diuretic and diaphoretic, and is an excellent beverage for treatment of febrile diseases. The juice can also be boiled to an extract with sugar and used for inflammatory sore throats. Black currant oil is a rich source of gamma linoleic acid, a precursor of prostaglandins, which are involved in many functions.

Although numerous epidemiological studies show that consuming fruits reduces the risk of cancer, there is little epidemiological evidence of berries' anti-cancer properties. There are also not many animal studies on berries' anti-tumor properties. Only one recent study by Wedge showed that the extracts from strawberry and blueberry significantly decreased the growth of cells in two aggressive cervical cancer cell lines (CaSki and SiHa) and two breast cancer cell lines (MCF-7 and T47-D) (Wedge, 2001). Most studies have concentrated on the anti- tumor properties of individual phytochemicals found in the berries and not on the whole fruit.

Anthocyanins, which account for 34% of total polyphenolics in the berries (Moyer *et al.*, 2002), have high antioxidant activity and are thought to be responsible for the anti-inflammatory and anti-aging property of berries (Joseph *et al.*, 1999). There are reports of the antitumor effects of anthocyanins in other foods (Yoshimoto *et al.*, 1999; Deguchi *et al.*, 2000; Hagiwara *et al.*, 2001; Koide *et al.*, 1996; 1997; Kamei *et al.*, 1995), but no data from berries.

The other two important groups are flavonols, represented by quercetin, myricetin; and polyphenolic acids, represented by caffeic, p-coumaric and ferulic acids. Studies have shown that quercetin and myricetin suppressed androgen-independent human prostatic tumor cells (PC-3) proliferation (Knowles *et al.*, 2000). Ferulic and caffeic acids have been found to inhibit chemical induced lung and tongue tumors (Castonguay *et al.*, 1993; Tanaka, *et al*, 1993).

In this preliminary work, the anti-mutagenicity of whole berry extracts was studied using the Salmonella mutagenicity assay. A phase I enzyme assay was conducted to explain the mechanism of antimutagenicity of berries.

CHAPTER 2

A COMPARISON OF WHOLE WHEAT, REFINED WHEAT, AND WHEAT BRAN AS INHIBITORS OF HETEROCYCLIC AMINES IN THE SALMONELLA MUTAGENICITY ASSAY AND IN THE RAT COLONIC ABERRANT CRYPT FOCUS ASSAY

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ABSTRACT

Refined wheat, unrefined whole wheat, and wheat bran were studied for their ability to protect against heterocyclic amines (HCA) *in vitro* and *in vivo*. Wheat bran, which binds HCA *in vitro*, as well as refined wheat and unrefined whole wheat, inhibited the mutagenic activities of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) when they were co-incubated and the supernatant (minus grain) was added to the Salmonella assay. The water-soluble fraction alone from refined and unrefined wheat, but not bran, also inhibited these mutagens *in vitro*. *In vivo*, AIN-93G diets containing refined wheat or unrefined wheat were examined for their ability to inhibit IQ-induced colonic aberrant crypt foci (ACF) in the F344 rat. A slight increase in the number of AC/ACF (aberrant crypts/ACF) was seen after 16 weeks in rats treated post-initiation with refined wheat ($p<0.05$), and fewer foci with 2 or 3 aberrant crypts (ACF-2) were found in rats given unrefined whole wheat *post*-initiation compared with animals treated with the same diet during the initiation phase ($p< 0.05$). There was no significant difference in the profile of IQ urinary metabolites or excretion of promutagens 0-48 hours after carcinogen dosing, and grains had no effect on hepatic cytochrome P4501A1 (CYP1A1), CYP1A2, aryl sulfotransferase, or *N*-acetyltransferase activities; however, a slightly higher UDP-glucuronosyl transferase activity was observed in rats fed unrefined wheat compared with refined wheat diets ($p<0.05$). Thus, despite their antimutagenic activities *in vitro*, only marginal effects were seen with refined and unrefined wheat *in vivo* with respect to hepatic enzyme activities, carcinogen metabolism, and IQ-induced ACF in the rat colon.

Key words: cereal grains, aberrant crypt foci, heterocyclic amines.

INTRODUCTION

Colorectal cancer is the third most common cancer diagnosed in Americans (American Cancer Society, 2000). Evidence from epidemiological and animal studies supports a relationship between diet and the risk of colorectal cancer (Giovanucci *et al.*, 1994; Slattery *et al.*, 1997; Potter, 1996). Cereals, including wheat, rice and corn, are major staples of the human diet. Certain case-control and prospective studies showed that there was a reduced risk for colorectal cancer associated with higher intake of whole grains (Jacobs *et al.*, 1995; Levi *et al.*, 1999). So far, few studies have examined the protective effects of whole grains against cancer development in animal models, although several reports have appeared on the effects of individual component in grains, such as bran. Zoran *et al.* (1997) reported that wheat bran diet reduced the incidence of colon tumors in rats given azoxymethane (AOM), and dietary supplementation with wheat bran also protected against the formation of colonic aberrant crypt foci (ACF) induced by 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) in the rat (Ferguson and Harris, 1996).

Some recent human studies, however, have raised questions about the association between reduced colorectal cancer risk and cereal fiber intake. Results from the Nurses' Health Study did not support the protection by dietary fiber against colorectal cancer or adenoma in nearly 90,000 nurses during 16 years of follow-up (Fuchs *et al.*, 1999). Alberts *et al.* (2000) also showed that a dietary supplement of wheat bran fiber did not protect against recurrent colorectal adenomas in 1303 people who had been diagnosed 5 years previously with one or more colorectal adenomas. One response to these findings has been a call for further research on the health benefits of whole grains, rather than the fiber component alone (Santana-Rios and Dashwood, 1999; Kestell *et al.*,1999; Ferguson and Harris, 2000).

Wheat accounts for one third of the total grain production worldwide (Pedersen *et al.*, 1989). There are three parts to each kernel, namely the endosperm, bran and germ. In the milling process, the bran and germ are separated

from the endosperm, and the starchy endosperm is ground to make flour. Therefore, whole wheat has less concentrated fiber content compared with bran, and refined wheat has the least fiber. It is not clear at present which constituent(s) in whole wheat offer significant protection against cancer. In addition to dietary fiber, which has received considerable attention, other components of whole grains, such as vitamins, minerals and various phytochemicals (tocotrienols, lignans, phytoestrogens, and phenolic compounds) may contribute to the inhibition against endogenous and exogenous carcinogen and mutagens (Thompson, 1994).

Among various carcinogens and mutagens to have been implicated in human colorectal cancer are the heterocyclic amines (HCA) formed in cooked meat and fish (Skog *et al.*, 1998, Sugimura, 1997). Some HCA, such as 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), have been judged by IARC as a possible human carcinogens, and IQ as a probable carcinogen (IARC,1993). The HCA are metabolized by phase I and phase II enzymes in the body; the activation pathway starts with hepatic cytochrome P4501A2 (CYP1A2), catalyzing *N*-hydroxylation of the exocyclic amine group, and subsequently *O*-acetyltransferase forms a nitrenium ion that interacts with DNA to generate covalent adducts. The detoxification pathway involves CYP1A1, which *N*-hydroxylates and also *O*-hydroxylates HCA in various ring positions, the latter pathway facilitating phase II conjugation and elimination of *O*-glucuronide and *O*-sulfate metabolites in the urine and feces (Davis *et al.*,1993; Windmill *et al.* 1997; Nowell *et al.*, 1999). Anti-carcinogens that operate as blocking agents during the initiation phase may change the activities of phase I and phase II enzymes in favor of detoxification of HCA, or bind directly to the procarcinogen or ultimate carcinogen(s). Post-initiation mechanisms would include all steps that favor increased apoptosis *versus* cell proliferation in the colon (Hayashi *et al.*, 1996).

In the male F344 rat, chronic administration of IQ or PhIP induces tumors at multiple sites, including adenocarcinomas of colon (Sugimura, 1997), and short-term treatment leads to the formation of colonic ACF (Tudek *et al.*, 1989; Takahashi *et al.*, 1991; Tachino *et al.*, 1995; Xu *et al.*, 1996; 1999). In the present study, the effects of whole unrefined wheat, refined wheat, and wheat bran on the mutagenicity of HCA were assessed using the Salmonella assay, and *in vivo* experiments were conducted in male F344 rats treated with IQ, including analysis of hepatic enzyme activities, carcinogen metabolism, and induction of colonic ACF.

MATERIALS AND METHODS

Chemicals

MeIQx, IQ, and PhIP were purchased from Toronto Research Chemicals (Ontario, Canada). Postmitochondrial supernatant (S9) from the livers of male Sprague-Dawley rats induced with aroclor 1254 was purchased from MolTox Inc (Boone, NC). Pepsin [EC 3.4.23.1] was purchased from Acros (New Jersey); pancreatin (CAS 8049-47-6) was purchased from Sigma Chemical Co. (St Louis, MO). Other chemicals and reagents were from sources described before (Liew *et al.*, 1995; Guo *et al.*, 1995; Xu *et al.*, 1996; Tachino *et al.*, 1994).

Wheat grains and bran

Refined wheat and unrefined whole wheat, as well as diets containing these cereals, were provided by General Mills Inc. (Minneapolis, MN). For the *in vivo* studies, AIN-93G base diet was modified as shown in Table 2.1. Wheat bran, used in the *in vitro* studies, was purchased from a local market.

Table 2.1 Diet ingredients and nutrient composition (5000g diet)

Diet	Refined wheat	Unrefined wheat	AIN-93G
Diet ingredients			
Flour	Refined soft white wheat flour 3075g	Whole soft white wheat flour 3075g	———
Sucrose	0g	307.4g	500g
Cornstarch	———	———	1987.4g
Dextrinized cornstarch	———	———	660g
Casein	781.7g	704.8g	1000g
Cellulose	372.3g	67.9g	67.9g
L-cysteine	15g	15g	15g
Choline bitartrate	12.5g	12.5g	12.5g
Vitamin mix	50g	50g	50g
Mineral Mix	175g	175g	175g
Corn oil	518.5g	592.4g	350g
Macronutrient content			
Carbohydrate	46.00 %	46.00 %	62.9%
Protein	20.00 %	20.00 %	20.00 %
Fiber	8.00 %	8.00 %	5.00 %
Fat	11.08 %	12.97%	7.00 %

In vitro studies

Treatment protocols

A. Pre-incubation of wheat grains with MeIQx Based on previous studies showing binding of HCA to cereals *in vitro* (Ryden and Robertson, 1997; Ferguson and Harris, 1996; Vikse *et al.,* 1992), wheat grains were incubated with MeIQx (1 μg/ml) in sodium phosphate buffer, pH 7.0, for 1 hour at 37°C. Samples were centrifuged at 12,000 *g* for 5 min, and supernatants were sterilized by passage through a 0.45 μm filter prior to addition to the Salmonella assay (see below). Some experiments compared the effects of grains on MeIQx, IQ and PhIP.

B. Pepsin and pancreatin digestion Wheat grains (1.5 g) or wheat bran (0.5 g) were added to 5 ml 0.02 M HCl solution (pH 1.8) containing 1% pepsin and 15 μg MeIQx. Samples were incubated at 37°C for 1 hour. After centrifugation at 12,000 *g* for 5 min, an aliquot of the supernatant (0.1 ml) was diluted to 0.3 ml with sodium phosphate buffer, pH 7.0, and sterilized by passage through a 0.45 μm filter. The remainder of the pepsin treated solution was neutralized with 0.1 M NaOH and added to 10 ml 1% pancreatin phosphate buffer, pH 7.0. After incubation at 37°C for 3 hours, samples were heated at 65°C to inactive the enzymes and centrifuged before passage through a 0.45 μm filter. Controls containing no enzyme and/or mutagen were included to verify that none of treatments were toxic to Salmonella, see below.

C. Effect of pH Wheat grains (0.5 mg) and MeIQx (1μg/ml) were added to 5 ml sodium phosphate buffer, pH 1.8, 5.5 or 7.0, and incubated at 37°C for 1 hour. The supernatant obtained after centrifugation at 12,000 *g* for 5 min was passed through a 0.45 μm filter and tested in the Salmonella assay.

D. Water-soluble fraction of grains Wheat grains (0.5 g) were added to sodium phosphate buffer pH 7.0 and incubated at 37°C for 1 hour in the absence of mutagen. After centrifugation as before, mutagen was added to the supernatant and the solution obtained by passage through a 0.45 μm filter was tested in the Salmonella assay. The amount of mutagen 'spiked' into this experiment was

equivalent to that from previous iterations of the experiment, in which HCA was mixed with the cereal directly, and an aliquot was subsequently taken for testing in the mutagenicity assay.

Salmonella mutagenicity assays

Mutagenicity tests were conducted using *Salmonella typhimurium* strain TA98, kindly provided by Dr. B.N. Ames (UC Berkeley). In order, 12 µl test sample from the incubation studies containing the equivalent of 12 ng MeIQx, 12 ng IQ, or 400 ng PhIP were added to 2 ml soft agar, followed by 0.2 ml Salmonella strain TA98 and 0.2 ml S9 mix (10% S9, 4 mg protein/ml S9 mix). Minimal glucose plates were incubated at 37°C for 48 hours. The number of His^+ revertant colonies was counted manually. Inhibitor doses reported here were nontoxic, as judged by normal growth of the background lawn and spontaneous reversion rates within the normal range (Maron and Ames, 1983).

***In vivo* studies**

Animals and Treatments

Twenty-eight-day-old weanling male F344 rats were purchased from the National Cancer Institute. Immediately upon arrival, rats were divided randomly into the groups shown in Figure 2.1 and given water and AIN-93G diet *ad libitum*. After 2 weeks, dosing with IQ or vehicle alone (controls) was started; during experiment weeks 3 and 4 each rat received 133.3 mg IQ/kg body weight or test vehicle every other day for 2 weeks by oral gavage. Rats were killed in week 16 and the colons were removed, fixed mucosal side up in 10% phosphate buffered formalin, stained with 0.2% methylene blue, and the ACF were scored as detailed previously (Tachino *et al.*, 1995).

According to the protocol shown in Figure 2.1, additional rats were included for the study of urinary metabolites and hepatic enzyme activities. After 2 weeks on the assigned diets, three rats in each diet group were killed before dosing with IQ. The livers were frozen in liquid nitrogen and stored at –80°C. Microsomal and cytosolic fractions were obtained by differential centrifugation.

Protein concentrations were determined using bovine serum albumin as standard (Lowry *et al.*, 1951).

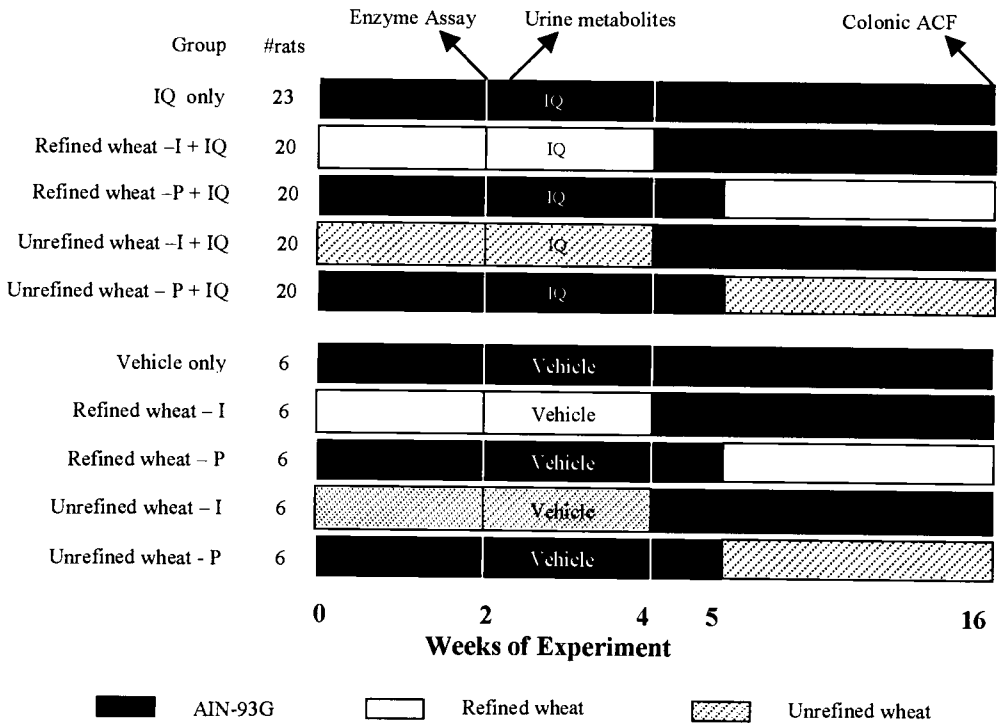

Figure 2. 1 *In vivo* study protocol. Liver enzymes were assessed after 2 weeks on the assigned diets; urinary metabolites were examined for the period 0-48 hours after the first of seven IQ doses (133 mg/kg body wt., each, by oral gavage); ACF were scored after 16 weeks (see Table 2.2). I, treatment during the initiation phase; P, post-initiation treatment.

Phase I enzyme activities

Liver microsomes were compared in assays for 7-ethoxyresorufin *O*-deethylase (EROD) and methoxyresorufin *O*-demethylase (MROD), which are mainly indicative of CYP1A1 and CYP1A2, respectively. A fluorometric assay was used to determine the activities of EROD and MROD (Xu *et al.*, 1997). The product in both assays, resorufin, was quantified by fluorescence using 540 nm excitation and 580 nm emission settings in a Hitachi model F-2500 spectrofluorimeter.

Phase II enzyme activities

UDP-glucuronosyltransferase (UDPGT) activities of the liver microsomes were measured according to the method of Bock *et al.* (1983). Aryl sulfotransferase (AST) activities of the liver cytosols were quantified as described by Matsui and Watanabe (1982). In both UDPGT and AST assays, enzyme activities were calculated from the loss of *p*-nitrophenol substrate, monitored spectrophotometrically at 405 nm. Hepatic *N*-acetyltransferase (NAT) activities were measured using *p*-aminobenzoic acid (PABA) as substrate, according to the method of Andres *et al.* (1985); the final concentrations of acetyl-CoA and PABA were 0.44 and 0.2 mM, respectively.

Rat urine metabolites

Urine samples collected for the period 0-48 hours after IQ dosing were centrifuged at 14,000 *g* for 5 min and the equivalent of 0.1 µl of the supernatant was tested in the Salmonella assay. The conditions of the mutagenicity assays were as described before (Dashwood and Liew, 1992), using Salmonella strain TA98 and rat liver S9 for metabolic activation. Urinary metabolites of IQ were identified by high-pressure liquid chromatography (HPLC), based on previously reported mobile phase and gradient conditions (Turesky *et al.*, 1986), but using a Shimadzu *VP* series instrument (autoinjector, UV-VIS detector, tandem pumps, and a system

controller) equipped with a 25 cm x 4.6 mm, 5 μm particle size, reverse-phase column (Supelcosil™ LC-18).

Statistical analysis

Results were expressed as mean ± SE within a group. Data were analyzed by ANOVA (Waller-Duncan K-ratio *t*-test) using the SAS statistical package, with significant difference considered at $p<0.05$ level.

RESULTS

Figure 2.2 shows results from experiments *in vitro* using different quantities of wheat grains in preincubation assays with MeIQx. When the supernatants (minus grain) were tested in the Salmonella assay, all of the grains inhibited the mutagenicity of MeIQx significantly. Refined wheat and unrefined whole wheat had a comparable inhibitory effect, with maximal inhibition of around 80% at the highest concentration tested in the *in vitro* preincubation assay (0.3 g/ml). Bran was more effective in these experiments, and produced close to 80% inhibition at a concentration of only 0.1 g/ml in the *in vitro* preincubation (Figure 2.2, open symbols).

Because cereals pass through various pH conditions along the length of the GI tract, the extent of inhibition was next examined for grains (0.1g/ml) pre-incubated with MeIQx at pH 1.8, 5.5, or 7.0 (Figure 2.3). In incubations at pH 1.8, there was no significant inhibition by refined wheat, moderate inhibition by unrefined whole wheat, and close to maximal inhibition by wheat bran. Inhibition by refined wheat and unrefined whole wheat improved as the pH was increased to 5.5 and 7.0, whereas inhibition by bran was unaffected by pH. The latter results were surprising since previous reports indicated that the binding of MeIQx to bran was maximal at pH 5.5 (Ryden and Robertson, 1996); the data in Figure 2.3 suggested that inhibition might be facilitated beyond the acid conditions of the stomach, such as in the small intestine and large bowel, but the potential role of digestive enzymes required additional study.

Thus, wheat grains were treated with pepsin and pancreatin *in vitro* and preincubated with MeIQx in order to determine whether enzyme digestion might alter the extent of inhibition in the Salmonella assay. Figure 2.4 shows that the overall extent of inhibition in groups treated with pepsin was in the range 48% to 68%, with no significant differences according to grain type or size (fine, medium, course). In the various test groups treated with pancreatin, inhibition was in the range 26% to 42%; only the wheat bran group was higher, statistically, *versus* the

MeIQx group ($p < 0.05$). Thus, the results indicated that enzymatic digestion did not further enhance the antimutagenic effects of cereals against MeIQx, and indeed may have attenuated the inhibitory activities under the experimental conditions used.

Figure 2.5 shows the antimutagenic effect of wheat grains and the water-soluble fractions (minus grains) when incubated with MeIQx, IQ and PhIP. In general, the inhibitory effects seen in Figure 2.2 were reproduced for all three mutagens, such that refined wheat and unrefined wheat were essentially equally potent, and wheat bran was the most effective (Fig. 2.5a). Figure 2.5b shows the contribution of the water-soluble fraction alone (minus cereals) in the antimutagenic activity. For the refined and unrefined cereals, but not bran, significant inhibition was detected against all three mutagens, despite the fact that the experiment, by design, avoided direct mutagen-cereal interaction.

When the results in Fig. 2.5b are subtracted from those in Fig. 2.5a for the 'complete' system, namely fiber fraction plus water-soluble fraction, it is evident that mutagen-cereal binding cannot account entirely for the inhibition of mutagenicity. Indeed, the results suggested that antimutagens released from refined and unrefined wheat contributed to the inhibitory mechanism(s) against MeIQx, PhIP, and IQ, and might thus be important *in vivo*.

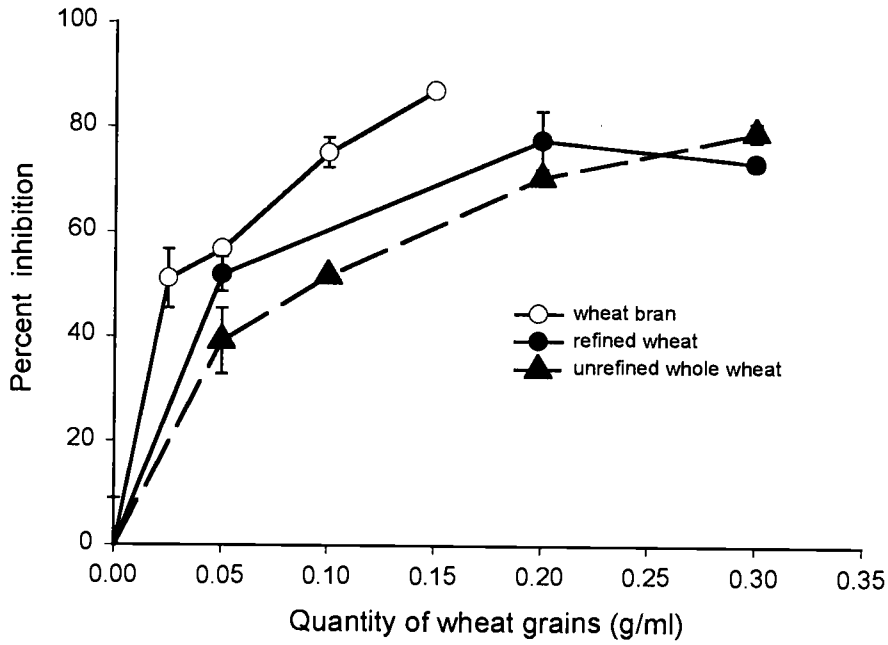

Figure 2.2 Inhibition of MeIQx mutagenesis by wheat grains. Grains were pre-incubated with MeIQx (1μg/ml) for 1 hr at 37°C and 12 μl of the supernatant (minus grains) was added to the Salmonella assay. Results are given as percent inhibition of mutagenicity (mean ± SE) using Salmonella strain TA98+S9, with triplicate plates for each dose tested. Percent inhibition was calculated thus:

$$\text{Inhibition (\%)} = 100 - \frac{\text{Induced revertants/plate in presence of grains}}{\text{Induced revertants/plate in absence of grains}} \times 100$$

Spontaneous revertant counts (mean±SE, n=3) were as follows: water control: 55±1.4; sodium phosphate buffer (no cereal): 63±4.7; refined wheat: 49±4.6; unrefined whole wheat: 69±7.5; wheat bran: 56±2.0. Positive control (12 ng MeIQx/plate): 607±49.0 (mean±SE, n=3).

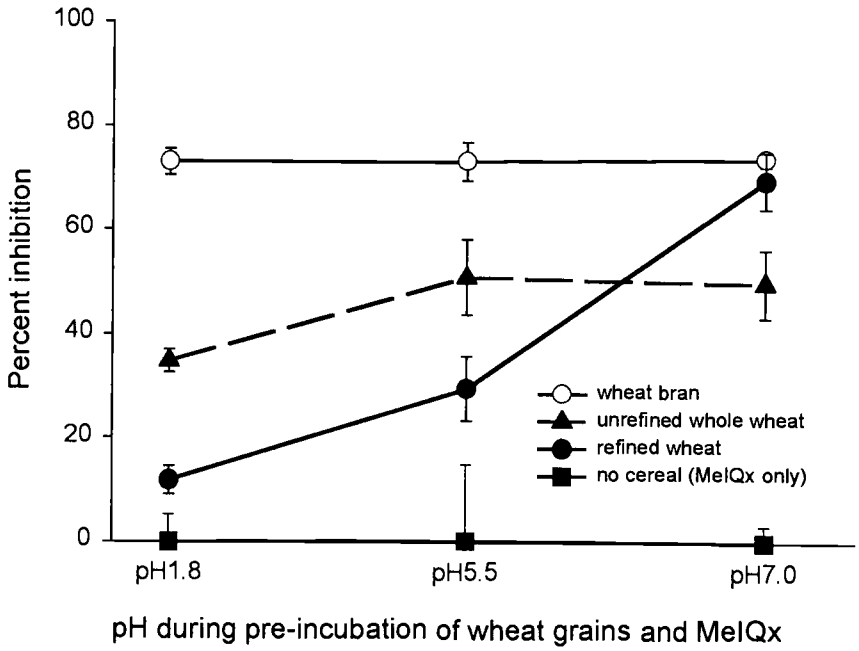

Figure 2. 3 Effect of pH on antimutagenic activities of cereals. Results (mean±SE, *n*=3) are given as percent inhibition of mutagenicity, calculated as described in Figure 2.2 legend. Negative controls (minus mutagen, in the presence and absence of grains) and positive controls containing MeIQx but no cereal were included in the pre-incubation assays, at each pH; results were in the range 82±4.1(mean±SE, *n*=12) and 664±37.9 (mean±SE, *n*=3), respectively.

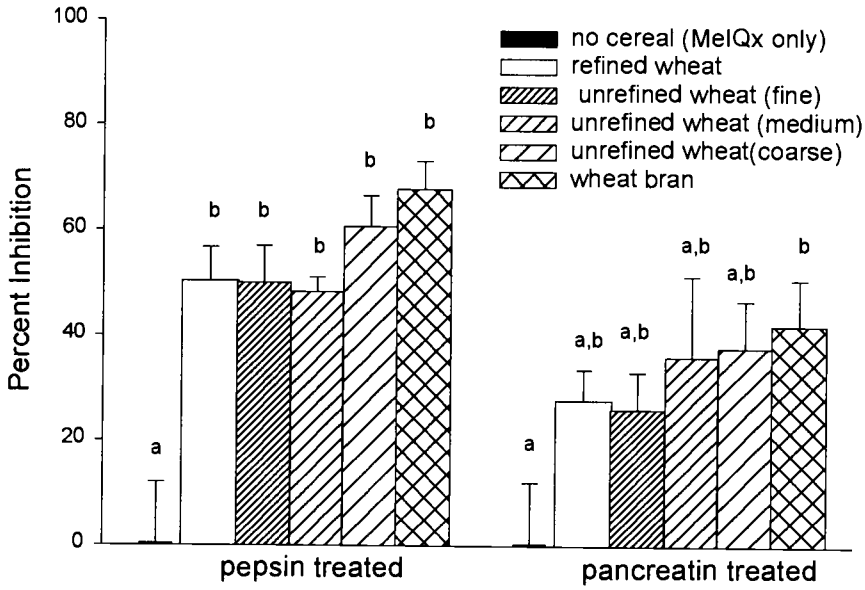

Figure 2. 4 Enzymatic digestion of wheat grains *in vitro*. Results (mean±SE, n=3) are given as percent inhibition of mutagenicity, as described in Fig. 2.2 legend. In the experiment shown, revertant counts for negative controls (minus mutagen) and positive controls were 88±15.3 (mean±SE, n=3) and 348±41.6 (mean±SE, n=3), respectively. Statistical analysis was applied separately to the groups treated with pepsin and the groups treated with pancreatin; bars with different letters were significantly different (p<0.05, ANOVA).

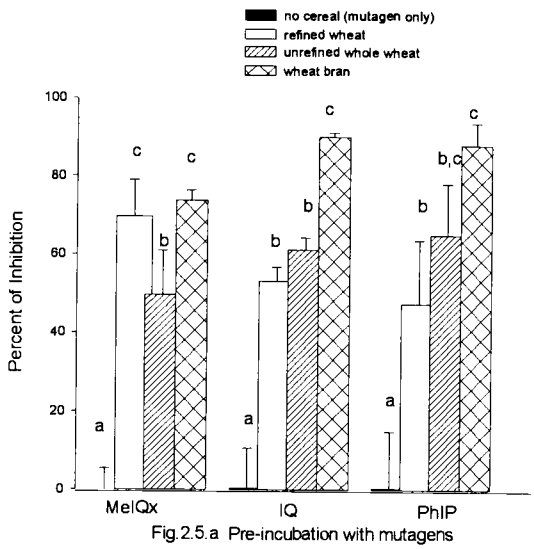

Fig.2.5.a Pre-incubation with mutagens

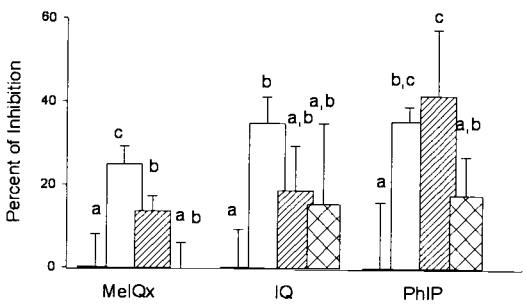

Fig.2.5.b Wheat grains leach out solution with mutagens

Figure 2. 5 Inhibition by cereal grains and their water-soluble eluates against HCA. (a) grains were incubated with mutagen, or (b) grains were incubated in the absence of mutagen, and the supernatant was mixed with mutagen, and an aliquot (minus grain) was added to the Salmonella assay. Results (mean±SE, n=3) are given as percent inhibition of mutagenicity, calculated as described in Fig. 2.2 legend. Negative controls (minus mutagen, in the presence or absence of various grains) were in the range 63±1.0 revertants/plate (mean±SE, n=5). Positive controls were as follows: 664±37.9 revertants/plate (mean±SE, n=3, 12 ng MeIQx/plate), 1545±90.0 revertants/plate (mean±SE, n=3, 12 ng IQ/plate), and 351±24.0 revertants/plate (mean±SE, n=3, 400 ng PhIP/plate). Within the data-set for each mutagen, bars with different letters were significantly different (p<0.05, ANOVA).

In vivo, no significant differences were detected among the various groups with respect to food and water intake or body weight gain throughout the 16-week study (data not shown). Table 2.2 and Figure2.6 show the ACF results. There was no difference in ACF incidence, ACF/colon, and AC/colon among all treatments. The only result in Table 2.2 that was significantly different was in the group post-treated with refined wheat, in which the size of ACF, as measured by average number of aberrant crypts per ACF, was increased compared with all other groups ($p<0.05$, see final column). When ACF were classified according to size category, as before (Tachino *et al.*, 1995), no differences were seen among treatments (Figure 2.6). The only exception was among ACF-2, which contain two or three aberrant crypts per focus; thus, rats given whole (unrefined) wheat diet during the post-initiation phase had fewer ACF-2 than rats given the same diet before and during carcinogen treatment ($p< 0.05$). These results suggested weak effects, at best, by whole wheat and refined wheat diets against IQ-induced ACF. However, more pronounced modulation with longer feeding cannot be ruled out.

Table 2.2 Effect of cereal-containing diets on IQ-induced ACF

Diet	ACF incidence*	ACF/colon	AC/colon	AC/ACF
AIN-93G	**13/13[a]**	**5.3 ± 0.75[a]**	**13.5 ± 2.46[a]**	**2.5 ± 0.28[b]**
Refined wheat-I	**14/16[a]**	**4.8 ± 0.72[a]**	**12.5 ± 1.89[a]**	**2.5 ± 0.17[b]**
Refined wheat-P	**14/14[a]**	**4.5 ± 0.67[a]**	**14.7 ± 1.90[a]**	**3.5 ± 0.21[a]**
Unrefined wheat-I	**14/14[a]**	**4.6 ± 0.68[a]**	**11.3 ± 1.57[a]**	**2.6 ± 0.20[b]**
Unrefined wheat-P	**13/14[a]**	**3.3 ± 0.58[a]**	**8.6 ± 2.17[a]**	**2.2 ± 0.25[b]**

ACF were scored in week 16. In the final 3 columns, data are given as means±SE; values with different superscripts differ significantly ($p< 0.05$, by ANOVA). ACF, aberrant crypt foci; AC, aberrant crypts. *No. rats with ACF/total rats per group. I = treatment during the initiation phase, P = post-initiation treatment, see Figure 2.1.

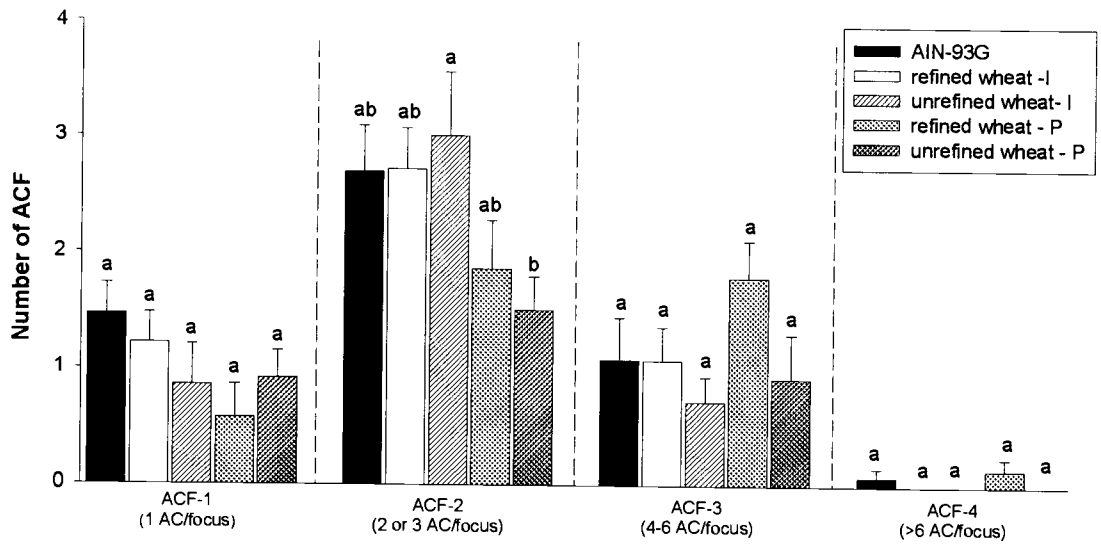

Figure 2. 6 Effect of Cereal Diets on IQ-induced ACF size category. Results are given as number within each size category (mean ± SE, n= 14-16; bars with different letters were significantly different (p<0.05, ANOVA). I, treatment during the initiation phase; P, post-initiation treatments with cereals (see Table 2.2 for further details).

Table 2.3 shows results for the phase I and phase II enzymes examined. Refined and unrefined wheat diets fed to rats for 2 weeks had no significant effect on CYP1A1 and CYP1A2 activities, as determined by EROD and MROD assays, respectively. Also, no significant differences were seen among test groups for AST or NAT activities. The activity of UDPGT was significantly higher in rats given whole wheat *versus* refined wheat ($p<0.05$, Table 2.3), but neither group was different from the control group given AIN-93G diet alone.

Table 2.3 Effect of diet on hepatic enzyme activities (nmol/min/mg protein)

Enzyme	AIN-93G	Refined wheat	Unrefined wheat
EROD	0.43 ± 0.10^{a}	0.29 ± 0.03^{a}	0.27 ± 0.021^{a}
MROD	0.19 ± 0.018^{a}	0.13 ± 0.016^{a}	0.16 ± 0.036^{a}
UDPGT	12.67 ± 1.08^{ab}	9.85 ± 2.11^{b}	16.17 ± 0.90^{a}
AST	0.43 ± 0.034^{a}	0.47 ± 0.037^{a}	0.51 ± 0.025^{a}
NAT	0.83 ± 0.054^{a}	0.79 ± 0.064^{a}	1.01 ± 0.138^{a}

*Data are mean±SE, $n=3$ from triplicate determinations: different superscripts indicate statistically significant difference ($p< 0.05$ by ANOVA). EROD, 7-ethoxyresorufin *O*-deethylase; MROD, methoxyresorufin *O*-demethylase; UDPGT, UDP-glucuronosyl-transferase; AST, Aryl sulfotransferase; NAT, *N*-acetyltransferase.

Figure 2.7a shows a representative HPLC profile of urinary metabolites excreted for the period 0-48 hours after IQ dosing. Only a small amount of parent compound, IQ, was detected in the urine, but several metabolites were observed. Based on our previous studies (Xu *et al.*, 1996) and data of Kestell *et al.* (1999), the major peaks were tentatively identified as follows: unknown (M1); IQ-5-*O*-glucuronide (M2); IQ-*N*-sulfamate (M3), IQ-5-*O*-sulfate (M4). Integration of these peaks showed that there was no effect of the test diets on the metabolism of IQ, compared with rats fed AIN-93G (Fig. 2.7b), and the excretion of promutagens

within each group of rats was not altered significantly by feeding refined or unrefined diets *versus* AIN-93G (Table 2.4).

Table 2.4 Rat urine mutagenicity data and the effect of cereal-containing diets

Revertants/Plate*	AIN-93G	Refined wheat	Unrefined wheat
Rat No. 1	$942 \pm 86.5^{d,e}$	$1192 \pm 64.6^{c,d}$	580 ± 85.4^{e}
Rat No. 2	1962 ± 28.1^{a}	$1364 \pm 77.4^{b,c}$	1508 ± 277.7^{b}
Rat No. 3	$1135 \pm 180.1^{c,d}$	$862 \pm 113.9^{d,e}$	687 ± 37.7^{e}
Per group of rats	1346 ± 312.7^{A}	1139 ± 147.3^{A}	925 ± 293.3^{A}

*0.1µl of urine from each rat was tested in triplicate for mutagenic activity using *Salmonella typhimurium* strain TA98+S9. The data were corrected for exact dose of IQ given and the volume of urine collected from each rat. Data, means±SE, n=3; different superscripts indicate statistically significant difference ($p< 0.05$ by ANOVA).

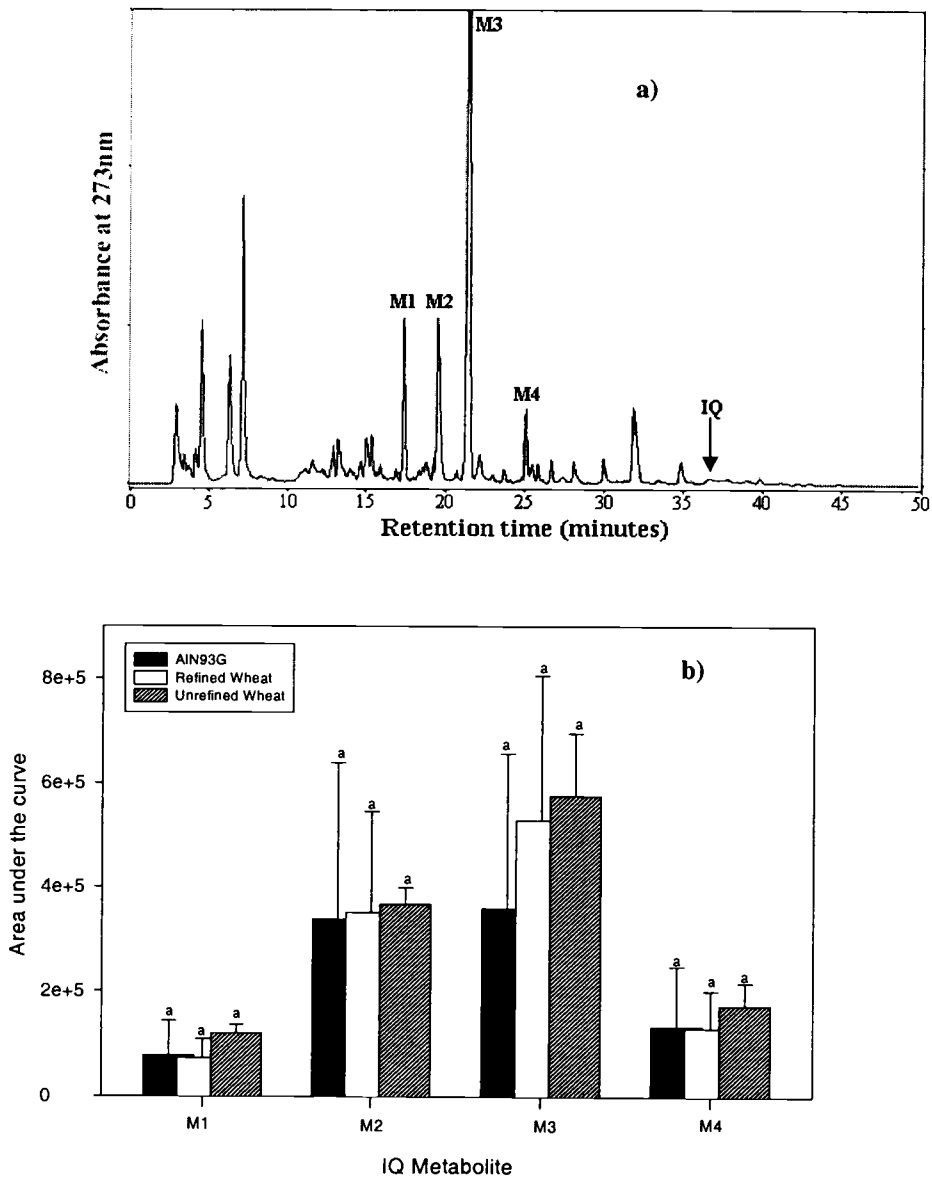

Figure 2.7 Effect of cereal diets on the excretion of IQ metabolites in the urine. a) Representative high-pressure liquid chromatography (HPLC) separation of IQ-urinary metabolites, for urine collected 0-48 hours after a single oral administration of IQ in rats fed AIN-93G diet (see Fig. 2.1). Similar profiles were seen for rats fed diets containing refined wheat or unrefined wheat (not shown). Major IQ metabolites were as follows: M1, unknown; M2, IQ-5-*O*-glucuronide; M3, IQ-*N*-sulfamate; M4, IQ-5-*O*-sulfate. b) Area under the curve data for IQ metabolites M1 – M4 in each treatment group (mean±SE, n=3). For each metabolite, no significant differences were detected among the various treatment groups (statistical analysis by ANOVA).

DISCUSSION

Several mechanisms have been proposed to explain the reported chemopreventive effects of cereals and dietary fiber in the colon, including the binding of bile acids, the decreased fecal transit time, the increased bulk of intestinal contents, and the release of fermentation products as mediators of apoptosis in colonic mucosa cells (reviewed in Santana-Rios and Dashwood, 1999). However, in this study, the initial hypothesis was that unrefined wheat and refined wheat would bind mutagens *in vitro* and 'intercept' such compounds in the GI tract *in vivo*. As a corollary, enzyme digestion was anticipated to influence mutagen/cereal interactions by increasing or decreasing the availability of putative binding sites. According to the binding hypothesis, increasing amounts of cereal would interact with greater levels of mutagen, and the free (unbound) fraction should exhibit correspondingly reduced mutagenic activity in the Salmonella assay. Results from this study supported such a mechanism for bran, in agreement with published data (Ryden and Robertson, 1997; Ferguson and Harris, 1996; Vikse *et al.*, 1992), but direct cereal/mutagen binding accounted only in part for the inhibition by whole and refined wheat against HCA *in vitro*. Indeed, when refined and unrefined wheat were incubated under aqueous conditions and the supernatant alone was mixed with HCA, inhibition on the order of 20%-40% was detected in the Salmonella assay. This corresponded to about half of the maximum inhibition of the entire system (aqueous fraction plus cereal fraction), and suggested that one or more constituents of the grains may be released into solution to act as antimutagens. We did not attempt to isolate and characterize these putative inhibitors in the present study. In the case of unrefined wheat, one might speculate that phenolics or other phytochemicals could be involved (see Introduction), either as inhibitors of the enzymes in S9 or by affecting the stability of activated mutagens. In the case of refined wheat, which in the present study was of the unsupplemented variety (no vitamins or other compounds added), the nature of the inhibitors remains unclear; during the milling process, refined wheat often includes

not only the starchy endosperm but also the aleuron layer, and the latter contains phytochemicals with demonstrated antioxidant activity *in vitro* (Miller *et al.*, 2000). It remains to be determined whether such compounds exhibit additional protective mechanisms, such as inhibition of carcinogen activation, scavenging of electrophilic intermediates of carcinogens, or complexing with carcinogens as 'interceptor molecules' (Hartman and Shankel, 1990).

In previous studies with interceptor molecules such as chlorophyllin and chlorophyll, the protocols used *in vivo* typically allowed for maximum interaction between the mutagen and inhibitor, either by co-gavage administration or via simultaneous dosing in the diet (Guo *et al.*, 1995; Brienholt *et al.*, 1995; Hasegawa *et al.*, 1995; Harttig and Bailey, 1998; Reddy *et al.*, 1999). However, based on preliminary data from the *in vitro* studies, the rodent protocol used here, by design, did not focus on direct mutagen/cereal interaction. Indeed, the hypothesis was that phytochemicals and other constituents released from whole wheat and refined wheat *in vivo* should operate independently of mutagen binding in the GI tract. In the *in vivo* experiments, only marginal effects were seen, at best, on IQ-induced ACF, the excretion of IQ urinary metabolites, and the activities of hepatic enzymes, indicating that the *in vitro* antimutagenic properties of refined and unrefined wheat did not translate to the whole animal situation. In terms of the blocking protocol, it might be argued that a 2-week treatment prior to IQ dosing might not be of sufficient time to allow for significant changes to have occurred in the levels or activities of carcinogen metabolizing enzymes. During the carcinogen treatment period, IQ dosing may have altered the expression of hepatic enzymes significantly, as reported previously in rats given AIN-76A, AIN-93G, and chow diets (Xu *et al.*, 1999), possibly obscuring any changes in enzyme activities associated with the dietary cereal intake. The present study did not examine changes in phase I or phase II metabolizing enzymes in the colonic mucosa (Lin *et al.*, 1994, Malfatti *et al.*, 1996), but such changes are not expected in light of the fact that the profile of

IQ urinary metabolites excreted urine 0-48 hours after a single dose of carcinogen remained unaffected by the test diets.

The duration of post-initiation treatment with refined and unrefined wheat diets, namely 11 weeks (Fig. 2.1), should have been sufficient to test whether phytochemicals or other constituents released from the cereals were capable of decreasing the overall incidence or multiplicity of IQ-induced ACF. No such inhibition was observed, although longer treatment might provide evidence for greater protection. The initial study design included terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) and bromodeoxyuridine (BrdU)-immunohistochemistry analyses (not shown); however, since there was no marked inhibitory effect of the cereals *post*-initiation in the ACF assay, we elected not to test for possible changes in apoptosis or cell proliferation in the colonic mucosa.

One important question, however, concerns the relative levels of the cereals and mutagens *in vitro* and *in vivo*, and whether these ratios correspond with typical human intakes. In several of the *in vitro* preincubation assays, 0.1 g/ml cereal grain was incubated with 1 μg/ml MeIQx or IQ. *In vivo*, the grains were 'diluted' by mixing with other components of the diet; typically, a 200 g rat consuming 15 g of diet ingested 9.225 g wheat per day (3075 g wheat flour/5000 g diet, see Table 2.1), and in weeks 3 and 4 this occurred during dosing with 26.6 mg IQ (133 mg/kg body wt by oral gavage). According to Clydesdale (1994), a person aged between 19 and 64 consumes an average of 122.54 g total grain per day, whereas the consumption of all HCA may be on the order of 1.8 μg/ person/day (Augustsson *et al.*, 1997). Based on these numbers, the corresponding weight ratios of grain:HCA would be approximately 67,000,000:1 in humans, 10,000:1 in the *in vitro* incubation experiments, and only 350:1 in the *in vivo* ACF studies. Thus, assuming that the weight ratio of grain to mutagen is important in the inhibitory mechanism, it is perhaps not surprising that marginal changes only were detected in the *in vivo* study.

If the prior assumption is correct, then one might speculate that cereals would exhibit greater inhibitory activity *in vivo* when the ratio of wheat to mutagen is increased in favor of the cereal. A limitation of many studies conducted *in vivo* is the necessity of using large doses of the initiating agent and test inhibitor, typically in a small 'population' of animals, in order to detect measurable changes in the end-points under investigation. This most likely mimics the human situation rather poorly, in which large numbers of individuals may be exposed chronically to dietary carcinogens and inhibitors at lower levels throughout the entire period of life. One possible approach would be to isolate and purify the individual antimutagen(s) released under aqueous conditions from the cereal grains and to test these compounds *in vivo* against human relevant doses of HCA (Malfatti *et al.*, 1999). Under these conditions, the weight ratio of cereal to mutagen would be augmented significantly and one might detect enhanced protection against HCA *in vivo*, as evidenced by reduced HCA-DNA adducts and increased excretion of detoxified HCA metabolites. Further studies of whole cereals and their constituents are now in progress.

Acknowledgements

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CHAPTER 3

ANTIMUTAGENIC POTENCY OF CRANDALL BLACK CURRANT (*RIBES AUREUM*) IN THE SALMONELLA MUTAGENICITY ASSAY AND ENZYME INHIBITION, A PRELIMINARY STUDY

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ABSTRACT

The fresh juice and extract of crandall blackcurrant (*Ribes aureum*) were not diectly mutagenic in the Salmonella mutagenicity assay. Berry extract or fresh juice at levels to 50 µl (22 mg berry) in a 500 µl pre-incubation system significantly inhibited mutagenicity of 2-amino-3-methylimidazo [4, 5-*f*] quinoline (IQ), a mutagen from cooked meat, by 32% when a liver S9 metabolism system was present ($p<0.05$). One hundred µl of crandall black currant extract gave 89% inhibition of IQ mutagenicity. However, the mutagenicity of 2- hydroxyamino-3-methylimidazo[4,5-*f*] quinoline (*N*-hydroxy-IQ), a direct-acting metabolite of IQ, was not affected. An *in vitro* fluorometric assay showed the activity of cytochrome P 450 (CYP) 1A1 and CYP 1A2 was decreased. Inhibition of CYP 1A2 activity may be an important mechanism of antimutagenicity of crandall black currant extract. Similar results were also observed with other berry samples.

Key words: crandall black currant, berries, Salmonella mutagenicity assay, IQ, CYP1A1, CYP1A2.

INTRODUCTION

Cancer is the second leading cause of death in the United States. Some epidemiological studies suggest that consuming vegetables and fruits is associated with reducing risk of cancer and other chronic diseases (Doll, 1990; Block, *et al.*, 1992; Willett, 1994). Berries are groups of small fruits that have been widely consumed in the world. The health benefit of berries is also recognized (Jennings, 1988; Joseph, *et al.*, 1999; Howell *et al.*, 1998). However, there is no epidemiological evidence that berries are effective in cancer prevention and few animal studies have been done. Most studies have concentrated on the anti- tumor properties of individual phytochemicals appeared in the berries (Alldrick, *et al.*, 1986; Castonguay *et al.*, 1998).

IQ is one of a group of amino acid pyrolysis products or heterocyclic amines found in cooked meat and fish (reviewed by Sugimura, 1997) and has been judged by IARC as a probably human carcinogen (IARC, 1993). IQ is highly mutagenic to *Salmonella typhimurum* TA98 in the presence of a liver S9 bioactivation system. IQ also causes multi-organ tumors in rodents (review by Sugimura, 1997).

The mutagenicity of IQ requires metabolic activation. IQ is oxygenated by hepatic cytochrome P450 (CYP) 1A2 in the liver to form *N*-hydroxy-IQ. *N*-hydroxy-IQ is conjugated by *O*-acetyltransferase (which is present in *Salmonella typhimurium* TA98 bacteria cytosol) to form *N*-acetoxy-IQ, which is spontaneous degraded to form a nitrenium ion capable of binding DNA to generate covalent adducts to initiate mutagenicity (Nagao *et al.*, 1983; Yamazoe *et al.*, 1989; Snyderwine *et al.*, 1992). IQ may also be detoxified by CYP1A1, which *N*-hydroxylates, but also *O*-hydroxylates IQ in various ring positions, the latter pathway facilitating phase II conjugation and elimination of *O*-glucuronide and *O*-sulfate metabolites in the urine and feces (Davis *et al.*, 1993; Windmill *et al.* 1997; Nowell *et al.*, 1999).

In this study, crandall black currant (*Ribes aureum)* was selected for the Salmonella mutagenicity assay to examine its anti- mutagenicity potency with IQ. An *in vitro* phase I enzyme assay was used to examine the mechanism of action.

MATERIALS AND METHODS

Chemicals

IQ was purchased from Toronto Research Chemicals (Ontario, Canada). *N*-Hydroxy-IQ was obtained from the National Cancer Institute Carcinogen Repository (Kansas City, NI), Postmitochondrial supernatant (S9) from the livers of male Sprague-Dawley rats induced with aroclor 1254 was purchased from MolTox Inc. (Boone, NC). Methoxyresorufin was from Molecular probes Inc. (Eugene, OR). Ethoxyresorufin and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Berry samples

Berry samples were kindly provided by Dr. Richard Moyer, visiting sabbatical Professor from King College, Bristol, Tennessee, working with Dr. Ronald Wrolstad, Distinguished Professor of Food Science and Technology, Oregon State University. Briefly, ripe fruit samples were placed immediately on ice in the field and frozen at -10 °C later that same day. Samples were prepared according to Rodriguez-Saona and Wrolstad (2001). The frozen fruits were further cooled in liquid nitrogen, then they were cryogenically milled to powder and weighed, and then the powder was extracted with acetone, followed by two additional extractions with 70:30 acetone/water. The pooled supernatants were partitioned with two volumes of chloroform and the aqueous extracts were stored at -70 °C for further analysis (Moyer *et al.*, 2002). The abbreviations of berries used here and amount of berry contained in a 50 µl extract are:

Blueberry:

G344: Cultivated highbush blueberry (*Vaccininium corymbosum*); 35 mg

Vac 45: Wild highbush blueberry; 26 mg

Blackberry:

Rubus 10: hybrid blackberry ORUS 1452-1; 25 mg

Rubus 35: hybrid ORUS 1369-3; 34 mg

Black currant:

Titiaia: Black currant (*Ribes nigrum*); 22 mg

Crandall: American Clove currant (*Ribes aureum*); 22 mg

Salmonella assays for berry antimutagenic activity

Mutagenicity tests were conducted using *Salmonella typhimurium* strain TA98. In order, sterile H_2O or berry samples (up to 100µl), 8 ng IQ or 4 ng *N*-OH-IQ, 0.2 ml Salmonella strain TA98 and 0.2 ml S9 mix (10% S9, 4 mg protein/ml S9 mix, when using IQ as mutagen) were added and then pre-incubated at 37°C for 20 minutes in a test tube, followed by the addition of 2 ml soft agar and pouring onto minimal glucose plates. The number of His^+ revertant colonies was counted manually after 48 hours incubation at 37°C in the dark (Maron and Ames, 1983).

Phase I enzyme assay in vitro

Berry extracts were co-incubated with S9 first to conduct 7-ethoxyresorufin *O*-deethylase (EROD) and methoxyresorufin *O*-demethylase (MROD) assays, which are mainly indicative of CYP1A1 and CYP1A2, respectively. The amount of S9 and berry extracts were at the same proportions as in the Salmonella mutagenicity assay. A fluorometric assay was used to determine the activities of EROD and MROD (Xu *et al.*, 1997). The product in both assays, resorufin, was quantified by fluorescence using 540 nm excitation and 580 nm emission settings in a Hitachi model F-2500 spectrofluorimeter.

Statistical analysis

Results were expressed as mean ± SE within a group. Data were analyzed by ANOVA (Waller-Duncan K-ratio *t*-test) using the SAS statistical package, with significant difference considered at $p<0.05$ level.

RESULTS

Mutagenicity and toxicity of berry extracts in the Salmonella mutagenicity assay

The extraction method used in this experiment maintains the maximal amount of anthocyanins, total phenolics and antioxidant capacity in the berry samples (Moyer *et al.*, 2002). The extraction procedure didn't cause any mutagenicity and toxicity in the Salmonella mutagenicity assay (data not shown). The spontaneous revertants per plate was 98 ± 3.4 (mean ± SE, n= 9, when S9 mix is present). Crandall black currant extract showed a similar effect on the TA 98 strains compared with same amount of fresh berry juice, as did the trend of inhibition effect on the IQ induced mutagenicity (Figure 3.1). Thus, berry extracts were used throughout the experiment.

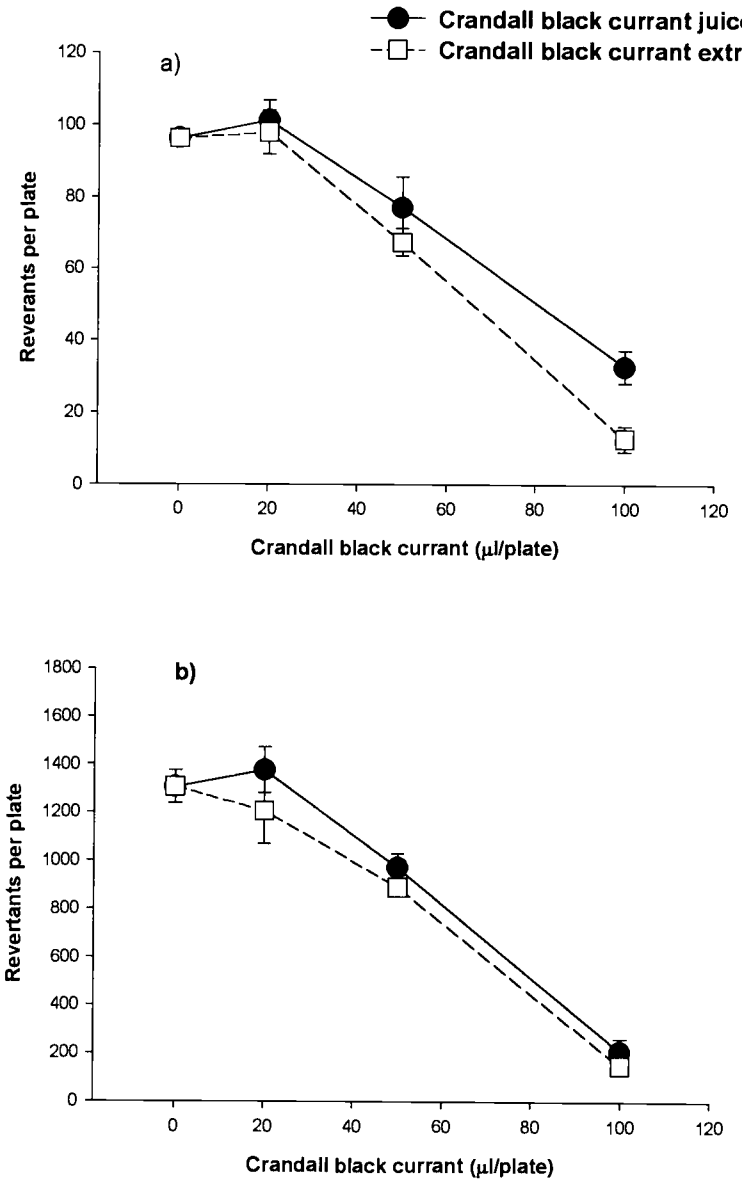

Figure 3.1 **Mutagenicity and antimutagenicity of crandall black currant juice and extract**. a) Mutagenicity of crandall black currant juice and extract. *Salmonella typhimurium* strain TA98 was pre-incubated with berry samples 20 min in the presence of a metabolic activation system (S9 mix), prior to the addition of soft agar. Data are given as means ± SE from the testing of triplicate plate. b) Antimutagenic activity of crandall black currant juice and extract towards IQ. *Salmonella typhimurium* strain TA98 was pre-incubated with IQ (8 ng/plate) plus berry samples 20 min in the presence of a metabolic activation system (S9 mix), prior to the addition of soft agar. Data are given as means ± SE from the testing of triplicate plate.

Figure 3.2 showed the result of mutagenicity of berries when the incubation system is only berry extract and TA 98 bacteria (i.e., no metabolite system (S9 mix)). The spontaneous revertant number is 19± 1.4 (mean ± SE, n= 3). For crandall black currant, two blueberry samples and one blackberry sample, the revertants per plate was around 12-31, average 22 ± 0.9 (mean ± SE, n= 34). For crandall blackcurrant alone, the number is 20 ± 2 (mean ± SE, n= 8). Berry extracts concentration reported here were nontoxic, as judged by normal growth of the background lawn and spontaneous reversion rates within the normal range (Maron and Ames, 1983). There was also no mutagenicity of berry extracts up to 100 μl per plate.

When a S9 metabolism component is supplied, the spontaneous revertants per plate is increased to 96± 2.7 (mean ± SE, n= 14). Figure 3.3 shows the revertants per plate of two blackberry extracts, two blueberry extracts, two black currant extracts (blackberry rubus 35, blackberry rubus 10, blueberry Vac 45, blueberry G344, titania black currant, crandall black currant). The revertants per plate decreased with increasing concentrations of all extracts. Berries were not mutagens in the Salmonella assay even in the presence of S9 enzymes.

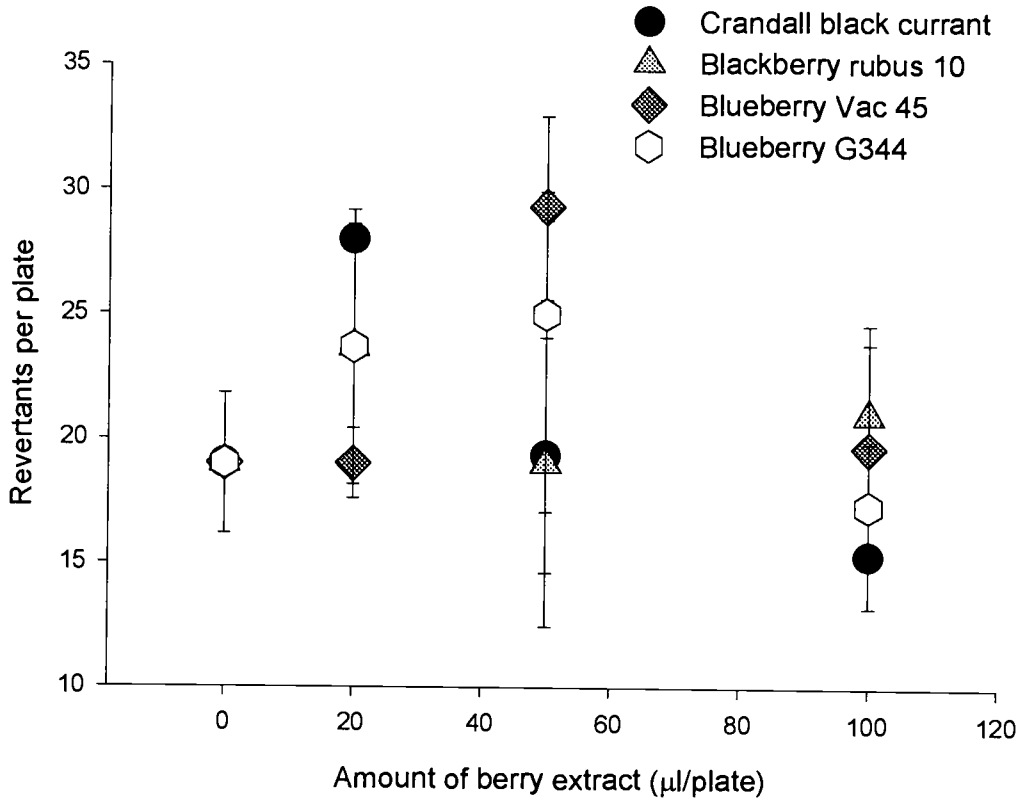

Figure 3.2 **Mutagenicity of berry extracts in the absence of S9.** *Salmonella typhimurium* strain TA98 was pre-incubated with berry extracts 20 min in the absence of a metabolic activation system (S9 mix), prior to the addition of soft agar. Data are given as means ± SE from the testing of triplicate plate.

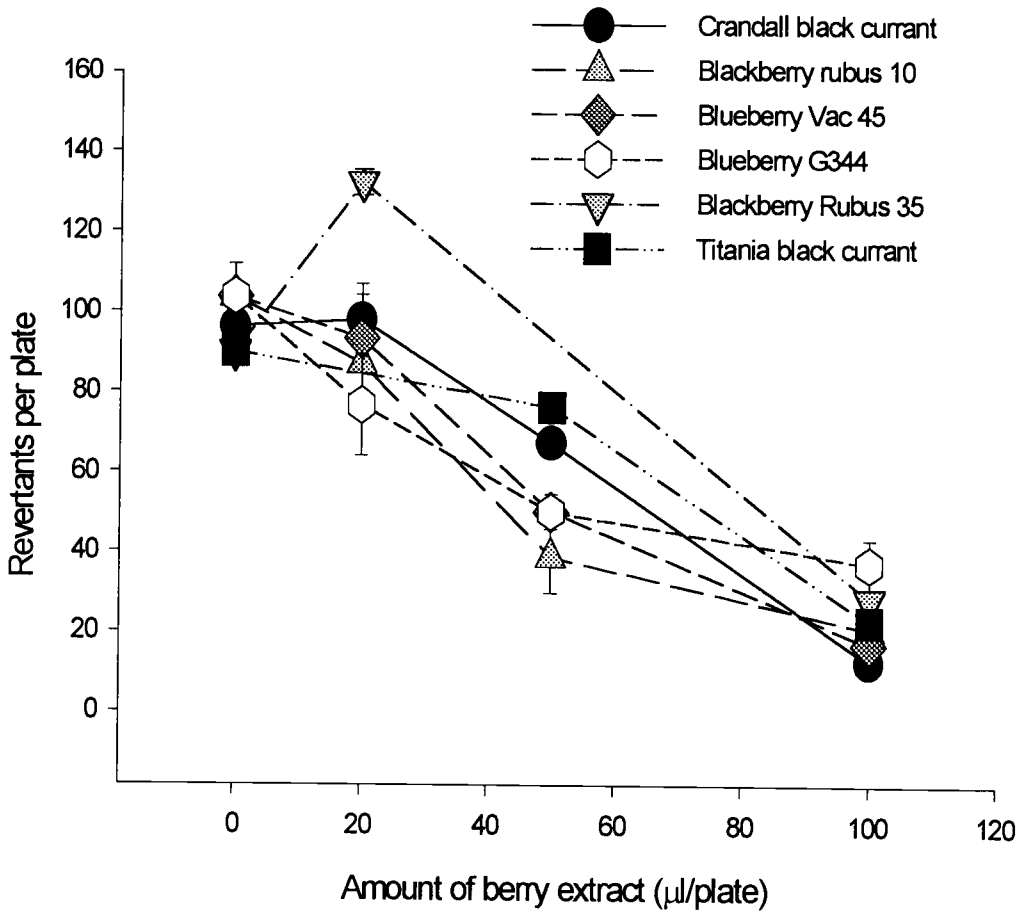

Figure 3.3 **Mutagenicity of berry extracts in the presence of S9.** *Salmonella typhimurium* strain TA98 was pre-incubated with berry extracts 20 min in the presence of a metabolic activation system (S9 mix), prior to the addition of soft agar. Data are given as means ± SE from the testing of triplicate plate.

Effect of berries on the mutagenicity of IQ and *N*-hydroxy-IQ on the Salmonella mutagenicity assay

N-hydroxy-IQ is a direct mutagen to *Salmonella typhimurium* TA98 and does not require addition of rat liver S9 for bioactivation. The following experiment compared the effect of berry extracts on the mutagenicity of *N*-hydroxy-IQ compared to IQ.

Figure 3.4 depicts the antimutagenicity effect of crandall black currant on the mutagenicity of *N*-hydroxy-IQ (in the absence of S9 mix) and IQ (in the presence of S9 mix). There was no statistically significant inhibition effect on the mutagenicity of *N*-hydroxy-IQ even at crandall black currant concentrations up to 100 µl per plate. Crandall black currant was effective in inhibition of IQ mutagenicity, 50 µl per plate of extract inhibited 32% of the mutigenicity of IQ, whereas 100 µl per plate of extract inhibited 89% of the mutigenicity of IQ. Similar results were also observed with other blackberry and black currant extracts (Figure 3.5).

Based on these results, we hypothesize that berries decrease the mutagenicity of IQ through inhibition of CYP 1A2 bioactivation, rather than by blocking the reactivity of *N*-hydroxy-IQ.

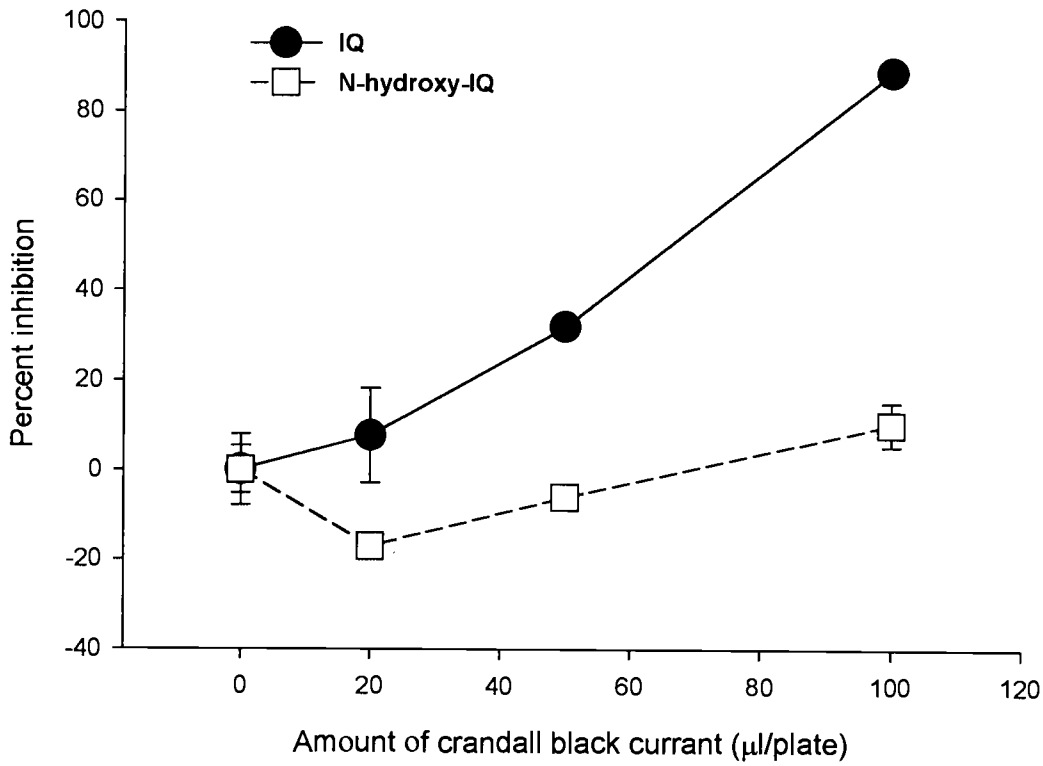

Figure 3.4 Inhibition of IQ or *N*-hydroxy-IQ mutagenicity by crandall black currant extract. *Salmonella typhimurium* strain TA98 was pre-incubated with IQ (8ng/plate) plus berry samples 20 min in the presence of a metabolic activation system (S9 mix) or TA98 was incubated with *N*-hydroxy-IQ (4 ng/plate) plus crandall black currant extract in the absence of S9, prior to the addition of soft agar. Results are given as percent inhibition of mutagenicity (means ± SE) with triplicate plates for each dose tested. Percent inhibition was calculated thus:

$$\text{Inhibition (\%)} = 100 - \frac{\text{Induced revertants/plate in presence of berry}}{\text{Induced revertants/plate in absence of berry}} \times 100$$

Negative control (H_2O only) were 19 ± 2.8 revertants/plate (means ± SE, n= 5, in the absence of S9 mix) and 96± 2.2 revertant/plate (mean ± SE, n= 3, in the presence of S9 mix), respectively. Positive control were 2732± 165 revertants/plate (means ± SE, n= 5, in the absence of S9 mix, 4 ng/plate *N*-hydroxy-IQ) and 1306± 68 revertant/plate (mean ± SE, n= 3, in the presence of S9 mix, 8 ng/plate IQ).

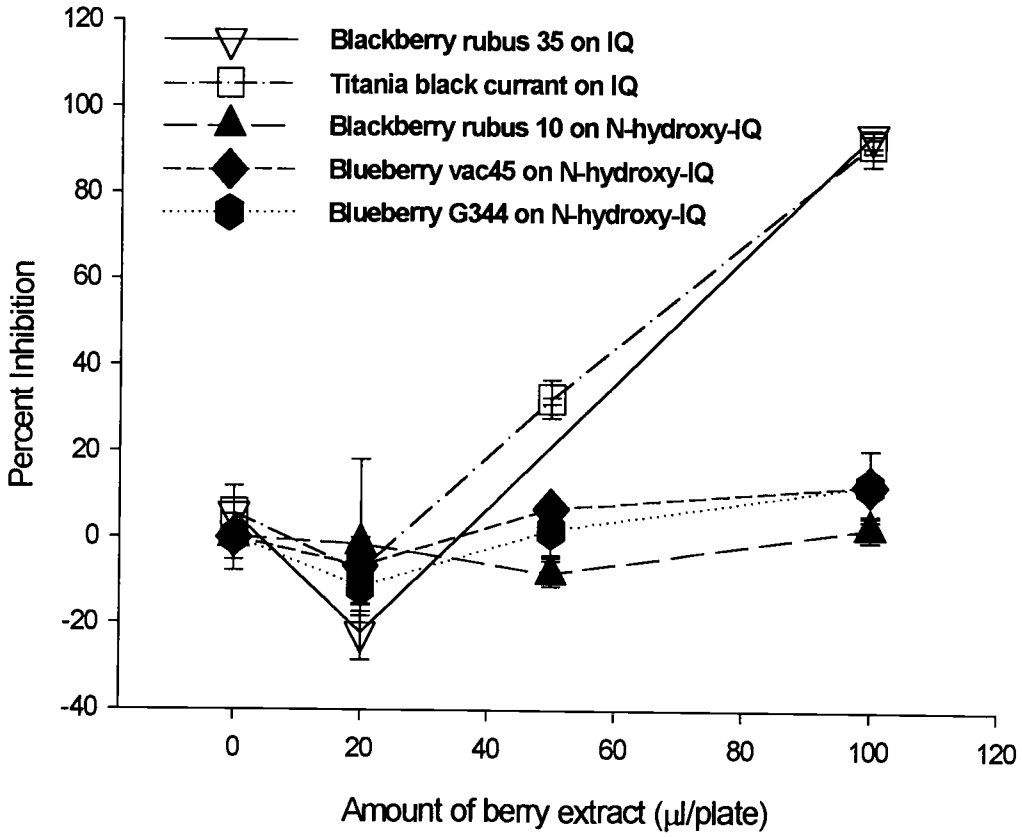

Figure 3.5 **Inhibition of IQ or *N*-hydroxy-IQ mutagenicity by berry extract.** *Salmonella typhimurium* strain TA98 was pre-incubated with IQ (8 ng/plate) plus berry samples 20 min in the presence of a metabolic activation system (S9 mix) or TA98 was incubated with *N*-hydroxy-IQ (4 ng/plate) plus berry extracts in the absence of S9, prior to the addition of soft agar. Results are given as percent inhibition of mutagenicity (means ± SE) with triplicate plates for each dose tested. Percent inhibition was calculated as described in the legend of figure 3.4.
Negative control (H_2O only) were 19 ± 2.8 revertants/plate (mean ± SE, n= 5, in the absence of S9 mix) and 96± 2.7 revertant/plate (mean ± SE, n= 14, in the presence of S9 mix), respectively. Positive control were 2732± 165 revertants/plate (mean ± SE, n= 5, in the absence of S9 mix, 4 ng/plat *N*-hydroxy-IQ) and 1625± 89 revertant/plate (mean ± SE, n= 9, in the presence of S9 mix, 8 ng/plate IQ).

EROD and MROD study *in vitro*

Figure 3.6 shows the EROD/MROD activity in the presence of crandall black currant. Both EROD activity (which represent CYP1A1 enzyme activity) and MROD activity (which represents CYP 1A2 enzyme activity) were decreased when berry was present. CYP 1A2 activity was inhibited at lower extract concentration than was CYP 1A1 activity. At a berry extract amount of 5 μl, co-incubated with 10 μl S9 (same proportion of 50 μl extract pre-incubated with 200 μl S9 mix in Salmonella mutagenicity assay), the CYP 1A2 activity was below the limit of detection. Similar results were also shown in another three extracts (blackberry Rubus 10, blueberry Vac 45, blueberry G344, data not shown). In the mutagenesis of IQ, CYP 1A2 predominates by catalyzing formation of *N*-hydroxy-IQ, whereas CYP 1A1 is capable of contributive to the detoxification pathway. Therefore the inhibition of CYP1A2 activity may be a major mechanism for the antimutagenicity of berries.

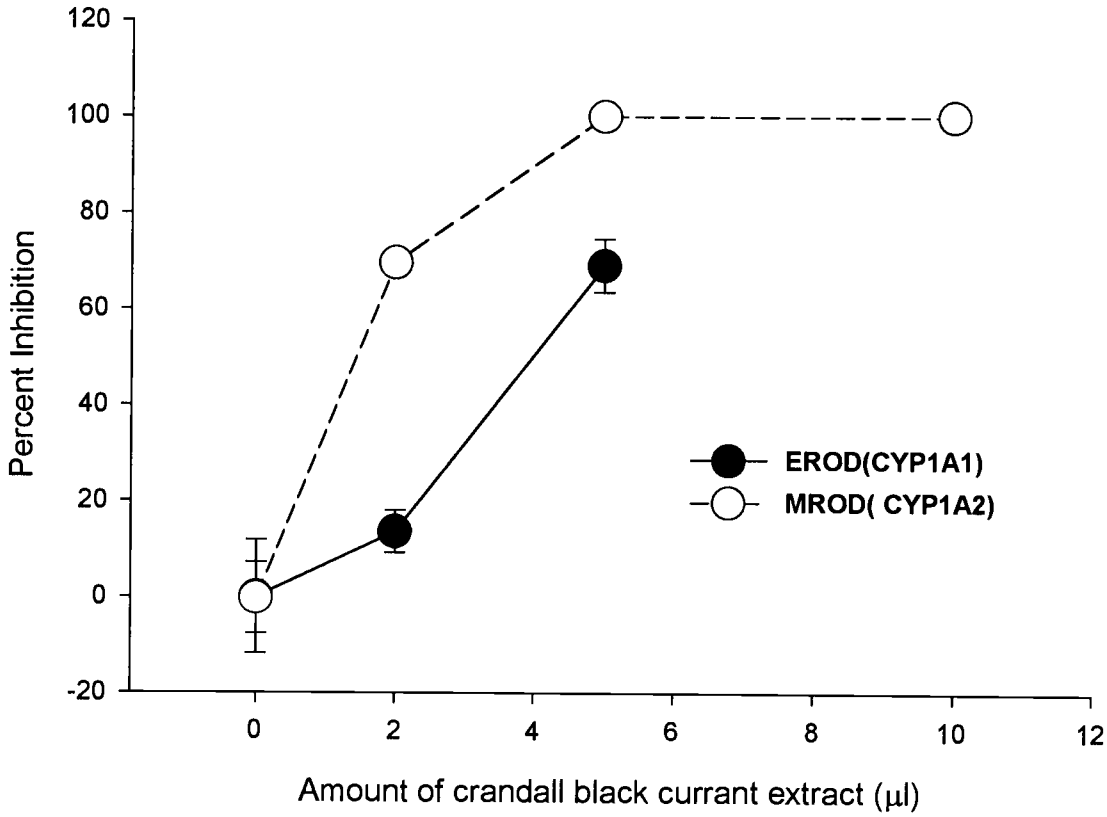

Figure 3.6 **Inhibition effect of crandall black currant extract on EROD/MROD activity *in vitro*.** When in the absence of crandall black currant extract, EROD and MROD activity (nmol/min/mg protein) was 7.92 ± 0.94 (mean ± SE, n= 2) and 1.28 ± 0.1 (mean ± SE, n= 3), respectively. Percent inhibition was calculated thus: 100 × (EROD/MROD activity in the absence of berry extract / EROD/MROD activity in the presence of berry extract). Data are mean ± SE, n=2 or 3.

DISCUSSION

In the process of seeking the mechanism by which fruit, vegetables and other foods' protect against cancer, studies usually concentrate on isolated components, yet it is important to remember that food compounds are not eaten in isolation, and the benefit of one or two components may not represent the benefit of whole food. Many phytochemicals have been found in berries. Several of them have been studied and related to the health benefit of berries. One class of chemicals found in berries is anthocyanins, responsible for the red, purple and blue colors of berries. Blueberries, blackberries, and blackcurrants are rich in anthocyanins (2.3g/kg in blueberry, 1.79 g/kg in blackberry, 2.07g/kg in blackcurrant, Moyer *et al.*, 2002). Anthocyanins average 34% of total polyphenolics in these berries (Moyer *et al.*, 2002). There are no studies on the antimutagenicity or antitumor potency of anthocyanins in berries, but some studies have already demonstrated the antimutagenicty or antitumor potency of anthocyanins in other foods (Yoshimoto *et al.*, 1999; Deguchi *et al.*, 2000; Hagiwara *et al.*, 2001; Koide *et al.*, 1996,1997; Kamei *et al.*, 1995). There is no report of anthocyanins affecting phase I and phase II enzymes which play a major role in the mutagenicity of IQ. The antimutagenicity of berries may be related to the high content of anthocyanins. Anthocyanins may also interfere with EROD/MROD assay because of their color.

Another important group of phytochemicals in berries are the flavonols represented by quercertin, myricetin *etc*.. Quercetin is present at 44 mg/Kg in black currant, myricetin at 71 mg/Kg in black currant; in blueberry, quercetin is present at 24mg/Kg, myricetin at 26 mg/kg (Hakkinen, *et al.*, 1999). Studies showed that quercetin and myricetin were mutagens in the Salmonella mutagenicity assay (Brown and Dietrich, 1979; Hardigree and Epler, 1978). Other studies also showed that quercetin and myricetin decreased the mutagenicty of IQ and other carcinogens (Alldrick *et al.*, 1986). The amount of quercetin and myricetin in the present study (0.968 μg and 1.562 μg of quercetin and myricetin in 50μl crandall black currant extract, respectively.) was well below the test mutagenicity amount of quercetin

and myricetin (100 μg, Hardigree and Epler, 1978). The revertants per plate were also inversely correlated with the amount of berry extract in our study.

A number of studies have examined quercetin and myricetin as inhibitors of CYP 1A1 and CYP1A2 (Siess, *et al.*, 1995; Kansanen *et al.*, 1996; Bear and Teel, 2000). In Kansanen's study, extracts corresponding to 1 mg of black currant (dry weight) caused 50% inhibition of aryl hydrocarbon hydroxylase (AHH) activity (CYP 1A1 activity). In Bear and Teel's Salmonella mutagenicity assay study, IQ and MeIQx induced mutagenesis in *S. typhimurium* TA 98 was significantly inhibited by rutin (glucoside form of quercetin) at concentrations as low as 0.25 mM and the inhibition of MROD activity by rutin correlated best with the inhibition of MeIQx and IQ induced mutagenesis (Bear and Teel, 2000). In our study, if all inhibition was due to quercetin, the inhibition effect of quercetin could be seen at concentrations as low as 6 μM.

Thus, besides quercetin and myricetin, which may be the major contributors of antimutagenicity of berry extracts, there may be other compounds which can interact with quercetin and myricetin to inhibit their mutagenicity, and, at the same time, also contribute to the antimutagenicity of berry.

Polyphenolic acids are another group of compounds found in berries. In Torronen's study of Finnish berries, an almost equal distribution of flavonols and hydroxycinnamic acids (one group of phenolic acids (caffeic, p-coumaric and ferulic acids)) were found in blackcurrant and ellagic acid was only a small portion of the total (Torronen *et al.*, 1997). These compounds were not mutagens in the Salmonella mutagenicity assay (Alldrick, *et al.*,1986). The antimutagenicity of caffeic acid and ellagic acid towards IQ has also been reported (Yoshimoto *et al.*, 1999; Ayrton *et al.*, 1992). Although there were some studies demonstrating that ellagic acid inhibited the enzyme activity of CYP 1A1(Barch *et al.*, 1994), considering the very low amount of ellagic acid in the blackcurrant, the effect of ellagic acid may be not important. There are controversial studies published on whether hydroxycinnamic acid can inhibit the activity of the CYP 1A subfamily

(Ayrton *et al.*, 1992; Kansasnen *et al.*, 1996). Therefore, the role of polyphenolic acids in the antimutagenicity of berries and the relation to CYP 1A1 and CYP 1A2 activity still needs further study.

Overall, the antimutagenicity of berries in this study may be related to quercetin and myricetin through inhibition of the phase I enzyme in the liver S9 activation system. Polyphenolic acids and other phytochemicals in the berries may also contribute to this antimutagenicity.

Mutagenesis and carcinogenesis are complex processes. A simple bacteria mutation test and an *in vitro* enzyme assay can only partially explain the mechanism. Many phytochemicals are found in berries and some of them have high activity as antioxidants. Antioxidants protect cells against oxidative damage, which may affect carcinogenesis. Some phytochemicals may also affect phase II enzymes, leading to detoxification of carcinogens (Kawabata *et al.*, 2000). For further study, an *in vivo* colonic aberrant crypt foci (ACF study) or other tumor study can be conducted in lab animal models to address the issue of the antitumor potency of berries. The individual components from berries can also be separated for further tumor study and the interaction effects between components can also be studied.

According to Augustsson *et al.* (1997), the consumption of all HCAs may be on the order of 1.8 μg/person/day. In our study, 0.022 g berry can significantly inhibit mutagenicity of 8 ng IQ. If our *in vitro* results can be extrapolated to human *in vivo* exposures, consumption of only 5 g berry per day should have a significant inhibition effect on the mutagenicity of 1.8 μg IQ, suggesting that berries will be good candidates in prevention against HCA-induced cancer.

In this experiment, when berries were pre-incubated with the *Salmonella typhimurium* strain TA98 in the presence of liver S9, we detected a trend of decreasing revertants per plate when berry extracts concentration were 50-100 μl in a 500 μl pre-incubation system. Since the growth of the background lawn is normal and the number is not significantly different compared to the no S9 system at high concentration, we conclude that berries are not toxic to the bacteria. Antimicrobial

properties of phenolic compounds from berries has been discussed by Puupponen-Pimia *et al.* (2001). Phenolic extract from black currant (*Ribes nigurum*, var. Öjeby) up to 7 mg (almost equal to 52 mg fresh black currant, Kahkonen *et al.*, 1999) gave no inhibition to Salmonellla *enterica* SH-5014 in an agar diffusion assay, but clear inhibition in liquid culture when the extract was present at 10 mg (equal to 75 mg fresh berry). In our Salmonella mutagenicity assay, the berry extract was in the range of 9- 44 mg. Since the bacteria strain is different, it's hard to compare toxicity. Other berry samples expressed different toxicity towards different bacteria species (Puupponen-Pimia *et al.*, 2001). So, if we continue to use the Salmonella assay to study mutagenicity or antimutagenicity of berry extracts, toxicity will be one issue to consider and a survivor test should be done to test each berry sample.

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CHAPTER 4

CONCLUSION

Cancer is the second leading cause of death in the United States. The antimutagenic potency and anti-tumor potency of wheat grains and berry extracts were studied against several heterocyclic amines in this study.

In the wheat grains study, wheat bran, which binds HCAs *in vitro*, as well as refined wheat and unrefined whole wheat, inhibited the mutagenic activities of IQ, PhIP and MeIQx when they were co-incubated and the supernatant (minus grain) was added to the Salmonella assay. The water-soluble fraction alone from refined and unrefined wheat, but not bran, also inhibited these mutagens *in vitro*. *In vivo*, AIN-93G diets containing refined wheat or unrefined wheat were examined for their ability to inhibit IQ-induced ACF in the F344 rat. A slight increase in the number of AC/ACF (aberrant crypts/ACF) was seen after 16 weeks in rats treated post-initiation with refined wheat ($p<0.05$), and fewer foci with 2 or 3 aberrant crypts (ACF-2) were found in rats given unrefined whole wheat *post*-initiation compared with animals treated with the same diet during the initiation phase ($p< 0.05$). There was no significant difference in the profile of IQ urinary metabolites or excretion of promutagens 0-48 hours after carcinogen dosing, and grains had no effect on hepatic CYP1A1, CYP1A2, aryl sulfotransferase, or *N*-acetyltransferase activities; however, a slightly higher UDP-glucuronosyl transferase activity was observed in rats fed unrefined wheat compared with refined wheat diets ($p<0.05$).

Our conclusion is, despite their antimutagenic activities *in vitro*, only marginal effects were seen with refined and unrefined wheat *in vivo* with respect to hepatic enzyme activities, carcinogen metabolism, and IQ-induced ACF in the rat colon.

In the study of berries, the fresh juice and extract of crandall black currant (*Ribes aureum*) were not mutagens in the Salmonella mutagenesis assay. Berry

extract or fresh juice at levels to 50 μl (22 mg berry) in a 500 μl pre-incubation system significantly inhibited the mutagenicity of IQ by 32% when rat liver S9 bioactivation system was present ($p<0.05$). One hundred μl of crandall black currant extract gave 89% inhibition of IQ mutagenicity ($p<0.05$). However, the mutagenicity of *N*-hydroxy-IQ, a direct-acting metabolite of IQ, was not affected. An *in vitro* fluorometric assay showed the activity of cytochrome P 450 (CYP) 1A1 and CYP 1A2 was decreased. Inhibition of CYP 1A2 activity may be an important mechanism of antimutagenicity of crandall black currant extract. Similar results were also observed with other berry samples.

From this study, we conclude that crandall black currant extract (may be other berries extracts, too) inhibit the mutagenicity of IQ and the inhibition of CYP 1A2 bioactivation may be a major mechanism. However, bearing in mind the result with cereals, showing anti-mutagenic effects *in vitro* but no inhibition *in vivo*, it is premature to conclude that berries will inhibit HCAs in the whole animal.

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