THE INFLUENCE OF WHEAT BRAN AND PHYTIC ACID ON EARLY BIOMARKERS OF COLON CARCINOGENESIS

By

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A thesis submitted in conformity with the requirements for the degree of Ph.D.
Graduate Department of Nutritional Sciences
University of Toronto

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Doctor of Philosophy, 2000
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University of Toronto

Abstract

It is unclear whether the previously observed colon cancer protective effect of wheat bran (WB) is due to its fiber and/or phytic acid (PA) components since pure PA (1-2%) has also been shown to be protective of colon cancer in rats. Thus, the objectives of this study were to determine whether WB, due to its fiber and/or its PA, alters early biomarkers of colon cancer risk (aberrant crypt foci (ACF) and indices of cell proliferation) and whether endogenous and exogenously added PA differ. Possible modes of WB and PA action were also investigated. Five groups of azoxymethane-treated male F344 rats were fed, for 100 days, a basal control diet (BD), BD supplemented with either 25% WB, 25% dephytinized WB (DWB), 25% DWB plus 1.0% PA or 1.0% PA. All treatment diets reduced (p<0.05) the number of sialomucin (SIM) ACF (by 43-66%), the degree of ACF luminal alterations (by 7-14%) and indices of cell proliferation (maximum 26-57%) versus the BD. Exogenous PA also reduced the number (by 31%) and size of ACF (by 39%) versus the BD. Some indices of cell proliferation were significantly increased (by 11-42%) upon dephytinization of WB. The treatment diets also increased the rate of apoptosis (by 135-217%) and cell differentiation (using lectin staining; by 144-168%) in various regions of the crypt.

There were no major changes in indices of colon mucosal and liver lipid peroxidation, colonic butyrate production or serum and femur calcium or iron levels. However, versus the BD, all treatment diets reduced colonic pH, the WB-containing diets reduced β-glucuronidase activity, the WB diet increased total colon SCFA production and the PA diet decreased serum zinc. It was also observed that SIM ACF are more advanced types of ACF. It is concluded that WB significantly reduced putative early biomarkers of colon cancer risk due, in part, to its PA and dietary fiber. Furthermore, endogenous and exogenous PA are both effective, but exogenous PA is more effective in a low fiber diet. These effects are related to decreased cell proliferation, colon pH and increased apoptosis and degree of cell.
differentiation. They do not appear to be due to an anti-oxidative mechanism or inhibition of mineral availability.
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<tr>
<td>AA</td>
<td>Atomic Absorption</td>
</tr>
<tr>
<td>AC</td>
<td>Aberrant Crypt</td>
</tr>
<tr>
<td>ACF</td>
<td>Aberrant Crypt Focus or Aberrant Crypt Foci</td>
</tr>
<tr>
<td>Al</td>
<td>Apoptotic Index</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AOAC</td>
<td>Association of Official Analytical Chemists</td>
</tr>
<tr>
<td>AOM</td>
<td>Azoxymethane</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous Polyposis Coli</td>
</tr>
<tr>
<td>BD</td>
<td>Basal Diet</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine Tetrahydrochloride</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DBA</td>
<td>Dolichos Biflorus Agglutinin</td>
</tr>
<tr>
<td>DCC</td>
<td>Deleted in Colon Cancer</td>
</tr>
<tr>
<td>ddiH₂O</td>
<td>Double distilled de-ionized water</td>
</tr>
<tr>
<td>DMH</td>
<td>1,2-Dimethylhydrazine</td>
</tr>
<tr>
<td>DWB</td>
<td>Dephytinized Wheat Bran</td>
</tr>
<tr>
<td>DWBPA</td>
<td>Dephytinized Wheat Bran plus Phytic Acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra-acetic acid</td>
</tr>
<tr>
<td>F344</td>
<td>Fischer 344</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial Adenomatous Polyposis</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>HIDAB</td>
<td>High Iron Diamine Alcian Blue</td>
</tr>
<tr>
<td>HNPCC</td>
<td>Hereditary Non-Polyposis Colon Cancer</td>
</tr>
<tr>
<td>IP</td>
<td>Inositol phosphates</td>
</tr>
<tr>
<td>IP₁₆</td>
<td>Lower Inositol Phosphates</td>
</tr>
<tr>
<td>IP₆</td>
<td>Phytic Acid; myo-inositol 1,2,3,4,5,6-hexakis-dihydrogen phosphate</td>
</tr>
<tr>
<td>LaO</td>
<td>Lanthanum Oxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MAM</td>
<td>Methylazoxymethane</td>
</tr>
<tr>
<td>MIPP</td>
<td>Multiple Inositol Phosphate Phosphatase</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metallo-Proteinases</td>
</tr>
<tr>
<td>PA</td>
<td>Phytic Acid; myo-inositol 1,2,3,4,5,6-hexakis-dihydrogen phosphate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
</tr>
<tr>
<td>PCNA-LI</td>
<td>Proliferating Cell Nuclear Antigen-Labeling Index</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>SCFA</td>
<td>Short Chain Fatty Acids</td>
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<tr>
<td>SEB</td>
<td>Surrogate End-point Biomarker</td>
</tr>
<tr>
<td>SIM</td>
<td>Sialomucins</td>
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<tr>
<td>SUM</td>
<td>Sulphomucins</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Tdt-mediated dUTP Nick End Labeling</td>
</tr>
<tr>
<td>WB</td>
<td>Wheat Bran</td>
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1.0 Introduction
1.0 Introduction

It has been suggested that diet is the most important environmental factor in the development of colon cancer with one high in fiber and low in fat associated with a decreased risk (Wynder et al, 1992). Epidemiological studies have shown that high fiber foods, such as whole grains and cereals may be protective against colon cancer (Greenwald et al, 1987; Howe et al, 1992; Helzlsouer et al, 1994; Steinmetz et al, 1994). In particular, several animal studies have shown that wheat bran (WB) has a colon cancer protective effect (Barbolt and Abraham, 1978, 1980; Reddy and Mori, 1981; Alabaster et al, 1993, 1995; McIntyre et al, 1993), attributed mostly to its high fiber content. Interestingly, many of the proposed protective mechanisms of WB fiber action, such as decreased transit time (Eastwood, 1992), increased fecal bulk (Weisburger et al, 1993) or colonic fermentation (Velazquez et al, 1996a), are analogous to those suggested for the protective effects of phytic acid (PA; myo-inositol 1,2,3,4,5,6-hexakis-dihydrogen phosphate) which is a major fiber-associated component of WB (Graf and Eaton, 1985; Thompson, 1995). In some observational studies, a colon cancer protective effect has been observed for high fiber foods rich in PA, such as WB, but not for low PA high-fiber foods (Graf and Eaton, 1985; Englyst et al, 1982).

PA consists of a myo-inositol ring with six phosphate moieties attached (Cheryan, 1980). It serves as the major storage form of phosphorus in the seed, as well as being a natural antioxidant by chelating and reducing the catalytic activities of many divalent transition metals (Rickard and Thompson, 1997). It is this chelating ability of PA that has been suggested to suppress iron-mediated oxidation in the colon, thereby reducing colon cancer risk (Graf and Eaton, 1985, 1993; Shamssuddin, 1992; Nelson, 1992). PA may also bind proteins and starch present in the diet and so affect their solubility, digestibility, absorption and function (Rickard and Thompson, 1997), which in turn may affect the colonic environment, for example, by stimulating short chain fatty acid (SCFA) production from fermentation of the bound starch which has reached the colon (Thompson, 1995).

Pure PA (1 - 2%) has been shown to significantly decrease the number and volume of colon tumours, when treatment was commenced prior to azoxymethane (AOM) carcinogen injection (Shamsuddin et al, 1988; Ullah and Shamuddin, 1990; Pretlow et al, 1992a) or when administered at up to five months post-initiation (Shamsuddin and Ullah, 1989). In these studies, it was postulated that the antineoplastic effects of PA may be mediated by its ability to regulate colonic cellular proliferation.
However, since these experiments used pure PA dissolved in the drinking water, results may not be representative of the effects of the PA naturally present. The effect of endogenous PA in WB or purified PA added to high or low fiber diets on colon carcinogenesis have not yet been systematically studied. In addition, to date, only a small number of studies have focused on the effects of PA (Pretlow et al, 1992a) or WB (Alabaster et al, 1995; Shivapurkar et al, 1995; Ishizuka and Kasai, 1996; Young et al, 1996) on aberrant crypt (AC) and foci (ACF) formation and characteristics. Much remains to be determined about the effects of WB and PA in a natural matrix on early markers of colon cancer risk and its possible mechanisms of action.

Thus, the overall hypothesis of this study was that WB will reduce the early biomarkers of colon cancer risk, in part, due to its PA content. The overall objectives were to determine (a) whether WB is protective of colon cancer using ACF characteristics and indices of colonic cell proliferation as early risk markers, (b) whether PA is the component responsible for these changes, (c) whether endogenous and exogenous PA are equally effective and (d) some of the possible physiological and molecular modes of action of WB and PA action.
2.0 Literature Review
2.0 Literature Review

2.1 Azoxymethane model for colon carcinogenesis

The use of chemicals capable of inducing colon cancer facilitates the study of colon carcinogenesis in vivo. AOM, a metabolite of 1,2-dimethylhydrazine (DMH), is one of the most widely used chemicals for the induction of colon cancer because it mimics the adenoma-carcinoma sequence in humans (Vivona et al., 1993). It has enhanced potency (requiring a smaller dose) and greater chemical stability than DMH (Druckrey, 1970). It is metabolized to methylazoxymethane (MAM) by the microsomal mono-oxygenase system in the liver, glucuronated and sent to the gastrointestinal tract through secretion in the bile (Fiala, 1977). In the colon, the MAM-glucuronide conjugate is acted upon by β-glucuronidase to release the aglycone (Fiala, 1977). The AOM may also be hydroxylated to MAM directly by the colon mucosa. The MAM, either spontaneously or through a tissue specific enzyme-catalyzed reaction, is metabolized to the ultimate carcinogen methylcarbonium ions (Fiala, 1975) which methylate DNA and initiate tumourigenesis (Hirose et al., 1996). Gennaro et al (1973) showed that looped sections of colon surgically transposed to different areas within the abdomen still developed tumours after AOM injection, indicating that colonic tissue is very sensitive to AOM treatment.

Colon cancer may be induced by multiple or single injections of carcinogen. The multiple injection method is desirable for animals with a low tolerance or lower lethal dose range for the specific carcinogen. Its major disadvantage is that the carcinogenic process is initiated several times over the course of the experiment and so a clear distinction between the initiation and promotion stages may not be apparent (Farber and Cameron, 1980). A single injection of a higher dose of carcinogen is more desirable for animals, such as rats, with a greater tolerance or higher lethal dose rating for the carcinogen and also when a clearer distinction between the initiation and promotion stages of cancer is needed. Carcinogen dose also affects the site of tumour development with a dose of 15 mg/kg of body weight of AOM producing tumours mostly in the distal colon whereas half this dose produces tumours more in the proximal colon (Ward et al, 1973).

In general, about 70% of colon tumours induced with AOM are adenocarcinomas (well differentiated, malignant tumours with invasion into the muscularis mucosa, may be mucinous, and may invade and metastasize, mostly to the mesenteric lymph nodes, lung and liver) and the rest adenomas
(benign, mostly with mild or moderate dysplasia) (Reddy, 1998). AOM treatment induces both H- and k-ras oncogene mutations and increases their expression (Singh et al, 1994). AOM tumours have also been shown to have mutations of the adenomatous polyposis coli (APC) gene (Caderni et al, 1997) and production of aberrant APC protein (Maltzmann et al, 1997), which has been implicated as a causal mutation in human colon cancer. Although most of the morphologic and molecular characteristics of tumours induced by AOM and sporadic human colon cancers are similar, there appear to be some important differences. For example, microsatellite instability, which is an alteration in length of short repetitive DNA sequences associated with defective DNA mismatch repair, occurs at a rate of 15–30% in sporadic human colon cancers but has been shown to be very rare in AOM induced rat intestinal, proximal (Walchle et al, 1999) and distal colonic tumours (Haag et al, 1999).

AOM is not universal in its ability to induce colon cancer. Some strains of mice (i.e. SWR/J and A/J) have been shown to be very susceptible to its effects while others (i.e. AKR/J) appear to be resistant, although all three strains do develop ACF upon AOM treatment (Papanikolaou et al, 1998). The reasons for this require much further research but may be related to an active inhibition or a delay in the tumourigenic process in the resistant strain. It is unknown whether these lesions in the resistant strain can be modulated by diet. However, the implications of this observation are that the use of individual rodent strains and particular carcinogens in diet chemoprevention studies must be thoroughly validated prior to the use of that model.

One of the most commonly used experimental models for the study of the etiology of human colon carcinogenesis is the Fischer-344 (or F344) rat. Similar to the mice models mentioned above, rat strains also differ in susceptibility to the effects of a chemical colon carcinogen (Teague et al, 1981). For example, F344 rats are more susceptible to tumour induction than Wistar rats. The use of F344 rats initiated with AOM is considered a valid model for the study of dietary chemoprevention (Alabaster et al, 1996) since lesions induced by AOM in F344 rats have similar histopathology and regional distribution to neoplastic lesions, adenomas and carcinomas observed in the colons of humans with or at high risk for colon cancer (Reddy, 1998).


2.2 Colon cancer development

The development of cancer is thought to be a multi-step process and has been divided into three stages, initiation, promotion and progression (Bodmer, 1996).

2.2.1 Initiation stage

The initiation stage occurs when the tissue or cells are exposed to a carcinogen (first hit theory) and acquire growth potential, either through defect in repair or elimination of apoptosis (Sancar, 1994) and undergoing changes that become permanent after a single cycle of proliferation (Farber, 1982a). These changes may be point mutations, deletions, amplification, miscoding, translocation and large rearrangements of DNA (Freeman et al, 1989) or changes in other cellular components such as RNA, proteins, glutathione and polysaccharides (Miller, 1978). The DNA affected by either activation of an oncogene or inactivation of a tumour suppressor gene, may be repaired by DNA repair mechanisms prior to cell replication or the cell may die. A single activated oncogene is insufficient to convert a normal cell into a neoplastic one (Ashendel, 1995) and often a number of requisite mutations must occur to cause a malignant phenotype to develop (Ilyas et al, 1999). Changes may also occur in DNA methylation patterns, thus deregulating gene expression (Kennedy, 1991). Oncogene cell proteins may bind to receptors inducing abnormal physiological changes and further alterations in gene expression and DNA synthesis (Marshall, 1987). The initiating event may also be potentiated by certain tumour-promoters (Tlsty et al, 1984) and inhibited by anti-carcinogenic substances, possibly from the diet (Heilbronn et al, 1985). Regular cellular processes, such as signal transduction and gene expression regulation are clearly altered (Ashendel, 1995). For as of yet unknown reasons, genetic damage and chromosomal instability are more prominent in the distal than in the proximal colon (Delattre et al, 1989). Initiation is thought to occur quickly (Appel et al, 1990) and is dose-related with no measurable threshold (Pitot, 1991).

Exposure to a colon carcinogen causes transformations in colonic epithelial cells which then migrate up the crypt to the luminal surface, as do normal or non-transformed cells (Chang, 1984). Exposure to a single dose of AOM (15 mg / Kg body weight) has been shown to increase apoptosis (after 8 hours) of altered cells followed by an increase in the rate of cell proliferation (Hirose et al, 1996), effectively fixing any mutations that have occurred.
2.2.2 Promotion stage

Initiated cells can multiply in preferential response to promoters and thus the second stage of carcinogenesis is promotion. Unlike initiation, promotion is a slow and on-going process which may not necessarily lead to the generation of malignant cells (Appel et al, 1990). Also contrary to initiation, the primary, selective effects of promotion on initiated tissue are considered reversible (Farber, 1982b). In the presence of promoting agents, an initiated cell or tissue will develop focal proliferations, such as polyps. (Farber, 1982b), from which colon cancer appears to develop (Morson et al, 1983). In the colon, some promoting agents may be supplied by the diet.

The promotion stage is characterized by hyperproliferation or proliferation in excess of the normal tissue replacement rate, of colonic epithelial cells (Bird et al, 1985) thus increasing the risk for neoplastic transformation. The proliferating regions of the crypts in the colon shift from the lower two thirds to the middle and upper areas (Deschner, 1982) where initiated cells may develop into tumour cells through a series of cellular changes that are not clearly understood but could be related to altered and increased activities of cellular compounds such as ornithine decarboxylase, growth factors and regulatory proteins, which are ordinarily involved in cell proliferation (Farber, 1982b; Kennedy, 1991). A single aberrant crypt (AC), composed of transformed or initiated cells, may thus be promoted to continue to grow by crypt multiplication and may develop into an adenoma or further to a malignant tumour at any point. Adenomas can gradually grow, changing from tubular to a villous architecture, often accompanied by gradually increasing levels of dysplasia (Ilyas et al, 1999).

2.2.3 Progression stage

This continued development of the focal proliferations into malignant tumours is the third stage of carcinogenesis known as progression (Appel et al, 1990). It is a period of autonomous growth and malignancy and no longer requires the influence of a promoter or promoting environment (Farber, 1982b) and so is distinguished from both initiation and promotion. This is a stage of biochemical changes which are directly related to the tumour’s increased rate of growth, degree of invasiveness and metastatic capability (Pitot et al, 1991). Although irreversible, the progression stage can be affected by extracellular and environmental factors (Pitot et al, 1991), quite possibly diet borne.
2.3 Molecular genetics of colon cancer

The histologically distinct steps of colon cancer (normal to ACF to early, intermediate and late adenomas to cancer) have been termed the adenoma to carcinoma sequence (Ilyas et al, 1999). Progression through this sequence is often characterized by a number of genetic mutations, which may be hereditary or influenced by environmental factors, particularly diet. One of the earliest gene mutations, detected upon transition of normal epithelium to benign adenoma, is the tumour suppressor gene APC, located on chromosome 5q21 and found in 60-80% of sporadic colorectal cancers (Bodmer et al, 1987; Powell et al, 1992). It is found as a germline mutation in Familial Adenomatous Polyposis (FAP; is seen in approximately 1% of total colorectal cancers; Groden et al, 1991). The APC gene protein is necessary not only in the regulation of colonic epithelium but also extra-colonic tissues, since FAP patients are prone to the development of a number of lesions outside of the colon (Phillips et al, 1994). Several different mutations of the APC gene have been identified with those in the center of the gene resulting in large numbers of polyps and those at either end of the gene producing few adenomas and a later onset of cancer (Brensinger et al, 1998). Identical germline APC mutations in FAP kindreds often have variable expression, suggesting that the APC gene is influenced by a number of modifier genes, whose activities may either enhance or resist tumourigenesis (Su et al, 1992; Shoemaker et al, 1998). Another gene mutation thought to occur later than APC in colon cancer development is the k-ras gene, which is involved in a large number of signal transduction and signaling pathways and which is found in approximately 50% of sporadic colorectal tumours (Ilyas et al, 1999). The majority of k-ras mutations are gain of function mutations on either codon 12 or 13 (Bos, 1989) and may only confer a selective advantage to cells that already have APC mutations (Jen et al, 1994).

The Deleted in Colorectal Cancer (DCC; on chromosome 18q) and p53 tumour suppressor (on chromosome 17p; found in approximately 70% of sporadic colorectal cancers) genes are also thought to be involved in colon cancer (Ilyas et al, 1999). However the p53 gene is thought to be much more important since its action is central to several apoptotic pathways (Lane, 1992), thus the selective advantage of this later stage mutation is resistance to apoptosis (Ilyas et al, 1999). P53 mutation may also be necessary for conversion to high grade dysplasia (Miyaki et al, 1995) and has been reported to be a causal factor in the transition of an adenoma to carcinoma (Baba, 1997). Genetic mutations involved in colon carcinogenesis are shown in Table 2.1.
<table>
<thead>
<tr>
<th></th>
<th>Frequency (%) †</th>
<th>Mutations</th>
<th>Microsatellite Instability (%)†</th>
<th>Mutated genes involved in % or tumours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sporadic colon cancer</td>
<td>≈ 90</td>
<td>Polygenic multifactorial</td>
<td>≈ 15</td>
<td>APC* 60-80%  K-ras* 50%  P-53* 55%  MMR † 70%</td>
</tr>
<tr>
<td>Familial adenomatous polyposis colon cancer</td>
<td>≈ 1</td>
<td>APC</td>
<td>0</td>
<td>100% 56% 56%  ?  ?</td>
</tr>
<tr>
<td>Hereditary nonpolyposis colon cancer</td>
<td>≈ 5 - 10</td>
<td>Mismatch repair genes, hMSH1&amp;2</td>
<td>&gt; 90</td>
<td>20% 7% 13% 60%  ?</td>
</tr>
</tbody>
</table>

† Bocker et al, 1999; * Konishi et al, 1996; † Ilyas et al, 1999.

There are also some new reports of other possible gene mutations in colon tumourigenesis, particularly on chromosome 18q which holds a number of tumour suppressor genes involved with Transforming Growth Factor β (TGF-β; Takagi et al, 1996; Eppert et al, 1996). Some colon tumours may also have other genetic defects, such as microsatellite instability, (genome-wide alterations in repetitive DNA sequences a result of defects in genes coding for the mismatch repair machinery; found in 15% of sporadic colon cancers and 80-90% of Hereditary Non-Polyposis Colorectal Cancer (HNPCC) which is thought to follow a pathway separate from the adenoma carcinoma sequence)(Lothe, 1997) and chromosomal instability (changes in the number of chromosomes, shifts in DNA content and rearrangements of DNA fragments)(Meuth, 1996). HNPCC, which represents 5-10% of all colorectal cancers (Bocker et al, 1999), is an inheritable form of colon cancer where patients inherit a single mutated allele for genes involved in mismatch repair of DNA sequences and require subsequent somatic mutation to inactivate the gene (Baba, 1997). Patients with HNPCC mostly exhibit microsatellite instability which causes abnormalities in TGF-β receptor and thus inhibits TGF-β mediated suppression of growth (Markowitz et al, 1995).

It is thus clear that colon carcinogenesis is polygenic and multifactorial, proceeding through an accumulation of specific genetic alterations with the majority of colon tumours following the classical stepwise pathway of mutations in APC, k-ras, DCC (or possibly others on chromosome 18q) and p53 genes. Since the functions of these genes are in one way or another relevant to cell growth and the cell cycle, it is possible that environmental factors, particularly diet, may modulate their aberrant expression and thus influence the growth and survival of mutated cells throughout the stages of malignant...
transformation from preneoplastic lesions to carcinoma. Thus, a study of the preneoplastic markers of colon cancer is necessary.

2.4 Preneoplastic markers of colon carcinogenesis

2.4.1 Aberrant crypt foci

As mentioned, colon tumourigenesis takes place over time with a number of phases from normal to malignant colonic epithelium (Bacus et al, 1999). AC and ACF have been identified as putative preneoplastic markers and have been used as early indicators of colon cancer risk (Bird, 1987). The AC can be distinguished from normal crypts by their increased size, elongated luminal opening, increased epithelial lining and increased pericryptal zone (distance between neighbouring crypts) with the number of crypts in a single ACF ranging from one to dozens. Although ACF have varying growth features (Lasko and Bird, 1995), their mean proliferative activity in the distal colon of rats has been shown to be three to four times greater than that in adjacent normal crypts and comparable to that in benign and malignant colon tumours (Pretlow et al, 1994). In addition, cells in the upper crypt portions of ACF have been shown to be mostly in the G1 phase as opposed to the normal G0 phase (Takahashi et al, 1991) further indicating the presence of proliferative defects in ACF. A correlation between the induction and distribution of ACF and tumours in the colon has been observed (Shivapurkar et al, 1992; Bird, 1995). Long term animal studies have shown that ACF increase in crypt multiplicity over time (Kristiansen et al, 1995) and develop into microadenomas (MA), which are putative precursor lesions of adenomas and colon cancer (Archer et al, 1992; Simons et al, 1992).

2.4.1.1 Genetic mutations in ACF

That ACF are valid preneoplastic markers of colon cancer is evident in the presence of k-ras oncogene mutations and expression, which are early events in many colon tumours (Stopera and Bird, 1992; Vivona et al, 1993; Yamashita et al, 1993; Pretlow et al, 1993; Yamashita et al, 1995).

In rats, codon 12 and 13 k-ras mutations have been found in 25% and 37% of large (≥ 4 crypts) ACF at 12 and 30 weeks post-initiation (Shivapurkar et al, 1994). This was equal to the level (29%) found in colon tumours in the same rats at 30 weeks. Thus, the k-ras mutation may be of significance as a marker of malignant potential in larger ACF. The k-ras mutation may also exist in different forms and thus may be essential and sufficient for ACF formation (Yamashita et al, 1995). Therefore, most ACF
may harbour particular k-ras mutations. For example GAT as well as GTT mutated base sequences have been observed on codon 12 in human ACF (Yamashita et al, 1995). Therefore, different mutations may possibly result in the formation of different lesions or in altered rates of promotion towards malignancy. This may be why not all ACF increase in size or exhibit marked atypia after extended periods of time (McLellan et al, 1991; Pretlow et al, 1992a).

Mutant p53 tumour suppressor gene products have also been characterized in ACF (Stopera and Bird, 1993) along with alterations in the activities of many important cellular enzymes (Pretlow et al, 1990; Pretlow et al, 1992a). Mutation of the p53 gene is the most common genetic change in the sequential development of colon cancer and its presence in ACF provides a strong link between these lesions and colon tumours (Stopera and Bird, 1993).

Furthermore, mutations of the adenomatous polyposis coli (APC) gene, which are responsible for intestinal tumours in patients with FAP and are also an early event in colon carcinogenesis, have been identified in some human ACF (Smith et al, 1994). Apparently, dysplastic ACF contain more APC mutations and hyperplastic ACF more k-ras mutations (Jen et al, 1994). Yamashita et al (1994; 1995) have shown that human ACF with high degrees of apical branching harbour a higher frequency of k-ras mutations. Furthermore, Otori et al (1995) have shown that some hyperplastic ACF develop into adenomatous ACF via increased cell proliferation and increased proliferative compartment, probably due to increased k-ras and other genetic mutations. In contrast, Davies and Rumsby (1998) who followed the development of ACF in rats for up to 84 weeks, found no evidence of mutations of k-ras, APC or p53, suggesting that other genes may also be involved in directing the abnormal growth of these lesions. However, they did show evidence for the progression of ACF to microadenomas, to polyps and to carcinoma, underscoring their involvement in the stepwise development of colon tumourigenesis.

2.4.1.2 Features of ACF

ACF are induced in a dose-dependent manner by carcinogens such as AOM (McLellan, 1990) and exhibit altered enzyme activities (Pretlow et al, 1990), and increased cell proliferation (McLellan, 1990). They persist throughout the carcinogenic period into the phase of tumour development (Pretlow et al, 1992a) and with increased time after carcinogen treatment significantly more ACF exhibit nuclear atypia or dysplasia (Pretlow, 1995). Some rat ACF have been shown to have elevated levels of
hexosaminidase enzyme (Pretlow et al, 1994) and decreased transforming growth factor (TGF) α and β expression (Thorup, 1997).

ACF can be identified in whole mount colons by methylene blue staining. The differential staining of ACF by methylene blue is indicative of altered cellular and tissue structures, suggesting some dysplasia, from mild to severe (Pretlow et al, 1992b), often similar to the degrees observed in microadenomas or adenomas (Caderni et al, 1995). Such varying degrees of dysplasia have been identified in both animal (Thorup, 1997) and human (Siu et al, 1997) ACF where the incidence of dysplasia ranges from 5% (Jen et al, 1994) to 74% (Roncucci et al, 1991a) and is more severe in ACF from FAP patients than those from patients with sporadic colon cancer (Di Gregorio et al, 1997). The identification of dysplasia, in addition to hyperplasia (seen as increase in cell bulk and extension of the proliferative zone), is indicative of the preneoplastic nature of certain populations of ACF. However, such subgroups have yet to be clearly defined. In human ACF, a strong correlation has been observed between the severity of dysplasia and a slit-shaped lumen, observed on whole mount specimens (Roncucci et al, 1991b).

In an effort to further characterize ACF, Caderni et al (1995) have shown that with increasing ACF size, there are more luminal and nuclear alterations as well as goblet cell reduction in the cells surrounding the lumen of the crypt, all of which are dysplastic features common to precancerous lesions. More importantly, Caderni et al (1995) have also shown that some ACF have alterations in mucin production also found in the normal mucosa of colon cancer patients and in dysplastic distal colon foci of carcinogen treated rats, namely the predominant production of sialomucins (SIM) instead of sulphomucins (SUM)(Filipe, 1975; Wargovitch et al, 1983; Sandforth et al, 1988). SIM are thought to be of oncofetal character (Uchida et al, 1997), reflecting the type of colonic mucin present in the fetal life of humans and rats while SUM are normal types of mucins produced in the human colon as well as the distal colon of rats. SIM production in a large number of colon tumours has been correlated with increased level of dysplasia (Uchida et al, 1997) and linked to increased aggressiveness, increased probability to metastasize and generally poorer prognosis for the patient (Green et al, 1993). This suggests that the type of mucin production may be a way of separating sub-groups of ACF that are more advanced towards tumourigenesis.
Cademi et al (1995) also showed that SIM production increases while SUM production decreases with increasing ACF size and multiplicity. Also, with increasing ACF size, the number of ACFs producing both SIM and SUM was increased, suggesting an increased promotion from SUM, the normal, to SIM, the abnormal, mucin production by advancing ACF. Although, Cademi et al (1995) suggest that SIM ACF are a more advanced population of ACF than SUM ACF, proof of this is lacking. It still needs to be confirmed that in ACF, SIM production is accompanied by a greater degree of pre-neoplastic features, such as increased dysplasia and rate of cell proliferation, than is SUM production.

2.4.1.3 Dietary modulation of ACF

ACF can be remodelled or eliminated, especially in response to particular agents, either promoters or inhibitors, in the diet (Bird, 1995). Their development can also be modulated by tumour inhibitors or enhancers (Pereira and Khoury, 1991) and by dietary modifications (Shivapurkar et al, 1992). For example, a high fat diet (20%) has been shown to produce more AC per colon (McLellan and Bird, 1988) and the amount of fat consumed is positively correlated to the number of medium sized (4-6 crypts) ACF (Lasko and Bird, 1995). Different types of fat caused variable effects on AC promotion and growth with more AC being produced by a high corn oil or high beef tallow diet than a high olive oil diet (Bird et al., 1989). Caloric restriction, which has been shown to be effective in reducing the development of colon tumours (Kumar et al, 1990) has also been shown to decrease the development of ACF over the long term with a lower fat intake associated with lowered AC multiplicity (Lasko and Bird, 1995). Shivapurkar et al (1995) found that a high risk high fat (20%) diet significantly reduced AC multiplicity when supplemented with vitamin E or β-carotene six weeks after carcinogen injection. The same diets were also able to significantly reduce the incidence of colon tumours and tumour multiplicity. The influence of high fiber diets on ACF is discussed in more detail in sections further on.

2.4.1.4 ACF parameters

Although it is well accepted that ACF are indeed valid preneoplastic markers (Bird, 1995), there is some controversy regarding which ACF parameters are best reflective of future colon cancer risk. Many different ACF parameters have been measured including, the number of AC, number of ACF, size of ACF, ACF multiplicity, the number of ACF per length of the colon, or per cm² of the colon, and the degree of luminal alterations of ACF. Although some researchers have found a correlation between the
induction and distribution of ACF and tumours in the colon (Shivapurkar et al, 1992; Bird, 1995), others have shown that the number of ACF at early (Magnuson et al, 1993) and longer (Young et al, 1996) time points after carcinogen treatment is not a predictor of tumour incidence or that there is a negative correlation between the number of ACF and tumour incidence when the two are measured at the same time (Carter et al, 1994). Yet others have shown that either the AC multiplicity (Magnuson et al, 1993) or the number of larger ACF, defined as either greater than 4 AC (Pretlow et al, 1992a; Lasko and Bird, 1995) or 8 AC (Shivapurkar et al, 1995) are predictive of tumour incidence under different experimental situations. However, Cademi et al (1995) observed that neither the number of ACF or their multiplicity correlated with tumour development, although rats with tumours had significantly more large (≥ 14 crypts) ACF than rats without tumours.

Thus, it is possible that only certain populations or types of ACF, probably those with the higher degrees of atypia, dysplasia or general defects advance along the adenoma carcinoma sequence of colon cancer (Bouzourene et al, 1999). However, it is not known how to identify those populations of ACF that might be more advanced, in part because only a few studies have compared ACF parameters measured in whole mount unsectioned colons to other ACF characteristics such as degree of dysplasia (Thorup, 1997). Thus, much controversy still exists regarding the particular ACF parameters observable in whole mount unsectioned colons, that may be suggestive of a greater degree of alterations (e.g. increased degree of dysplasia or rate of cell proliferation) within an ACF and that may thus be used to identify more advanced populations of ACF.

Despite the incomplete understanding of ACF as early biomarkers of colon cancer risk, the use of ACF parameters has been invaluable in the study of diet-borne tumour inhibitors or enhancers in carcinogen treated rats.

2.4.2 Cell proliferation, apoptosis and differentiation

In the adenoma carcinoma or HNPCC sequences of colon cancer described above, the most important events appear to be loss of growth control (i.e. increased cell proliferation) and alterations in apoptotic rate, leading to increased cell bulk. For example, mutation of k-ras oncogene can result in lessened control of cell proliferation (Konkel, 1988) while mutation of the BAX gene, shown to occur in HNPCC (Rampino et al, 1997), and over-expression of cyclo-oxygenase 2 or of bcl-2 (Krishnan et al, 1998), can decrease the rate of apoptosis. Alterations in the rate or extent of cell differentiation may also
affect cell proliferation and apoptosis rates. A reduced degree of cell differentiation has been observed in colon adenomas, carcinomas and HNPCC syndromes (Sam et al, 1990). As a result, cell proliferation, apoptosis and differentiation have been proposed to be valid surrogate endpoint biomarkers of colon carcinogenesis (Krishnan et al, 1998).

Increased cell proliferation has been linked to the genesis of a number of neoplasias (Henderson and Preston-Martin, 1990; Moore and Tsuda, 1998) such as that of the colon (Moore and Tsuda, 1998) and so its lowering can be associated with a protective effect. The evidence for colon cancer is derived from several lines, (i) those at low risk for colon cancer have lower rate of colonic cell proliferation than recurrent colon adenoma patients (Bostick et al, 1997), (ii) some drugs shown to decrease incidence of colon cancer or to inhibit development of colon adenomas, such as sulindac or other NSAIDS, may act by reducing the rate of cell proliferation (Muskat et al, 1994), (iii) susceptibility of rats to AOM or DMH carcinogen treatment is dependent on increased rate of cell proliferation (Deschner et al, 1988), (iv) inhibitors of enzymes (e.g. β-hydroxymethylglutarate CoA reductase) known to be involved in the process of cell proliferation, decrease tumour development (Rao et al, 1991; Narisawa et al, 1996), and (v) a stepwise increase in cell proliferation and expanding proliferative compartment has been observed during neoplastic progression of ACF.

In the colon carcinogenesis process, the interplay between cell proliferation, apoptosis and differentiation is very important (Butler et al, 1999). The inhibition of apoptosis has been shown to play an important role in the genesis of colon adenomas and carcinomas (Bedi et al, 1995) and has been suggested to be a risk factor for colon cancer (Garewal et al, 1996). If colonic epithelial cells no longer respond to DNA damage by undergoing apoptosis, then mutations possibly leading to colon cancer may be acquired and fixed through further proliferation. Similar to apoptosis, delay or failure in differentiation has been proposed to be important in assessing the dietary colon cancer risk (Yang et al, 1996b). The study of these cellular events may shed light on some of the mechanisms of dietary modulation of colon carcinogenesis.
2.5 Structure and composition of wheat bran

WB is perhaps the most popular source of dietary fiber (Bennett and Cerda, 1996). In fact, in the first fiber reference in medical literature, Hippocrates wrote about WB “to the human body it makes a great difference whether the bread be made of fine flour or coarse, whether of wheat; with the bran or without the bran” (Burkitt, 1995). Now, WB is used extensively in breads and cereals and, despite the use and exploitation of a large number of other dietary fibers, remains a major fiber source throughout the world.

WB is a fraction of the whole wheat grain and is widely eaten by humans. There are many cultivars of wheat, which can be grouped depending on their hardness and colour, with the most common being ‘bread wheat’ and ‘durum wheat’ (Halverson and Zeleny, 1988). The wheat grain is composed of several layers: the pericarp, the seed coat, the endosperm (aleurone layer and starchy endosperm) and the embryo. The bran layer where the WB is from, refers to the outer layers of the wheat, the pericarp, seed coat and the aleurone (Esau, 1965). Often WB contains some of the starchy endosperm as well, with the harder varieties containing less (since they split mostly along the cell walls of the endosperm) and the softer varieties containing more (since they do not break cleanly and the starchy endosperm is less clearly separated; Blakeney et al, 1979; Bass, 1988).

WB is a rich source of dietary fiber with some varieties, particularly the hard red kind, containing more than 40% (Ferguson and Harris, 1997). The WB dietary fiber is composed of complex polysaccharides, celluloses and hemicelluloses, and also lignin (Fincher and Stone, 1986; Schwartz et al, 1988), which protects the cell walls from degradation by colonic bacteria (Ferguson and Harris, 1999). The WB fiber is associated with a number of other components such as ferulic acid (Basic and Stone, 1981), lignans (Thompson, 1994), phenolic acids (vanillic acid, p-coumaric acid, protocatechuic acid, syringic acid, p-hydroxybenzoic acid, caffeic acid, genisitic acid)(Onyeneho and Hettiarachchy, 1992), flavonoids (mostly flavones and flavonols, catechin, proanthocyanidin)(Feng and McDonald, 1989), vitamin E, β-carotene (Verhagen et al, 1997), β-sitosterol (Lupton and Turner, 1999) and, perhaps most importantly, phytic acid (PA; Cosgrove, 1989; Stone 1996). A number of these compounds may have individual effects on colon cancer. For example, ferulic acid has been shown to be a strong antioxidant (Rice-Evans et al, 1997); lignans, either in pure form or from flaxseed, a rich dietary source, have been
shown to inhibit a number of ACF parameters (Jenab and Thompson, 1996); some of the phenolic acids may inhibit nitrosamine carcinogen formation (Pignatelli et al, 1982) or prevent carcinogen binding to critical targets (Wattenberg, 1985); some flavonoids may affect the activities of phase I and II enzymes, potentially affecting metabolic activation of dietary carcinogens (Uda et al, 1997) and β-sitosterol, a phytoestrogen, may be able to bind colonocyte nuclear estrogen receptors and has been shown to activate transcription of a tumour suppressor gene (Issa et al, 1994; Waliszewski et al, 1997).

2.6 Dietary fiber, wheat bran and colon cancer

World wide, cancer is the leading cause of death in adults aged 25 to 64 (Yang et al, 1996a), with approximately 35% of all the cancer mortality in North America and probably around the world being attributable to dietary factors (Reddy, 1995; Doll and Peto, 1981). Despite the gaps in knowledge about diet and cancer, many public health agencies in a large number of nations have formulated dietary cancer prevention strategies and made dietary recommendations to increase fruit, vegetable and dietary fiber intake to the general public (Cannon, 1992), since dietary modulation is nonetheless believed to have the greatest potential for impact in cancer prevention (Ames et al, 1995).

The most important role of dietary fiber may be its colon cancer protective effect. Colon cancer is a major health problem in North America and is the second leading cause of cancer death (Miller et al, 1993). It is a disease of affluence with rates being much higher in developed regions such as North America, Western Europe and Australia while less developed regions, such as India and China show much lower rates, with the largest differences between East and West being about 20 fold (Potter, 1996). Most of that is due to diet, with a high fat “Westernized” diet being promotive and a low fat, high fiber diet being protective.

2.6.1 Epidemiological studies: total dietary fiber and colon cancer

Many epidemiological studies have found that dietary fiber intake is negatively associated with mortality from colon cancer (Burkitt, 1971; McKeown-Eyssen and Bright-See, 1984; Bingham et al, 1985; West et al, 1989; Trock et al, 1990a, 1990b; Thun et al, 1992; Helzlsouer et al, 1994; Steinmetz et al, 1994; Ghadirian et al, 1997). In combined and meta-analyses of case control studies, strong inverse relationship between dietary fiber intake and colon cancer incidence were found in 55 (Greenwald et al,
1987), 23 (Trock et al, 1990b), 12 out of 13 (Howe et al, 1992), 13 (Dwyer, 1993) and 13 out of 19 studies (Pilch et al, 1987). Trock et al (1990a) showed a colon cancer protective odds ratio of 0.57 comparing the highest to the lowest quintiles of fiber intake in 16 case control and 37 observational studies. In most cases, the magnitude of the protection was similar for all parts of the colon, for men and women and for all different age groups.

Similar colon cancer protective effects of high dietary fiber intake have been observed in individual studies in China (Hu et al, 1991; Guo et al, 1993), in Russia (Zaridze et al, 1992) and in Argentina (Iscovitch et al, 1992). Perhaps in one of the longest studies to date, Jansen et al (1999) found, in a 25 year ecological epidemiologic study spanning seven countries, that a 10 gram increase in daily dietary fiber intake was associated with a 33% lower 25 year colorectal cancer mortality risk. Comparing different population groups at high and low risk for colon cancer, Reddy et al (1978) found that the three fold lesser incidence of colon cancer amongst rural Finns compared to New Yorkers could be due to differences in the intake of dietary fiber – twice as much amongst the Finns versus the New Yorkers. Likewise, Kuhnlein et al (1981) showed that vegetarians, considered at lower risk for colon cancer, had significantly lower levels of fecal mutagens than non-vegetarians.

However, many epidemiological studies have also found no significant association between fiber intake and colon cancer risk (Drasar and Irving, 1973; Haenszel et al, 1973, 1980; Phillips, 1975; Lyon and Sorensen, 1978; Bingham et al, 1979; Martinez et al, 1981; Helms et al, 1982; Miller et al, 1983; Minowa et al, 1983; Pickle et al, 1984; Tajima and Tominaga, 1985; Potter and MacMichael, 1986; Walker et al, 1986; Giovannucci et al, 1994). Hill and colleagues (1979) have shown a colon cancer risk enhancing effect of fiber rich foods while, in a prospective study of nurses, Fuchs et al (1999) reported no colon cancer protective effects from either total dietary fiber or dietary fiber from cereals, fruits or vegetables. Slattery et al (1988) have shown that fruit and vegetable intake lowered colon cancer risk whereas grains had no effect, suggesting varying effects for different types of fibers.

2.6.2 Epidemiological studies: cereal fiber and colon cancer

Epidemiological studies are often complicated by varying definitions of dietary fiber making it hard to define the exact type of dietary fiber which may pose the most protective effect. Epidemiologic studies which determined the type of dietary fiber which may have stronger protective effects showed dietary fiber intake from cereals and pulses to be the most effective (Irving and Drasar, 1973; Armstrong
and Doll, 1975; Howell, 1975; Schrauzer, 1976; Yanai et al, 1979; Liu et al, 1979; Bingham et al, 1979; McKeown-Eyssen and Bright-See, 1984; Macquart-Moulin et al, 1986; Kune et al, 1987; La Vecchia et al, 1988; Tuyns et al, 1988; Peters et al, 1989; Freudenheim et al, 1990; Willett et al, 1990; Bidoli et al, 1992) with statistically significant correlation coefficients ranging from -0.31 (Drasar and Irving, 1973) to -0.96 (Bingham et al, 1979). In addition, Hill (1997), reviewed over 55 epidemiological studies involving cereals, cereal fiber and colon cancer risk and found that, in general, cereals were very protective. These results are in contrast with other studies which found no effect (Martinez et al, 1975; Manousos et al, 1983; Miller et al, 1983; Benito et al, 1990; Centonze et al, 1994) or even a promotive effect (Potter and McMichael, 1986; Freudenheim et al, 1990; Meyer and White, 1993). The disparity in results with cereal or cereal fiber intake suggests that, like different dietary fibers, not all cereal dietary fiber sources may contribute equally to effects on colon carcinogenesis. In most populations tested around the world, cereal intake accounts for between one third to over a half of the total dietary fiber intake, with vegetables and fruits accounting for the rest (Wolever and Jenkins, 1997). This suggests that in most epidemiological studies looking at total dietary fiber and colon cancer risk it may be possible to assume that cereals are contributing considerably to the total dietary fiber measurements.

2.6.3 Epidemiological and clinical studies: wheat bran and colon cancer

Of the cereal dietary fiber sources, WB appears to show a greater protective effect, particularly for early biomarkers of colon cancer risk. Experiments on both normal and high colon cancer risk humans consuming different amounts of WB (summarized in Appendix Table 11.1), have shown decreases in the levels of fecal mutagens (Reddy et al, 1987; Reddy et al, 1989; Reddy et al, 1994), fecal bile acids (Reddy et al, 1987; Reddy et al, 1989; Lampe et al, 1992; Reddy et al, 1992; Alberts et al, 1996) and colonic cell proliferation (Alberts et al, 1990; Rooney et al, 1994) as well as increases in fecal butyrate concentration (Kashtan et al, 1992), changes considered risk reducing or protective of colon cancer. In contrast, there are human clinical trials which observed no difference in rate of cell proliferation with WB supplementation for various lengths of time (Gregoire et al, 1992; Kashtan et al, 1992; Alberts et al, 1997). These apparent inconsistencies in the results with WB supplementation suggest that better controlled studies and more information about the endpoint biomarkers are required.

Although prospective (Giovannucci et al, 1992) and case control (Little et al, 1993; Sandler et al, 1993) studies have found significant relationships between total dietary fiber intake and adenomatous
polyp recurrence, only a few studies have tested the effects of WB supplementation per se. In the FAP Trial, De Cosse et al (1989) found a dose dependent negative correlation between the intake of wheat fiber from WB in excess of 11 g/day and polyp number ratios. This effect was maintained even after adjustments for vitamin intake. The Australian Polyp Prevention Project (MacLennan et al, 1995) evaluated the long term effect of WB supplementation on polyp recurrence after resection of colorectal adenomas and found a significant interaction between dietary fat reduction and WB intake and reduction of adenomas greater than 10 mm in diameter after 24 and 48 months. In the ongoing Arizona Phase III Polyp Prevention study, supplementation of the diet of post polypectomy patients with 13.5 g/day of WB has so far not shown any significant effects (Earnest et al, 1999).

There are some inherent problems and issues with epidemiological studies including the high degree of collinearity among major nutrients, interactions with other dietary components, the feasibility of food composition tables, the use of food frequency questionnaires and the appropriate definition of dietary fiber and fiber components from different types of food (Potter, 1996; Harris and Ferguson, 1999). This suggests that more research is necessary in identifying the exact definition and appropriate mode of measurement of dietary fiber and the appropriate risk markers or experimental endpoints. It must also be noted that there are many other confounding factors in epidemiological studies such as the intake of fat, flavonoids, phytoestrogens, antioxidants, vitamins, iron, calcium, as well as obesity and physical activity among others (Cummings and Bingham, 1998). Thus, to control for some of these variables, the effects of dietary fiber and WB supplementation on colon cancer, numerous animal studies have been conducted.

2.6.4 Animal studies: wheat bran and colon cancer


The variabilities in results may be attributed to a number of factors including (a) the level of WB used, which ranged from 4% (Alabaster et al, 1993) to 45% (Maziya-Dixon et al, 1994), (b) the level of fat used, from 2% (Fleiszer et al, 1978) to 35% (Nigro et al, 1979; Nigro and Bull, 1987), (c) the type and percent of fiber in the control group (ranging from grain based non-purified to fiber free), (d) the use of different species (rat vs. mouse) and different strains, with better protective effects being observed in F344 rats, (e) type, dose, number of injections and timing of the carcinogen used, (f) length of feeding period, ranging from 15 (Bauer et al, 1979) to 48 weeks (Cruse et al, 1978) and (g) the use of appropriate statistics. However, the most consistent protective effects (decrease in tumour incidence and/or frequency) were observed with the use of high WB (≥20%) levels supplemented with a low fat diet (4 - 6%; Barbolt and Abraham, 1978; Chen et al, 1978; Fleiszer et al, 1978; Nigro et al, 1979; Abraham et al, 1980; Barbolt and Abraham, 1980; Nigro and Bull; 1987; Iwane, 1989). In contrast, all but one of the equivocal studies (Tatsuta et al, 1988) used a high fat diet with varying levels of WB.

The lower levels of WB may not result in a protective effect since it may be below or close to the threshold required for a significant effect. On the other hand, higher levels may be counterproductive, causing irritation and increasing the colonic cell proliferation. Diets with high fat levels may enhance tumourigenesis masking any WB protective effects, particularly if they are slight or moderate (Shivapurkar et al, 1992; Steinbach, 1993). The effect of fat is highlighted by the study of Nigro and Bull (1987) who found no significant effect of WB supplementation with a high fat diet, but did see a significant decrease in the number of tumours in a lower fat model (5%). Human clinical studies have shown significant interactions between WB fiber and reduced fat in the inhibition of adenomatous polyp recurrence (MacLennan et al, 1995). The most reasonable percent fat content level which does not mask the protective effects of WB appears to be 4 - 7%.

Some of the experiments had basal diet or control groups using semi-purified diets with 0% fiber (Wilson et al, 1977; Chen et al, 1978; Bauer et al, 1979; Nigro et al, 1979; Watanabe et al, 1979; Barnes et al, 1983; Jacobs, 1983; Clapp et al, 1984; Calvert et al, 1987; Tatsuta et al, 1988; Iwane,
1989; Sinkeldam et al, 1990) while others used a grain based non-purified basal diet (Barbolt and Abraham, 1978; Cruse et al, 1978; Fleiszer et al, 1978; Abraham et al, 1980; Barbolt and Abraham, 1980; Fleiszer et al, 1980) or even a 1% WB basal diet (Alabaster et al, 1993, 1995, 1997; Shivapurkar et al, 1995). Some of the studies even used 20% cellulose (Takahashi et al, 1999) or 6% oat bran (Zoran et al, 1997a) as their control or comparison groups, making interpretation of any WB protective effects observed very difficult. Individual components in the non-purified control diets, which are also often high in fiber, could have had their own effects on colon carcinogenesis and so the use of such basal diets appears to be inappropriate. On the other hand, the use of a fiber free control diet may also be inappropriate since such diets have been shown not only to produce very little feces and to increase transit time but also to slow down mucosal cytokinetics as well as cause changes in the intestinal microflora which may affect the fecal bile acid profile (Klurfeld, 1990) and normal intestinal function. Thus, a low fiber (5%) diet, composed of a pure source of fiber such as cellulose, as opposed to a fiber free diet, non-purified diet or another type of dietary fiber, is more appropriate for the control group.

It appears that WB fiber is colon cancer protective under certain experimental conditions, with better results being achieved with the supplementation of a high level of WB to a low fat diet in the Fischer 344 rat. In addition, the use of a low fiber control diet is more appropriate than fiber-free or unpurified fiber diet.

The termination timepoint of the study may also be of significance. Nakaji et al (1996), performed an endoscopic study where the number of tumours were counted in each rat each week for 30 weeks throughout the study. They found that although there was lower induction of tumours and decreased number of tumours up to the 24th week of treatment with the WB diet, at sacrifice, there were no significant differences between the WB and control diets. This suggests that the timepoint for termination of a study is of vital importance and may impact the results dramatically.

Also of interest is the study of Alabaster et al (1993) which shows a synergism between WB and other dietary fiber sources, i.e. psyllium. They supplemented a high fat, low calcium diet (20% and 0.18% w/w respectively) with 1, 4 and 8% dietary fiber from WB and / or psyllium for 25 weeks with feeding commencing 2 weeks prior to the carcinogen injection. They found that both colon tumour number and multiplicity had a decreasing trend with increasing levels of dietary fiber from WB or psyllium alone. However, their equal combination produced a significant protective effect. Alabaster et
al (1996), reported further that such synergism has also been observed with WB and rice bran and WB and oat bran, suggesting that consumption of a variety of different types of dietary fibers may reduce colon cancer risk more so than a single source of dietary fiber. In another interesting study, Young et al (1996) showed that WB supplementation can reduce the potato starch potentiated increase in colon tumourigenesis down to basal levels, suggesting that WB supplementation can effectively eliminate the promoting effect of some other dietary constituents.

2.6.5 Animal studies: wheat bran and early biomarkers of colon cancer risk

A number of studies (summarized in Appendix Table 11.3) have used various early colon cancer risk markers, such as cell proliferation or ACF, as endpoints. Like the tumourigenesis studies, these experiments also used various amounts of WB, ranging from 2.5% (Floor et al, 1991) to 20% (Jacobs and White, 1983; Floor et al, 1991; Ishizuka and Kasai, 1996), and various amounts of fat, from 1% (Floor et al, 1991) to 20% (Alabaster et al, 1995; Shivapurkar et al, 1995). Five of the studies (Pence et al, 1985; Robblee et al, 1989; Floor et al, 1991; Lupton and Kurtz, 1993; Shivapurkar et al, 1995) were equivocal and ten showed protection via increase in fecal bile acid excretion (Gestel et al, 1994) and decreases in cell proliferation (Jacobs and White, 1983; Boffa et al, 1992; Folino et al, 1995), number of intestinal and colonic polyps (Hioki et al, 1997) and in the number of ACF per rat (Alabaster et al, 1995, 1997; Ferguson and Harris, 1996; Ishizuka and Kasai, 1996; Compher et al, 1999). To date seven studies have used WB along with ACF as an early risk marker (Alabaster et al, 1995; Shivapurkar et al, 1995; Alabaster et al, 1997; Ferguson and Harris, 1996; Ishizuka and Kasai, 1996; Young et al, 1996; Compher et al, 1999). All but three of these studies (Ferguson and Harris, 1996; Ishizuka and Kasai, 1996; Compher et al, 1999) continued their experiments to the tumour progression stage, the results of which have been discussed above. The objectives of the study by Alabaster et al (1995) were not to see the effects solely of WB on colon carcinogenesis but rather the effects of the combination of WB and β-carotene. They used two levels (1 and 8%) of WB supplemented with various amounts (from 0 to 20 mg) of β-carotene and found that WB showed a dose-response protective effect in the short term (6 weeks), significantly reducing the total number of ACF per rat. However, they did not perform other important analyses such as the size of ACF, type of ACF mucin production or degree of luminal alterations. Although their ACF results were significant between the 1 and 8% WB diets with no added
\( \beta \)-carotene, they only used 5 rats per group. In addition, for their statistical calculations they used a two way analysis of variance followed by the student’s t-test. However, a multiple group comparisons test was not performed, putting into question the statistical validity of the results.

The study by Shivapurkar et al (1995) tested the effects of WB as well as \( \beta \)-carotene, folic acid and vitamin E, on pre-existing ACF. Five rats from each group were killed at 10, 14 and 18 weeks post initiation for the study of only ACF multiplicity. Like the study by Alabaster et al (1995) other analyses of ACF were not performed. They found no significant changes in ACF multiplicity between the WB and the high fat control diet, although a multiple group comparison’s test was not performed. Similarly, the objectives of Alabaster et al (1997), were not focused on effects of WB components but rather to observe if there are differences between unprocessed WB and two common breakfast cereals containing WB. Although they showed no differences between the unprocessed WB and the breakfast cereals, they did show that 4 and 8% WB diets reduced the number of ACF versus a 1% WB diet. All the studies from the Alabaster research group analyzed only one or two ACF parameters and did not perform other important analyses such as the size of ACF, type of ACF mucin production or degree of AC luminal alterations. As discussed earlier there is some controversy regarding which ACF parameters are best reflective of colon cancer risk. The measurement and significant change of more than one parameter will increase the likelihood that a real effect is being observed. Neither of these studies has focused solely on the protective effects of WB or its components and all suffer from the problem of level and source of dietary fiber in the control group, discussed above.

The study of Young et al (1996), focused on the promotion of colon carcinogenesis by diets containing potato starch. The WB diet contained appreciable amount of potato starch and was not significantly different from the control diet in effect, expressed as number of ACF. In addition, they found an increase in the rate of cell proliferation, attributed to the potato starch. The inclusion of potato starch is a confounding factor and together with the lack of measurement of multiple ACF parameters, does not allow direct conclusions to be drawn on the protective effects of WB or its components in this study.

Compher et al (1999) studied the effect of 20% WB supplementation in a high fat (20%) diet at the initiation (1 week post carcinogen) and promotion (8 weeks post carcinogen) stages. Although they found a decrease in ACF at the promotion stage, their results may be questionable since they only used 2
cm portions of either proximal or distal colon sections for analysis of ACF. Any ACF present in other sections of the colon were not accounted for and so a whole picture of ACF parameters was not presented. The study by Ishizuka and Kasai (1996) is also questionable because of the small amount of information provided. Like the other studies listed above, they only discussed one or two ACF parameters and although they saw a decrease in number of ACF, they also observed a significant increase in ACF multiplicity. Likewise, the study of Ferguson and Harris was also very small (n=3 per group) and certainly needs confirmation.

Evidently more research on the effects of WB on colon carcinogenesis and its early biomarkers is necessary. However, under certain conditions, WB is protective of colon tumourigenesis, with better results being achieved with the use of a high WB/low fat diet in the F344 rat. Despite the observed protective effects of WB fiber in animal studies, the mechanisms of its action are mostly speculative and unknown.

2.7 Mechanisms of action of wheat bran

Studies have shown that WB has the ability to reduce experimental colon carcinogenesis as well as some risk markers of colon cancer such as ACF and rate of cell proliferation. Over the past few decades many mechanisms for the protective effects of WB and dietary fibers in general have been hypothesized (Klurfeld, 1997). They include (a) physical dilution of the gut contents thus diluting the effects of carcinogens (Schneeman, 1987), (b) shortening transit times thus decreasing contact between the colonic mucosa and tumour initiators or promoters (Eastwood, 1990), (c) alterations in the mutagenicity of intestinal contents, (d) alterations in mucosal cytokinetics (Klurfeld, 1992), (e) increased fermentation producing butyrate with protective effects on gut epithelium (Newmark and Lupton, 1990), (f) alteration of bile acid metabolism (Nagengast et al, 1993), (g) stimulation of bacterial growth and change in its metabolic activity (Jacobs, 1988), (h) alteration in sterol metabolism, gut hormones and decrease in epidermal growth factors (Schaudies et al, 1991), and (i) effect on the production, absorption and excretion of putative carcinogens and promoters (Reddy, 1988). Some of the important mechanisms that relate to WB action are discussed further below:
2.7.1 Bulking and transit time

Some sources of dietary fibers, particularly insoluble ones such as WB, have a fecal bulking effect through their physical presence, increased bacterial mass and absorption of water (Gazzaniga and Lupton, 1987). One gram of WB fiber has been suggested to increase stool bulk by 5.7 g (Black, 1996). This may dilute or adsorb any mutagens or carcinogens present (Schneeman, 1987; Harris et al, 1993; Kestell et al, 1999) thus lowering the concentrations effective in initiating or promoting carcinogenesis. For example, WB has been shown to dilute the concentration of secondary bile acids (Reddy et al, 1978; Galloway et al, 1986), which are known to be strong promoters of colon carcinogenesis (Klurfeld, 1997). Another effect of increased bulk is accelerated transit thus decreasing contact between the colonic mucosa and tumour initiators or promoters (Eastwood, 1990; Kestell et al, 1999). WB has been shown to be much more effective than other dietary fiber sources (Lupton and Meacher, 1988). The increased bulk is partly due to greater bacterial production and can cause an alteration in its metabolic activity (Jacobs, 1988), affecting enzymes such as β-glucuronidase.

2.7.2 Metabolic activity of colonic flora

β-glucuronidase is one of the major bacterial enzymes implicated in the activation of carcinogens, mutagens and genotoxic substances. It is an inducible enzyme with a wide substrate specificity and is believed to be largely responsible for the hydrolysis of glucuronide conjugates in the large intestine (Reddy, 1992). Many compounds, both endogenous and from the diet, are conjugated with glucuronic acid or sulfate in the liver and secreted into the intestinal tract for disposal via the bile (Chipman, 1982). In the colon, the glucuronide biliary conjugates may be hydrolyzed by β-glucuronidase, leading to the release of the parent compound, which is considerably more toxic than its glucuronide conjugate (Weisburger, 1971). The parent compound may then again undergo enterohepatic circulation, thus re-exposing the colon to any carcinogenic or mutagenic properties that it may possess (Goldman, 1982, Rowland et al, 1985).

Since β-glucuronidase is involved in the metabolism of procarcinogens to carcinogens (Weisburger, 1971) and its activity may play a role in the etiology of colon cancer (Reddy et al, 1974; Gorbach and Goldin, 1992), the modification of its activity by diet has been suggested to have important consequences for the health of the colon. This is particularly important since WB has been shown to

The potential of WB to modulate colonic bacterial activity is also highlighted by the study of Reddy et al (1994), showing that WB supplementation can reduce bacterial diacylglycerol (DAG) production. DAG is thought to be mitogenic to the colonic epithelium and influence colonic mucosal protein kinase C (PKC) activity.

2.7.3 Carcinogen binding and bile acids

Because of the lignified cell walls in its dietary fiber (Ferguson and Harris, 1999), WB can also bind carcinogens or tumour promoters (Klurfeld, 1997) such as primary and secondary bile acids (Jacobs, 1988; Hill, 1991). This does not allow them to contact the epithelial surface or to undergo enterohepatic circulation. For example, WB has been shown to effectively bind a number of colon carcinogens such as DNP (Harris et al, 1998), MelQx (Ryden and Robertson, 1995) and DMH (Smith-Barbaro et al, 1981).

Although all of these mechanisms may provide a means whereby WB may be protective of colon cancer, no firm conclusions can be made due to some contradictions in the literature. For example, Glober et al (1977) have shown that Japanese immigrants to Hawaii had much lower incidence of colon cancer than their Hawaiian born sons, but both groups exhibited similar stool bulk and transit times and, presumably, similar dilution potential of putative carcinogens, bile acids and bacterial enzymes. In addition, Calvert et al (1987) showed that 10% WB produced less tumour yield in rats than a fiber free diet, even though the two diets were adjusted to produce the same levels of fecal bile acids. These studies suggest that there are other mechanisms of WB action.
2.7.4 Colonic fermentation, production of short chain fatty acids

A large proportion of soluble dietary fibers and some of insoluble dietary fibers are fermented to short chain fatty acids (SCFA), mostly acetate, propionate and butyrate (Holtug et al, 1992). They are involved in sodium absorption, epithelial cell nutrition, proliferation and differentiation, and thus play a major role in the normal functions of the colon (Velazquez and Rombeau, 1997). Although composed of mostly insoluble dietary fibers, WB has been shown to undergo some fermentation (McIntyre et al, 1993). In contrast to more rapidly fermented fibers which produce rapidly absorbed SCFA in the proximal colon, the slow fermentation of WB tends to increase total SCFA and also butyrate concentrations in the distal colon. WB feeding has been shown to increase the molar concentration of butyrate in rats (Lupton and Kurtz, 1993; McIntyre et al, 1993; Mathers and Tagny, 1994; Folino et al, 1995; Compher et al, 1999) and in humans (Kashtan et al, 1992). Thus, it has been suggested that WB could be protective against colon cancer through the fermentation of some of its fiber by anaerobic bacteria in the colon (Kritchevsky, 1999).

Of all the SCFA, butyrate is the most important fuel source for and has a protective effect on the colon epithelial cells (Roediger, 1982). It suppresses glucose oxidation in these cells and is also involved in the modulation of nucleic acid metabolism, for example, the regulation of gene expression and cell growth. (Cummings and Macfarlane, 1991). Butyrate has been shown in-vitro to reversibly prolong the doubling time and to slow down the growth rates of human colorectal cancer cell lines (Kim et al, 1982; Sakata, 1987); to reduce DNA synthesis (Borenfreund et al, 1980); to inhibit the enzyme histone deacetylase thus allowing greater access of repair enzymes to the DNA, and promoting chromatin stability during cell division (Smith, 1986); to induce apoptosis in human colon cancer cell lines (Hague et al, 1993); to induce terminal differentiation in leukemia cells and keratinocytes (Staiano-Coico et al, 1992); and to suppress colon tumour formation (McIntyre et al, 1993). Krupitza et al (1995) have shown that butyrate can block the expression of the c-myc oncogene as well as influence components of the intracellular signaling network in ovarian carcinoma cells. Thus, the possibility exists that butyrate may act to block the expression of other oncogenes, particularly those involved in colon cancer such as k-ras. It is clear that butyrate production may play a critical role in colonic health and also in colon cancer.

However, the extent of that role is not clear due to some controversy brought to light by some recent studies. Caderni et al (1998) have shown that rats fed butyrate pellets, which significantly
increase butyrate concentrations in the distal colon, showed increased colonic cell apoptosis but not significantly different ACF parameters or rate of cell proliferation from control rats. This study suggests that butyrate can modulate cell processes such as apoptosis, but that any protective effects may be seen only in the long term and not at the early biomarker stage. However, Zoran et al (1997a) have shown that WB, compared to oat bran, produced significantly less butyrate in the distal colon while having significantly decreased number of tumours, suggesting butyrate had no long term effects either. If butyrate production is a key factor in protection against colon carcinogenesis, it would be expected that production of more butyrate by oat bran would result in greater protection. However, the study of Zoran et al (1997a) is unique since they found significantly higher amount of butyrate in the distal colon with oat bran feeding. Other studies (McIntyre et al, 1993) showed that butyrate from oat bran fermentation is produced and absorbed mostly in the cecum and barely affects distal colonic butyrate levels or that WB yields the highest amount of butyrate (Adiotomre et al, 1990). In addition, direct rectal or cecal instillation of butyrate has been shown to reduce the size and number of distal colonic tumours (D'Argenio et al, 1996; Medina et al, 1998). Part of the answer to this controversy may lie in the comparison of the in-vitro and in-vivo activities of butyrate. As discussed above, most of the effects of butyrate have been observed in-vitro. In-vivo, butyrate has had some paradoxical effects, for example, having been shown both to inhibit colonic cell proliferation in the proliferative compartments of colonic crypts (Boffa et al, 1992; Lupton and Kurtz, 1993; Velazquez et al, 1996b), and to stimulate it (Sakata, 1987; Folino et al, 1995). Some of this may be explained by the general understanding that butyrate has a trophic (growth promoting) effect on normal cells and a growth inhibiting effect on neoplastic colonocytes (Velazquez et al, 1997). However, the full in-vivo effects of butyrate need further research. Although none of these studies used a colon carcinogen, colonocytes from carcinogen treated animals have been shown to metabolize butyrate differently (Lupton, 1995) and to have a higher intracellular pH (Zoran et al, 1997b) and so may behave differently than those from normal animals. Since different fibers produce varying proportions of butyrate (Ferguson and Harris, 1996), it is possible that WB produces just the right proportion of butyrate to the other SCFA to obtain a protective effect (Kritchevsky, 1998). Thus, similar to the issues discussed in reviewing the WB and colon cancer studies (Appendix Table 11.2), in-vivo studies on the effect of butyrate may produce confounding results depending on the experimental protocol.
2.7.5 Colonic pH

Since acidification of the colonic contents has been proposed to be protective of colon cancer (van Dokkum et al., 1983), one mechanism for the protective effect of the SCFA in general could be their contribution to the acidification of the colonic contents. The production of SCFA in the colon, whether from the fermentation of WB fiber or starches within the matrix of WB, may cause reductions in pH (Newmark and Lupton, 1990). Walker et al. (1986) have found that variations in cancer risk amongst different populations in South Africa were only significantly related to fecal pH and not dietary fiber intake. Others have shown lowered fecal pH associated with WB intake (Lampe et al., 1992; Folino et al., 1995). Higher pH of colon contents has been linked in epidemiological studies to an increased risk for colon cancer (Macdonald et al., 1978; Malhotra, 1982; Walker et al., 1986; Heaton, 1983). Change in pH can affect the composition and enzyme activity of the microflora and absorption of particular compounds (Mallett et al. 1989; Newmark and Lupton, 1990). For example the activity of 7-α-dehydroxylase, which is involved in the synthesis of secondary bile acids from primary bile acids, is inhibited by pH below 6.5 (Thornton, 1981). In addition, with lowered pH, the promotive ability of the primary and secondary bile acids is decreased due to lowered solubility of free bile acids (Bruce, 1987), and increased availability of calcium to bind them (Newmark and Lupton, 1990). Furthermore, reduced pH may also reduce the absorption of ammonia by epithelial cells since it is trapped by anions (Bown et al., 1975). Ammonia has been suggested to increase the rate of cell proliferation and thus enhance the risk for colon cancer (Visek, 1978; Clinton et al., 1987). It must be noted, however, that very low pH values may stimulate cell proliferation and so may actually be a risk factor for colon cancer (Newmark and Lupton, 1990).

2.7.6 Cell proliferation, apoptosis and differentiation

WB fiber may also be exerting its protective effects by lowering the rate of cell proliferation. In a recent study, Alberts et al. (1990) found that patients who had received resections for colorectal cancers, and so were at high risk for the disease, showed a total of 22% decrease in rectal mucosal cell rate of proliferation when taking daily WB supplements, suggesting that WB fiber can inhibit DNA synthesis. A decrease in the rate of cell proliferation could also have been a factor in DeCosse et al.’s (1989) observation of a decrease in adenomatous polyps in the low sigmoid colon and rectum of WB
supplemented patients suffering from familial polyposis. Several animal studies have also shown a decrease in the rate of cell proliferation with WB supplementation (Jacobs and White, 1983; Boffa, 1992; Folino et al, 1995). In addition, Compher et al (1999), have shown that WB supplementation inhibits the rise in colon cell proliferation normally observed in rats upon carcinogen injection.

In the colon carcinogenesis process, the interplay between cell proliferation, apoptosis and differentiation is very important (Butler et al, 1999). The inhibition of apoptosis has been shown to play an important role in the genesis of colon adenomas and carcinomas (Bedi et al, 1995) and has been suggested to be a risk factor for colon cancer (Garewal et al, 1996). If colonic epithelial cells no longer respond to DNA damage by undergoing apoptosis, then mutations possibly leading to colon cancer may be acquired and fixed through further proliferation. Compher et al (1999) have recently shown an increase in apoptosis by WB supplementation at the initiation stage of colon carcinogenesis. Similar to apoptosis, delay or failure in differentiation has been proposed to be important in assessing the dietary colon cancer risk (Yang et al, 1996b). Both the microfloral environment of the colon (Bry et al, 1996) and diet (Yang et al, 1996b; Chang et al, 1997), which can affect the flora, have been shown to modulate the degree of cell differentiation in the colon. In addition, the feeding of some known differentiating agents has been shown to significantly decrease some ACF parameters (Wargovich et al, 1995). Very little else is known about the effects of WB on apoptosis and differentiation and, with regard to the recent suggestion that their measurement may have greater prognostic significance in the assessment of dietary effects on tumour incidence and colon cancer risk than measurements of cell proliferation alone (Chang et al, 1997), more research is needed in this area.

2.7.7 The insulin hypothesis

Based on the similarities in increased risk of colon cancer and diabetes with obesity, physical inactivity and high fat diet, a number of different research groups (McKeown-Eyssen, 1995; Giovannucci, 1995; Bruce and Corpet, 1996) have suggested that hyperinsulinemia, acting as a growth factor, may promote colon carcinogenesis. Both case control and cohort epidemiological studies have provided evidence for a link between colon cancer and non-insulin dependent diabetes mellitus (Steenland et al, 1995; La Vecchia et al, 1997; Weiderpass et al, 1997). Others have shown an association between colon cancer, polyps and increased fasting glucose and serum triglycerides (Bird et al, 1996). Colon cancer patients have more glucose intolerance and insulin resistance (Yam et al, 1996)
and diabetics show an excess mortality from colon cancer (Kim, 1998). In animal models, growth of ACF (Corpet et al, 1997) and colon tumours (Tran et al, 1996) have been shown to be increased with insulin injection. In addition, Koohestani et al (1998), comparing diets with different levels and types of fat, have shown that increased ACF growth is related to glucose intolerance, higher fasting insulin, triglyceride levels and energy consumed, all also being risk factors of diabetes.

2.7.8 Other potential mechanisms

Some dietary constituents, particularly fibers, may induce changes from the normal appearance of the colonic mucosa. Of several dietary fiber sources, WB has been shown to induce the least amount of ultrastructural colonic disruption and the most amount of mucin production (Klurfeld, 1990). Greater mucin release works to effectively protect the colonic mucosa from the luminal contents (Satchithanandam et al, 1996). It has been shown to adsorb carcinogens (Ng et al, 1992), inhibiting their interaction with colon cells. Thus, the ability of WB to induce greater mucin production to protect the mucosa from the colonic milieu and to not disturb the colonic morphology (and thus, to not induce compensatory cell proliferation) may also be involved in its mechanisms of action.

WB may also be colon cancer protective by altering local levels of certain peptide growth factors, such as epidermal growth factor, which has been shown to be reduced with WB supplementation (Schaudies et al, 1991).

Recently there has been some question as to whether the cancer protective effect of WB is only due to its fiber component (Ferguson and Harris, 1998). The inverse association of colon cancer and the degree of WB fiber intake could also be the result of the action of other components closely associated with the fiber. In other foods which are good sources of fiber, trace amounts of anticarcinogens with established properties are found (Steinmetz and Potter, 1991) such as phenolic compounds, sulfur containing compounds, flavones, phytoestrogens, lignans and vitamins with antioxidant properties and particularly PA (Potter, 1992). PA is perhaps the most abundant (some sources contain as much as 4.5%) and most promising phytochemical in WB (Graf and Eaton, 1985). However, none of the studies listed in Tables 11.1, 11.2 or 11.3 have considered the possibility that the PA in WB could be in part responsible for its protective effects.
2.8 Phytic acid:

2.8.1 Structure and occurrence

PA is myo-inositol hexa-phosphate (Figure 2.1; Graf and Eaton, 1993) and is an ubiquitous plant component often complexed with calcium, potassium or magnesium in cereals or as crystalline globule inside protein bodies in legumes, nuts and oilseeds (Reddy et al, 1982). In many cereals and oilseeds, PA constitutes up to 1 to 5% of the weight and serves as the chief storage form of phosphorus (between 60 – 90% of total phosphorus) as well as a natural antioxidant by chelating and reducing the catalytic activities of many divalent transition metals (Graf et al, 1984; 1987). Some of these are nutritionally important such as calcium, magnesium, copper, iron, zinc, cobalt and manganese (Graf and Eaton, 1990) and so PA is thought to be an anti-nutrient because it may reduce their bioavailability (Brune et al, 1989). In wheat, PA is found primarily in the aleurone or bran layer, mostly as a mixed calcium-magnesium-potassium salt (Lott and Ockenden, 1986). PA may also bind other positively charged compounds such as proteins and starches (Figure 2.2) and reduce their availability.

2.8.2 Synthesis and breakdown

PA is generally stable (Graf and Eaton, 1990), but during germination, it is broken down by endogenous phytase enzyme into a mixture of lower inositol phosphates (also referred to as IP₁ through IP₅; PA being IP₆), inositol and inorganic phosphate (Cosgrove, 1989). A number of different PA synthetic pathways have also been identified in a number of seeds (Scott and Loewus, 1986). However, unlike plant cells, mammalian cells were thought not to be capable of converting the lower inositol phosphates to PA and that the only intracellular source of lower inositol phosphates was from the breakdown of phosphatidylinositol-4,5-bisphosphate (PIP₂) to IP₃ and its rephosphorylation to IP₄₋₆ (Shamsuddin et al, 1997).

PA has been shown to be rapidly absorbed and to be converted to lower inositol phosphates in various murine and human cells in-vitro (Vucenik and Shamsuddin, 1994) and in-vivo (Sakamoto et al, 1993a), suggesting that it can provide lower inositol phosphates for participation in the intracellular inositol phosphate pool. In contrast to intestinal phytase, dietary phytase is thought to contribute considerably to PA breakdown in the digestive tract (Sandberg and Andersson, 1988). However, the
Figure 2.1: Basic structure of PA
Figure 2.2: Potential PA interactions with proteins, minerals and starches. (A) indicates divalent mineral interactions, (B) and (C) indicate interaction with protein and (D) shows interaction with starch.
influence of PA interactions with different food components on the extent of its breakdown and absorption in the digestive tract are not known.

Inositol, the parent compound of PA, is a common dietary component with about 1 g of daily consumption (Harland and Morris, 1995) and can be produced in the testis, mammary gland, brain, liver and kidney from D-glucose (Harland and Morris, 1995). Thus, it may be a source for the intracellular production of IPs and PA. In fact, it has been shown that stimulation of HL-60 cells by chemotactic peptides results in greatly increased intracellular PA and IP₃ concentrations (Pittet et al, 1989), indicating production of higher IPs probably from either inositol or the lower IPs. In addition, Shears et al (1998) have shown that certain mammalian cell lines contain the enzyme necessary for the conversion of IP₃ to PA and also the enzyme multiple inositol phosphate phosphatase (MIPP), which is hypothesized to be able to interconvert a variety of IPs.

Dietary PA may be hydrolyzed by either dietary, intestinal or bacterial phytases (Rickard and Thompson, 1997). Such breakdown can alter the mineral, protein or starch binding abilities of PA. These abilities can also be influenced by whether the PA is present naturally in a foodstuff (e.g. WB) whether it might be complexed to other food components and not able to further react, or whether it has been added pure (e.g. as a sodium salt), which may be highly reactive with other dietary components such as minerals, proteins and starches (Rickard and Thompson, 1997).

2.8.3 Phytic acid: interactions with proteins

By virtue of its structure and highly negative charge at wide range of pHs, PA is capable of binding proteins. The interactions of PA with proteins are shown in Figure 2.2. It can form strong electrostatic linkages with basic amino acids at low pH (such as those in the stomach), precipitating most below pH 5 (Cheryan, 1980). At pHs above 5, PA and proteins complexes dissociate from each other since they are both negatively charged. However, PA can still bind protein by using polyvalent cations to form metal bridge (Cheryan, 1980). Thus, in the digestive system, PA can potentially bind both dietary proteins, inhibiting their digestion and absorption, and digestive enzymes such as trypsin (Singh and Krikorian, 1982) or α-amylase (Thompson, 1986), indirectly inhibiting the proper digestion and absorption of dietary proteins and starches. Added PA has been shown to significantly decrease the in-vitro digestibility of protein from a variety of sources, such as beans, peas, flour or pure proteins, suggesting that PA can influence protein digestibility (Carmovale et al, 1988). However, several in-vitro
(Serraino et al, 1985; Reddy et al, 1988) and in-vivo human (Reinhold et al, 1973) and animal (Yoshida et al, 1982; Thompson and Serraino, 1986) experiments have not supported the notion that PA interferes with protein digestibility, suggesting that more research is needed before a firm conclusion can be reached.

2.8.4 Interactions with minerals

Due to its ability to bind and chelate divalent minerals, PA may affect mineral absorption to varying degrees (Burk and Solomons, 1985). In fact, it has generally been thought that the effects of PA on mineral absorption and balance may be greater than that of fiber particularly with respect to zinc, for which PA has a very high affinity (Oberleas and Harland, 1977; Davies, 1978) and forms a very stable and insoluble complex with (Evans et al, 1988). The mineral interactions of PA are shown in Figure 2.2. A number of studies in both animals (Davies and Olpin, 1979; Morris and Ellis, 1980; Lo et al, 1981) and humans (Reinhold et al, 1973; Morris and Ellis, 1989; Turnlund et al, 1984) indicate that PA plays a strong role in inhibiting zinc utilization and growth. Increased PA intake may not necessarily lead to decreased absorption of all minerals. For example, by binding zinc, PA may in fact increase the absorption of copper which competes with zinc at the site of intestinal absorption (Lee et al, 1988). Other factors may also affect the degree of PA mineral binding and decrease mineral bioavailability. For example, the presence of dietary, endogenous or bacterial phytase may all act to hydrolyze PA in the gastrointestinal tract thereby potentially releasing any bound minerals. Also, the presence or lack of absorption enhancers such as ascorbic acid, vitamin D3 and meat protein may also play a role in affecting mineral bioavailability and absorption particularly in situations of combined high PA and marginal mineral intakes (Rickard and Thompson, 1997). Although purified PA can affect mineral availability, its role when associated naturally with the dietary fiber and whether the fiber itself has any inhibitory role is still under debate.

Studies trying to differentiate the role of fiber from that of PA have shown that when the amount of PA is maintained while the level of WB is altered, there is no significant effect on Ca, Zn, Fe or Mg balance (Andersson et al, 1983) suggesting that the fiber plays little role in the alteration of mineral bioavailability. Similar results have been obtained by Fairweather-Tait (1982) for iron. On the other hand, Morris et al (1988) have shown no significant difference between whole or dephytinized WB on mineral bioavailability suggesting that the PA does not play a role. Even other studies have shown that
observed interferences with mineral bioavailability are unrelated to either WB fiber or PA (Ranhotra, 1979). In addition, some early studies show that longer term intake of high PA diets can lead to adaptation where initial negative balances become positive after several weeks (Walker et al, 1948), suggesting that even if PA had any detrimental effects on mineral nutriture, that they would disappear over time and not affect long term mineral balance and bioavailability.

2.8.5 Toxicity

The LD₅₀ of PA in rats has been shown to be 450-500 mg/Kg of body weight in males and 480 mg/Kg of body weight in females (Ichikawa et al, 1987). Several studies have shown that PA, fed at the 10% level, can significantly reduce body weight, while having only mild effects at the 4% level (Shigihara et al, 1984). A long term (up to 108 week) carcinogenicity study in normal, non-carcinogen injected rats fed various levels of PA showed no significant differences in the number and distribution of tumours between control and PA fed rats (Hiasa et al, 1992). However, the study did show that rats, particularly females, on high levels of PA intake (2.5%) developed greater number of renal papillomas arising from renal necrosis and calcification. Although there is some question as to whether such necrosis and calcification are just a feature of old age in rats (Yasukata et al, 1985), the lesions probably came about due to the PA. Hirose et al (1991) also showed that PA encouraged the appearance of urinary bladder papillomas. They found it to be due to an elevation of urinary pH and Na⁺ concentrations caused by sodium phytate (Takaba et al, 1994). Thus, there is some concern that administration of high levels of PA may induce some cancers. PA may also be toxic if infused intravenously, particularly due to its calcium binding ability (Gersonde and Weiner, 1981), although the extent of the toxicity depends greatly on the rate of infusion and the total dose of PA given.

2.8.6 Phytic acid and colon cancer

Since PA is a major phytochemical component of WB, it has been suggested that the colon cancer protective effects of WB could be due to its substantial content of endogenous PA. Graf and Eaton (1985) suggest that the international data may show that there is a greater negative correlation between PA and colon cancer than between fiber and colon cancer. For example, although Denmark and Finland have very similar amounts of total dietary fiber intake, Denmark has a much higher incidence of colon cancer (Jensen, 1982) as well as 20-40% less PA intake than Finland (Englyst et al, 1982; Graf
and Eaton, 1985) whose diet includes higher intakes of rye and WB which contain high levels of PA (Graf, 1983).

Pure PA (1%), provided in the drinking water, has been shown to significantly decrease both the number of tumours and the tumour volume of AOM treated rats when treatment was commenced in the pre-initiation phase (Shamsuddin et al, 1988; Ullah and Shamsuddin, 1990). When administered to AOM treated rats, up to five months post-initiation, PA (2%, supplied in the drinking water) again significantly reduced the number of colon tumours, tumour size and the mitotic rate, when compared to the control group (Shamsuddin and Ullah, 1989), suggesting that PA can have anti-initiative and anti-promotive colon cancer protective effects. Pretlow et al (1992a) also found that post-initiation administration (1 week) of PA (2%, supplied in the drinking water) reduced both the number of colon tumours (by 58%) and the tumour volume (by 76%), which are comparable to the above studies. PA has also been shown to be colon cancer protective (Shivapurkar et al, 1996; Nelson et al, 1989) and to reduce the number of ACF (Challa et al, 1997; Corpet et al, 1997) when provided in the diet. In addition, Pretlow et al (1992a) observed a significant increase in larger (≥ 4 crypts per focus) ACF with time (up to 36 weeks) in the control group versus the PA treated group, suggesting that only larger ACF progress towards tumours. The total number of ACF, however, was not significantly different between the treatment and control groups. None of these studies have reported observations of the physical size of ACF, degree of luminal alterations and number of SIM ACF. The total number of ACF was also not significantly different in AOM treated rats fed a high fat diet with or without PA (1.2%) supplementation for five weeks (Mullan, 1990). The number of AC and ACF, however, were significantly lower when PA was added to either calcium or iron supplemented high fat diets (Mullan, 1990). These studies suggest that although some protective effects of PA can be observed, time periods longer than five weeks and the consideration of ACF factors other than the number are necessary for an adequate assessment.

2.8.7 Phytic acid and other cancers

PA has been shown to affect tumourigenesis in other tissues as well as the colon. Supplementation of PA (1.2%) in the diet has reduced cell proliferation in the mammary gland, with the reductions being more dramatic when the PA was added to diets supplemented with high levels of iron and calcium (Thompson and Zhang, 1991). Vucenik et al (1995) have shown that PA, supplemented in
the drinking water, can effectively decrease mammary tumour incidence in DMBA treated rats. Similar effects have been observed by Shivapurkar et al (1996). PA has also reduced the growth of subcutaneously transplanted mice fibrosarcoma cells and the number of pulmonary metastases established after their injection (Vucenik et al, 1992). Antitumour effects have also been shown in rat fibrosarcoma (Jariwalla et al, 1988), lung adenomas (Estensen and Wattenberg, 1993) and skin 2-step papillomas (Ishikawa et al, 1999). Dietary supplementation of PA (2%) has also been shown to inhibit hepatocellular carcinomas in the liver as well as eosinophilic foci which are putative preneoplastic lesions in the pancreas (Hirose et al, 1991). Vucenik et al (1998b) have recently shown that PA, delivered by injection directly into the tumour, can inhibit human rhabdomyosarcoma, an aggressive soft tissue tumour cell line, growth in immunodeficient mice, as well as HepG2 liver cancer cells in a similar model (Vucenik et al, 1998a).

2.8.8 Phytic acid and cancer: in-vitro studies

Several in-vitro cell culture studies support the antineoplastic action of PA. It inhibited cell growth and increased cell differentiation and maturation of HT-29 human colon carcinoma cells (Sakamoto et al, 1993b), K562 human erythroleukemia cell lines (Shamsuddin et al, 1992), human PC-3 prostate adenocarcinoma (Shamsuddin and Yang, 1995), human rhabdosarcoma (Vucenik et al, 1998b), human HepG2 liver cancer (Vucenik et al, 1998a), estrogen receptor positive MCF-7 and estrogen receptor negative MDA-MB-231 human breast carcinoma cells (Shamsuddin et al, 1996) and mouse 3T3 fibroblast cells (Babich et al, 1993). The increase in maturation and reversion to normal phenotype observed in most of these cell lines indicates that the effect of PA is a therapeutic one and not a cytotoxic one.

2.9 Phytic acid and colon cancer protection: mechanisms of action

It is clear from the above discussion that PA can have a cancer protective effect on a variety of different tissues under different experimental conditions and in different models of cancer. However, the mechanisms behind the actions of PA are not clear.

2.9.1 Protein interactions

PA may potentially bind important proteins and enzymes within the cell and also result in alteration in the growth characteristics of the cell. For example, PA at concentrations between 4–13 μM
has been shown to inhibit three different species of serine/threonine protein phosphatases in-vitro (Larsson et al, 1997) and a number of other intracellular proteins (O’Rourke et al, 1996; Yamaguchi et al, 1996; Ali et al, 1995). In addition, PA may bind and inhibit matrix metalloproteinases (MMPs), either by direct binding or by chelating zinc which is a vital cofactor for MMPs. MMPs are thought to be critically involved in degradation of the extracellular matrix around tumours, leading to increased tumour growth, invasion, metastatic spread and angiogenesis (Ferrante et al, 1999). However, these effects may be different in-vitro and in-vivo (Shears, 1998). Thus, all these possible modes of action require more study.

A number of cell surface PA binding proteins (e.g. clathrin adaptor complex AP2, AP180) have been identified in recent years (Theibert et al, 1991; DeCamilli et al, 1996), suggesting that extracellular PA can be internalized. These could be a means by which PA can alter cell function and affect cell growth, differentiation and apoptosis.

2.9.2 Starch interactions

Similar to its binding of proteins, PA can also bind and hinder the proper digestion and absorption of starches (Thompson, 1993). It can bind to starch by hydrogen bonding or indirectly via proteins it is bound to (Thompson, 1986, 1993) or inhibit the proper digestion and hence absorption of starch by binding amylase or enzyme cofactors such as Ca^{2+} (Rickard and Thompson, 1997). In fact, a negative correlation has been observed between the level of PA intake from cereal and legume foods and the glycemic index (Yoon et al, 1983), suggesting impaired absorption of dietary starch with increasing levels of PA. Furthermore, re-addition of PA to dephytinized navy bean flour caused a reversion of the increased rate of digestion and blood glucose response seen upon dephytinization (Thompson et al, 1987). The undigested and unabsorbed starch reaches the colon and contributes to increased fecal bulk or is fermented to SCFA and decreases pH, all of which may have protective effects on the colon (Rickard and Thompson, 1997), as discussed earlier. Any starch that is not digested and absorbed in the small intestine would lessen the glucose absorption at that site thus decreasing the insulin response. This is of consequence not only in diabetics, but also in light of the insulin hypothesis of colon carcinogenesis described earlier, whereby lesser insulin response may equate to decreased tumour growth promotion.
The extent of these interactions depends on the ratio of starch or protein to PA and the overall PA content of the diet. It must be noted that these components may also interact with other dietary compounds. For example, the inhibitory effects of PA on starch digestibility and absorption have been reversed by the addition of calcium, which is preferentially chelated by the PA. The PA is then unable to bind to or interact with starches in the ingested food (Thompson, 1989). Thus, the effects of pure PA added to a diet may be different from the same amount of PA present within the matrix of a foodstuff.

2.9.3 Mineral interactions

PA can bind to polyvalent cations forming chelates with varying stability (Cu³⁺ > Zn²⁺ > Ni²⁺ > Co³⁺ > Mn²⁺ > Fe³⁺ > Ca²⁺) (Graf and Eaton, 1990) and solubility across a wide pH range (Graf and Eaton, 1993). The latter depends both on the pH and on the ratio of PA to cation, with 1:1 or lower ratios being completely soluble while greater metal to PA ratios can precipitate out at varying concentrations (Graf and Eaton, 1984). Thus, PA can have a biphasic effect on mineral solubility, with the minerals being soluble at higher ratios of PA (with PA-mineral complexes containing 1 cation) and at higher mineral ratios (where the minerals are mostly unchelated) (Graf and Eaton, 1990). In general, calcium and magnesium salts of PA are soluble at lower pH and insoluble at higher pH, while an iron-phytate chelate tends to be insoluble at low pH and increase in solubility (up to 50%) at higher pH’s, varying depending on the concentration of PA to iron (Cheryan, 1980). The chelation of cations by dietary PA is of particular significance inside the cell where cations such as zinc or calcium are required as cofactors for enzymes and metalloproteins involved gene regulation and expression (O’Haloran, 1993). For example, calcium is involved in a number of cell cycle events such as initiation of the cell cycle, stimulation of DNA synthesis and regulation of a number of enzymes (Hunt and Groff, 1990) and magnesium is involved in several cellular processes and participates in many important enzymatic reactions such as synthesis and degradation of DNA (Brady et al, 1987; Wester, 1987). Zinc serves as a component in many metallo-enzymes such as DNA and RNA polymerase, deoxythymidine kinase and ribonuclease (DiSilvestro and Cousins, 1983). Their chelation may alter the activities of various enzymes and thus have a general effect on the functions, growth, differentiation and apoptosis of cells. For example, zinc availability can alter thymidine kinase activity, which is essential for DNA synthesis and cell division, while magnesium is involved with enzymes in the secondary messenger pathways of the cell and calcium is involved in IP₃ receptor modulation, as described above. This may be a
mechanism in the recently observed protective effects of PA on human rhabdomyosarcoma in athymic mice (Vucenik et al., 1998b). The PA was injected directly into the tumour daily and could have chelated minerals within the matrix of the tumour necessary for its growth. The ability of PA to chelate minerals particularly iron, confers on it a potential anti-oxidative property which will be discussed in detail in the next section.

2.9.4 Participation in the inositol phosphate pool

If PA can enter the cell, it can participate in the inositol phosphate pool and be converted to lower inositol phosphates. Shamsuddin et al (1992) have shown in-vitro that PA added to the media of K-562 erythroleukemia cells results in a 41% increase and 26% decrease in intracellular IP3 and IP2 respectively, indicating both absorption and conversion of PA. Similarly, Barker et al (1995) have shown in-vitro that PA can be absorbed and converted to IP3 by WRK-1 rat mammary epithelial cells.

Interestingly, Shamsuddin et al (1989) have shown that the colon cancer protective effects observed with PA supplementation are strengthened in the presence of myo-inositol, the parent compound of PA. Similar observations have been made in metastatic and mammary models of cancer (Vucenik et al, 1992, 1993, 1995), with the PA + myo-inositol group showing significantly greater reduction of tumour burden, number and multiplicity than PA or inositol alone. In light of these observations and since PA can be converted to lower inositol phosphates within mammalian cells (Vucenik and Shamsuddin, 1994), it is possible that its cancer protective effects are being mediated by lower inositol phosphates or myo-inositol itself. However, Shamsuddin and Yang (1995) observed no difference in growth inhibition upon addition of PA or extracts of PA dephosphorylated for various lengths of time (producing increasing concentrations of the lower inositol phosphates) in human PC-3 prostate cancer cells, indicating that the observed effects were due to PA and not the lower inositol phosphates. Likewise, intracellular inhibition of PA dephosphorylation (which was accompanied by decreased intracellular concentration of lower inositol phosphates and increased PA concentration), resulted in no difference in growth inhibition compared to cells in which the rapid dephosphorylation of PA was not inhibited (Shamsuddin and Yang, 1995), again suggesting that PA is more responsible for any observed effects than the lower inositol phosphates.

Myo-inositol, either present in the diet or from PA breakdown, is actively absorbed by the colonic mucosa via its own distinct carrier (Holub, 1992). Inside the cell, myo-inositol is sequentially
phosphorylated to phosphatidylinositol-4,5-bisphosphate (PIP₂) which is involved in signal transduction, intracellular calcium mobilization and PKC activation by producing IP₃ and DAG (Holub, 1992). Thus, inositol produced from PA breakdown may also participate in the IP pool, contribute to lower IP production and modulate signal transduction mechanisms. This possibility is strengthened by the observation that myo-inositol protects against mammary cancer (Vucenik et al, 1995), lung cancer (Wattenberg and Estensen, 1996) as well as airway epithelial cells in-vitro (Jyonouchi et al, 1999).

Both PA and the lower IPs, are found in almost all mammalian cell systems (Szwergold et al, 1987; Vallejo et al, 1987) with concentrations within the cell ranging from near zero up to 10 mM (Shamsuddin et al, 1997). Despite the observations stated above, the modulation of these inositol compounds by the entrance of dietary PA and lower IPs derived from it into the inositol phosphate pool within cells, may have dramatic implications for their health and well being since the lower IPs act as second messengers in intracellular signal transduction systems (Berridge and Irvine, 1989). For example, IP₃, or inositol-1,4,5-trisphosphate, is a second messenger causing mobilization of intracellular Ca²⁺ from the endoplasmic reticulum and is involved in many cellular processes including mitosis and cell proliferation (Menniti et al, 1993); IP₄, produced from the phosphorylation of IP₃, is involved in the induction of Ca²⁺ sequestering (Berridge and Irvine, 1989) and in control of plasma membrane Cl⁻ channel activation and so may affect osmoregulation and pH balance (Yang et al, 1999). The release of calcium via IP₃ receptor gated Ca²⁺ release channels is often a cyclic or calcium burst phenomenon (Taylor, 1998), after which the IP₃ is quickly deactivated by dephosphorylation. Increased concentrations of PA from the diet, and the lower IPs derived from it, may cause constant calcium release and increase in intracellular calcium concentration, possibly causing changes in cellular activities such as proliferation or apoptosis. An increase in intra-cellular Ca²⁺ concentration has been associated with anti-proliferative and differentiation-inducing effects in colon cancer cell lines (Buset et al, 1986).

Conversely, increased calcium or IP₃ concentrations may inactivate the IP₃ receptor (give it a lesser ability to conduct calcium; Taylor, 1998) and thus alter all the cellular processes that it controls. This suggests that feedback inhibition of the IP₃ receptor by dietary phytate may be a possible mechanism of its action. Another suggestion for the role of dietary PA and the lower inositol phosphates derived from it, may be that high concentrations of IP₃ may alter the conformation of the receptor causing it to allow a leak of calcium only 10% the initial rate (Khodokhah and Ogden, 1995). This may
result in altered intracellular calcium concentration and hence cell growth signals, particularly in lieu of the fact that the IP$_3$ receptor requires obligatory calcium binding for activation (Taylor, 1998) and thus may be activated less. This issue is very complex, especially since several subtypes of the IP$_3$ receptor have recently been identified, each of which is differentially expressed and regulated in different cell types (Taylor, 1998). This indicates that dietary PA and lower IPs derived from it may have varying effects on different aspects of the IP$_3$ regulated cell signaling mechanisms in the same cell and across different types of cells. More research on inositol phosphate receptors and time dependent changes in intracellular calcium concentrations is warranted.

In addition, because of their possible effects on intracellular calcium concentration, PA, IP$_3$ and IP$_4$ may modulate some isoforms of PKC, a family of calcium dependent enzymes involved in many cellular processes such as cell proliferation and differentiation (Davidson et al, 1994). PKC is also modulated by DAG, a breakdown product of PIP$_2$ which also produces IP$_3$ (Zhou and Erdman, 1995). If dietary PA can influence IP$_3$ levels and modulate PIP$_2$ breakdown, it may thus alter DAG production and hence PKC activity. IP$_3$ and IP$_6$ may also act as secondary messengers since Menniti et al (1993) have shown them to be involved in neuronal stimulation.

PA may also be acting to inhibit the activity of phosphatidylinositol-3-kinase (Huang et al, 1997a), which has been implicated in the growth promotion of a number of cancer cell lines (Huang et al, 1996, 1997b) and whose activity is shown to be higher in colorectal tumours (Phillips et al, 1998). Furthermore, inositol phosphates could also be involved in the anchoring of proteins such as enzymes or antigens to the cellular membrane (Low, 1987) while inositol lipids are thought to be involved in the control of cell surface receptors or proto-oncogene encoded proteins (Mitchell et al, 1990). The full functions, secondary messenger roles and intracellular importance of inositol phosphates still need much further clarification. Thus with so many roles within the cell, it is conceivable that the dietary modulation of inositol phosphates by PA may play an as of yet undiscovered role in colon cancer prevention or inhibition.

### 2.9.5 Antioxidant effects

Graf and Eaton (1985) have suggested that the protective effects of dietary fiber, particularly grain fiber – the most important source of which is WB, may be due to its PA content. They were the
first to suggest that PA may be capable of suppressing free radical generation and lipid peroxidation, because of its structure and iron chelating potential.

2.9.5.1 Free radical generation and iron

In general, the process of production of activated oxygen species commences with the formation of the superoxide radical through the addition of an electron to molecular oxygen. The superoxide radical can, by gaining a further electron and protons, form hydrogen peroxide which can then break down to form hydroxyl radicals ($\text{HO}^\bullet$) in the presence of catalytic transition metal ions, particularly iron (Graf and Eaton, 1985).

Babbs (1990) hypothesized that the colonic milieu may be capable of producing free radicals and showed that abundant amounts of highly reactive $\text{HO}^\bullet$ can be produced by suspensions of feces under aerobic conditions. In fact, individuals consuming less dietary fiber, have been shown to produce significantly more $\text{HO}^\bullet$ (Erhardt et al, 1997). Such oxygen derived free radicals, generated in fecal material close to colonic epithelium, may play a role in the etiology of colon carcinogenesis by causing either direct cellular and genetic damage, or by promoting the conversion and oxidation of procarcinogens to carcinogens or mitogenic tumour promoters. Free radicals generated in excess close to cellular membranes may attack the fatty acid side chain of the membrane phospholipid, particularly those with several double bonds, producing lipid hydroperoxides (Halliwell and Gutteridge, 1989). Accumulation of lipid hydroperoxides in a membrane may reduce its fluidity and permeability, allowing the cell to become more susceptible to carcinogenic damage. In addition, breakdown of the lipid hydroperoxides to cytotoxic aldehydes may damage cell growth receptors situated at cellular membranes and thus promote DNA synthesis and cell proliferation in colonic epithelium (Bull et al, 1984, 1988), in itself a risk factor for colon carcinogenesis. The production of hydroxyl radicals and other oxidants can also increase direct genotoxic damage by causing DNA strand breaks (Nelson, 1992).

Several epidemiological studies have shown a positive link between iron nutriture and overall cancers (Stevens et al, 1986, 1988; Selby et al, 1988) and colon cancer risk (Wurzelmann et al, 1996). Iron has been associated with both the initiation and promotion stages of carcinogenesis due to its role in the Fenton reactions or the Haber-Weiss cycle ($\text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow ^{\text{Fe}}\text{HO}^\bullet + \text{OH}^- + \text{O}_2$), enhancing oxidative damage (Nelson, 1992). The formation of hydroxyl radicals within the colon depends on these
superoxide driven and iron dependent reaction pathways. The necessary iron can be diet derived (Babbs, 1990) while the superoxides can be formed via the respiratory activity of bacteria such as E. coli (Hassan and Fridovich, 1979) or via the lipoxygenase activity of normal or sloughed colon epithelial cells (Craven et al, 1986). Furthermore, iron may also react with these free radicals to form other free radicals or reactive iron-oxygen species (Comporti, 1993) that may also be damaging to the colonic epithelium.

2.9.5.2 Phytic acid and iron chelation

As can be seen from the above discussion, removal of iron may inhibit the production of HO• and thus may be protective. Removal of iron may be instigated by using certain chelators (Nelson, 1992). However, not all chelating agents act appropriately. For example, ethylenediamine tetra-acetic acid (EDTA) increases hydroxyl radical production by solubilizing iron. For iron to participate in Fenton chemistry it must be soluble (Owen et al, 1998). EDTA chelates iron but still allows water to occupy a coordination site, making the complex water soluble (Graf et al, 1984). Other chelating agents, i.e. PA, chelate iron without solubilizing it making it completely bio-unavailable (Graf et al, 1984) and incapable of participating in free radical generating pathways. PA also catalyzes the oxidation of ferrous to ferric iron, thus removing the substrate for the Fenton reaction (Graf et al, 1987). A correlation has been observed between fecal PA and iron content, indicating that PA is capable of binding iron within the digestive tract (Owen et al, 1998).

The lower IPs have also been suggested to be involved in iron chelation, with both IP3 and IP5 (Hawkins et al, 1993) completely stopping iron catalyzed hydroxyl radical formation. Phillipy and Graf (1997) have shown that the 1,2,3-triphosphate grouping (that present in IP3 and higher inositol phosphates) is responsible for chelating the six coordination sites of trivalent iron and the inhibition of hydroxyl radical formation. This is important since, in the digestive tract, PA may be naturally broken down, either via food borne (Sandberg and Svanberg, 1991) or intestinal phytases (Bitar and Reinhold, 1972), into the lower IPs. That breakdown, however, does not mean that its antioxidative functions are eliminated. In fact, breakdown products of the PA in WB by wheat phytases may contain the 1,2,3-triphosphate cluster (Lim et al, 1973), which is necessary for the iron chelation.

Graf et al (1984) have observed that the in-vitro addition of PA to a superoxide radical generating system inhibits the Fenton reaction at PA to iron ratios of up to 0.25. However, Rimbach and
Pallauf (1998) observed an inhibition of hydroxyl radical formation only at PA/iron ratios greater than 5 and did not observe a scavenging effect of PA on superoxide radicals. It is not known why there is such variability in the active ratios of PA/iron, but it could be related to differences in the in-vitro radical generating systems and the sensitivity of the assays. However, the method of Rimbach and Pallauf (1998), known as ESR-spin trapping, is a much newer technique considered to be the best for the measurement of hydroxyl radical production (Yamazaki and Piette, 1990). Such inconsistencies suggest that much more corroborating data using new techniques is necessary to fully assess the full antioxidative potential of PA.

Also in-vitro, PA has been shown to completely inhibit iron-catalyzed lipid peroxidation as measured through the appearance of thiobarbituric reactive substances (TBARS; Graf et al, 1987) and the production of reactive oxygen species (Owen et al, 1997). However, the latter has only been shown to be true for EDTA deplete systems, suggesting that EDTA may be a stronger chelator of iron than PA. This is important since it shows that the inhibitory effects of PA may only be strong in the absence of other chelating agents. Thus, in the in-vivo situation, if dietary iron is already chelated or bound, and with the mostly anaerobic environment of the colon, the in-vitro antioxidant effects of PA may not be as strongly reproducible. In fact, Rimbach and Pallauf (1998) observed no effect of PA on liver oxidant or antioxidant status even in a high iron situation.

PA has also been shown to decrease the incidence and number of colon tumours in rats fed high levels of iron (Nelson et al, 1989). Addition of PA (1.2%) to high iron or calcium diets has reduced the labeling index in the colon of carcinogen treated mice (Thompson and Zhang, 1991) and has reduced the numbers of ACF and the rate of lipid peroxidation in carcinogen treated rats on high iron diets (Mullan, 1990). PA supplementation has favorably affected the outcomes of carcinogen treated rats on other high mineral diets as well. For example, lowered tumour incidence and rate of growth and increased latent period and survival time were observed in rats fed a high magnesium diet along with PA, and injected with fibrosarcoma producing cells (Jariwalla et al, 1988). Interestingly, the form of PA tested in this study was not sodium phytate, as used in the other studies, but rather pentapotassium dimagnesium phytate, the type found commonly in grains (Reddy et al, 1982).

PA has also been shown to reduce the hydroxyl radical formation and subsequent DNA damage that accompany amosite-asbestos exposure in-vitro (Kamp et al, 1995a) and pulmonary inflammation
and fibrosis after amosite-asbestos exposure in-vivo (Kamp et al, 1995b). Since the effects of asbestos, both in-vitro and in-vivo, are thought to be mediated by iron catalyzed free radicals (Kamp et al, 1995a), the ability of PA to lessen hydroxyl radical formation and DNA damage as well as the associated pulmonary inflammation and fibrosis, is further evidence of PA's antioxidant functionality.

Thus, PA and to an extent, lower IPs derived from it, can act as preventive antioxidants by chelating and insolubilizing iron to prevent its participation in free radical generating pathways.

2.9.6 Cell proliferation, apoptosis and differentiation

It has been found that PA can normalize the carcinogen-induced increase in mitotic rate in the non-tumor section of colonic epithelium of rats (Shamsuddin et al, 1988; Ullah and Shamsuddin, 1990), suggesting that PA affects tumour growth through changes in the rate of cell division. Similar decreases in the rate of colonic cell proliferation were obtained in mice (Shamsuddin et al, 1989). Previous to this, Nielsen et al (1987) had observed a negative relationship between the intake of dietary PA and the rate of cell proliferation throughout the colon of non-carcinogen treated rats. Similarly, PA has been shown to decrease the rate of cellular proliferation in vitro (Shamsuddin et al, 1992; Sakamoto et al, 1993b). Again, it can be seen that the protective effects of WB could be due to its PA and thus much further experimentation is necessary.

2.9.7 Other possible mechanisms

PA may also have an effect on host defense mechanisms by modulating natural killer cell activity. Enhanced NK killer cell activity has been shown in-vivo in carcinogen injected mice treated with PA and in-vitro when splenocytes from normal mice were treated with PA (Baten et al, 1989). Such immune system effects may be another means by which PA can exert its actions.

Being an acid, PA could additionally lower the colonic luminal pH. In fact, Nielsen et al (1989) observed that PA supplementation (1.2%) significantly reduced cecal pH.

PA has also been shown to compete for binding sites on the IGF-II receptor (Kar et al, 1994), which is over-expressed in certain tumours and cell lines (Sullivan et al, 1995; Lamonerie et al, 1995). Increased IGF-II levels have been linked to an increased risk for colorectal cancer (Manousos et al, 1999). In fact, Manousos et al (1999) suggest that IGF receptor activation plays a role in later stages of carcinogenesis, suggesting that IGF receptor inhibition, possibly by PA, may be a favourable event. PA has also been shown to bind the basic fibroblast growth factor receptor (bFGF), sterically blocking its
binding domain and thus disrupting its signals (Morrison et al, 1994). These are examples of PA modulating cellular response at the receptor level, suggesting that dietary PA may be able to similarly modulate normal and neoplastic cells of the colon.

In short, PA may be able to have its cancer protective effects by a large number of different and plausible mechanisms which require further research in order to be accepted or refuted.

2.10 Summary

Colon cancer is a major public health concern, especially in North America and Western Europe. A high WB (20-28%)-low fat (4%) diet has been associated with a decreased risk of colon cancer but the specific mechanisms of the fiber action are still unclear. WB is rich in PA (Graf and Eaton, 1993), which has been shown to have an anti-neoplastic function in models of experimental colon carcinogenesis, mammary carcinogenesis, transplanted metastatic fibrosarcoma, hepatocellular carcinoma and in-vitro cell culture. PA, may also partake in many of the proposed mechanisms of WB fiber action, such as increased stool bulk, increased SCFA and butyrate production, decreased pH, bile acid concentration and rate of cell proliferation. In addition, PA may be further protective by chelating iron and other minerals thus reducing the potential for lipid peroxidation and preventing oxidative damage. The interactive roles of WB fiber and its associated PA remain unclear and unelucidated.

Evidently, WB and PA both play a role in dietary colon cancer protection. Since WB is a rich source of PA (Graf and Eaton, 1993), it is difficult to distinguish between the effects of WB or those of the PA associated with it, particularly since they share many potential mechanisms whereby they may be protective, alone or in combination. For example, as discussed, both WB and PA may (i) increase stool bulk, (ii) provide substrate for fermentation to SCFA, particularly butyrate, (iii) alter bacterial metabolism, and (iv) reduce pH. Thus, it is probable that some of the proposed protective mechanisms of WB are due to the actions and properties of its PA. For each of these mechanisms, it is important to be able to distinguish whether WB fiber or PA or a combination of both is responsible for any protective effects of WB. In addition, many unanswered questions about the precise role of PA when given in the natural matrix of a foodstuff, and its mechanisms of action remain to be elucidated. As a result, extensive experimentation on the colon cancer protective effects of WB as well as PA in the natural
matrix of the WB fiber is necessary. Such overlapping of the properties and capabilities of WB and PA require that the effects of the two be elucidated and distinguished from each other.

Extensive studies on the effects of PA within the natural matrix of the cereal grain have not previously been performed. In our laboratory, several experiments have been conducted on the role of PA and fiber on early risk markers of colon carcinogenesis. These studies determined the effects of WB, and dephytinized WB (DWB) with or without pure PA (Shih, 1991); the effects of PA in a high fat diet supplemented with either calcium or iron (Mullan, 1990); and the effects of PA on colon cell proliferation indices and bacterial enzyme activities (Nielsen, 1987). Although the data show that PA may be colon cancer protective, more conclusive results based on longer term experiments and more indepth ACF parameters are required to further elucidate both the level of protection offered and the precise mechanisms involved. Experiments by other researchers (Shamsuddin, 1995) have used pure PA dissolved in the drinking water of rats. The results of these studies are variable and can be affected by simple changes such as neutralization of the pH of the PA-drinking water (Ullah and Shamsuddin, 1990). In addition they may not accurately reflect the action of PA present in foods, such as WB.

In addition, due to the presentation of recent opinions about the advancement of different populations of ACF (Cademi et al, 1995), an indepth analysis and comparison of different ACF parameters is necessary in order to establish whether indeed certain populations of ACF, based on certain molecular, morphological and histological characteristics, are more deformed and hence more advanced than other types of ACF. It is also of interest to establish whether these different ACF populations can be modulated and their characteristics modified by diet, in line with other risk markers such as cell proliferation, apoptosis and degree of differentiation.
3.0 Hypotheses, Objectives and Experimental and Thesis Organization
3.0 Hypotheses, Objectives and Experimental and Thesis Organization

3.1 Overall hypothesis

WB will reduce the early biomarkers of colon cancer risk, in part, due to its PA content. Endogenous PA will be as effective as exogenous PA. These effects will be due to decreased cell proliferation, increased apoptosis, increased cell differentiation, increased colonic butyrate production, inhibited lipid peroxidation and decreased β-glucuronidase activity.

3.2 Overall objective

To determine (a) whether WB is colon cancer protective using ACF characteristics and indices of colonic cell proliferation as early risk markers, (b) whether PA is responsible for any observed effects and (c) whether endogenous and exogenous PA are equally effective and (d) some of the physiological and cellular changes induced by WB and PA action.

3.3 Specific objectives

a) To establish a method for the large scale dephytinization of WB.
b) To determine the effects of WB, DWB, DWB plus pure PA (DWBPA), and pure PA on early biomarkers of colon carcinogenesis i.e. ACF parameters, number of sialomucin producing ACF and rate of colonic cell proliferation.
c) To determine whether PA is the component of WB responsible for any observed effects.
d) To determine whether there is a difference between endogenous and exogenous pure PA added to the diet.
e) To determine whether SIM ACF are more advanced ACF than other types of ACF.
f) To determine some of the physiological and molecular changes that may take place, particularly in the colon, and how they relate to the effects of WB and/or PA on colon carcinogenesis, by analyzing the rate of colonic cell apoptosis and cell differentiation; the extent of lipid peroxidation in colonic mucosa, liver and urine; colon pH; cecal and colonic short chain fatty acid production, profiles and molar ratios; cecal β-glucuronidase activity; serum and femur mineral status and fasting glucose and insulin levels.
3.4 Organization of the experiments and thesis

The organization of the experiments and thesis is summarized in Figure 3.1. To achieve the above objectives, a method for the large scale preparation of DWB was developed in Experiment 1 (Chapter 4).

The prepared DWB was then used in subsequent studies. In Experiment 2 (Chapter 5) groups of 30 rats were injected with AOM colon carcinogen and one week subsequent fed either a basal diet (BD), or BD supplemented with either WB, DWB, DWBPA or PA for 100 days after which they were sacrificed. Half the rats in each group were processed for analysis of ACF parameters and rate of cell proliferation (Experiment 2, Chapter 5). The other half of the rats were used in Experiment 5 (Chapter 8) where some of the modes of action of the treatment diets were explored. The colon tissues used in Experiment 2 (Chapter 5) were also used to determine the effect of the diets on rate of apoptosis, degree of differentiation and colon morphology (Experiment 4; Chapter 7). Experiment 3 (Chapter 6) was performed in order to determine the validity of the sialomucin producing (SIM) ACF parameters measured in Experiment 2. It involved a separate animal study where colons were collected, various ACF populations microdissected and analyzed for degree of dysplasia and rate of cell proliferation. The overall results of these experiments are discussed and conclusions drawn in Chapter 9.
Experiment 1: Preparation of dephytinized wheat bran

Experiments 2 and 5:
BD / WB / DWB / DWBPA / PA diets fed to groups of 30 carcinogen injected animals each for 100 days

15 rats / gp

Colons collected for:

Experiment 2: The influence of phytic acid and wheat bran on early risk markers of colon carcinogenesis
ACF parameters and rate of cell proliferation measured

Colons also used for:

Experiment 4: Phytic acid in wheat bran affects colon morphology, cell differentiation and apoptosis
Colon morphology, apoptosis and differentiation rates measured

Colons mucosa, cecal / colon contents, serum, femurs, livers collected for:

Experiment 5: Some modes of action of wheat bran, dephytinized wheat bran and phytic acid
Analyzied indices of lipid peroxidation, serum and femur minerals, pH and SCFA production and β-glucuronidase activity

Figure 3.1: Organization of the experiments and the thesis
4.0 Experiment 1: Preparation of Dephytinized Wheat Bran
4.0 Experiment 1: Preparation of Dephytinized Wheat Bran

4.1 Introduction

To determine the role of PA in colon carcinogenesis, there was a need to prepare DWB so that the effect of WB fiber in the absence of PA and the effect of pure PA added back to the WB fiber (i.e. PA not in its natural matrix) could be determined.

Phytase is an enzyme which breaks down PA into inositol and free phosphates. WB contains endogenous phytase (Cheryan, 1980) and thus may be capable, under the right conditions, of hydrolyzing its own PA. Previously in our laboratory, Shih (1991) prepared DWB by incubating WB in a 10% aqueous dispersion at 37.5 °C with constant gentle stirring for 16 hours, followed by freeze drying. Because different batches of WB may contain different amounts of phytic acid and phytase enzyme, there was a need to determine the optimum conditions for dephytinization of our batch of WB. In addition, the efficacy of such a method of dephytinization on a larger scale needed to be confirmed.

The objectives of this study were (a) to determine, on a laboratory scale, the optimum conditions (i.e. time, percent dispersion) for the dephytinization of our batch of WB, (b) to prepare DWB on a semi-pilot scale using the conditions found in the first objective and (c) to determine the PA and proximate composition of the WB and DWB.

4.2 Materials and methods

4.2.1 Experimental design

Freshly prepared WB from hard red wheat was obtained from King Milling Company (MI). This type of WB was selected because it is commonly used in commercial WB products. In addition, it has been shown to be more colon cancer protective and to contain more dietary fiber than bran from other wheat varieties (Maziya-Dixon et al, 1994). The experimental design is shown in Figure 3.1. WB was dispersed in 1 L of double distilled de-ionized water (ddiH₂O) at various levels (10-20%), incubated at 37 °C for various time periods (5-25 hrs.), freeze dried and then analyzed for PA. The best condition which resulted in the lowest concentration of PA was used in the semi-pilot scale preparation of DWB.
**1 L volumes:**

Percent Dispersion of wheat bran:

- 10.0
- 12.5
- 15.0
- 18.0
- 20.0

- Analysis for phytic acid performed at each time point

**Figure 4.1: Design of Experiment 1**
4.2.2 Dephytinization methods

4.2.2.1 Laboratory scale

WB contains natural phytase that can breakdown PA over time at the optimum temperature (37°C). Incubation of WB at various dispersions activates this phytase. One liter dispersions of WB at 10, 12.5, 15, 18 and 20% level in 1500 mL beakers were placed in a water bath maintained at 37°C and samples stirred for 5, 10, 12, 15, 18 and 25 hours. At the end of each time period, the content of each beaker was shell frozen, freeze dried and kept at -20°C until analyzed for PA levels. Duplicate samples were prepared and analyzed at each time point.

4.2.2.2 Semi-pilot scale

The dephytinization was performed at the pilot plant of the Guelph Food Technology Center at the University of Guelph (Guelph, ON). A large pasteurization vat with a steam jacket for temperature control and mixing device was used. Based on the results of the laboratory scale experiment, 70.4 Kg of WB was dispersed in 560 L of double distilled deionized water (ddiH2O) and kept at 37°C with constant stirring for 18 hrs. The temperature was automatically monitored and adjusted using a digital thermometer, as needed. At the end of the incubation period, the steam jacket of the pasteurization vat was flushed with ice water to quickly cool the DWB dispersion, which was then poured into over 200 individual freeze drying trays and placed in a -20°C walk in freezer to freeze. Once frozen, the trays were transported to Freeze Drying Incorporated (Oakville, ON) and freeze dried for 24 hrs in an industrial freeze dryer in a single batch.

4.2.3 Phytic acid analysis

4.2.3.1 AOAC method

The PA was analyzed by the method of the Association of Official Analytical Chemists (AOAC; 1986). All glasswares used were acid washed with 10% nitric acid. Briefly, duplicate WB and DWB samples (2.0g) were placed in individual 125 mL Erlenmeyer flasks and 40 mL of 2.4% HCl was added. The flasks were covered and magnetically stirred for 3 hours at room temperature. Samples were
then filtered through Whatman #1 paper under vacuum. To 1.0 mL filtrate was added 1.0 mL of Na₂EDTA-NaOH reagent (prepared by adding 10.23 g of 0.11 M Na₂EDTA and 7.50 g of 0.75 M NaOH and diluted to 250 mL with ddiH₂O) and diluted to 25.0 mL with ddiH₂O. The solutions were mixed well and transferred to the columns.

Chromatography columns (20 cm, Fisher Scientific, Nepean, ON) were prepared by adding 3 mL of ddiH₂O and 0.5 g of a H₂O slurry of AG1-X8 100-200 mesh chloride form of anion exchange resin (Bio-Rad Laboratories, Richmond, CA) and allowed to settle. The columns were then washed with 15 mL of 0.7 M NaCl, followed by 15 mL of ddiH₂O. After addition of the samples, the columns were washed with 15 mL of ddiH₂O and then eluted with 15 mL of 0.7 M NaCl solution and the eluant was collected in microKjeldahl digestion flasks to which 0.50 mL of H₂SO₄, 3.0 mL of HNO₃ and 3 glass beads were then added. Duplicate blank samples were prepared using 1.0 mL 2.4% HCl with 1.0 mL of Na₂EDTA-NaOH reagent, diluted to 25.0 mL and added to the column and treated exactly as described above.

The flasks were boiled until after a cloud of thick yellow vapour filled the neck of the digestion flask. The flasks were heated for 5 more minutes on medium heat, 5 minutes on low heat, allowed to cool, added 10 mL of ddiH₂O to dissolve the formed salt, heated on low temperature for 10 more minutes and allowed to cool. The contents were then transferred quantitatively to a 50 mL volumetric flask and 2.0 mL of molybdate solution (prepared by adding 12.5 g of ammonium molybdate, 200 mL of ddiH₂O, 50 mL of 10 N H₂SO₄ and diluting to a volume of 500 mL with ddiH₂O), 1.0 mL of sulfonic acid solution (prepared by adding 0.16 g of 1-amino-2-naphthol-4-sulfonic acid, 1.92 g of Na₂SO₃, 9.60 g of NaHSO₃ and diluted to 100 mL with ddiH₂O) were added, mixed well at each stage and diluted to 50 mL with ddiH₂O. The solution was allowed to stand for exactly 15 minutes and then read at 640 nm (Beckman DU-7 Spectrophotometer, Beckman Instruments, Fullerton, CA).

A standard curve was prepared by pipetting exactly 1.0, 3.0 and 5.0 mL of phosphate standard solution (prepared by adding 0.350 g of dried, desicated acid phosphate, 500 mL of ddiH₂O, 10.0 mL of 10 N H₂SO₄ and diluted to 1000 mL with ddiH₂O) into a 50 mL volumetric flask. To each standard
volume, 20.0 mL of ddiH₂O, 2.0 mL of molybdate solution, 1.0 of sulfonic acid reagent were added and then diluted to volume with ddiH₂O. The resulting solution was let stand for 15 minutes and then read at 640 nm wavelength as described above. The standard curve was used to calculate the mg/g concentration of PA in the WB and DWB samples.

4.2.3.2 HPLC method

The WB and large volume prepared DWB were also analyzed for PA and lower iP content by the HPLC method of Rounds and Nielsen (1993). Briefly, 0.5g samples of WB and DWB were placed in 10 mL of 0.5M HCl, sonicated for 1 min. and centrifuged (Sorvall SS-3 Centrifuge, Dupont Corp., Newtown, CT) at 15000 rpm for 15 minutes. One mL of the supernatant was removed and diluted to 20 mL with ddiH₂O and poured onto an anion exchange SAX column (Analytichem Int., Harbor City, CA). The column was washed with 10 mL of 0.05M HCl and the bound inositol polyphosphates eluted with 2 mL of 2M HCl. The samples were dried and the residue resolubilized in 1 mL of ddiH₂O with 15μL of tetrabutylammonium hydroxide. 20μL of this was injected into an HPLC unit (Model 810, SpecraPhysics Inc., San Jose, CA) equipped with PRP-1 5μm reverse phase column (Hamilton Co., Reno, NE) and UV-Vis and RI detectors. A 60:40 methanol:0.035M formic acid mobile phase at pH 4.3 and a 0.9mL/min. flow rate was used.

4.2.4 Chemical composition of WB and DWB

The WB and DWB were analyzed for dietary fiber, fat, total carbohydrate, protein, ash and moisture. Total dietary fiber was determined by the method of Prosky et al (1984). Fat content was determined by AOAC methods (1984). Total carbohydrate was determined by difference from the calculated percentage of all of these methods.

Protein content was determined by a modified MicroKjeldahl method. Duplicate samples (0.25g) of WB and DWB were weighed out on cigarette paper and placed in MicroKjeldahl tubes. A Kjeltabs tablet (Tecator Corp., Sweden) and 5 mL of concentrated H₂SO₄ were then added to the tube. The tubes were digested for 2 hrs, cooled for 1 hr, 25 mL of ddiH₂O added to and then read in a protein analyzer (Kjeltec Auto 1030, Tecator Corp., Sweden).
Ash was analyzed by heating duplicate samples (5.0 g) of WB and DWB in a muffle furnace preheated to 660°C for 36 hours. Moisture content was determined by drying duplicate samples (2.0 g) of WB and DWB in an oven preheated to 105°C for 12 hrs.

4.3 Results, discussion and conclusion

The PA content of samples dispersed at different levels and incubated at various time periods are shown in Table 4.1. Evidently, the 10 and 12.5% dispersions provided the best results. Dispersions ≥ 15% were very thick and difficult to stir. Difficulty in stirring resulted in lack of homogeneity and generally poorer dephytinization. The optimum time for dephytinization appears to be 18 hrs since no appreciable further reduction was obtained with longer incubation time in the 10 and 12.5% dispersions. Increased incubation time, with constant stirring, may result in undesirable mechanical breakdown of the WB. Although there was little difference between the 10 and 12.5% dispersions, the 12.5% level was chosen because it meant more WB could be dephytinized in the same volume, reducing overall workload and cost of incubation and freeze drying for the larger semi-pilot scale dephytinization. Thus the DWB was subsequently prepared on a large scale using 12.5% dispersion (70.4 Kg wheat bran / 560 L of ddH₂O) for 18 hours incubation.

<table>
<thead>
<tr>
<th>Incubation Period (hrs)</th>
<th>10</th>
<th>12.5</th>
<th>15</th>
<th>18</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.10 ± 0.04</td>
<td>4.10 ± 0.04</td>
<td>4.10 ± 0.04</td>
<td>4.10 ± 0.04</td>
<td>4.10 ± 0.04</td>
</tr>
<tr>
<td>5</td>
<td>2.26 ± 0.06</td>
<td>2.30 ± 0.08</td>
<td>2.43 ± 0.08</td>
<td>3.32 ± 0.04</td>
<td>3.82 ± 0.05</td>
</tr>
<tr>
<td>10</td>
<td>0.52 ± 0.02</td>
<td>0.58 ± 0.02</td>
<td>0.66 ± 0.03</td>
<td>1.53 ± 0.04</td>
<td>2.51 ± 0.06</td>
</tr>
<tr>
<td>15</td>
<td>0.19 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>0.31 ± 0.02</td>
<td>1.21 ± 0.03</td>
<td>1.97 ± 0.07</td>
</tr>
<tr>
<td>18</td>
<td>0.11 ± 0.02</td>
<td>0.12 ± 0.01</td>
<td>0.26 ± 0.03</td>
<td>0.98 ± 0.03</td>
<td>1.62 ± 0.05</td>
</tr>
<tr>
<td>25</td>
<td>0.10 ± 0.01</td>
<td>0.11 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.52 ± 0.02</td>
<td>0.68 ± 0.02</td>
</tr>
</tbody>
</table>

Values at each time point and % dispersion are means of duplicate measurements ± SEM. Analysis for the zero time values was shared for all % dispersions.

The DWB prepared by the semi-pilot scale contained 0.127 % PA (1.27 mg PA/g) while the undephytinized WB contained 4.10% (41.0 mg PA/g). The HPLC analysis of the WB (Table 4.2) shows
negligible content of lower IPs. The DWB had negligible amounts of PA and IP₅ but did have very minor amounts of IP₄ and IP₃.

<table>
<thead>
<tr>
<th>Table 4.2: PA and lower IP's in WB and DWB</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA and lower IP's (%)</td>
</tr>
<tr>
<td>WB</td>
</tr>
<tr>
<td>-----</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>DWB</td>
</tr>
</tbody>
</table>

Values are means of duplicate measurements ± SEM.

The proximate composition of WB and DWB (Table 4.3) showed a significant difference in moisture content between WB and DWB. The compositions were used in designing the diets used for Experiment 2.

<table>
<thead>
<tr>
<th>Table 4.3: Chemical composition of WB and DWB.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent Composition (%)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Wet basis:</td>
</tr>
<tr>
<td>Ash</td>
</tr>
<tr>
<td>Carbohydrate</td>
</tr>
<tr>
<td>Fat</td>
</tr>
<tr>
<td>Fiber</td>
</tr>
<tr>
<td>Moisture</td>
</tr>
<tr>
<td>Protein</td>
</tr>
</tbody>
</table>

Dry Basis:

| Ash                                        | 6.79 ± 0.04 ³ | 6.41 ± 0.07 ³ |
| Carbohydrate                               | 32.21 ± 0.54 ³ | 31.81 ± 0.70 ³ |
| Fat                                        | 1.32 ± 0.11 ³ | 1.09 ± 0.16 ³ |
| Fiber                                      | 43.11 ± 0.98 ³ | 44.13 ± 1.15 ³ |
| Protein                                    | 16.56 ± 0.93 ³ | 16.57 ± 1.00 ³ |

Values are means of duplicate measurements ± SEM. Values within a row with different superscripts indicates a significant difference via Students t-test, p < 0.05.
5.0 Experiment 2: The Influence of Phytic Acid in Wheat Bran on Early Risk Markers of Colon Carcinogenesis
5.0 Experiment 2: The Influence of Phytic Acid and Wheat Bran on Early Risk Markers of Colon Carcinogenesis.

5.1 Introduction

Epidemiological studies have shown that high fiber foods such as whole grains and cereals may be protective against colon cancer (Greenwald et al, 1987; Howe et al, 1992; Helzlsouer et al, 1994; Steinmetz et al, 1994; Slavin et al, 1997). Specifically, many animal studies have shown that WB has a colon cancer protective effect (Barbolt and Abraham, 1978, 1980; Reddy and Mori, 1981; Alabaster et al, 1993, 1995; McIntyre et al, 1993) which was attributed primarily to its high fiber content. Interestingly, many of the proposed protective mechanisms of WB fiber, such as decreased colonic transit time (Eastwood, 1992), increased colon content bulk (Weisburger et al, 1993) or fermentation (Velazquez et al, 1996a), are analogous to those suggested for the protective effects of PA. PA is a major fiber-associated component of WB (Graf and Eaton, 1985). In some epidemiological studies, a colon cancer-protective effect has been observed for fiber-containing foods rich in PA, such as WB, but not for low PA fiber foods (Englyst et al, 1982; Graf and Eaton, 1985).

PA serves as the major storage form of phosphorus in the seed, as well as being a natural antioxidant by chelating and reducing the catalytic activities of many divalent transition metals (Rickard and Thompson, 1997). It is this chelating ability of PA that has been suggested to suppress iron-mediated oxidation in the colon, thereby reducing colon cancer risk (Graf and Eaton, 1985, 1993; Nelson, 1992; Shamsuddin, 1992). PA may also bind proteins and starches present in the diet and so affect their solubility, digestibility, function and absorption (Rickard and Thompson, 1997), which in turn may affect the colonic environment, for example, by stimulating short chain fatty acid (SCFA) production from fermentation of the trapped starch.

Pure PA (1% - 2%) has been shown to significantly decrease the number and volume of colon tumours in rats when fed prior to azoxymethane (AOM) carcinogen injection (Shamsuddin et al, 1988; Ullah and Shamsuddin, 1990; Pretlow et al, 1992a) or when fed up to five months post-initiation (Shamsuddin and Ullah, 1989). In these studies, it was postulated that the antineoplastic effects of PA
may be mediated by its ability to regulate colonic cellular proliferation. However, these experiments used pure PA dissolved in the animal’s drinking water and so may not be representative of the effects of the PA naturally present in many foods. The effects of endogenous PA in WB or purified PA added to high or low fiber diets on colon carcinogenesis have not yet been systematically studied. In addition, to date, only a small number of studies have focused on the effects of PA (Pretlow et al, 1992a) or WB (Alabaster et al, 1993; Shivapurkar et al, 1995; Ishizuka and Kasai, 1996; Young et al, 1996) on AC and ACF formation and characteristics. Much remains to be determined about the effects of WB and PA in a natural matrix on early markers of colon cancer risk.

Thus, it is hypothesized that WB, due to its endogenous PA and fiber, as well as exogenously added pure PA will reduce early preneoplastic biomarkers of colon cancer risk, namely ACF and rate of cell proliferation. The primary objectives of this study were to determine (a) if WB can reduce early biomarkers of colon cancer risk such as ACF characteristics and indices of colonic cell proliferation, (b) if PA is the component of WB responsible for any observed effects and, (c) if there is a difference between endogenous and pure exogenous PA added to the diet. A secondary objective was to determine the use of the high iron diamine alcian blue (HIDAB) staining method for SIM and SUM producing ACF along with a grading system to measure the degree of luminal alterations of AC as a means of further characterizing AC and ACF.

5.2 Materials and methods

5.2.1 Experimental design and diets

The experimental design is illustrated in Figure 5.1. Seventy five male Fischer 344 rats (40 days old, Charles River Inc., Montreal, Canada) were individually maintained in stainless steel cages at an ambient temperature of 22-24°C on a 12 h light dark cycle. They were acclimatized for 2 weeks on the AIN-93G basal diet (BD) (Reeves et al, 1993) and then injected i.p. with 15 mg/Kg body wt of the colon carcinogen azoxymethane (Sigma Chemical Co., St. Louis, MO). One week later they were randomized into five groups with 15 rats per group, such that the mean weight of each group was equal.
Day -14  Acclimatization

Day -7  Injection with AOM (15 mg/Kg BW)

Day 0  Randomization into 5 groups of 15 rats each:

- basal diet (BD;AIN-93G;control)
- BD + 25% undephytinized wheat bran (WB)
- BD + 25% dephytinized wheat bran (DWB)
- BD + 25% dephytinized wheat bran + 1% PA
- BD + 1% PA (PA)

Day 100  Sacrifice and tissue collection

Figure 5.1: Design of Experiment 2
Power analysis for sample size with a minimum detectable difference of 20% with a desired power of at least 0.80, 5 groups and an alpha value of 0.05, provided a recommended sample size of 15 rats per group.

They were fed ad libitum either the BD (BD group), or BD supplemented with either 25% WB (WB group), dephytinized WB (DWB group), 25% DWB plus 1.0% PA (DWBPA group) or 1.0% PA (PA group). All diet ingredients were ordered from Dyets Inc., except the WB (King Milling Co., MI). The 25% WB level was chosen so as to provide a 1.0% level of PA in the diet. All diets were based on the AIN-93G diet (Reeves et al, 1993) with the WB, DWB, DWBPA and PA diets corrected for the protein, fat, fiber and moisture content contributed by the added WB, DWB or PA as estimated from their composition given in Chapter 4 (Table 5.1). Since DWB has some residual PA, the level of PA added to the DWBPA diet was adjusted such that the overall concentration of PA in the diet was 1.0%.

Rats were supplied with fresh diet every 2 days. Diets were prepared biweekly and stored at −20°C until needed. In use diets were refrigerated at 4°C. Food intake and weight were monitored weekly. After 100 days treatment, the rats were sacrificed by CO₂ gassing. All the major organs (liver, heart, lungs, kidneys, small intestine, colon and spleen) underwent gross pathological examination and were weighed. The colons were excised and prepared for examination of ACF as described below.

5.2.2 Aberrant crypt formation

ACF formation was analyzed according to the method of McLellan and Bird (1988). Briefly, the colons were split open lengthwise, washed with saline, flushed with physiological saline (0.9%, pH 7.0) and cut into two equal length sections (proximal and distal). The proximal section was that closest and the distal colon was the section furthest from the cecum. The colon sections were then fixed flat between two layers of filter paper in 10% buffered formalin for a minimum of 3 days. The fixed colon sections were placed in 0.2% methylene blue (Fisher Chemical Co., Fairlawn, NJ) dissolved in 0.9% saline solution for 15-30 min, immediately after which they were immersed (to prevent drying) mucosal side up in physiological saline solution in a glass culture dish attached to the microscope stage and viewed
with a light microscope (Labophot, Nikon Corp., Japan) for ACF. Examples of ACF and the parameters measured are shown in Figure 5.2.

Table 5.1: Nutrient Composition of the diets used

<table>
<thead>
<tr>
<th>Diet Ingredients</th>
<th>Nutrient Composition of the Diets (g/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BD†</td>
</tr>
<tr>
<td>Casein</td>
<td>20.00</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10.000</td>
</tr>
<tr>
<td>Cellulose</td>
<td>5.000</td>
</tr>
<tr>
<td>Soybean Oil</td>
<td>7.000</td>
</tr>
<tr>
<td>TBQH</td>
<td>0.001</td>
</tr>
<tr>
<td>Vitamin Mix</td>
<td>1.000</td>
</tr>
<tr>
<td>L-cystine</td>
<td>0.300</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.250</td>
</tr>
<tr>
<td>Water added †</td>
<td>0.000</td>
</tr>
<tr>
<td>WB added</td>
<td>0.000</td>
</tr>
<tr>
<td>DWB added</td>
<td>0.000</td>
</tr>
<tr>
<td>Pure PA added *</td>
<td>0.000</td>
</tr>
<tr>
<td>Endogenous PA ‡</td>
<td>0.000</td>
</tr>
<tr>
<td>Total</td>
<td>100.000</td>
</tr>
</tbody>
</table>

† based on the AIN93G diet (Reeves et al, 1993).
‡ double distilled de-ionized water; value takes into account lower moisture content of the DWB due to freeze drying.
* pure PA equals 1.010%; values take into account purity and moisture content of sodium phytate dded.
‡ accounts for endogenous PA in WB or residual PA in DWB, value not included in total sum.

The colon sections were examined at a magnification of 45X for the number of ACF and at a magnification of 100X for ACF size by counting the number of squares covered on a grid by the ACF. The ACF were distinguished from surrounding normal colonic crypts by their increased size, elongated luminal opening, thickened epithelial lining and increased pericryptal space (MacLellan and Bird, 1988). The exact position of each ACF along the colon was carefully recorded. In addition, the degree of luminal alteration or severity of deformation of the shape of the luminal outline of each AC was also recorded according to the method of Caderni et al (1995). Briefly, each AC was given a grade from 1 to 3 based on whether the luminal shape was mildly altered (enlarged and round; grade 1), moderately altered (elliptical; grade 2) or severely altered (slit like, irregular or cribriform; grade 3). All colons were scored blind by the same observer.
Figure 5.2: ACF and ACF parameters of interest
a) shows an ACF (pointed out by the arrow) identified by its increased size, thickened luminal opening and epithelial lining and increased pericryptal space. b) shows the physical ACF size or surface area covered; c) shows the multiplicity or number of AC per ACF. This ACF has a multiplicity of 5; d) shows the degree of luminal alterations of each AC in an ACF; e) & g) show individual ACF stained with methylene blue; f) & h) show the same ACF stained with HIDAB showing the characteristic blue colour of SIM staining while surrounding normal crypts are brown indicating SUM; i) shows a HIDAB staining of SUM ACF, brown colour.

ACF= aberrant crypt foci; AC= aberrant crypt; HIDAB= high iron diamine alcian blue; SIM= sialomucin; SUM= sulphomucin
5.2.3 Determination of the type of mucous produced in the aberrant crypt foci

After scoring the ACFs using the methylene blue method, the colon sections were stained for type of mucous production, i.e. SIM or SUM, according to the HIDAB method of Cademi et al (1995). Examples of SIM and SUM ACF are shown in Figure 5.2. Briefly, the sections were removed from the 10% buffered formalin, individually rinsed in distilled water, and then immersed in high iron diamine solution (20 mg of N-N’-dimethyl-p-phenylene diamine, 120 mg of N-N’-dimethyl-m-phenylene diamine in 50 mL of distilled water plus 1.4 mL of 60% ferric chloride, all from Sigma Chemical Co., St. Louis, MO) in a petri dish protected against light for 18 hours at room temperature. The colons were rinsed three times in distilled water, stained with 1% alcian blue (Sigma Chemical Co., St. Louis, MO) in 3% acetic acid for 30 minutes, rinsed three times in 80% ethanol and once in distilled water and then observed for ACF in the same manner as with the methylene blue staining described above. Since the exact position of each ACF had previously been recorded, the type of mucin produced by each AC could readily be noted. Dark brown staining of the crypts implied SUM production while bright or dark blue staining indicated predominantly SIM production. As was observed by others (Cademi et al, 1995), in normal crypts, SIM production predominates in the upper proximal section while SUM production predominates in the lower proximal and all the distal section. However, the SIM producing ACF data presented here are only those that were surrounded by normal crypts producing SUM. Those ACF in the proximal regions where SIM production was the norm, were not counted while ACF in the latter regions of the proximal colon where SUM production was the norm were included in the results and statistical analyses.

5.2.4 Cell proliferation by PCNA method

Subsequent to measurement of all the ACF parameters, the distal colon sections were wrapped in ‘swiss roll’ style, embedded in paraffin blocks and sliced at 5 μm thickness with two sections from each rat on each slide. The tissue sections were deparaffinized with two changes of toluene and rehydrated in a graded series of ethanol from 100% to 70% and then distilled water. Endogenous
peroxidase activity was blocked by immersing the slides in 3% H₂O₂ in water for 10 minutes with a subsequent double washing in distilled water. To expose the proliferating cell nuclear antigen (PCNA) protein, tissue sections were microwaved for 30 min in citric acid buffer (pH 6.0). The slides were allowed to cool for 30 min while immersed in the buffer and then treated with a 0.025% triton-phosphate buffered saline (PBS) solution for 3 minutes. The primary antibody (PCNA Ab-1, Calbiochem Inc.) was placed onto the tissue sections and incubated in a humid chamber for 2 hrs. The slides were again washed three times with 0.025% triton-PBS solution for 3 minutes, treated with the secondary links antibody (Dako Inc., Carpenteria, CA) in a humid chamber for 60 minutes, washed three times with 0.025% triton-PBS solution for 3 minutes, treated with a streptavidin complex (Dako Inc., Carpenteria, CA) for 30 minutes, washed three times with distilled water for 3 minutes, treated with AEC substrate/chromagen (Dimension Labs, Mississauga, ON) for 30 minutes in a warm humid chamber, washed three times with distilled water for 3 minutes, treated with haemotoxylin for 20 seconds, washed with distilled water, tap water and eventually mounted with crystal mount.

For the labeling index (LI) evaluation, 24 crypts were counted along the entire distal colon section of each rat. All counting was performed at 400X magnification. Only whole longitudinally sectioned crypts that showed the entire column length from the lumen down to the muscularis mucosa were counted. Incomplete crypts or those with more than 2 missing cells were not counted. Only cells with a strong dark brown color, indicating a cell in S phase, were considered labeled (Lin et al 1996). The numbers of these labeled cells and the total number of cells in each crypt were counted. In addition, the position and height of each cell were recorded. Each crypt was divided into 5 equal length compartments and LIs were calculated as percent number of labeled cells for the whole crypt and the top 40% of the crypt. The phi index (Lipkin et al, 1983), which is the ratio of labeled cells in the top 40% of the crypt to the labeled cells in the entire crypt and which is thought to be the best discriminator of increased colon cancer risk, was also calculated for each crypt. All samples were counted blind by the same observer.
5.2.5 Statistical Analyses

All data were analyzed by one way ANOVA followed by the Tukey’s pairwise multiple comparisons test or Kruskal-Wallis non-parametric ANOVA followed by Dunn’s pairwise nonparametric multiple groups comparisons test using the SigmaStat statistical software package (Jandel Scientific).

5.3 Results

5.3.1 Animal weights

There were no significant differences in initial or final weight, weight gain or food intake (Table 5.2). This indicates that none of the diets was more palatable than the others and that any effects seen are not due to variations in food intake or weight differences.

Table 5.2: Initial, final weight, weight gain and average food intake.

<table>
<thead>
<tr>
<th>Diet Groups</th>
<th>Initial weight (g)</th>
<th>Final weight (g)</th>
<th>Weight gain (g)</th>
<th>Food Intake (g)</th>
<th>Feed Efficiency Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD</td>
<td>146.8 ± 2.9</td>
<td>327.3 ± 4.2</td>
<td>180.5 ± 4.8</td>
<td>14.2 ± 1.3</td>
<td>12.6 ± 0.5</td>
</tr>
<tr>
<td>WB</td>
<td>146.1 ± 2.5</td>
<td>317.6 ± 4.7</td>
<td>171.5 ± 4.7</td>
<td>14.1 ± 1.0</td>
<td>12.1 ± 0.4</td>
</tr>
<tr>
<td>DWB</td>
<td>149.6 ± 2.8</td>
<td>317.5 ± 3.5</td>
<td>167.9 ± 5.0</td>
<td>14.9 ± 1.4</td>
<td>11.5 ± 0.7</td>
</tr>
<tr>
<td>DWBPA</td>
<td>146.5 ± 2.3</td>
<td>318.4 ± 3.7</td>
<td>171.9 ± 4.6</td>
<td>13.4 ± 1.1</td>
<td>12.9 ± 0.6</td>
</tr>
<tr>
<td>PA</td>
<td>147.9 ± 2.0</td>
<td>316.4 ± 2.7</td>
<td>168.2 ± 4.0</td>
<td>13.2 ± 1.1</td>
<td>12.7 ± 0.4</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n=30 per group; There were no significant differences. For definition of abbreviations see Table 5.2. BD = basal diet, control group; WB = 25% wheat bran diet; DWB = 25% dephytinized wheat bran diet; DWBPA = 25% dephytinized wheat bran plus 1.0% added PA; PA = 1.0% added PA.

5.3.2 Aberrant crypt formation

No significant differences were seen in the total number of ACF in the proximal and distal colons (Figure 5.3), although in both sections all treatment groups tended to have lower total numbers of ACF than the BD. However, considering the whole colon, i.e. proximal plus distal sections, a significant decrease (p < 0.01) in the number of ACF was observed in the PA group versus the BD.

The size of ACF in the proximal section did not differ significantly amongst the groups (Figure 5.4). In the distal section, the PA group had significantly smaller size of ACF than both the BD (p < 0.02) and WB (p < 0.04) groups but did not differ significantly from the other groups.

Because there are differences in colon lengths which approached significance (p < 0.08), the concentration of ACF per cm of colon length was estimated. The number of ACF per cm length of colon
was less in all the treatment groups versus the BD in both the proximal and distal colon sections but the difference did not reach significance (Figure 5.5). However, the WB and PA groups had significantly lower number of ACF per cm than the BD (p < 0.03 and p < 0.02 respectively) group in the whole colon.

Figure 5.6 shows the number of SIM ACF with the total number of ACF provided in outline for reference. When compared to Figure 5.3, it is clear that with the BD group, over 50% of all the ACF are SIM producing, while the treatment groups have less than a third of the total ACF producing SIM. PA significantly decreased the number of SIM ACF in both the distal and proximal colon sections while all the treatment groups significantly decreased the number of SIM producing ACF in the whole colon.

All the WB-containing diets as well as the PA diet significantly reduced the degree of luminal alterations in both the proximal and distal colon sections as well as the colon as a whole (Table 5.3).

| Table 5.3: Degree of luminal alterations of the aberrant crypts in the various colon sections |
|-----------------------------------------------|---------------|---------------|
|                  | Proximal       | Distal        | Whole         |
| BD               | 2.50 ± 0.09a   | 2.62 ± 0.08a  | 2.57 ± 0.07a  |
| WB               | 2.06 ± 0.07b   | 2.32 ± 0.04b  | 2.27 ± 0.04b  |
| DWB              | 1.97 ± 0.08b   | 2.26 ± 0.07b  | 2.21 ± 0.06b  |
| DWBPA            | 1.97 ± 0.07b   | 2.40 ± 0.03b  | 2.32 ± 0.05b  |
| PA               | 2.04 ± 0.11b   | 2.34 ± 0.06b  | 2.29 ± 0.05b  |

Values are means ± SEM, n=14 per group; values with different superscripts are significantly different, p<0.05. For the definition of abbreviations see Table 4.2. For the determination of the degree of luminal alterations, each AC was given a grade from 1 to 3 based on whether the luminal shape was mildly altered (enlarged and round; 1), moderately altered (elliptical; 2), or severely altered (slit like; 3); refer to Figure 5.2.
Figure 5.3: Number of aberrant crypt foci in the proximal, distal and whole colon sections
Figure 5.4: Total size of ACF in the proximal, distal and whole colon sections.
Figure 5.5: Number of ACF per cm of colon in the proximal, distal and whole colon sections.
Figure 5.6: Number of SIM ACF in the proximal, distal and whole colon sections. Outlines indicate the total number of ACF.
5.3.3 Cell proliferation

Figure 5.7 shows the PCNA-LI in the various crypt compartments. The BD group had consistently higher PCNA-LI than all the treatment groups. The PCNA-LI was lower in the topmost compartments. Table 5.4 shows the indices of cell proliferation in the distal colon. The BD group had the highest rate of cell proliferation, which was significantly higher than all the treatment groups with the exception of DWBPA. The PCNA-LI of the DWB and PA groups, although not significantly different from each other, were significantly higher than that of the WB group. All the treatment groups had significantly lower positions of the topmost labeled cell versus the BD, particularly the WB group, which also had significantly lower values than the DWB and PA groups.

The PCNA-LI in the top 40% of the crypt (Table 5.4) followed a similar pattern, again with the BD diet having a rate of cell proliferation significantly higher than all the treatment groups. In addition, the WB group was significantly lower than the DWB and PA groups. In the distal colon, all the treatment groups with the exception of PA had a significantly lower $\phi h$ index versus the BD group (Table 5.4).

Table 5.4: Whole crypt PCNA-LI, PCNA-LI of the top 40% of each crypt, position of the topmost labeled cell and $\phi h$ index in the distal colon.

<table>
<thead>
<tr>
<th>Groups</th>
<th>PCNA-LI</th>
<th>PCNA-LI in the top 40%</th>
<th>Position of Topmost Labeled Cell</th>
<th>$\phi h$ Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD</td>
<td>58.74 ± 1.08$^a$</td>
<td>18.45 ± 1.38$^a$</td>
<td>17.95 ± 0.39$^c$</td>
<td>0.107 ± 0.007$^a$</td>
</tr>
<tr>
<td>WB</td>
<td>48.41 ± 1.06$^c$</td>
<td>7.97 ± 0.99$^d$</td>
<td>22.62 ± 0.38$^a$</td>
<td>0.052 ± 0.006$^b$</td>
</tr>
<tr>
<td>DWB</td>
<td>54.35 ± 0.93$^b$</td>
<td>13.71 ± 1.48$^{bc}$</td>
<td>19.79 ± 0.39$^b$</td>
<td>0.081 ± 0.009$^b$</td>
</tr>
<tr>
<td>DWBPA</td>
<td>55.89 ±1.01$^{ab}$</td>
<td>9.69 ± 0.91$^d$</td>
<td>21.47 ± 0.39$^{ab}$</td>
<td>0.063 ± 0.005$^b$</td>
</tr>
<tr>
<td>PA</td>
<td>53.61 ± 0.96$^b$</td>
<td>12.69 ± 1.19$^{bc}$</td>
<td>19.88 ± 0.36$^b$</td>
<td>0.085 ± 0.007$^{ab}$</td>
</tr>
</tbody>
</table>

Values are means ± SEM on a sample size of 24 crypts per rat with 6 rats per group; values with different superscripts within a column are significantly different, $p < 0.05$. Position of topmost labeled cell is denoted as percent of all cells on either side of the crypt from the top cell down to the first labeled cell i.e. percentage of all cells that are unlabeled from the top of the crypt to the first labeled cell. Thus a high value indicates a greater percentage of unlabeled cells at the top side of the crypts and thus a lowered position of the topmost labeled cell. The $\phi h$ value is the number of labeled cells in the top two crypt compartments divided by the number of labeled cells in the entire crypt. For definition of abbreviations see Table 5.2.
Figure 5.7: Proliferating cell nuclear antigen labeling index (PCNA-LI) in the various crypt compartments.

For measurement of the PCNA-LI, each crypt was divided into 5 equal compartments with 1 = topmost compartment; 1+2 = top 40% of the crypt; 5 = bottom compartment.
5.4 Discussion

This study has shown that WB, DWB and DWB with added PA can reduce some early biomarkers of colon cancer such as the number of SIM ACF in the whole colon and the degree of luminal alterations, i.e. the degree of observed deformity of the ACF lumen. These treatment diets can significantly decrease cell proliferation in the entire crypt and in the top 40% of the crypt as well as generate a smaller proliferative zone and a lower $P$ index, which is thought to be the best cell proliferation discriminator for determining colon cancer risk (Lipkin et al, 1983). This study has also shown that PA added to a low fiber diet can significantly decrease the number of ACF (distal and whole colon) as well as the size of ACF (distal and whole colon) versus the BD, suggesting a potential reduction in colon cancer risk. If PA was the only active component in WB, it would be expected that the dephytinization of WB would cause an increase in ACF parameters. However, such an effect was not observed while addition of exogenous PA had a significant effect only when added to a low fiber diet. Since no differences were observed in the initial weight, final weight, weight gain, food intake and feed efficiency ratio of the rats, none of the diets was likely more palatable than the others and thus, the effects seen are not due to variations in food intake or body weights.

ACF formation was used as an early indicator of reduction in cancer risk since ACF are believed to be valid preneoplastic markers of colon cancer (Bird, 1995). Although, there are some conflicting opinions on whether ACF are reliable endpoints in experimental colon carcinogenesis, they have been shown to have much increased proliferative activity than that in normal adjacent crypts (Pretlow et al, 1994) as well as mutations in the p53 tumour suppressor gene (Stopera and Bird, 1993) and in the k-ras oncogene (Stopera et al, 1992), both of which are common to many colon cancers.

It has been suggested that WB fiber has some colon cancer protective effects (Black, 1996) based on many experiments over the past few decades (Appendix Table 11.2). In this experiment, WB-containing diets lowered the number and size of ACF compared to the control, but the changes were not significant. However, the WB diet did decrease ACF concentration (number of ACF per cm) over the
length of the colon. Also, the WB-containing diets significantly reduced the number of SIM ACF, which have been suggested to be better neoplastic markers than other types of ACF (Cademi et al., 1995). The shift to SIM production by ACF is important since SIM are produced by a large percentage of colon tumours and are linked to increased aggressiveness, increased probability to metastasize and a generally poorer prognosis for the patient (Green et al., 1993). Thus, SIM ACF are likely further advanced along the stepwise adenoma-carcinoma sequence of colon cancer, although this still needs to be experimentally confirmed by comparing the characteristics of SIM ACF versus other types of ACF. However, the fact that all the WB-containing diets as well as PA decreased this parameter suggests that these diets may potentially modulate colon cancer risk from an early stage.

The differential staining of ACF by methylene blue can also provide information on tissue structural changes which are features of precancerous lesions. Thus it has been suggested that the severity of the degree of luminal alterations may relate to the degree of dysplasia present in a particular AC (Cademi et al., 1995). In this study, we found a strong reduction in the luminal deformity and alterations by all the treatment groups in the proximal and distal sections and in the colon as a whole. Thus, the WB diets likely slowed the development of deformity and consequently the advancement towards neoplasticity for the ACF observed.

WB is thought to exert its colon cancer protective effect by several mechanisms including physical dilution of gut contents, shortening transit times, alterations in the mutagenicity of intestinal contents, alterations in mucosal cytokinetics, increased fermentation producing butyrate, and effect on the production, absorption and excretion of putative carcinogens (Ferguson and Harris, 1996). Interestingly, many of these mechanisms appear to be analogous to those suggested for the protective effects of PA, adding to the speculation that PA may be the component of WB which is contributing to its colon cancer protective effects. For example, WB fiber increases the colon content bulk considerably (a point observed in this experiment; data shown in a later chapter) causing a dilution and adsorption of any mutagens and carcinogens present (Ferguson and Harris, 1996).
PA is a negatively charged molecule and is thus capable of binding proteins and starches (Rickard and Thompson, 1997) leading to their malabsorption and subsequent fermentation to SCFA in the colon, increasing bacterial and fecal bulk, and acting like a soluble fiber. Since WB is a rich source of PA, it is difficult to distinguish between the effects of the WB fiber and those of the PA associated with it. In this study, very little difference in effect was seen upon the dephytinization of the WB used or upon the addition of exogenous PA back to DWB, even though the exogenous PA added to the low fiber diet was very effective in reducing ACF parameters. This suggests that both endogenous and exogenous PA probably interact with the fiber in WB making them less available for binding with other dietary components. On the other hand, in the low fiber diet, PA may be more free to interact with dietary components other than fiber, such as starches and proteins, leading to a more pronounced protective effect as described above.

WB may be exerting its protective effects by reducing the rate of cell proliferation. Increased cell proliferation has been linked to the genesis of a number of neoplasias (Henderson and Preston-Martin, 1990) including colon cancer (Deschner, 1988). Measurement of cell proliferation may be considered a valid intermediate biomarker for colon cancer risk. In fact, a stepwise increase in cell proliferation has been observed during neoplastic progression of colon lesions such as ACF and increased cell proliferation and expanding proliferative compartment are known as characteristic features of colon carcinogenesis (Shpitz et al, 1997).

Several studies have shown a decrease in the rate of cell proliferation with WB supplementation (Jacobs and White, 1993; Folino et al, 1995). In this experiment, WB clearly decreased the overall PCNA-LI as well as the PCNA-LI in the top 40% of the crypt and the $\phi$h index. An effect of endogenous PA was evident when the WB and DWB groups were compared for the PCNA-LI and the PCNA-LI in the top 40% of the crypt. However, the addition of pure PA back to the DWB (DWBPA group) resulted in PCNA-LI that did not differ significantly from either group. This suggests that the endogenous PA is acting to decrease the rate of cell proliferation and that this effect is conditional to the role of PA in its natural matrix since the pure PA added back to the DWB did not decrease the PCNA-
LIs to the level of the WB. The effect of dephytinization is also apparent in the position of the topmost labeled cell with WB producing a significantly smaller proliferative zone than the DWB, not to mention the BD group. Here too, the addition of pure PA back to the DWB (DWBPA) did cause a reduction in the proliferative zone but not down to the level of the WB. Thus, it is apparent that the endogenous PA is having a protective effect on mucosal cell kinetics but the relatively short time course of this study may not have allowed for these effects to be translated into decreased ACF parameters. Interestingly, although the LI and the position of the topmost labeled cell for pure PA added to a low fiber diet (PA group) were lower relative to the BD group, they were significantly higher than the WB group but not different from either the DWB or DWBPA groups. The differential effect of endogenous PA and the pure PA added to the low fiber diet is also evident in values of the $\phi$h index, which is thought to be the best discriminator of colon cancer risk (Lipkin et al, 1983), which were decreased relative to the BD by all the WB containing diets but not by the PA diet. Thus, with respect to these results and the decreases observed in the ACF parameters with the PA group, it is possible to suggest that endogenous PA and the PA added to the low fiber diet, although both effective, may be so due to different mechanisms, which require further elucidation. In addition, the significant decreases in overall LI and of the LI in the top 40% of the crypts brought about by the WB-containing diets is further indication of a decreased risk of colon carcinogenesis associated with their consumption, although all these data need to be confirmed in colon tumourigenesis studies.

Although generally showing a protective effect of WB fiber, experiments to date have not studied the effect of PA within the matrix of WB as a possible contributor to the effects observed. Pure PA added to the drinking water has been shown to be protective in a number of experiments (Shamsuddin et al, 1988; Shamsuddin and Ullah, 1989; Ullah and Shamsuddin, 1990; Pretlow et al, 1992a) but since the major source of intake of PA is in the natural matrix of fibers in which it is present and not as a pure additive in drinking water, studies such as this one are necessary. However, it is still unclear how WB and PA may be affecting ACF parameters and modulating cell proliferation or whether they affect other important cellular events. Thus research on other cellular functions and some of the
physiological mechanisms affected by WB and PA is necessary. In addition, although circumstantial evidence suggests that SIM ACF are better early bio-markers of colon cancer risk than other types of ACF, this needs to be confirmed by direct comparison of the characteristics of these different ACF populations.

5.5 Conclusions

It is concluded that based on some of the methylene blue ACF characteristics, WB does not seem to be protective of early preneoplastic putative biomarkers of colon cancer, but based on decreases in the degree of luminal alterations, the number of SIM ACF and indices of cell proliferation, WB appears to be protective. WB, with or without endogenous or exogenous PA, along with the exogenous PA added to the low fiber diet, reduced the level of cell proliferation, the proliferative zone and the $\phi h$ index, suggesting a protective effect. It can also be concluded that, while having a role, endogenous PA is not the sole active component in WB and that exogenous PA is most effective when added to a low fiber diet. Although with the dephytinization of WB, significant changes in ACF parameters were not observed, significant changes in the indices of cell proliferation indicate a reduced effectiveness of WB upon the removal of its PA. Furthermore, it appears that the measurement of SIM producing ACF and grading of luminal alterations may be effective ways of further characterizing ACF as preneoplastic markers of colon cancer. However, in order to further validate the results of this experiment, there is a need to experimentally confirm whether there are indeed differences in growth and degree of deformity between SIM and SUM ACF.
6.0 Experiment 3: Relationship of Aberrant Crypt Foci Parameters to Degree of Dysplasia and Rate of Cell Proliferation
6.0 Experiment 3: Relationship of Aberrant Crypt Foci Parameters to Degree of Dysplasia and Rate of Cell Proliferation

6.1 Introduction

ACF have been used to identify modulators of colon cancer risk and to discover the finer details of the stepwise development of colon cancer. Although it is well accepted that they are indeed valid preneoplastic markers (Bird, 1995), there is some controversy regarding which ACF parameters are best reflective of future colon cancer risk. Many different ACF parameters have been measured including the number of aberrant crypts (AC), number of ACF, size of ACF, ACF multiplicity, the number of ACF per length of the colon, or per cm$^2$ of the colon, and the degree of luminal alterations of ACF. Although some researchers have found a correlation between the induction and distribution of ACF and tumours in the colon (Shivapurkar et al, 1992; Bird, 1995), others have shown that the number of ACF at early time points after carcinogen treatment is not a predictor of tumour incidence (Magnuson et al, 1993) or that there is a negative correlation between the number of ACF and tumour incidence when the two are measured at the same time (Carter et al, 1994). Yet others have shown that either the AC multiplicity (Magnuson et al, 1993) or the number of larger ACF, defined as either greater than 4 AC (Pretlow et al, 1992a; Lasko and Bird, 1995), 8 AC (Shivapurkar et al, 1995) or 14 AC (Caderni et al, 1995) are predictive of tumour incidence under different experimental situations.

Thus, it is possible that only certain populations or types of ACF, probably those with the higher degrees of atypia, dysplasia or general defects advance along the adenoma carcinoma sequence of colon cancer (Bouzourene et al, 1999). However, it is not known how to identify those populations of ACF that might be more advanced, in part because only a few studies have compared ACF parameters measured in whole mount unsectioned colons to other ACF characteristics such as degree of dysplasia (Thorup, 1997). Thus, much controversy still exists regarding the particular ACF parameters observable in whole mount unsectioned colons, that may be suggestive of a greater degree of alteration (e.g. increased degree of dysplasia or rate of cell proliferation) within an ACF and that may thus be used to identify more advanced populations of ACF.
As discussed in Chapter 5, ACF differ in their type of mucin production with some being SIM, while others produce SUM (Sandforth et al, 1988; Caderni et al, 1995). SIM production in a large number of colon tumours has been correlated with increased level of dysplasia (Uchida et al, 1997). It has thus been suggested that SIM ACF are further advanced than SUM ACF (Caderni et al, 1995). In Chapter 5, it was shown that WB or PA containing diets reduced the number of SIM ACF identified in whole mount colons. Therefore, it is important to determine the differences that exist between SIM and SUM ACF and to see if SIM ACF are indeed further advanced.

It is hypothesized that SIM ACF will have greater degree of abnormalities such as dysplasia and rate of cell proliferation and are thus more advanced early biomarkers than SUM ACF. The main objective of this study was to see if features of ACF parameters detected on whole mount unsectioned colons, particularly SIM and SUM production, can be used to identify different populations of ACF with varying degrees of abnormality and defect (determined by degree of dysplasia and rate of cell proliferation). The parameters of greatest interest are the overall type of mucin production in the ACF, ACF multiplicity, physical size and the average degree of AC luminal alterations in ACF.

### 6.2 Materials and methods

#### 6.2.1 Experimental design

Three male Fischer 344 rats (40 days old, Charles River Inc., Montreal, Canada) were maintained in individual stainless steel cages at an ambient temperature of 22-24°C on a 12 h light dark cycle. They were acclimatized for 2 weeks on the AIN-93G basal diet (BD; Reeves et al, 1993), previously described in Table 5.1 (Chapter 5). The animals were then injected i.p. with 15 mg/Kg body wt of the colon carcinogen azoxymethane (Sigma Chemical Co., St. Louis, MO). After 100 days treatment the rats were sacrificed by CO₂ gassing and the colons were collected for examination of ACF.

#### 6.2.2 Identification of aberrant crypt formation

ACF were identified according to the methods outlined in Chapter 5. The exact position of each ACF along the colon was carefully recorded along with the ACF multiplicity (number of AC per ACF),
physical size of ACF and the degree of luminal alteration or severity of deformation of the shape of the luminal outline of each AC as shown in Figure 5.2. All colons were scored blind by the same observer.

6.2.3 Determination of the type of mucin produced in the ACF

After the scoring of ACFs using the methylene blue method, the distal colon sections were stained for type of mucous production according to the HIDAB method of Caderni et al (1995), described in Chapter 5. Since the exact position of each ACF had previously been recorded, the type of mucin produced by each aberrant crypt could readily be noted. Dark brown staining of the crypts implied SUM production while bright or dark blue staining indicated predominantly SIM production. Only ACF that were surrounded by normal crypts producing SUM were considered.

6.2.4 Microdissection and preparation of ACF

Seventy seven ACF were randomly chosen from the distal sections of the 3 different HIDAB stained whole mount colons. These ACF had varying (i) types of mucin production (SIM, SUM or a mixture of the two), (ii) size (physical area of the colon covered by the ACF), (iii) multiplicity and (iv) degree of luminal alterations.

ACF were located under a microscope at a magnification of 100-400X and microdissected, along with adjacent mucosa of normal appearance, from the colon section. The location of each ACF on the microdissected section of tissue was mapped out in detail. The microdissected sections were then individually embedded in paraffin and the tissue blocks serially sectioned (4-6 μm thickness) perpendicular to the luminal surface to obtain longitudinally sectioned crypts. Longitudinal sections provide a view of the crypts from top to bottom allowing changes in cell proliferation and dysplasia to be accurately assessed throughout the crypt and not at just one level as is the case with horizontal cross sections. The sections were mounted on microscope slides and every fifth slide was deparaffinized in two changes of toluene, rehydrated in a graded series of ethanol to distilled water and then stained with hematoxylin and eosin (H&E) for identification of the ACF. Using the detailed location map of each microdissected section drawn earlier, ACF were located on the H&E stained slides. Slides adjacent to the one bearing the section of the recovered ACF were also either stained with H&E for determination
of degree of dysplasia or used for immunostaining for the PCNA determination of rate of cell proliferation.

6.2.5 Assessment of degree of dysplasia

Dysplasia can be defined as a preneoplastic change in the epithelium, consisting of cellular atypia, abnormal differentiation and disorganized architecture and is associated with an increased risk for cancer (Siu et al, 1997). The degree of dysplasia was scored blindly on the H&E stained sections. At least 2 sections per ACF were assessed using a semi-quantitative scale from 1 to 3 based on descriptions given in previous publications (Lane et al, 1971; Stemmerman et al, 1973, 1986; Shamsuddin and Trump, 1981; Decaens et al, 1983; Roncucci et al, 1991a, 1991b; Thorup, 1997; Siu et al, 1997). If the degree of dysplasia differed between the AC assessed in each ACF, then the degree assigned was determined by the AC with the highest degree of dysplasia. Each slide was also viewed and ranked by an experienced pathologist. The criteria were as follows:

Dysplasia Ranking of 1 (Non dysplastic foci): Crypts have increased crypt height with slight dilation. Cells exhibit normal nuclei with regular nucleus location, orientation, shape, color, number of nucleoli, no visible nuclear vesiculation or stratification. There is some loss of mucin with normal looking goblet cells and apical localization of mucus. There may also be presence of slight basophilicity and hypercellularity. Dysplasia Ranking of 2 (Mild to moderate dysplastic foci): Crypts have greater increased crypt height and more dilation of the crypt. Cells exhibit moderately enlarged nuclei with irregular location, orientation, shape and darker colour with some focal nuclear stratification and slight vesiculation with no mitotic bodies observable. There is greater loss of cytoplasm and mucin production with abnormal location of mucin. Crypts are moderately basophilic and more hypercellular. Dysplasia Ranking of 3 (Moderate to severe dysplastic foci): Crypts have increased height with severe dilation. Cells exhibit enlarged nuclei with irregular location, loss of cell polarity, abnormal color and shapes, some pleomorphism, extensive stratification and more vesiculation with prominent or multiple nucleoli. Crypts have very irregular mucin pattern and greater loss of mucus production. Crypts are strongly basophilic and very hypercellular. ACF with a dysplasia rankings of 1 to 3 are illustrated in Figure 6.1.
Figure 6.1: ACF dysplasia rankings. 'a, b & c' show ACF with dysplasia rankings of 1 to 3 respectively. Note the increasing degree of crypt dilation, basophilicity, and nuclear and morphological irregularity with increasing dysplasia. Pictures 'd, e & f' show the PCNA staining for the respective ACF in pictures 'a, b & c'. Note the increased brown nuclear staining and increased proliferative zone within the ACF versus the normal surrounding crypts. Pictures 'a' & 'd' clearly show SUM and 'c' & 'f' clearly show SIM production.
6.2.6 Assessment of cell proliferation

The rate of cell proliferation was assessed using the PCNA method described earlier in Chapter 5. The PCNA-LI of the ACF was determined by counting as many whole crypts as possible. Multiple sections were immunostained with the PCNA method until whole AC from the ACF were visible. For the evaluation of the PCNA-LI for normal tissue surrounding the ACF, 12 normal crypts were counted along both sides of the identified ACF. Only whole longitudinally sectioned crypts that showed the entire column length from the lumen down to the muscularis mucosa were counted. Both normal and ACF PCNA-LI were counted on the same slide. All counting was performed at 400X magnification. LIs were calculated as percent number of labeled cells at the top 40% of the crypt. The $\phi h$ index (Lipkin et al., 1983), which is the ratio of labeled cells in the top 40% of the crypt to the labeled cells in the entire crypt was also calculated for both normal and aberrant crypts. Examples of ACF stained with PCNA are shown in Figure 6.1.

6.2.7 Immunohistochemical determination of mutated k-ras

Immunohistochemical determination of mutated k-ras was attempted using an antibody (Oncogene Research Products, Cambridge, MA) directed towards residues 5-16 of the protein product of the k-ras gene with the aspartic acid mutation on codon 13. The tissue sections neighbouring the identified ACF were deparaffinized with two changes of toluene and rehydrated in a graded series of ethanol from 100% to 70% and then distilled water. Endogenous peroxidase activity was blocked by immersing the slides in 3% $H_2O_2$ in water for 10 min with a subsequent double washing in distilled water. To expose the mutated k-ras protein, tissue sections were microwaved for 30 min in citric acid buffer (pH 6.0). The slides were allowed to cool for 30 min while immersed in the buffer and then treated with 0.025% Triton-PBS. The primary antibody (pan-ras$^{\text{asp13}}$ Ab-1, Oncogene Research Products, Cambridge, MA) was diluted to a concentration of 10 $\mu g$ / mL and placed onto the tissue sections and incubated in a humid chamber for 1 hr. The slides were washed three times with 0.025% Triton-PBS for 3 minutes, treated with the secondary links antibody (Dako Inc., Carpenteria, CA) in a humid chamber for 60 min, washed three times with 0.025% Triton-PBS for 3 minutes, treated with a streptavidin
complex (Dako Inc., Carpinteria, CA) for 30 minutes, washed three times with distilled water for 3 minutes, treated with AEC substrate/chromagen (Dimension Labs, Mississauga, ON) for 30 min in a warm humid chamber, washed 3 times with distilled water for 3 minutes, dipped in haematoxylin for 20 seconds, washed with distilled water, tap water and eventually mounted with crystal mount. Human colon cancer tissues known to be positive for this k-ras mutation were used as positive controls and run with each round.

6.2.8 Statistical analyses

Using the Sigmastat statistical software package (Jandel Scientific, San Rafael, CA), all data were analyzed by one and two way analyses of variance followed by the Tukey’s multiple comparisons test or Kruskal-Wallis non-parametric ANOVA followed by Dunn’s non-parametric multiple comparisons test. Simple and multiple linear regressions were also performed. Differences with p < 0.05 were considered significant.

6.3 Results

ACF had a significantly higher LI in the top 40% of the crypt and \( \phi_h \) index of cell proliferation than surrounding normal crypts (Table 6.1).

<table>
<thead>
<tr>
<th></th>
<th>ACF</th>
<th>Normal Crypts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labeling Index (top 40% of crypt)</td>
<td>50.68 ± 3.18 (^a)</td>
<td>5.85 ± 1.23 (^b)</td>
</tr>
<tr>
<td>( \phi_h ) Index</td>
<td>0.24 ± 0.01 (^a)</td>
<td>0.04 ± 0.01 (^b)</td>
</tr>
</tbody>
</table>

Values are means ± SEM. \( n=77 \). Values with different superscripts within a row are significantly different from each other, \( p < 0.001 \). The \( \phi_h \) index is defined as the ratio of labeled cells in the top 40% of the crypt to the labeled cells in the entire crypt.

Although the multiplicity of SIM ACF was higher than SUM ACF, the difference did not reach significance (Table 6.2). However, SIM ACF, along with ACF producing a mixture of SIM and SUM had a significantly greater physical size and more degree of luminal alterations than ACF producing only SUM. Significant differences in the degree of dysplasia between SIM and SUM ACF with multiplicities of 4 or more were observed.
Table 6.2: Relation of type of ACF mucin production to ACF multiplicity, size of ACF, degree of luminal alterations, and degree of dysplasia in ACF with multiplicity of 4 or more.

<table>
<thead>
<tr>
<th>Type of Mucin Production in ACF</th>
<th>Multiplicity of ACF</th>
<th>Multiplicity of ACF ≥ 4</th>
<th>Size of ACF (x10^2mm^2)</th>
<th>Avg. Degree of Luminal Alterations</th>
<th>Degree of Dysplasia (for ACF with mult. ≥ 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulphomucins</td>
<td>2.87 ± 0.32 a</td>
<td>4.00 ± 0.00 b</td>
<td>2.78 ± 0.29 b</td>
<td>2.18 ± 0.20 b</td>
<td>1.71 ± 0.18 b</td>
</tr>
<tr>
<td>Sulpho and sialomucins</td>
<td>3.41 ± 0.27 a</td>
<td>4.67 ± 0.33 ab</td>
<td>4.68 ± 0.42 a</td>
<td>2.74 ± 0.10 a</td>
<td>2.17 ± 0.40 ab</td>
</tr>
<tr>
<td>Sialomucins</td>
<td>3.73 ± 0.26 a</td>
<td>5.04 ± 0.25 a</td>
<td>5.16 ± 0.40 a</td>
<td>2.82 ± 0.10 a</td>
<td>2.52 ± 0.14 a</td>
</tr>
</tbody>
</table>

Values are means ± SEM. n = 77. Values with different letters in a column indicates a significant difference, p < 0.05.

In addition, all SIM ACF had a significantly higher degree of dysplasia than SUM ACF (Table 6.3). ACF producing both SIM and SUM had an intermediate level of dysplasia which was not significantly different from either SIM or SUM ACF. LI in the top 40% of the crypt and $\phi$h index were both significantly higher in SIM ACF and in ACF producing both SIM and SUM than in SUM ACF.

Groups of ACF with a multiplicity of 2 to 3 and 4 or more had a significantly higher degree of dysplasia, LI in the top 40% of the crypt and $\phi$h index than single crypt ACF (Table 6.3). There were no significant differences between ACF with a multiplicity of 2 and 3 compared to those with a multiplicity of 4 or more.

Large (ACF with a physical size greater than 6.0 x 10^2 mm^2) and medium sized ACF (those with a physical size between 4.0 and 6.0 x 10^2 mm^2), had significantly greater degree of dysplasia and LI in the top 40% of the crypt than smaller ACF (those with a physical size between 1 and 3.9 x 10^2 mm^2; Table 6.3). There were no differences in the $\phi$h index between the ACF based on physical size.
Table 6.3: Relation of type of mucin production in ACF, ACF multiplicity, size of ACF and degree of luminal alterations to degree of dysplasia, ACF labeling index in the top 40% of the crypt and the ACF $\phi_h$ index.

<table>
<thead>
<tr>
<th>ACF Parameters</th>
<th>Risk Indicators</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Degree of Dysplasia</td>
</tr>
<tr>
<td></td>
<td>ACF Labeling Index (top 40% of crypt)*</td>
</tr>
<tr>
<td></td>
<td>ACF $\phi_h$ Index *</td>
</tr>
<tr>
<td>Type of Mucin Production in ACF</td>
<td></td>
</tr>
<tr>
<td>Sulphomucin</td>
<td>1.60 ± 0.31 b 25.01 ± 8.74 b 0.12 ± 0.04 b</td>
</tr>
<tr>
<td>Sulpho- and sialomucin</td>
<td>2.12 ± 0.15 * 50.54 ± 6.49 * 0.23 ± 0.03 *</td>
</tr>
<tr>
<td>Sialomucin</td>
<td>2.32 ± 0.10 * 49.50 ± 3.47 * 0.22 ± 0.02 *</td>
</tr>
<tr>
<td>ACF Multiplicity</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.43 ± 0.20 b 8.08 ± 7.48 b 0.05 ± 0.03 b</td>
</tr>
<tr>
<td>2-3</td>
<td>2.09 ± 0.10 * 47.97 ± 4.45 * 0.21 ± 0.02 *</td>
</tr>
<tr>
<td>≥ 4</td>
<td>2.31 ± 0.13 * 49.85 ± 4.31 * 0.23 ± 0.02 *</td>
</tr>
<tr>
<td>Size of ACF ($\times 10^{mm^2}$)</td>
<td></td>
</tr>
<tr>
<td>Small (1-3.9)</td>
<td>1.72 ± 0.11 b 31.69 ± 5.94 b 0.14 ± 0.03 a</td>
</tr>
<tr>
<td>Medium (4-6.0)</td>
<td>2.27 ± 0.11 * 52.15 ± 3.56 * 0.24 ± 0.02 a</td>
</tr>
<tr>
<td>Large (&gt;6.0)</td>
<td>2.64 ± 0.17 * 55.29 ± 6.33 * 0.25 ± 0.03 a</td>
</tr>
<tr>
<td>Degree of Luminal Alterations</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.33 ± 0.24 b 21.11 ± 7.77 b 0.11 ± 0.03 b</td>
</tr>
<tr>
<td>2</td>
<td>1.95 ± 0.12 ab 44.58 ± 7.61 ab 0.20 ± 0.03 ab</td>
</tr>
<tr>
<td>3</td>
<td>2.35 ± 0.09 * 48.77 ± 3.59 * 0.22 ± 0.02 a</td>
</tr>
</tbody>
</table>

Values are means ± SEM; means with different letters in a column section indicates a significant difference, $p < 0.05$. * Labeling index values represent absolute difference of ACF labeling index and labeling index of normal tissue surrounding ACF.

ACF with an average degree of luminal alterations of 3, had significantly greater degree of dysplasia, LI in the top 40% of the crypt and $\phi_h$ index than ACF with a degree of luminal alterations of 1 (Table 6.3). ACF with a degree of luminal alterations of 2, had intermediate levels of dysplasia and LI indices and were not significantly different from either ACF with degree of luminal alterations of 1 or 3.

Table 6.4 shows regression coefficient and $p$ values for the ability of the ACF parameters measured, whether by themselves or in various combinations, to predict indicators of risk analyzed, i.e. degree of dysplasia, LI in the top 40% of the crypt and the $\phi_h$ index. The data show that all significant associations included the type of mucin. Absence of type of mucin in the independent variables of the regression analysis, resulted in the absence of predictive value for the parameters in that test. The best prediction of degree of dysplasia, LI in the top 40% of the crypt and the $\phi_h$ index was obtained when all
the parameters measured were included as independent variables of the regression analysis. In addition, no k-ras mutations were observed in any ACF.

Table 6.4: Type of mucin, ACF multiplicity, size of ACF, degree of luminal alterations and their various combinations as predictive parameters for the risk indicators degree of dysplasia, ACF labeling index in the top 40% of the crypt and the $\Phi_h$ index.

<table>
<thead>
<tr>
<th>Predictive Parameters</th>
<th>Risk Indicators</th>
<th>Degree of Dysplasia</th>
<th>ACF Labeling Index (top 40% of crypt)</th>
<th>$\Phi_h$ Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of mucin</td>
<td></td>
<td>r = 0.386</td>
<td>r = 0.313</td>
<td>r = 0.361</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p &lt; 0.001</td>
<td>p = 0.008</td>
<td>p = 0.002</td>
</tr>
<tr>
<td>ACF multiplicity</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Size of ACF (x10^2 mm^3)</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Degree of luminal alterations</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Type of mucin x ACF multiplicity</td>
<td></td>
<td>r = 0.386</td>
<td>r = 0.313</td>
<td>r = 0.361</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p = 0.003</td>
<td>p = 0.032</td>
<td>p = 0.011</td>
</tr>
<tr>
<td>Type of mucin x Size of ACF</td>
<td></td>
<td>r = 0.392</td>
<td>r = 0.313</td>
<td>r = 0.363</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p = 0.002</td>
<td>p = 0.030</td>
<td>p = 0.010</td>
</tr>
<tr>
<td>Type of mucin x Degree of luminal alterations</td>
<td></td>
<td>r = 0.392</td>
<td>r = 0.362</td>
<td>r = 0.470</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p = 0.002</td>
<td>p = 0.009</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>ACF multiplicity x Size of ACF</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Size of ACF x Degree of luminal alterations</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>ACF multiplicity x Degree of luminal alterations</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Type of mucin x ACF multiplicity x ACF size</td>
<td></td>
<td>r = 0.412</td>
<td>r = 0.382</td>
<td>r = 0.364</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p = 0.004</td>
<td>p = 0.015</td>
<td>p = 0.027</td>
</tr>
<tr>
<td>Type of mucin x ACF multiplicity x Degree of luminal alterations</td>
<td></td>
<td>r = 0.393</td>
<td>r = 0.362</td>
<td>r = 0.470</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p = 0.007</td>
<td>p = 0.025</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Type of mucin x Size of ACF x Degree of luminal alterations</td>
<td></td>
<td>r = 0.404</td>
<td>r = 0.363</td>
<td>r = 0.471</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p = 0.005</td>
<td>p = 0.025</td>
<td>p = 0.001</td>
</tr>
<tr>
<td>ACF multiplicity x Size of ACF x Degree of luminal alterations</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Type of mucin x ACF multiplicity x Size of ACF x Degree of luminal alterations</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are regression coefficients for simple linear regression or multiple linear regression with respective p values. n=77 ACF.
6.4 Discussion

This study has shown that ACF have significantly higher indices of cell proliferation than normal mucosa and that there are statistically significant relationships between the type of mucin production of ACF and their cell proliferation parameters and degree of dysplasia. SIM ACF have been shown to have significantly higher degree of dysplasia and cell proliferation abnormalities indicating that they have more alterations than SUM ACF and thus may be further advanced towards tumourigenesis and be better markers of colon cancer risk. In addition, this study has shown that the combined measurement of type of mucin produced in ACF with either ACF multiplicity, ACF size and/or degree of AC luminal alterations may be the best predictor of greater ACF alterations as assessed by degree of dysplasia and rate of cell proliferation.

Alterations in mucin production, particularly production of SIM and loss of SUM, in the distal colon have long been thought to be characteristic of 'transitional mucosa' adjacent to carcinomas (Filipe and Branfoot, 1974). Although observations of dysplastic foci of the colon with increased SIM production have been mentioned in much earlier literature (Filipe, 1975; Sandforth, 1988), only one study to date has attempted to look at the type of mucin production in ACF and relate it to the degree of dysplasia (Thorup, 1997). Thorup (1997) found that reduction in SUM and, to a lesser extent increase in SIM were predictive of degree of dysplasia. However, that study used the HIDAB technique for the identification of SIM and SUM within ACF on slides of the sectioned ACF coupled to a scoring technique for the amount of mucin. In our study, we did not assess the amount of mucin, but rather the predominant type of mucin produced in ACF as observed in the whole mount unsectioned colon as a marker of greater cellular alterations within ACF. Since one of the goals of this study was to relate ACF parameters easily observed in whole mount unsectioned colons to the degree of dysplasia and rate of cell proliferation, we identified the overall type of ACF mucin produced in whole mount colons using just three possible conditions: predominance of SIM, predominance of SUM or mixture of SIM and SUM.

In this study, SIM ACF had significantly higher dysplasia grading, higher indices of cell proliferation, size and degree of AC luminal alterations than SUM ACF, irrespective of their
multiplicity. Since their discovery, all ACF have been suggested to have some degree of dysplasia (Sandforth, 1988; McLellan et al, 1991; Thorup, 1997). Irregularity of crypt architecture, changes in nuclei location, size and shape, reductions in number of goblet cells and alterations of mucin structure and glycosylation, at the very least, satisfy criteria for epithelial dysplasia. In fact, as Cademi et al (1995) have mentioned, even differential staining of ACF by methylene blue is indicative of abnormal changes in cellular and tissue structures. In this study the degree of dysplasia observed in ACF was graded and the results related to ACF parameters that can be observed in whole mount colons so that different populations of ACF with varying levels of advancement could be distinguished from each other. The fact that increased dysplasia and rate of cell proliferation were related to type of mucin production, ACF multiplicity, size and luminal deformity, indicates that these parameters can be used together (as opposed to the observation of just one or two of the parameters) for an overall assessment of the colon cancer risk involved in an experimental treatment.

It is interesting that of all the parameters measured in the whole mount colons, only the type of mucin produced in ACF, alone or in combination with one or more of the other parameters, was significantly predictive of the degree of dysplasia and rate of cell proliferation in ACF. Although, the regressions are statistically significant, the coefficient is low (maximum was $r = 0.473$; Table 6.4), indicating that there are other possible contributing factors (e.g. rate of apoptosis or degree of differentiation), in addition to the degree of dysplasia and rate of cell proliferation, that need further study.

This study indicates that the measurement of more than one or two ACF parameters in whole mount colons is required. This is an important point since the overall state of an ACF contributes to all of the ACF parameters measured. For example, if only one parameter is used, e.g. ACF multiplicity (as has been suggested by a number of authors (Pretlow et al, 1992a, Magnuson et al, 1993), the researcher might assume that an ACF with a multiplicity of 4, small physical size, normal mucin production and few luminal alterations, has the same characteristics (degree of dysplasia and rate of cell proliferation) as an ACF with a multiplicity of 4 but spanning a large area with overall abnormal mucin production and
extensive luminal alterations. In our study, ACF multiplicity of 4 or more, was not significantly different in degree of dysplasia and rate of cell proliferation from ACF with multiplicities of 2 and 3, while SIM ACF of any multiplicity had higher amounts of dysplasia and proliferation abnormalities than SUM ACF. In addition, SIM ACF with multiplicities of 4 or more, had significantly greater degree of dysplasia than SUM ACF with multiplicities of 4 or more (Table 6.1). Thus, the type of ACF mucin production, as an indicator for greater degree of dysplasia and higher rate of cell proliferation, should also be considered in future experiments.

SIM and the mixture of SIM and SUM ACF had significantly higher LI in the top 40% and h index, than SUM ACF suggesting that SIM ACF have an expanding proliferative zone. This is particularly important since an expansion of the proliferative zone is a known characteristic of colon carcinogenesis (Shpitz et al, 1997), indicating that these ACF are more advanced types of ACF.

The results show very little significant difference between ACF producing SIM and those producing a mixture of SIM and SUM, although the numbers for all of the parameters measured were higher for SIM ACF. This suggests that induction of SIM production occurs in line with other changes throughout the crypt and that predominantly SIM ACF and mixture of SIM and SUM ACF may both be used as markers of increased cell proliferation indices. However, ACF producing a mixture of SIM and SUM did not have significantly higher levels of dysplasia than SUM ACF and so may not be appropriate for estimation of degree of dysplasia. It must also be noted that in the extreme gradings for all the parameters measured, i.e. ACF multiplicity of 4 or more, large physical size of ACF and dysplasia ranking of 3, the bulk of ACF were SIM producing (approximately 64%, 79% and 82% respectively, data not shown). Thus, more emphasis must be placed on the measurement of SIM ACF in the distal colon.

Another easily quantifiable feature of ACF on whole mount colons is the degree of AC luminal alterations. Caderni et al (1995) suggest that degree of luminal alterations may be related to the degree of dysplasia present within an ACF and have observed a correlation between the degree of luminal alterations and ACF multiplicity. This study has shown that ACF with SIM or a mixture of SIM and
SUM production had significantly higher indices of cell proliferation and degree of luminal alterations than SUM ACF. In addition, ACF with luminal alteration grading of 3 had significantly higher degree of dysplasia and rate of cell proliferation than ACF with luminal alteration grading of 1. These results suggest that grading of the degree of AC luminal alterations may be an adequate way of estimating large variations in the degree of dysplasia and rate of cell proliferation within ACF.

The lack of positive results in the immunohistochemical staining for the protein product of the mutated k-ras gene indicates either the absence of mutation or low rate of expression of the mutated oncogene. This requires further investigation using more advanced techniques. However, Davies and Rumsby (1999) also failed to see expression of mutated k-ras after 12 weeks in ACF from rats treated with a single dose of AOM.

6.5 Conclusions

In conclusion, this study has shown that ACF have significantly higher rate of cell proliferation than normal crypts and that there are statistically significant relationships between SUM production in ACF and increased degree of dysplasia and rate of cell proliferation versus SUM ACF. SUM ACF have more abnormalities than SUM ACF and thus may be further advanced types of ACF. In addition to SUM production, larger ACF size, multiplicity and more luminal alterations within ACF have been shown to be reflective of greater cellular alterations and abnormality. Thus, it appears that the measurement and consideration of several ACF parameters, particularly type of mucin production, is required for estimating the degree of abnormality within ACF.
7.0 Experiment 4: Phytic Acid in Wheat Bran Affects Colon Morphology, Cell Differentiation and Apoptosis
7.0 Experiment 4: Phytic Acid in Wheat Bran Affects Colon Morphology, Cell Differentiation and Apoptosis

7.1 Introduction

Numerous animal studies have consistently shown that WB has a colon cancer protective effect at early (Jenab and Thompson, 1998; Chapter 5) and late stages of tumourigenesis (Appendix Table 11.2). This effect of WB has been attributed both to its high dietary fiber and PA content. Many of the proposed protective mechanisms of WB and PA action, such as decreased transit time (Eastwood, 1992), increased bulk (Weisburger et al, 1993), and fermentation (Velazquez et al, 1996a) appear to be shared.

In addition, our previous study (Chapter 5) has shown that WB and PA diets are protective of early biomarkers of colon cancer by reducing the PCNA-LI of cell proliferation. However it is still unclear whether these diets also affect other important processes involved in the maintenance of the colonic epithelium, i.e. cell differentiation and apoptosis, which need to be considered in the evaluation of colon cancer risk. The importance of these factors is highlighted by the recent suggestion that their measurement may have greater prognostic significance in the assessment of dietary effects on tumour incidence and colon cancer risk than measurements of cell proliferation alone (Chang et al, 1997).

In the colon carcinogenesis process, the interplay between cell proliferation, differentiation and apoptosis is very important (Butler et al, 1999). It is clearly conceivable that increased or uncontrolled proliferation, failure to properly differentiate and delay in apoptosis may collectively lead to changes in colonic and crypt morphology and architecture, increased cell bulk and a heightened probability for the ‘fixation’ of any mutations that may be present. The importance of apoptosis, as a means of elimination of damaged cells and in the maintenance of colon tissue homeostasis (the balance between cell proliferation and cell death and the maintenance of constant crypt length), is slowly coming to light. In fact, the inhibition of apoptosis has been shown to play an important role in the genesis of colon adenomas and carcinomas (Bedi et al, 1995) and has been suggested to be a risk factor for colon cancer (Garewal et al, 1996). If colonic epithelial cells no longer respond to DNA damage by undergoing apoptosis, then mutations possibly leading to colon cancer may be acquired and fixed through further
proliferation. Apoptotic death may be initiated, inhibited or modulated by the presence or absence of certain endogenous stimuli such as growth factors, hormones, and cytokines (Butler et al, 1999) or exogenous factors such as drugs, ionizing radiation (Ijiri and Potten, 1983) and, most importantly, diet (Hong et al, 1997; Zheng et al, 1999).

Similar to apoptosis, delay or failure in differentiation has been proposed to be important in assessing the dietary colon cancer risk (Yang et al, 1996b). Both the microfloral environment of the colon (Bry et al, 1996) and diet (Yang et al, 1996b; Chang et al, 1997), which can affect the flora, have been shown to modulate the degree of cell differentiation in the colon. In addition, the feeding of some known differentiating agents has been shown to significantly decrease some ACF parameters (Wargovich et al, 1995). The more strongly colon cells are stimulated to differentiate, the less likely they are to proliferate and, hypothetically, the higher their rate of apoptosis. Techniques assessing changes in types of mucin produced and their carbohydrate profiles can accurately display the state of differentiation in the colon.

Despite the vast amount of information on the colon cancer protective effects of WB and PA, there is very little known about how these diets affect colonic crypt morphology, rate of cellular apoptosis and degree of differentiation. Since changes in cell apoptosis along with lack of or delay in differentiation are important processes in the promotion of the colon carcinogenic process it is necessary to determine the effects of WB and PA on these cellular functions.

It is hypothesized that WB will increase cell apoptosis and differentiation and favourably affect colonic crypt morphology, due, in part, to its PA. The objectives of this study were to determine (a) whether changes in cell apoptosis, differentiation and colonic crypt morphology play a role in the previously observed inhibitory effects of WB and its PA on early biomarkers of colon cancer, (b) whether PA is the component of WB responsible for these effects and (c) if there is a difference between endogenous and pure exogenous PA added to the diet.
7.2 Materials and methods

7.2.1 Experimental design

The distal colon sections used in this study were collected from the study performed in Experiment 2 (Chapter 5). Sections (5 μm thickness) were sliced from ‘swiss roll’ wrapped, paraffin embedded colon sections, with two sections from each rat on each slide. Adjacent slides from 10 animals per group, chosen at random, were taken and processed for the measurement of rate of apoptosis, degree of cell differentiation and colon morphometry. All slides were coded and the observer blinded as to the dietary groups to which the animals belonged. For each analysis, a minimum of 24 crypts were counted per colon. All counting was performed at 400X magnification. Only whole longitudinally sectioned crypts that showed the entire column length from the lumen down to the muscularis mucosa were counted. Incomplete crypts or those with more than 2 missing cells were not counted.

7.2.2 Measurement of apoptosis

The rate of apoptosis was measured via the Tdt-mediated dUTP nick end labeling (TUNEL) technique using the in-situ cell death detection kit from Roche Molecular Biochemicals (Mannheim, Germany) which labels DNA strand breaks produced during apoptosis. The tissue sections were deparaffinized, rehydrated and washed with PBS. The slides were then microwaved for 5 minutes on low power in citric acid buffer, allowed to cool, washed twice in PBS, treated with proteinase K (20μg/mL in 10mM Tris/HCl) for 15 minutes at room temperature, rinsed twice with PBS, incubated with 3% H₂O₂ in methanol for 10 min at room temperature and again rinsed twice with PBS. They were then incubated for 60 min at 37° C in a humid chamber with 50 μl of TUNEL reaction mixture consisting of terminal deoxynucleotidyl transferase and the nucleotide, rinsed three times with PBS, incubated for 30 min at 37°C in a humid chamber with converter-POD peroxidase conjugated antibody and rinsed three times with PBS. Slides were incubated for 10 min at room temperature with 100 μl of diaminobenzidine tetrahydrochloride (DAB) substrate solution, counterstained with haematoxylin for 20 seconds, rinsed in distilled water, tap water and mounted with Permount. The apoptotic index (AI) was
calculated as the percent number of labeled cells for the whole crypt and top 40% and bottom 60% of each crypt. Examples of crypts stained with TUNEL are shown in Figure 7.1.

7.2.3 Measurement of cell differentiation – lectin histochemistry

The method was modified from the method of Boland et al (1982). The tissue sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by immersing the slides in 0.3% H$_2$O$_2$ in methanol for 10 min with a subsequent double washing in distilled water (3 mins.). The slides were then treated with a 0.025% triton-PBS solution for 3 minutes. The biotinylated Dolichos biflorus agglutin (DBA; 10 µg/ml; Vector Laboratories Inc., Burlingame, CA) was placed onto the tissue sections and incubated in a humid chamber for 30 minutes. The slides were washed with PBS for 3 minutes, then treated with streptavidin complex (Dako Inc., Carpenteria, CA) for 30 minutes and washed three times with distilled water for 3 minutes. The colour was developed by adding 100 µl of 0.5 mg of DAB (Vector Laboratories Inc., Burlingame, CA) /ml PBS with the addition of 0.015% H$_2$O$_2$ immediately prior to staining for 7 min. The slides were washed three times with distilled water for 3 minutes, counterstained with haematoxylin 20 seconds, rinsed in distilled water, tap water and mounted with Permount. To test staining specificity, 0.2 M N-acetyl-D-galactosamine (Sigma Chemical Company, St. Louis, MO), which inhibits lectin binding, was added to a control slide for each run.

The lectin scoring index was calculated according to the method developed by Chang et al (1997). This method determines a score that ranges on a scale of 0 to 4 based on the percent of cells stained and also the intensity of staining (Appendix Table 11.5). For example, crypts with similar percent of positive stained cells but different intensity of the stain colour would receive different scores, with a higher score indicating greater number of stained cells and greater intensity and hence, higher degree of differentiation. Examples of crypts stained with DBA are shown in Figure 7.1.

7.2.4 Measurement of cell differentiation – determination of sulpho- and sialomucins

The HIDAB method (Spicer, 1965) was used. Briefly, the tissue sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by immersing the slides in 0.3% H$_2$O$_2$ in
Figure 7.1: Apoptosis and differentiation methods. 'a' shows a crypt stained with TUNEL. 'b' shows a closeup of top of the same crypt. Note the brown nuclear staining indicating positive staining, hence a cell undergoing apoptosis (indicated by arrows). 'c' shows a crypt stained with lectin DBA staining. Note the brown colour indicating binding of the lectin to carbohydrate residues. Staining is not nuclear. The lectin score (Chang et al, 1997) was based on the percentage of cells stained and also the intensity of the staining. 'd' shows lectin DBA staining of an ACF with surrounding normal mucosa to demonstrate differences in stain intensity. Note the difference in staining intensity from strong in the surrounding normal mucosa to very light within the ACF. 'e' shows HIDAB staining for SIM and SUM production. Brown staining indicates SUM, blue indicates SIM and a mix of blue and brown indicates both SIM and SUM production. Note the two crypts on the right producing more SIM while those on the left are producing more SUM, as is the norm in the distal colon. 'f' shows HIDAB staining of an ACF with surrounding normal mucosa. Note that the normal mucosa produces mainly SUM while the ACF is producing only SIM or a combination. TUNEL= Tdt mediated dUTP nick end labeling, DBA= Dolichos biflorus agglutinin, ACF= aberrant crypt foci, HIDAB= high iron diamine alcian blue, SIM= sialomucin, SUM= sulphomucin.
methanol for 10 min with a subsequent double washing in distilled water. The slides were then immersed in high iron diamine solution (20 mg of N-N'-dimethyl-p-phenylene diamine, 120 mg of N-N'-dimethyl-m-phenylenediamine in 50 mL of distilled water plus 1.4 mL of 20% ferric chloride, (all from Sigma Chemical Co., St. Louis, MO)) in a covered slide rack protected against light for 18 hours at room temperature. The slides were then rinsed three times in distilled water, stained with 1% alcian blue (Sigma Chemical Co., St. Louis, MO) in 3% acetic acid for 30 minutes, rinsed three times in 80% ethanol and once in distilled water. Dark brown staining of the crypt goblet cells implied SUM production, bright or dark blue staining indicated predominantly SIM production, while a mixture of brown and blue stain indicated the production of both SUM and SIM. Since these were distal colon sections, it was anticipated that SUM production would be the predominant type of staining observed. The number of cells displaying each type of mucin produced were counted in each crypt and a ratio of the number of SIM to SUM producing cells calculated. Examples of crypts stained with HIDAB are shown in Figure 7.1.

7.2.5 Colonic mucosal morphometry

The tissue sections were deparaffinized, rehydrated and stained with haematoxylin and eosin. Crypt cell height, defined as the full height of the crypt in number of cells, and crypt density, defined as the number of crypts per mm of colon determined via a micrometer, were counted. At least 18 measurements of crypt density were taken at various parts of the distal colon. Colon length was determined at the time of sacrifice and the data used to calculate ratios of colon length to crypt height and to crypt density.

7.2.6 Statistical analyses

All data were analyzed by one or two way ANOVA followed by the Tukey’s pairwise or Dunn’s pairwise nonparametric multiple comparisons tests using the SigmaStat statistical software package (Jandel Scientific, San Rafael, CA).
7.3 Results

7.3.1 Apoptosis

A significant increase versus the BD was seen in the AI for the WB, DWB and PA groups in whole crypt and the bottom 60% of the crypt (Table 7.1). In the bottom 60% of the crypt, the DWBPA group was significantly lower than the PA but not different from the other groups. All the treatment groups had a significantly higher AI than the BD in the top 40% of the crypt. Analysis via 2 way ANOVA showed a significant effect of dietary fiber, PA and their interaction in the whole crypt and the top 40% of the crypt but not in the bottom 60% of the crypt.

<table>
<thead>
<tr>
<th>Apoptotic Index</th>
<th>Diet Groups</th>
<th>BD</th>
<th>WB</th>
<th>DWB</th>
<th>DWBPA</th>
<th>PA</th>
<th>Fiber</th>
<th>PA</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Crypt</td>
<td></td>
<td>1.84 ±</td>
<td>5.84 ±</td>
<td>5.44 ±</td>
<td>4.34 ±</td>
<td>6.59 ±</td>
<td>0.010</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.24 b</td>
<td>0.54 a</td>
<td>0.69 a</td>
<td>0.46 ab</td>
<td>0.54 a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Top 40%</td>
<td></td>
<td>3.29 ±</td>
<td>8.71 ±</td>
<td>8.92 ±</td>
<td>7.76 ±</td>
<td>7.86 ±</td>
<td></td>
<td>&lt; 0.001</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.51 b</td>
<td>0.66 a</td>
<td>1.37 a</td>
<td>0.87 a</td>
<td>0.65 a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bottom 60%</td>
<td></td>
<td>0.80 ±</td>
<td>2.94 ±</td>
<td>2.57 ±</td>
<td>1.61 ±</td>
<td>5.28 ±</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.24 c</td>
<td>0.64 ab</td>
<td>0.39 ab</td>
<td>0.15 bc</td>
<td>0.51 a</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SEM. Apoptotic index = (no. of apoptotic cells / total no. of cells) x 100. Values with different superscripts within a row are significantly different by one way ANOVA followed by a multiple groups comparisons test, p < 0.05. p values indicate results for a 2 way ANOVA on the effects of fiber, PA or their interaction using the BD, WB, DWB and PA groups. NS, not significantly different. n=10 rats per group. BD, basal diet, control group; WB, 25% wheat bran diet; DWB, 25% dephytinized wheat bran diet; DWBPA, 25% dephytinized wheat bran plus 1.00% added PA; PA, 1.00% added PA.

7.3.2 Colon cell differentiation

All the dietary treatment groups had significantly higher lectin scores in the whole, top 40% and bottom 60% of the crypt (Table 7.2). In addition, 2 way ANOVA showed a significant effect of dietary fiber, PA and their interaction in the whole crypt, the top 40% and the bottom 60% of the crypt.

The BD group showed a significantly much higher production of SIM and lower production of SUM in the distal colon than any of the treatment groups (Table 7.3). The ratio of SIM to SUM showed a similar pattern being significantly higher than the treatment diet groups (Table 7.3). There were no differences in the percentage of cells producing a mixture of SIM and SUM amongst the various dietary groups. An effect of fiber, PA and their interaction was observed for the percentage of cells producing SIM and for the overall ratio of SIM to SUM. An effect of fiber and PA but not their interaction was
observed for the percentage of cells producing SUM. No significant effects of fiber and PA were observed for cells producing a mixture of the types of mucins.

### Table 7.2: Lectin scores in the distal colon of the various dietary groups.

<table>
<thead>
<tr>
<th>Lectin Scores</th>
<th>Diet Groups</th>
<th>p values for effect of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BD</td>
<td>WB</td>
</tr>
<tr>
<td>Whole</td>
<td>0.94 ± 2.52</td>
<td>2.30 ± 2.59</td>
</tr>
<tr>
<td>Crypt</td>
<td>0.12 ± 0.20</td>
<td>0.21 ± 0.15</td>
</tr>
<tr>
<td>Top 40%</td>
<td>1.03 ± 2.55</td>
<td>2.15 ± 2.61</td>
</tr>
<tr>
<td>Bottom</td>
<td>0.97 ± 2.37</td>
<td>2.39 ± 2.63</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Values with different superscripts within a row are significantly different by one way ANOVA followed by a multiple groups comparisons test, p < 0.05. Values indicate results for a 2 way ANOVA on the effects of fiber, PA or their interaction using the BD, WB, DWB and PA groups. NS, not significantly different.

### Table 7.3: Type of mucin production in the distal colon of the various dietary groups.

<table>
<thead>
<tr>
<th>Diet Groups</th>
<th>p values for effect of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BD</td>
</tr>
<tr>
<td>% SIM cells</td>
<td>26.22 ± 6.20</td>
</tr>
<tr>
<td>% SUM cells</td>
<td>38.44 ± 60.38</td>
</tr>
<tr>
<td>% both SIM</td>
<td>35.34 ± 34.42</td>
</tr>
<tr>
<td>and SUM cells</td>
<td>3.90 ± 2.11</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Values with different superscripts within a row are significantly different by one way ANOVA followed by a multiple groups comparisons test, p < 0.05. Values indicate results for a 2 way ANOVA on the effects of fiber, PA or their interaction using the BD, WB, DWB and PA groups. NS, not significantly different. n = 10 rats per group. SIM, sialomucin; SUM, sulphomucin. For definitions of other abbreviations see Table 7.1.

#### 7.3.3 Mucosal morphometry

Although all the treatment groups had reduced crypt height, measured in number of cells, only the DWB and PA groups were significantly lower (Table 7.4). Two way ANOVA did not show an individual effect of dietary fiber or PA but did show a significant effect of their interaction on reduction of crypt cell height. The WB, DWB and PA groups all had significantly lower crypt density, defined as the number of crypts per mm of colon. A significant effect of both fiber and PA was observed for the crypt density. There were no significant differences observed for colon length, but there was a
significant effect of fiber affecting colon length. There were no significant differences amongst the various diet groups in the ratio of crypt density to height. The ratio of colon length to crypt cell height was significantly higher in the DWB and DWBPA than the BD group, with a significant effect of fiber and interaction of fiber and PA but not PA alone being observed. The ratio of colon length to crypt density was significantly higher in the WB and DWB than the BD group with only a significant effect of fiber being observed using the two way ANOVA.

Table 7.4: Distal colon mucosal morphometry measurements.

<table>
<thead>
<tr>
<th>Diet Groups</th>
<th>p values for effect of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fiber</td>
</tr>
<tr>
<td>Crypt Height (no. of cells)</td>
<td></td>
</tr>
<tr>
<td>BD</td>
<td>37.27</td>
</tr>
<tr>
<td>WB</td>
<td>34.31</td>
</tr>
<tr>
<td>DWB</td>
<td>33.05</td>
</tr>
<tr>
<td>DWBPA</td>
<td>32.69</td>
</tr>
<tr>
<td>PA</td>
<td>16.27</td>
</tr>
<tr>
<td>Crypt density*</td>
<td></td>
</tr>
<tr>
<td>BD</td>
<td>18.83</td>
</tr>
<tr>
<td>WB</td>
<td>15.25</td>
</tr>
<tr>
<td>DWB</td>
<td>15.75</td>
</tr>
<tr>
<td>DWBPA</td>
<td>16.72</td>
</tr>
<tr>
<td>PA</td>
<td>16.27</td>
</tr>
<tr>
<td>Colon length</td>
<td></td>
</tr>
<tr>
<td>BD</td>
<td>12.99</td>
</tr>
<tr>
<td>WB</td>
<td>13.56</td>
</tr>
<tr>
<td>DWB</td>
<td>14.14</td>
</tr>
<tr>
<td>DWBPA</td>
<td>14.63</td>
</tr>
<tr>
<td>PA</td>
<td>12.96</td>
</tr>
<tr>
<td>Ratios:</td>
<td></td>
</tr>
<tr>
<td>Crypt density:height</td>
<td></td>
</tr>
<tr>
<td>BD</td>
<td>0.52 ± 0.46</td>
</tr>
<tr>
<td>WB</td>
<td>0.46</td>
</tr>
<tr>
<td>DWB</td>
<td>0.49</td>
</tr>
<tr>
<td>DWBPA</td>
<td>0.52</td>
</tr>
<tr>
<td>PA</td>
<td>0.51</td>
</tr>
<tr>
<td>Colon length:height</td>
<td></td>
</tr>
<tr>
<td>BD</td>
<td>0.35 ± 0.40</td>
</tr>
<tr>
<td>WB</td>
<td>0.40</td>
</tr>
<tr>
<td>DWB</td>
<td>0.44</td>
</tr>
<tr>
<td>DWBPA</td>
<td>0.45</td>
</tr>
<tr>
<td>PA</td>
<td>0.41</td>
</tr>
<tr>
<td>Colon length:density</td>
<td></td>
</tr>
<tr>
<td>BD</td>
<td>0.71 ± 0.91</td>
</tr>
<tr>
<td>WB</td>
<td>0.91</td>
</tr>
<tr>
<td>DWB</td>
<td>0.91</td>
</tr>
<tr>
<td>DWBPA</td>
<td>0.89</td>
</tr>
<tr>
<td>PA</td>
<td>0.81</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Values with different superscripts across each row are significantly different, p < 0.05; p values in table indicate results for a 2 way ANOVA on the effects of fiber, PA or their interaction using the BD, WB, DWB and PA groups. NS, not significantly different. n=8 rats per group. For definitions of other abbreviations see Table 7.1. * crypt density is defined as the number of crypts per mm of colon.

7.4 Discussion

This experiment has shown that WB, DWB, DWBPA and PA diets can increase the cell apoptosis and differentiation and affect colon morphometry. These observations are particularly important since, coupled to the previously observed concomitant reduction in rate of cell proliferation caused by these diets (Experiment 2; Chapter 5), they provide a mechanism whereby WB and PA may be exhibiting their protective effects on early biomarkers of colon carcinogenesis.

It has previously been shown that the rate of apoptosis and degree of differentiation are important prognostic indicators of colon tumour development and colon cancer risk and may be even more predictive than measurements of the rate of cell proliferation (Chang et al, 1997). It is probable
that all these three mechanisms function together to limit the growth and abnormal expansion of cells in the colon. Apoptosis is key for the timely death of terminally differentiated or DNA damaged cells. Suppression of apoptosis may lead to an increased life span of cells or to an accumulation of genetic damage (Williams and Smith, 1993; Kerr et al, 1994), while the converse may be true for the promotion of apoptosis. Since it has recently been suggested that disruption of apoptosis may be an early event in carcinogenesis (Hoffman and Lieberman, 1994), the results of this study that WB, DWB, DWBPA and PA can all significantly increase the rate of apoptosis versus the control group at 100 days, suggests that this is an important mechanism in their colon cancer protective effects. Compher et al (1999) have recently shown an increase in apoptosis at the initiation stages of colon carcinogenesis but not at 56 days post-initiation with 20% WB feeding. However, their results are limited by the small size of the colon section studied and the small number of crypts counted (10 versus 24 in this study).

In this study, the WB diet was used to show the effects of both WB fiber and its endogenous PA while the DWB diet was designed to show the effect of WB fiber without endogenous PA and the PA diet was designed to show the effect of exogenous, pure PA without the interference of WB fiber. Thus, it is of particular interest that there was no significant difference in rate of apoptosis or degree of differentiation between the WB, DWB and PA groups. This suggests that both WB fiber and PA, whether added exogenously or present endogenously (within the matrix of WB fiber), can affect apoptosis. In fact, our data show a significant effect of WB fiber, PA and their interaction on rate of apoptosis in the whole crypt and top 40% of the crypt.

It is unclear whether the mechanisms of this modulation are the same for WB and PA. Although it has been suggested that WB and PA share a number of the same proposed colon cancer protective mechanisms of action (e.g. physical dilution of gut contents, shortening transit times, alterations in mutagenicity of the intestinal contents, production of butyrate), PA from the diet may also participate in cellular inositol phosphate pools in the colon cells and affect secondary messenger pathways (Shamsuddin et al, 1997; Shears, 1998), thus altering cell growth and gene expression. In fact, pure PA has been shown in-vitro to inhibit growth and promote apoptosis in a number of cancer cell lines.
(Sakamoto et al, 1993b; Shamsuddin and Yang, 1995; Yang and Shamsuddin, 1995; Shamsuddin et al, 1996). It may accomplish this by up-regulating the expression of the p53 gene (Saeid and Shamsuddin, 1998), which has been shown to be intimately involved in the type of apoptosis that occurs in times of cellular stress or genetic insult (Gottlieb and Oren, 1998).

Colonic butyrate levels may be increased by both WB (Mathers and Tagny, 1994; Folino et al, 1995) and PA, by causing starch malabsorption leading to increased colonic fermentation and production of butyrate and other short chain fatty acids (Thompson et al, 1989). Butyrate has been shown to induce differentiation (Roediger, 1982; Augeron and Laboisse, 1984) and also to promote apoptosis via a p53 independent pathway (Hague et al, 1993). The mechanism of butyrate induced apoptosis in the colon may be related to the expression of the bcl-2 gene, which blocks apoptotic cell death (Butler et al, 1999), or the bak gene, which is an apoptosis promoter (Kiefer et al, 1995). Hague et al (1997) suggested that butyrate may be able to induce apoptosis in the colon by either decreasing the levels of bcl-2 protein or by inducing the expression of bak. Thus, increased production of butyrate due to WB and PA feeding, and its subsequent modulation of bcl-2 or bak genes, may be responsible for the increased levels of apoptosis that we have observed in this study with WB and PA feeding. This may be particularly true for the PA diet since it had the highest rate of apoptosis in the bottom 60% of the crypt, where bcl-2 is most highly expressed (Butler et al, 1999).

WB may also be increasing the rate of apoptosis via a more simple mechanism. It is known that detachment of cells from the top of crypts may induce apoptosis since contact between colonocytes and the extra cellular matrix is essential for their survival (Butler et al, 1999). WB fiber has a great sloughing ability and thus could be inducing more apoptosis by causing more cells to detach or become less firmly attached from the tops of the crypts.

This study also showed that all the treatment diets could induce a greater state of differentiation in the colon. We used two different methods, lectin immunohistochemistry and profile of mucin production to analyze the degree of differentiation. Lectins are glycoproteins that bind to particular terminal carbohydrate residues on the cell surface. The lectin used in this study, DBA, is known to
increase in staining intensity due to increased binding, as cells become more differentiated (Boland et al., 1982; Hong et al., 1997) and is suitable for use as a marker of differentiation in normal appearing colonic epithelium (Boland et al., 1992). Un- or less differentiated cells do not show as much lectin staining since the terminal carbohydrate moieties are incomplete or deleted. Those at higher risk of colon cancer, such as FAP and HNPCC patients, have been shown to have reduced levels of lectin binding in their rectal tissues (Boland et al., 1992; Sams et al., 1990). Many studies have found the use of DBA lectin binding a powerful tool as a marker of degree of differentiation (Chang et al., 1997; Hong et al., 1997).

The normal distal colon of both humans and rats is known to secrete predominantly SUM with lesser production of SIM or a mixture of SUM and SIM, while greater production of SIM is found in some early lesions of colon cancer in the distal colon (Caderni et al., 1995) suggesting that glycoconjugate modifications occur early in process of colorectal carcinogenesis, and also in colon adenomas and adenocarcinomas (Filipe and Branfoot, 1974). In addition, changes in mucin profile of the distal colon and an increase in the ratio of SIM to SUM have been observed in individuals with increased colon cancer risk (Yang et al., 1996b). Thus, analysis of changes in mucin profile and the ratio of SIM to SUM give insight into the level of cellular differentiation caused by these diets.

These results indicate a great shift towards an abnormal mucin profile or a less differentiated state for the control group (BD diet) while the WB and PA containing diets remained essentially normal, i.e. a much higher amount of SUM production. In general, it is thought that cells that are more differentiated will proliferate less (Smith et al., 1998). The fact that WB and PA diets can maintain a higher degree of colon cell differentiation than the BD group may play a role in their observed colon cancer protective effects. In Experiment 2 (Chapter 5) a concomitant decrease in the rate of cell proliferation along with decreases in ACF parameters in these same rats was seen. Thus, the ability of these diets to reduce cell proliferation may be linked to their ability to induce more differentiation.

It is unclear how the treatment diets may be affecting differentiation, but pure PA in-vitro has been shown to increase the differentiation of colon cancer cell lines (Shamsuddin and Yang, 1995; Yang and Shamsuddin, 1995; Shamsuddin et al, 1996). Likewise, butyrate, a product of WB fermentation, has
also been shown to induce differentiation and reduce growth in several colon cancer cell lines (Augeron and Laboisse, 1984; Tanaka et al, 1989). Increased production of butyrate may be inhibiting the activity of histone deacetylases (Boffa et al, 1978) leading to histone hyperacetylation which may result in cell cycle arrest and more differentiation. Both PA, through the IP pool and as a secondary messenger, and butyrate may directly participate in the modulation of the expression of genes involved in cellular differentiation. The exact mechanisms of WB and PA action on induction of colon cellular differentiation require further study.

All the treatment diets reduced the crypt height measured in number of cells but only the DWB and PA groups were significantly lower. The BD group had significantly more crypts packed into 1 mm (crypt density) than the WB, DWB and PA groups. There was also a significant effect of WB fiber and PA on crypt density. Since these diets also decreased the rate of cell proliferation (Experiment 2; Chapter 5) and increased the rate of apoptosis in this experiment, it is possible that the lower number of cells and crypts per mm are due to greater cell loss and less rapid replenishment of new cells. This is entirely possible since Hong et al (1997), noticed a similar (but opposite) event: an increase in crypt cell height and number of crypts per mm of colon with a decrease in apoptosis and increase in cell proliferation.

The ratio of crypt density to crypt height and the other ratios were calculated since they may give an indication of the three dimensional state of growth of the colon, e.g. how the diets were affecting crypt height in relation to colon length or number of crypts per mm. Presumably, similar to the principle behind the rate of cell proliferation being a colon cancer risk factor, the more growth there is within the colon or the greater the cell bulk, the more probability that a mutation will occur and carry on to a tumour. Although this experiment has shown that WB-containing and PA diets affect cell apoptosis and differentiation within the colon, it is still unknown what physiological changes they may induce or inhibit within the colon and other organs.
7.5 Conclusions

It is concluded that WB, due both to its dietary fiber and endogenous PA component, and pure PA added to a low fiber diet can significantly increase the rate of apoptosis and degree of differentiation in the distal colon. They can also have favourable effects on colon morphology, reducing crypt cell height and crypt density. These results, coupled with the previously observed reductions in ACF and cell proliferation (Experiment 2; Chapter 5) showed that WB, its fiber and PA can affect early events in colon carcinogenesis. However, the exact mechanisms of that modulation need further elaboration.
8.0 Experiment 5: Some Other Modes of Action of Wheat Bran, Dephytinized Wheat Bran and Phytic Acid
8.0 Experiment 5: Some Other Modes of Action of Wheat Bran, Dephytinized Wheat Bran and Phytic Acid

8.1 Introduction

Since WB and PA have been found to be protective of early biomarkers of colon carcinogenesis in Experiment 2, it is necessary to establish some mechanisms whereby they may be affecting the carcinogenic process. Although many studies have shown a protective effect of WB on colon tumourigenesis (Wilson et al, 1977; Barbolt and Abraham, 1978; Chen et al, 1978; Fleiszer et al, 1978, 1980; Nigro et al, 1979; Watanabe et al, 1979; Abraham et al, 1980; Barbolt and Abraham, 1980; Reddy and Mori, 1981; Reddy et al, 1981; Barnes et al, 1983; Calvert et al, 1987; Alabaster et al, 1993; McIntyre et al, 1993; Alabaster et al, 1995), very few have tested the physiological mechanisms whereby WB fiber may be protective. None have tried to relate the protective effects of WB to its PA and none have tried to differentiate the mechanisms due to WB fiber and to PA. We hypothesize some physiological mechanisms for the protective effect of PA which are summarized on Figure 8.1.

Initiated cells may develop into tumour cells through a series of cellular changes some of which may be related to oxidative damage of the cells within the colonic crypts. PA has been called a potent antioxidant because of its ability to chelate minerals, particularly iron, which can catalyze lipid peroxidation via the Fenton reaction (Graf and Eaton, 1993). The inhibition of free radical production through iron chelation may prevent the initiation or promotion of colon carcinogenesis. Although, in-vitro, pure PA has been shown to have a high affinity for iron and to inhibit oxidative reactions (Graf and Eaton, 1990), its effect on lipid peroxidation in-vivo is not clear. Also, due to the many complex interactions between PA and other dietary compounds and potential PA hydrolysis in the gastrointestinal tract, the anti-oxidative effect of WB or its PA remains unclear. Thus, it is of interest to determine whether inhibition of lipid peroxidation is indeed a mechanism whereby WB or PA may protect against genetic damage.
Figure 8.1: Hypothesized modes of action for the protective effect of PA
PA's ability to chelate zinc may also potentially reduce the rate of DNA synthesis and eventually cell proliferation. Zinc has been shown to form the strongest mineral complex with PA (Evans and Martin, 1988) and is thought to be the mineral most adversely affected by PA (Torre and Rodriguez, 1991). PA has been shown to reduce the availability of zinc in humans (Morris et al, 1984,) and rats (Davis and Nightingale, 1975). In-vitro, decreased in cell culture media zinc levels has been shown to reduce the activity of DNA-synthesizing enzymes (Prasad et al, 1996) and cell proliferation (Lawson et al, 1998; Thornton et al, 1998) and to induce apoptosis (Sakabe et al, 1998) in different cell lines as well as to reduce malignant invasion of colon cancer in mice (Carter et al, 1997). Thus, PA's ability to chelate zinc may be related to its ability to decrease the rate of colonic cell proliferation. If PA is chelating iron and zinc and inhibiting their absorption, then serum and femur levels of these minerals will be lower in rats treated with diets that contain PA versus those that do not.

Starch malabsorption caused by PA may also lead to increased fermentation in the cecum and colon resulting in increased stool bulk, lowered cecal and colonic pH and increased production of SCFA, particularly butyrate. Increased stool bulk may dilute the concentration of putative mutagens or promoters such as bile acids in the GI tract resulting in less mucosal contact and hence, less damage. The increased level of fermentation and consequent production of butyrate may also have colon cancer protective effects since butyrate has been shown in-vitro to decrease the rate of cell proliferation and to induce apoptosis in colon cancer cell lines (Hague et al, 1993). Thus, if PA, through increased production of butyrate, is able to induce terminal differentiation and apoptosis of transformed colonic cells, this may be a cellular mechanism whereby it may protect against colon carcinogenesis.

In addition to dietary proteins, PA may also be able to bind various bacterial enzymes inhibiting or lowering their activity. Bacterial enzymes such as β-glucuronidase have been implicated in the colon carcinogenic process and a reduction of their activity may have protective effects.

Thus, the objectives of this study were to determine some potential physiological effects of WB, DWB and PA on (a) indices of lipid peroxidation, i.e. the levels of urinary, liver and colonic mucosal
thiobarbituric acid reactive substances (TBARS) and conjugated dienes, (b) indices of iron, zinc and calcium availability, i.e. femur and serum levels, (c) indices of cecal and colonic fermentation, i.e. pH, SCFA production, and (d) cecal β-glucuronidase activity.

8.2 Materials and Methods

8.2.1 Experimental design and sample collection

This study followed the same protocol as, and conducted concurrently with, Experiment 2 (Chapter 5). Animals were carcinogen injected, randomized into groups of 15 rats and fed either the BD, WB, DWB, DWBPA or PA diets for a period of 100 days. Two weeks prior to sacrifice, the animals were transferred individually to metabolic cages and urine was collected for 3 consecutive days. Daily urine samples were pooled and frozen at -20°C until analysis for TBARS. Animals were sacrificed by CO₂ gassing and blood was collected by cardiac puncture for serum analysis of minerals. Cecal and colon contents were collected after cutting the cecum and colon lengthwise on a glass plate placed on a bed of mixed ice and dry ice. They were quickly weighed, placed individually in labeled vials, placed on dry ice and subsequently stored at -70°C until needed for analysis of pH and SCFA. The colons were flushed and rinsed fully with ice cold PBS buffer and placed on another glass plate cover on a bed of mixed ice and dry ice. The mucosa was carefully scraped off using the sharp edge of a clean, new, glass microscope slide. It was quickly weighed, mixed and divided into 3 equal aliquots, flash frozen in liquid nitrogen and subsequently stored at -70°C until needed for analysis of conjugated dienes.

Livers were also collected, flash frozen in liquid nitrogen and subsequently stored at -70°C until needed for analysis of TBARS. The femurs were collected, placed on dry ice and stored at -20°C. They were subsequently thawed, defleshed and used for analysis of mineral content.

8.2.2 Measurement of TBARS

Frozen urines were thawed in a refrigerator for 20 mins. Duplicate 1.5 mL samples were centrifuged at 13 000 rpm in a microcentrifuge (Biofuge A, Heraeus Separations Technik Corp.,
Germany). 1.0 mL supernatants were removed and placed into clearly labeled, glass culture tubes. 1.0 mL of blank solution (0.9% saline), standards and positive control samples of a known amount of 1,1,3,3-tetraethoxypropane (TEP), which reacts with TBA, were also pipetted into clearly labeled glass culture tubes. To each tube was added 4 mL of hot TBA solution (TBA in acid phosphate buffer; prepared immediately prior to use). The tubes were vortexed quickly to mix the contents and placed on a block heater at 78°C for 30 minutes. The tubes were allowed to cool for 10 mins and the absorbance of the samples was read at 532 nm in a spectrophotometer (Beckman Instruments DU-7 Spectrophotometer, Beckman Instruments, Fullerton, CA). A standard curve was prepared using different volumes of a 23 µg/mL solution of TEP, run concurrently with the samples as described above.

Analysis of TBARS in liver was performed in the same manner, except that liver tissues (1 g) were homogenized in ice cold 1.5% KCl solution (1:10 mg weight of sample / mL buffer volume) using a polytron (PT3100 at the 5 setting, Brinkmann Corp., Switzerland). Homogenates were centrifuged at 3000 rpm (RC-5B Refrigerated Superspeed Centrifuge, Dupont Company, Wilmington, DE) at 4°C for 10 mins. TBARS were then analyzed as described above using 1.5 mL of supernatant and expressed as µg per g of liver, per whole liver and per mg of protein. Liver protein was measured using a bicinchoninic acid kit (Pierce Chemical Company, Rockford, IL).

8.2.3 Measurement of conjugated dienes

Conjugated dienes, an intermediate product of lipid peroxidation, in colonic mucosa were determined by a modified method of Deschner et al (1986). Samples of frozen mucosa (approximately 50 mg) were removed from storage at -70°C, accurately weighed and immediately placed in a 2:1 chloroform : methanol (plus 0.2% BHT) solution at a concentration of 1g sample/10 mL chloroform-methanol solution. The sample mixture was then homogenized on ice in a manual microhomogenizer. The mixture was quickly filtered through Whatman no. 10 paper and the filtrate collected on ice. To precipitate the protein in solution, a 0.9% saline solution was added to the filtrate at a concentration of 2 mL of saline solution/10mL of filtrate and then centrifuged at 1000g for 10 min. at 4°C (RC-5B Refrigerated Superspeed Centrifuge, Dupont Company, Wilmington, DE) to separate the chloroform
(which contained the lipid fraction) and the saline solution phases. The chloroform phase was accurately pipetted from the bottom of the saline/methanol phase into a clean preweighed tube on ice and evaporated under nitrogen. Additional chloroform was added and evaporated until the lipid solution was clear and not cloudy. The final lipid sample was accurately weighed and then dissolved in spectroanalyzed cyclohexane. The optical density was determined against a cyclohexane blank at 235 nm. At this wavelength, the absolute absorbance is a measure of the level of conjugated dienes.

The results were expressed as nmol of conjugated dienes per g of colon weight, per g of total fat, per g of colon mucosa and per mg of total protein. Mucosal protein was measured using a bicinchoninic acid kit (Pierce Chemical Company, Rockford, IL).

8.2.4 Femur, serum and diet mineral levels

Femurs were defleshed and cleaned by washing in ddHi2O. They were weighed, placed in individual preweighed labeled crucible, dried in an oven at 100°C overnight, allowed to cool in a dessicator, and re-weighed to obtain the dry weight. The femurs were then ashed in a muffle furnace at 525°C for 48 hrs and allowed to cool in a dessicator. Upon cooling, the crucible was weighed and the weight of ash determined by difference. The ash was then dissolved by adding 2 mL of ddHi2O and 2 mL of 6N HCl. The dissolved ash was then separated into 3 equal aliquots, one each for analysis of Ca, Fe and Zn. Each aliquot was further diluted as necessary with 0.6M HCl. For the calcium determinations, Lanthanum oxide (LaO) was added to the samples to a final concentration of 1.0%. A Varian (model no. AA1275 GTA95, Varian Canada Inc., ON) atomic absorption spectrophotometer with air-acetylene flame was used. The bulb wavelengths for calcium, iron and zinc were 422.7, 248.3 and 213.9 nm respectively using slit widths of 0.5, 0.2, 1.0 nm respectively and a 2.0-2.5 L/min flowrate of acetylene with a 13.5 L/min flowrate of oxygen.

Known concentrations of calcium, iron and zinc in 0.6M HCl (with 1% LaO for calcium) from certified atomic absorption standard solutions (Fisher Scientific, NJ) were used as references. All equipment and glasswares used were acid washed and rinsed three times in ddHi2O prior to use.
Serum mineral levels were determined by placing 1 mL of serum in a clean, acid washed test tube and diluted to 10 mL with ddH₂O. Duplicate aliquots (1 mL) of the diluted serum were used directly for analysis of iron and zinc by atomic absorption spectrometry. Aliquots used for analysis of the calcium determinations were further diluted to contain a final LaO concentration of 1.0%. The serum minerals were detected by atomic absorption spectrophotometry as described above.

Dietary mineral levels were determined in the same manner as described for the femur minerals.

8.2.5 pH and short chain fatty acid production

Cecal and colon contents were allowed to thaw at room temperature for 15 minutes. The pH was then assessed using a flat surface pH electrode (Beckman Instruments Inc., Fullerton, California). A 0.35 g sample was then weighed, diluted 1:5 with 0.2% CuSO₄, homogenized for 15 seconds with a polytron (Brinkmann Instruments, Switzerland), and then centrifuged for 10 minutes at 13,000 rpm in a centrifuge (RC5B Refrigerated Superspeed Centrifuge, Dupont Company, Wilmington, DE). The supernatant was analyzed for SCFA by the HPLC method of McBurney and Thompson (1987). The supernatant was filtered through a 0.22 μm filter (Millipore, Bedford, MA) and 30 μL of filtrate were injected directly onto a Bio-Rad HPX-87H organic acid column (Bio-Rad, Melville, New York). The sample was eluted with 0.005 M H₂SO₄ at a flow rate of 0.6 mL per minute and the absorbance measured at 240 nm. The C₁ - C₆ SCFA standards were obtained from Supelco Incorporated (Bellefonte, California).

8.2.6 Determination of β-glucuronidase activity

Cecal samples (0.50 g) were homogenized with a polytron (Brinkmann Instruments, Switzerland) in 10 mL of phosphate buffered saline (PBS), pH 7.0, for 30 seconds. The homogenized extract was sonicated (2 bursts, 30 seconds each) in a sonicator (Branson Ultrasonics, Danbury, CT) and centrifuged at 10,000 rpm in a centrifuge (RC-5B Refrigerated Superspeed Centrifuge, Dupont Company, Wilmington, DE), for 20 minutes at 4°C to remove particulate matter. The supernatant was collected and stored in 1 mL aliquots at -70°C until needed for analysis.
Extracts were thawed for 15 minutes at room temperature, after which 0.1 mL was incubated with 0.1 mL phenolphthalein-β-D-glucuronide (Sigma Chemical Company, St. Louis, MO) in 0.8 mL of PBS (pH 7.0) for precisely 1 hour at 37°C. After incubation, the reaction was terminated with the addition of 2.5 mL alkaline glycine solution, 1.0 mL 5% trichloracetic acid (TCA) solution and 1.5 mL distilled water. This solution provides the optimum pH for the colour development of the released phenolphthalein. The colour was allowed to develop for 10 minutes and absorbance was measured at 540 nm. The phenolphthalein released was estimated based on a phenolphthalein standard curve prepared as follows:

Increasing volumes of phenolphthalein stock solution (0 - 1.0 mL), corresponding to increasing concentrations of phenolphthalein (1 - 20 μg), were mixed with 1.0 mL of 5% TCA, 1.0 mL PBS buffer (pH 7.0), 2.5 mL alkaline glycine solution and 0.5 mL distilled water. The colour was allowed to develop for 10 minutes and absorbance was measured at 540 nm (Beckman Instruments DU-7 Spectrophotometer, Beckman Instruments, Fullerton, CA).

The specific and total activities of β-glucuronidase were determined. The specific activity is the nmol of phenolphthalein released per mg of cecal protein per minute while the total activity of β-glucuronidase is the nmol of phenolphthalein released per cecum per minute. Protein was determined using a bicinchoninic acid kit from Pierce Chemical Company (Rockford, IL).

8.3 Results
8.3.1 Lipid peroxidation, mucosal conjugated dienes and liver and urine TBARS

Table 8.1 shows the level of colonic mucosal lipid peroxidation. No significant differences nor trends were observed amongst groups regardless of how the data were expressed, i.e. nmol of conjugated dienes produced per gram of colon weight, per gram of colon mucosa, per gram of total fat in colon mucosa or per mg of protein in the colon mucosa.
Table 8.1: Level of colonic mucosal lipid peroxidation measured by conjugated diene production.

<table>
<thead>
<tr>
<th>Diet group</th>
<th>nmol/g colon wt</th>
<th>nmol/g total fat</th>
<th>nmol/g colon mucosa</th>
<th>nmol/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD</td>
<td>0.71 ± 0.10</td>
<td>201.95 ± 26.41</td>
<td>11.04 ± 0.98</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>WB</td>
<td>0.42 ± 0.05</td>
<td>206.72 ± 26.76</td>
<td>10.46 ± 0.65</td>
<td>0.18 ± 0.02</td>
</tr>
<tr>
<td>DWB</td>
<td>0.62 ± 0.11</td>
<td>201.33 ± 16.61</td>
<td>13.75 ± 1.25</td>
<td>0.24 ± 0.02</td>
</tr>
<tr>
<td>DWBPA</td>
<td>0.47 ± 0.09</td>
<td>232.98 ± 35.62</td>
<td>12.63 ± 0.84</td>
<td>0.24 ± 0.02</td>
</tr>
<tr>
<td>PA</td>
<td>0.64 ± 0.09</td>
<td>211.68 ± 18.45</td>
<td>12.63 ± 0.83</td>
<td>0.21 ± 0.03</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n=10 per group; there were no significant differences.

No significant differences were observed amongst groups when the data were expressed as μg of TBARS produced per gram of liver, in the whole liver or per mg of protein in the liver (Table 8.2).

Table 8.2: Level of liver TBARS produced.

<table>
<thead>
<tr>
<th>Diet group</th>
<th>μg/g liver</th>
<th>μg/whole liver</th>
<th>μg/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD</td>
<td>19.99 ± 1.44</td>
<td>197.75 ± 14.28</td>
<td>0.129 ± 0.100</td>
</tr>
<tr>
<td>WB</td>
<td>20.07 ± 1.23</td>
<td>196.57 ± 14.39</td>
<td>0.128 ± 0.009</td>
</tr>
<tr>
<td>DWB</td>
<td>19.06 ± 1.18</td>
<td>187.78 ± 10.35</td>
<td>0.128 ± 0.010</td>
</tr>
<tr>
<td>DWBPA</td>
<td>20.53 ± 1.39</td>
<td>212.94 ± 14.67</td>
<td>0.139 ± 0.009</td>
</tr>
<tr>
<td>PA</td>
<td>18.17 ± 1.05</td>
<td>171.93 ± 10.87</td>
<td>0.121 ± 0.005</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n=10 per group; there were no significant differences.

Daily total urinary TBARS were similar for all treatment diets except that of the DWBPA group which was significantly higher than the BD group (Table 8.3).

Table 8.3: Level of urinary TBARS produced.

<table>
<thead>
<tr>
<th>Diet group</th>
<th>Urinary TBARS production μg in urine/24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD</td>
<td>8.60 ± 0.75&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>WB</td>
<td>11.64 ± 0.87&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>DWB</td>
<td>12.72 ± 1.70&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>DWBPA</td>
<td>14.74 ± 1.70&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PA</td>
<td>12.56 ± 2.49&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n=12 per group; Values with different superscripts within a column are significantly different, p<0.05.

8.3.2 Femur, serum and diet mineral levels

Table 8.4 and 8.5 show the femur and serum mineral levels respectively. There were no significant differences in serum and femur iron or calcium levels. Although the WB containing diets
contained higher levels of iron than the BD and PA diets (Table 8.6), this did not translate into higher levels of femur and serum iron for these groups.

The femur zinc data show that DWB group has significantly higher values than the PA group but neither is significantly different from any of the other groups.

<table>
<thead>
<tr>
<th>Table 8.4: Femur mineral levels</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diet groups</strong></td>
</tr>
<tr>
<td>BD</td>
</tr>
<tr>
<td>WB</td>
</tr>
<tr>
<td>DWB</td>
</tr>
<tr>
<td>DWBPA</td>
</tr>
<tr>
<td>PA</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n=14 per group; values with different superscripts within a column are significantly different, p<0.05.

The DWB did not have nearly as much PA and thus less of its zinc was inhibited and so this group shows a higher femur zinc concentration than the PA group. The significant difference between the DWB and PA groups is probably due mostly to the higher level of these minerals in the DWB with little inhibition from the much smaller amount of PA present in the diet.

<table>
<thead>
<tr>
<th>Table 8.5: Serum mineral levels</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diet groups</strong></td>
</tr>
<tr>
<td>BD</td>
</tr>
<tr>
<td>WB</td>
</tr>
<tr>
<td>DWB</td>
</tr>
<tr>
<td>DWBPA</td>
</tr>
<tr>
<td>PA</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n=14 per group; values with different superscripts within a column are significantly different, p<0.05.

Serum zinc values for the BD, WB and DWB groups did not differ significantly from each other (Table 8.5). However, the DWBPA group was significantly lower than the BD and DWB groups, while the PA group had significantly lower serum zinc levels than the BD, WB and DWB groups. The DWBPA and PA groups did not differ significantly from each other. Similar to the pattern observed with the femur minerals, the PA containing diets had lower levels than the BD or DWB groups.
Although, there were some significant differences between the PA and BD groups, all the values still lie within normal ranges for male F344 rats of this age.

Table 8.6: Diet iron, zinc and calcium mineral composition.

<table>
<thead>
<tr>
<th>Diet group</th>
<th>Iron (mg/Kg)</th>
<th>Zinc (mg/Kg)</th>
<th>Calcium (mg/Kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD</td>
<td>23 ± 4</td>
<td>30 ± 1</td>
<td>4340 ± 350</td>
</tr>
<tr>
<td>WB</td>
<td>87 ± 6</td>
<td>38 ± 3</td>
<td>4048 ± 36</td>
</tr>
<tr>
<td>DWB</td>
<td>100 ± 7</td>
<td>42 ± 1</td>
<td>4110 ± 36</td>
</tr>
<tr>
<td>DWBPA</td>
<td>100 ± 7</td>
<td>42 ± 1</td>
<td>4110 ± 36</td>
</tr>
<tr>
<td>PA</td>
<td>23 ± 4</td>
<td>30 ± 1</td>
<td>4340 ± 350</td>
</tr>
</tbody>
</table>

Values are mean ± SD; n=3 samples. Data measured using atomic absorption spectroscopy. No statistics were performed.

8.3.3 Colonic pH

All treatment groups had a significantly lowered colonic pH than the BD group (Table 8.7). The WB and DWBPA groups had a significantly lower pH than the DWB group. Neither of these groups was significantly different from the PA group. An effect of dephytinization is observed since the WB diet produced a significantly lower pH than the DWB group.

Table 8.7: Colon pH.

<table>
<thead>
<tr>
<th>Diet group</th>
<th>Colon pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD</td>
<td>6.94 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>WB</td>
<td>6.05 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>DWB</td>
<td>6.34 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DWBPA</td>
<td>6.06 ± 0.03&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>PA</td>
<td>6.20 ± 0.08&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n=12 per group; Values with different superscripts within a column are significantly different, p<0.05.

8.3.4 Cecal and colonic short chain fatty acids

Table 8.8 shows the cecal content weight of PA is higher than those of the other groups. However, this does not carry over into the colon where the content weights for the PA group are comparable to those of the BD group.
Table 8.8: Cecal and colon content weights.

<table>
<thead>
<tr>
<th>Diet Groups</th>
<th>Cecal contents (g)</th>
<th>Colon Contents (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD</td>
<td>2.105 ± 0.082c</td>
<td>0.815 ± 0.057b</td>
</tr>
<tr>
<td>WB</td>
<td>2.382 ± 0.063ab</td>
<td>1.273 ± 0.088a</td>
</tr>
<tr>
<td>DWB</td>
<td>2.384 ± 0.049a</td>
<td>1.342 ± 0.084a</td>
</tr>
<tr>
<td>DWBPA</td>
<td>2.120 ± 0.057bc</td>
<td>1.335 ± 0.068a</td>
</tr>
<tr>
<td>PA</td>
<td>2.664 ± 0.107a</td>
<td>0.762 ± 0.078b</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n=12 per group; values with different superscripts are significantly different, p<0.05.

Cecal SCFA production (μmol / entire cecal contents) is shown in Table 8.9. Acetate and propionate production did not differ amongst the various diet groups. However, the butyrate production of the WB group was significantly higher than the BD and PA groups. There were also no significant differences in total production of acetate, propionate and butyrate.

Table 8.9: Cecal short chain fatty acid production (μmol / entire cecal contents)

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>Acetate (C2)</th>
<th>Propionate (C3)</th>
<th>Butyrate (C4)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD</td>
<td>116.02±17.91</td>
<td>18.41±2.27</td>
<td>30.32±7.04b</td>
<td>164.75±22.67</td>
</tr>
<tr>
<td>WB</td>
<td>81.31±13.80</td>
<td>18.39±3.19</td>
<td>87.30±13.80a</td>
<td>187.00±29.64</td>
</tr>
<tr>
<td>DWB</td>
<td>105.93±15.78</td>
<td>18.79±2.44</td>
<td>66.08±9.80ab</td>
<td>190.79±20.43</td>
</tr>
<tr>
<td>DWBPA</td>
<td>80.84±20.70</td>
<td>17.77±4.39</td>
<td>57.04±12.98ab</td>
<td>155.65±37.36</td>
</tr>
<tr>
<td>PA</td>
<td>86.76±16.96</td>
<td>23.50±4.66</td>
<td>31.28±4.08b</td>
<td>141.54±23.47</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n=9 per group; values with different superscripts within a column are significantly different, p<0.05.

Colonic SCFA production (μmol / entire colon contents) is shown in Table 8.10. Total colonic production of acetate, propionate and butyrate (Table 8.10) was highest in the WB containing groups, with WB significantly higher than the BD and PA diets and DWB significantly higher than the PA. The production of acetate was significantly higher in the WB and DWB groups compared to the PA group. The DWB was also significantly higher than the BD group. The propionate and butyrate were not significantly different among the different groups.
Table 8.10: Colon short chain fatty acid production (μmol / entire colon contents).

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>Acetate (C2)</th>
<th>Propionate (C3)</th>
<th>Butyrate (C4)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD</td>
<td>25.80±2.77bc</td>
<td>2.60±0.52</td>
<td>8.88±1.18</td>
<td>37.27±3.81bc</td>
</tr>
<tr>
<td>WB</td>
<td>47.61±7.47ab</td>
<td>5.10±1.94</td>
<td>20.12±5.28</td>
<td>72.82±11.50a</td>
</tr>
<tr>
<td>DWB</td>
<td>49.18±7.64c</td>
<td>6.51±0.87</td>
<td>15.90±3.36</td>
<td>71.59±11.10ab</td>
</tr>
<tr>
<td>DWBPA</td>
<td>37.47±5.25abc</td>
<td>5.71±1.16</td>
<td>17.58±3.54</td>
<td>60.76±9.31abc</td>
</tr>
<tr>
<td>PA</td>
<td>19.57±2.91c</td>
<td>2.96±0.94</td>
<td>10.96±1.66</td>
<td>33.48±3.94c</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n=9 per group; values with different superscripts within a column are significantly different, p<0.05.

Table 8.11 shows the molar ratios for acetate, propionate and butyrate in the cecum and colon.

The BD group had the highest cecal molar ratio of acetate which was significantly different from WB, DWB and DWBPA groups. The WB group also produced a significantly lower ratio of acetate versus the PA group. The cecal molar ratio of propionate was highest in the PA group and was significantly different from BD, WB, DWB and DWBPA groups. The WB containing diets had the highest cecal molar ratios of butyrate with all three groups being significantly higher than the BD group. The WB group was also significantly higher than the PA group. There were no significant differences between the groups for the colonic molar ratios of acetate, propionate and butyrate.

Table 8.11: Cecal and colonic acetate, propionate and butyrate molar ratios.

<table>
<thead>
<tr>
<th>Diet group</th>
<th>Cecal</th>
<th>Colonic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acetate</td>
<td>Propionate</td>
</tr>
<tr>
<td>BD</td>
<td>68.58a</td>
<td>11.94b</td>
</tr>
<tr>
<td>WB</td>
<td>43.49a</td>
<td>9.78b</td>
</tr>
<tr>
<td>DWB</td>
<td>55.15bc</td>
<td>10.21b</td>
</tr>
<tr>
<td>DWBPA</td>
<td>51.78bc</td>
<td>11.68b</td>
</tr>
<tr>
<td>PA</td>
<td>59.23ab</td>
<td>16.54a</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n=9 per group; values with different superscripts within each column are significantly different, p<0.05.

8.3.5 Cecal β-glucuronidase activity

The specific and total activities of β-glucuronidase are shown in Table 8.12. The specific and total activity of β-glucuronidase was significantly lower in the WB-containing groups than the BD while the PA group did not differ significantly from the BD group.
### Table 8.12: Specific and total activity of β-glucuronidase in cecal contents.

<table>
<thead>
<tr>
<th>Diet Groups</th>
<th>β-glucuronidase specific activity</th>
<th>β-glucuronidase total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD</td>
<td>6.80 ± 0.32</td>
<td>141.30 ± 6.91</td>
</tr>
<tr>
<td>WB</td>
<td>3.41 ± 0.30</td>
<td>95.35 ± 4.27</td>
</tr>
<tr>
<td>DWB</td>
<td>3.56 ± 0.12</td>
<td>118.30 ± 3.90</td>
</tr>
<tr>
<td>DWBPA</td>
<td>3.14 ± 0.15</td>
<td>89.73 ± 4.04</td>
</tr>
<tr>
<td>PA</td>
<td>5.31 ± 0.18</td>
<td>127.30 ± 4.48</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n=15 per group; values with different superscripts within a column are significantly different, p<0.05; specific activity is defined as nmol phenolphthalein released per min per mg protein; total activity is defined as nmol phenolphthalein released per min per g cecal contents.

---

**8.4 Discussion**

**8.4.1 Lipid peroxidation, mucosal conjugated dienes and liver and urine TBARS**

This study has shown no significant differences in level of colonic mucosal and liver lipid peroxidation amongst the different dietary groups, indicating that inhibition of lipid peroxidation may not be a mechanism of WB or PA action.

A large number of studies use indices of oxidative damage to lipids or tissues (Thompson et al, 1999) as determinants of anti-oxidative activity and as surrogate biomarkers for reduction of cellular injury and hence cancer risk. Lipid peroxidation chain reactions can produce exponential numbers of cell damaging reactive compounds. Since pure PA and endogenous PA in WB are purported to have antioxidant effects (Graf and Eaton, 1985) by binding iron and potentially reducing the production of reactive oxygen species, it was of interest to see if levels of lipid peroxidation, directly within the colon or in the liver, could be reduced by these diets.

The lack of significant differences in the colon mucosal and liver lipid peroxidation indices suggests that this may not be a protective mechanism for PA or for WB fiber at these sites. However, it must be noted that due to technical and sample availability limitations, other tests for oxidative status such as reactive iron availability assays (Mumby et al, 1998), 8-hydroxydeoxyguanosine assays for DNA damage (Thompson et al, 1999) or HPLC analyses (Halliwell and Chirico, 1993) of TBARS were not performed. Such sensitive tests may show subtle differences between the diets. In addition, many of
the suggested antioxidative effects of PA have been observed in-vitro (Graf and Eaton, 1985; 1993; 1995; Owen et al, 1998) while in-vivo, it has only been shown to be effective in reducing lipid peroxidation under high iron conditions (Nelson et al, 1989; Porres et al, 1999). Since this experiment did not have a high iron or iron overload situation, changes in the colonic or liver oxidative status brought about by dietary PA chelation of iron may have been subtle and thus not detectable. It must be noted that even those in-vivo studies using high iron levels found no significant difference with PA supplementation in hepatic TBARS (Porres et al, 1999). In addition, with the exception of some studies on isolated dietary fibers (Thampi et al, 1991), there have been no studies on the effect of WB or WB fiber on colon lipid peroxidation. Thus, further research on the in-vivo antioxidant effects of WB and PA is necessary.

The urinary TBARS, which indicate total body lipid peroxidation, showed an increase for the DWBPA diet but not for the WB, DWB or PA diets, compared to the BD. This suggests that some factor other than DWB or PA in the DWBPA diet may be stimulating overall lipid peroxidation. The reason for the increased urinary TBARS is not clear and requires further study.

Thompson et al (1999) suggest that tests for urinary malondialdehyde (MDA), which is the end product of lipid peroxidation and the major TBARS component, may not be as sensitive a marker for detecting whole body levels of lipid peroxidation as previously thought. In particular, they suggest that dietary sources of MDA, possibly from PUFA within the diet may complicate the results. This is a possibility in our experiment since the oil source within the diets was 7.0% soybean oil, a rich source of PUFA. In addition, other urinary compounds from lipid (Diplock et al, 1998) or non-lipid origin (Gutteridge and Tickner, 1978) may also react with TBA in the TBARS test. Nonetheless, the urinary TBARS method has been shown to change in response to changes in exposure to oxidative stress (Peng et al, 1995) and is thought to be adequate for the study of antioxidant effects of fixed diets (Diplock et al, 1998), such as those fed in this study.
8.4.2 Femur and Serum mineral levels

There were no significant differences in serum or femur iron or calcium levels, indicating that WB and its PA are not inhibiting the absorption of these minerals. Thus, little difference may exist in oxidative potential as it relates to iron levels between animals consuming these diets, as was observed in the lipid peroxidation data. Likewise, observed cellular changes may not be related to differences in levels of calcium, which is involved in a number of cell cycle events such as initiation of the cell cycle, stimulation of DNA synthesis and regulation of a number of enzymes.

However, significant differences were observed in femur and serum zinc levels amongst the dietary groups. Zinc serves as a component in many metallo-enzymes such as DNA and RNA polymerase, deoxythymidine kinase and ribonuclease (DiSilvestro and Cousins, 1983). Thus, theoretically, removal of zinc, as in the case of the PA diet, may cause cellular growth inhibition and may be related to PA’s protective effects. But, the differences observed in this study can be explained by differences in dietary levels and so inhibition of zinc absorption may not be the mechanism whereby PA reduced the rate of cell proliferation in Experiment 2 (Chapter5) or induced apoptosis and differentiation in Experiment 4.

The femur zinc data show that DWB group has significantly higher values than the PA group but neither is significantly different from any of the other groups. This result may be explained when one reviews the zinc content of the various diets (Table 8.6). WB, DWB and DWBPA had the highest dietary zinc levels while the WB and DWBPA groups had substantially more PA content than the DWB group. The $[\text{PA}] [\text{Ca}] / [\text{Zn}]$ molar ratio, a measure of dietary zinc bioavailability, for the BD, WB, DWB, DWBPA and PA groups are 0, 2.7, 0.4, 2.5 and 3.7, respectively. The higher this ratio, the less the availability of zinc due to the higher PA content of the particular diets. Thus, even though the WB and DWBPA groups had levels of zinc comparable to the DWB diet, their higher PA level probably inhibited absorption of some of the zinc which is why these groups have femur zinc levels similar to the BD group. The DWB did not have nearly as much PA and thus less of its zinc was inhibited and so this group shows a higher femur zinc concentration. The PA group had a zinc level comparable to that of the
BD but a much higher [PA][Ca]/[Zn] molar ratio (3.7 versus 0). Thus, it would be expected that the PA diet inhibit the absorption of some of the zinc in this group causing it to have a lower femur zinc level. The significant difference between the DWB and PA groups is due mostly to the higher level of zinc in the DWB with little inhibition from the much smaller amount of PA present in the diet. These results also show that there is little difference in endogenously present or exogenously added PA. Although removal of the endogenous PA from WB allowed an increase in the femur zinc, the increase was not significant. Likewise addition of pure PA back to the DWB caused a non-statistically significant drop in the femur zinc level, reducing it to the level of the WB diet, again suggesting little difference between endogenous and exogenously added PA in binding minerals.

With regard to serum minerals, similar to the pattern observed with the femur minerals, the PA containing diets had lower levels than the BD or DWB groups. Although all the values still lie within normal ranges for male F344 rats of this age, decreased serum zinc by the PA diet versus the BD may be indicative of decreased cellular zinc availability and thus decreased growth. Since the serum zinc levels of the WB group were significantly higher than the PA group and close to significantly higher (p < 0.059) than the DWBPA group, it may be possible that the endogenous PA has lesser inhibitive activity on zinc absorption than the exogenously added pure PA, although it must be kept in mind that the WB, DWB and DWBPA diets had higher zinc content than either the BD or PA diets (Table 8.6). This difference may have contributed to differences in serum zinc levels. It is also possible that PA does inhibit zinc absorption but the increased mineral levels in the WB-containing diets (Table 8.6) compensate for the loss.

Although increased PA has been associated with increased excretion of calcium (Owen et al, 1996), the lack of difference in femur or serum calcium levels indicates that in this study, dietary levels were sufficient to counter any losses induced by PA. This study has also shown that there were no significant differences amongst the treatment diets in serum and femur iron, suggesting that iron inhibition may not have been a mechanism of action for decrease in early risk markers of colon cancer.
observed in Experiment 2 (Chapter 5). In addition, any significant differences observed in serum or femur zinc levels are probably due to differences in diet zinc content.

8.4.3 Colonic pH

This study has shown that the WB, DWB, DWBPA and PA diets can significantly reduce colonic pH compared to the BD diet. This suggests that both the WB fiber as well as other dietary components, such as the starches potentially bound by PA, contribute to fermentation and lowering of the colon pH. The reduction in pH may be due to increased SCFA production which has been suggested to be protective of colon carcinogenesis (Newmark and Lupton, 1990) by causing alterations in the metabolic activity of colonic flora (Mallett et al, 1989), altering bile acid metabolism (Thornton, 1981) and inhibiting ammonia production and absorption (Clinton et al, 1987). This study shows that for the WB containing diets and PA, the lowered pH is in agreement with the protective effect observed.

An effect of dephytinization is observed since the WB diet produced a significantly lower pH than the DWB group. This is due to the PA present in the WB since the addition of pure PA to DWB (DWBPA group) reduced the pH comparable to the level of the WB group. The effect of dephytinization observed shows that the contribution of pure PA and of WB fiber to reduction of pH in colon can be cumulative with both the fiber and PA individually and additively contributing. Pure PA, in the absence of fiber (PA group) was also able to decrease the pH significantly. This may be due to increased fermentation of starches bound by PA, breakdown of PA and production of free phosphate or the acidic nature of PA.

The colonic pH was lower in all treatment groups than the BD, indicating possible differences in the degree of fermentation and of SCFA production and thus may be a factor in the reduction of early risk markers of colon cancer observed in Experiments 2 (Chapter 5) and 4 (Chapter 7).

8.4.4 Cecal and colonic short chain fatty acids

This study has shown a significant increase in cecal butyrate production in the WB diet and significant increases in colonic acetate and total SCFA production in the WB and DWB diets versus the BD and PA diets. These data suggest that the protective effects of WB, its fiber and PA, on early
biomarkers of colon cancer risk observed in Experiment 2 and 4 may not be mediated through increased colonic production of butyrate.

Some studies have linked the protective effect of fiber to the production of butyrate (McIntyre et al., 1993). Butyrate has been shown, in vitro, to induce differentiation and apoptosis (Hague et al., 1993), to prolong doubling time and slow down the growth and proliferation rates of human colorectal cancer cell lines (Kim et al., 1982; Sakata, 1987; Boffa et al., 1992; Lupton and Kurtz, 1993). It is also thought to be involved in modulation of gene expression and changes in cell growth (Cummings and Macfarlane, 1991) as well as in reduction of DNA synthesis (Borenfreund et al., 1980) and in the inhibition of the enzyme histone deacetylase thus allowing greater access of repair enzymes to the DNA and promoting chromatin stability during cell division (Smith, 1986). Although colonic butyrate production in the WB-containing diets was numerically higher, the results were not statistically significant. This suggests that perhaps with greater statistical power an effect of increased butyrate production in the colon may be observed. Thus, more study is necessary before the protective potential of butyrate in the colon in this model is discounted.

The PA can bind a variety of starches and proteins, inhibiting their absorption and allowing their passage into the cecum and colon where they may be fermented. This is observed for the PA group in the cecum, where the weight of its contents are higher than any of the other groups (Table 8.8). This does not carry through into the colon where the content weights for the PA group are comparable to those of the BD group (Table 8.8). Thus, the difference in weight may be attributable to increased fermentation and thus production of SCFA. However, the cecal SCFA production from the PA group is in fact lower than that of the other groups, although not significantly.

Irrespective of significant differences, if the above argument about the ability of the PA group to bind starches and increase fermentation in the cecum were true, then at least a higher not lower level of SCFA production would be observed. Thus the PA group could merely be taking more moisture into the cecum which is quickly absorbed, thereby reducing the colonic contents weight of this group back down to the level of the BD group. Since the PA group does not have increased production of SCFA versus
the BD group, the decrease in colonic pH of the PA group is probably due more to PA breakdown and production of free phosphate than to fermentation of trapped starch.

The total SCFA production is much higher in the cecum (Table 8.9) than in the colon (Table 8.10). This is as expected since most of the fermentation in the rat digestive system takes place in the cecum and proximal colon. McIntyre et al (1993) have shown that in rats the fermentation of WB continues into the colon while that of other fibers does not. Thus it would be expected that the WB containing diets have higher SCFA production in the cecum and colon versus the BD and PA groups. However, the differences here were not statistically significant. McIntyre et al (1993) also suggest that since WB fiber is more slowly fermented throughout the length of the cecum and colon, it may cause increased production of butyrate in the distal colon and that may be responsible for the protective effects of WB observed in previous studies. Other studies, however, suggest that WB has a rapidly fermented portion, which is fermented in the cecum and contributes to increased SCFA production and concentration at that site, and an unfermentable residue which does not affect colonic SCFA but merely increases stool output (Edwards, 1995). In our study, the cecal production of butyrate produced by the WB containing diets is indeed significantly higher than that of the BD and PA groups. However, there are no significant differences between the groups in the production of butyrate in the colon – where its cancer protective effects are thought to take place. A similar observation was made by Zoran et al (1997a), who found a lower concentration of butyrate in the distal than in the proximal colon with WB feeding. In addition, they found a correlation between increased butyrate production and higher colon tumour yield when comparing WB to oat bran. These observations suggest that the production of colonic butyrate, may not play an important role in the protective effects of WB in this model.

For a typical diet, the molar ratios of acetate, propionate and butyrate produced are generally 60:25:15 or 30:20:10 (Breves and Stuck, 1995). However, in this experiment, the molar ratios did not match this typical profile. In fact, for both the cecum and the colon, the molar ratio of the propionate was lowest in all the groups, while those of acetate and butyrate where higher than the norm. Such a disparity between the propionate and butyrate values was also observed in the study of Zoran et al
(1997a), in rats fed 6.0% WB (65:11:21). In the same study, rats fed 6.0% oat bran had a molar ratio of 45:15:35 which more closely resembles many of the molar ratios obtained by our study. Since all the WB containing diets used in our study have higher WB (25%) than the Zoran et al (1997a) study, it is conceivable that they caused a greater amount of fermentation than the level of WB used there, thus resulting in different SCFA molar ratios. In fact, the molar ratios produced by 6.0% of the more readily fermented oat bran group in that study are close to the levels we obtained in our study. Thus, the different molar ratios obtained in our study (25% WB) and the Zoran et al (1997a) study (6.0% WB) may just be due to different levels of fermentation.

Another study (McIntyre et al, 1993), tested the effect of 10.0% concentration of various dietary fibers including WB on cecal fermentation and colon carcinogenesis. The acetate and butyrate molar ratios obtained in our study are somewhat similar to those obtained by McIntyre et al (1993). They also noted a fall in the molar ratio of propionate (versus butyrate) with WB feeding, a point also observed in our study and by Edwards and Eastwood (1992). The difference in values could merely be due to the level of WB fed (25% versus 10%), while the fall in propionate molar ratio could be a just a feature of WB feeding. Boffa et al (1992) found that the colonic molar ratios of acetate, propionate and butyrate with a 20% WB diet was 13:1:1 in Sprague Dawley rats. This disparity between our results (both the BD and the other diets) and those of others discussed above, versus the typical (3:2:1) SCFA profile expected for a particular diet (Breves and Stuck, 1995) may just be a feature of age, different experimental conditions, diet mixtures and bacterial populations, different methods of detection, as well as due to possible variabilities in animal versus human profiles.

This study has shown that WB diet can increase cecal butyrate and colonic acetate and total SCFA compared to the BD and PA diets. Since alterations in colonic butyrate may be more important than cecal levels (Velazquez et al, 1996), these results suggest that the effects of WB and PA on early risk markers of colon carcinogenesis, in this model, may not be mediated through increased colonic production of butyrate. This study has also shown an increase in total colonic SCFA in the WB group
versus the BD, suggesting that total SCFA may be, in part, responsible for the decrease in colonic pH observed in the WB group.

8.4.5 Cecal β-glucuronidase activity

This study has shown that the WB-containing diets can significantly reduce the specific and total activity of cecal β-glucuronidase. This indicates a possible protective mechanism whereby reduction of activity may result in reduced enterohepatic circulation of toxic dietary compounds and hence decreased mucosal exposure. However, this effect may be due more to WB fiber alone and not due to its PA since there were no significant differences between WB and DWB and the addition of pure PA to DWB had no effect. The β-glucuronidase enzyme is involved in the glucuronide-deconjugation and resulting enterohepatic circulation of numerous dietary toxins, mutagens and carcinogens (Rowland et al, 1985). One of the proposed protective effects of high fiber diets is reduction of β-glucuronidase activity (Mallett et al, 1986) which has been shown to be decreased (Reddy et al, 1992) by WB diets under different experimental conditions. Thus, modulation of β-glucuronidase activity may be one mechanism that differentiates the effect of WB fiber from that of pure exogenous PA.

8.5 Conclusions

No significant differences were observed amongst any of the dietary groups in colonic mucosal conjugated dienes or liver TBARS indicating that these indices of lipid peroxidation are not inhibited by WB, its fiber and PA components or pure PA, suggesting that this may not be a mechanism for their observed effects on early colon cancer risk markers (Experiment 2, Chapter 5). WB and its PA, as well as pure exogenous PA did not affect femur or serum iron levels showing that iron absorption was not inhibited and that the diet groups did not differ in terms of oxidative potential due to the catalytic effects of iron. There were no significant differences in femur and serum calcium levels among the diet groups. This shows that calcium absorption and its availability for cellular growth processes was not inhibited and thus may not be the mechanism whereby WB and PA reduced the rate of cell proliferation in Experiment 2 (Chapter 5) or affected the rate of apoptosis and differentiation in Experiment 4 (Chapter
7). Although differences in femur and serum zinc levels amongst the dietary groups can be mostly explained by differences in dietary levels, significantly lower serum zinc in the PA group versus the BD group may have caused decreased zinc availability for cellular growth processes.

In addition, it is concluded that WB-containing and PA diets can significantly decrease colonic pH, possibly due to increased total SCFA production for the WB-containing diets but not the PA diet which showed no increase in total SCFA production. Thus, the decrease in colon pH may be related to the early biomarker protective effects of the diets observed earlier (Chapters 5 and 7). No significant differences were observed in colonic butyrate production suggesting that it may not be related to the effects of WB, DWB, DWBPA and PA on ACF parameters, cell proliferation, apoptosis and differentiation observed earlier (Chapters 5 and 7). However, total colonic SCFA production was increased by the WB versus the BD and PA groups, indicating that this may be a mechanism of WB but not PA action. Furthermore, it is concluded that significant decreases in the specific and total activity of cecal β-glucuronidase enzyme by the WB containing diets indicate a possible mode of action. However, this may be related to WB fiber alone and not to its PA since there were no significant differences between WB and DWB nor with addition of pure PA in the DWBPA group. Thus, the protective effects of PA on early biomarkers of colon cancer risk appear to be unrelated to β-glucuronidase activity.
9.0 General Discussion and Conclusions
9.0 General Discussion and Conclusions

9.1 General discussion

WB has been shown in a large number of previous studies (Appendix Table 11.2) to be colon cancer protective. However, until now, the effects of PA within the matrix of WB or pure PA added to a high or low fiber diet have not been clarified. In this thesis, studies were conducted to determine this effect and to elucidate some of the possible modes of action of WB, its fiber and PA components as well as pure PA.

9.1.1 Preparation of dephytinized WB (Chapter 4) and the influence of WB and PA on early risk markers of colon cancer risk (Chapter 5)

In Experiment 1 (Chapter 4), for the first time, a protocol for the large scale dephytinization of WB was developed and the DWB was used in subsequent experiments.

In Experiment 2 (Chapter 5), feeding studies of BD or BD supplemented with WB, DWB, DWBPA or PA were conducted to differentiate the role of WB and endogenous and exogenous PA. In this study, a number of different ACF parameters were analyzed with the view that one or two parameters (as are often measured in many studies) may not be reflective of the true effects of particular diets. Classical ACF parameters such as number of ACF and size of ACF were measured along with newer measures such as ACF density (the number of ACF per cm colon length), degree of luminal alterations (luminal deformity) of each AC and number of SIM ACF. The latter measure is of interest because it has been suggested (Caderni et al, 1995) to separate ACF populations based on extent of cellular alterations, i.e. ACF with SIM production have more alterations and are thus more advanced than those producing normal SUM. This study has shown for the first time that WB and PA diets can cause significant reductions in the number of SIM ACF. This suggests that had this study been allowed to continue beyond 100 days, the animals in the BD group may have developed more tumours sooner than those in the treatment groups, since it is thought that only certain, greatly altered and abnormal ACF may advance to colon tumours (Bird, 1995; Caderni et al, 1995).
Although this study (Chapter 5) showed little difference in total number of ACF amongst the diet groups, the WB and PA diets did significantly reduce the overall state of advancement (number of SIM ACF) and degree of luminal alterations of the ACF. Since ACF are continuously remodeled, do appear and disappear, an effect on more advanced lesions may be of greater consequence in the determination of dietary effect on colon cancer risk since the more advanced lesions are probably more likely to progress to colon tumourigenesis (Caderni et al, 1995).

A problem of studies on colon carcinogenesis and dietary fiber is the energy content of the diet. The diets used in this experiment were formulated to be isocaloric using the Atwater method (Atwater, 1910) which assigns energy values of 4 Kcal/g, 9 Kcal/g and 4 Kcal/g for protein, fat and carbohydrate (which includes all dietary fiber) respectively. The Atwater calculations assume that dietary fiber may provide calories due to its fermentation to SCFA. However, the method may overestimate available energy from higher fiber diets since not all dietary fibers may contribute energy, particularly from cereal sources such as WB, and the dietary fiber may interfere with the absorption and digestion of other food components (Miles, 1992). Thus, theoretically, the caloric values of the diets may not be entirely the same. As a result it is possible, as it is in any similar study, that some of the observed effects on ACF and other parameters may, to a degree, be due to differences in caloric intake. Since there is little information on modulation of early risk markers of colon cancer by the energy content of high and low dietary fiber or WB diets, studies comparing varying caloric and fat contents in high and low fiber diets containing both rapidly and non-fermentable dietary fiber sources will be of interest in the future.

An important part of the design of this study (Chapter 5) was that pure PA was provided in the diet while most previous carcinogenesis studies used pure PA either injected directly into the animal (Vucenik et al, 1998a) or provided in drinking water (Shamsuddin and Ullah, 1989; Ullah and Shamsuddin, 1990; Pretlow et al, 1992). However, as already discussed, PA has great potential to complex and interact with a variety of dietary components. Thus, PA provision in the diet may result in a different effect than when given as a bolus or dissolved in drinking water, which may be consumed ad libitum by the animal without concomitant consumption of diet. In addition, the PA present within the
matrix of WB (endogenous PA) may also act differently than pure PA (exogenous PA) in the diet or the drinking water. PA in WB is present within the matrix of the fiber, complexed with starches and proteins and bound to minerals (Ferguson and Harris, 1999). Thus, it may not be as free to interact with the colonic mucosa as pure PA, which might be less tightly complexed with other dietary components. Future studies need to address this issue and compare the effects of PA given in the diet versus in the drinking water or injected into the animal. Another form of PA delivery is intrarectal administration, but this method has not yet been studied. This method may be of interest in the direct delivery of PA solutions to the distal colonic mucosa where most ACF and tumours are observed without interactions that may take place in the small intestine between PA and other food components or due to absorption of PA from that site.

This study (Chapter 5) has shown an inhibitory effect of WB fiber and its associated PA, along with pure exogenous PA, in the diet on SIM ACF. However, although it was hypothesized that SIM ACF are more advanced towards carcinogenesis than other types of ACF, the evidence was lacking and it was necessary to perform an experiment to provide further support for this hypothesis.

9.1.2 Relationship of ACF parameters to degree of dysplasia and rate of cell proliferation (Chapter 6)

The goal of Experiment 3 (Chapter 6) was to see if SIM ACF have greater degree of abnormalities such as dysplasia and rate of cell proliferation and are thus more advanced early biomarkers than SUM ACF. In Experiment 3 (Chapter 6) it was shown for the first time that SIM ACF have a larger physical size, more luminal alterations, greater degree of dysplasia, increased PCNA-LI in the top 40% of the crypt and increased \( \phi h \) index than SUM ACF. These factors all suggest that, SIM ACF do indeed have more cellular alterations than SUM ACF, are a more advanced population of ACF and are thus more likely to progress to tumours faster.

The criteria for grading the level of dysplasia were combined from numerous publications (Lane et al, 1971; Stemmerman et al, 1973, 1986; Shamsuddin and Trump, 1981; Decaens et al, 1983; Roncucci et al, 1991; Thorup, 1997; Siu et al, 1997) covering both pre- and neoplastic colonic lesions.
Since the grading of the level of dysplasia is somewhat subjective, the validity of these rankings were confirmed by similarity in the rankings as read by an experienced pathologist. Nevertheless, because the identification and analysis of numerous slides from individual ACF is very time consuming, future experiments may benefit from digital image analysis as an important tool to improve the recording and analysis of the dysplasia gradings and comparison of different ACF slides.

Due to the results of this study, future experiments can utilize SIM ACF to determine the effects of diets or dietary compounds on a population of ACF that are more advanced towards tumourigenesis. This may allow for a better assessment of the effect and mechanisms of action of dietary compounds on ACF growth and advancement. However, future studies should be performed to identify other cellular and genetic differences that may exist between SIM and SUM ACF and to follow their development to tumours. Such information will allow better grouping of ACF into different populations associated with variable risk for colon carcinogenesis and will allow better conclusions to be drawn from short term ACF assays.

9.1.3 PA in WB affects colon morphology, cell differentiation, apoptosis and cell proliferation

(Chapters 5 and 7)

One of the objectives of Experiments 2 (Chapter 5) and 4 (Chapter 7) was to determine if there is a difference between endogenous and pure exogenous PA added to the diet. The results showed an effect of dephytinization on cell proliferation parameters, but not on apoptosis or cell differentiation. Increase in cell proliferation is thought to be the first stage of colon carcinogenesis (Terpstra et al, 1987) and is one of the earliest responses of colonic epithelium to carcinogenic insult (Hirose et al, 1996), prior to the development of any ACF or neoplastic lesions. Thus, the increase in PCNA-LI observed upon dephytinization of WB (DWB group) in Experiment 2 may have lead to increased ACF had the study been continued for a longer period of time.

Although hyperproliferation often takes place in a setting of decreased differentiation and apoptosis, these events are not always linked together (Boland et al, 1992; von Wangenheim and Peterson, 1998). Thus, decrease in PCNA-LI may not necessarily be accompanied by increased cell
differentiation or apoptosis. In fact, it has been suggested that a proliferation arrest is not by itself sufficient to induce differentiation (von Wangenheim and Peterson, 1998), suggesting that WB and PA, which both decreased proliferation and induced differentiation, may influence different cell signaling pathways in order to affect both of these cellular events. The same is true for the effect of dephytinization on cell proliferation and apoptosis, since apoptosis and differentiation are more closely linked to each other than to cell proliferation and share some of the same induction steps (von Wangenheim and Peterson, 1998).

9.1.4 Cell proliferation, apoptosis and differentiation: early biomarkers or mechanisms of action (Chapters 5 and 7)

Another important issue is whether cell proliferation, differentiation and apoptosis are merely cellular surrogate end-point biomarkers (SEB) or actual important mechanisms whereby WB, its fiber and PA act. A number of recent publications (Mori et al, 1999; Krishnan et al, 1999), suggest that these factors can be considered as SEB. However, all three factors play pivotal roles in the life cycle of a cell which is influenced by their induction or modification. Tissue growth is not regulated solely by cell proliferation, but also by the degree of differentiation and the resulting rate of apoptosis (Wyllie, 1997). The above studies also suggest that their control is important for cancer prevention. From all the possible modes of action of WB and PA analyzed in this study, the modulation of cell proliferation, induction of cell differentiation and stimulation of apoptosis appear to be the major means whereby these diets affect the ACF parameters measured and thus may also be considered mechanisms of action. Future studies need to focus on the molecular mechanisms of how WB and PA can alter cell proliferation, differentiation and apoptosis, perhaps through in-vitro methods. It needs to be determined whether the mechanisms include, for example, stimulation of tumour suppressor genes, deactivation of serine-threonine enzyme families involved in the processes of cell proliferation (Grana and Reddy, 1995), inhibition of transcription factors causing greater differentiation (Grander, 1998), or modulation of genes (e.g. bcl2 activity), intra-cellular signals (Wyllie, 1997) or enzymes such as the metallo-dependent cysteine proteases (Whyte, 1996) involved in apoptosis.
In contrast to butyrate and its apparent paradoxical behaviour of influencing the growth of normal cells while stimulating the differentiation and apoptosis of transformed cells (Velazquez, 1996), WB and PA seem to affect both normal and abnormal cell types. In this study (Chapters 5 and 7) the cell proliferation, differentiation and apoptosis measurements were made in normal appearing colonic crypts and not ACF. The fact that ACF parameters such as number of SIM ACF and degree of luminal alterations, were decreased by these diets indicates that cells in the ACF were also affected by the decrease in cell proliferation and increase in differentiation and apoptosis. Thus WB and PA appear to affect the physiology of the colon as a whole. However, it must be noted that since cancer cells are more rapidly proliferating and less differentiated than normal cells, the WB and PA may affect them to a greater degree than they would normal cells. Future studies should concentrate on the longer term effects of WB and PA, to see if they can alter these parameters during more advanced stages of carcinogenesis and to measure these parameters in actual tumours of rats treated with WB and PA diets.

9.1.5 Some other modes of action of WB, DWB and PA (Chapter 8)

A study (Experiment 5, Chapter 8), run in parallel with Experiment 2 (Chapter 5) was conducted to determine some potential physiological effects of WB, DWB and PA on indices of lipid peroxidation, mineral availability, cecal and colonic fermentation and cecal \( \beta \)-glucuronidase activity.

It is thought that increased butyrate production, from fermentation of WB fiber and starch trapped by PA (Rickard and Thompson, 1997), is a major mechanism of the protective effect of WB (McIntyre et al, 1993; Kritchevsky, 1999). In this study, WB significantly increased butyrate production versus the BD and PA diets in the cecum. It would be expected that increased butyrate production would mostly be of consequence in the distal colon since that is the site where most of the ACF are found and the site at which the effects on cell proliferation, differentiation and apoptosis were measured in this study. However, despite variations in the actual values there were no significant differences in colonic production of butyrate amongst the dietary groups. Although it appears that levels of butyrate may not play a strong role in the protective effects of WB and PA observed in this study, the role of butyrate production as a mode of WB and PA action warrants further research. However, the WB did
significantly increase total colonic SCFA versus the BD diet, suggesting that this may be a mode of WB, but not exogenous PA, action. Furthermore, all the treatment diets significantly decrease colonic pH indicating that this may be related to their protective effects on ACF parameters, rate of cell proliferation, apoptosis and cell differentiation (Chapters 5 and 7).

This study is the first to address all of the above potential modes of WB and PA action within the same experiment. Future studies are needed to elaborate on these potential mechanisms of action and to see if they are subtly affected by WB, endogenous or exogenous PA. For example, a large experiment in which colonic and fecal SCFA production and concentration are measured in WB or PA fed animals at various time points and correlated with ACF parameters and tumor development, may provide better insight into these potential mechanisms of action.

In this study, only serum and femur minerals were measured as indices of effect of WB and PA on mineral availability. However, these values may only have been affected if the animals had marginal mineral status. Since the diets provided adequate minerals (Reeves et al, 1993) and since the WB itself is a good source of minerals, any absorption inhibition of minerals such as iron in the digestive system would probably not have been reflected in these values. The significant effect was the reduction of serum zinc by the PA diet versus the BD diet. Although this suggests that inhibition of zinc may have caused a reduction in cell growth and perhaps ACF parameters, the values were still within normal range for the animals (Charles River Laboratories on-line database).

There were no significant differences in femur or serum iron, suggesting that the potential catalytic effect of iron on lipid peroxidation was not inhibited. Future experiments should concentrate on better measures of iron availability such as total amount of iron bound by PA in the diet or tests for catalytically reactive iron in the colon contents or feces which may contribute to free radical generation and subsequent oxidative damage. However, even these tests may not be of much relevance since colonic mucosa showed no significant differences in the level of conjugated dienes, which is an indirect measure of degree of lipid peroxidation. Thus, it is probably unlikely that WB or PA acted via an antioxidant mechanism in this study.
An additional potential mechanism of PA action is its involvement in the inositol phosphate pool and cell signal transduction, but this is very difficult to study in the in-vivo situation and was not investigated here. It is possible that endogenous and exogenously added PA may both participate in the inositol phosphate pool and thus affect cell proliferation, differentiation and apoptosis. PA may modulate intracellular calcium mobilization (Menniti et al., 1993; Shamsuddin et al., 1997) or up-regulate p53, involved in some apoptotic pathways, and WAF1 gene expression, whose protein product is growth inhibitory (Saied and Shamsuddin, 1998). It is unknown how or where within the cell PA or lower IP’s from a dietary source may be stored or how they may act. For example, in normal cells without any PA in the diet or media, the only source of IP3 for signal transduction and calcium mobilization is from the breakdown of PIP2 upon stimulation (Holub, 1992). Entry of PA or lower IPs into the cell may increase their internal concentration allowing them to have functions that may not be possible at the lower concentrations normally found within cells. Examples of these may be binding and deactivation of enzymes or minerals necessary as enzyme cofactors. These points are hypothetical, but need to be addressed in future experiments. For example, treatment of a variety of colon cancer lines with various concentrations of pure lower IPs and the monitoring of their state of growth, differentiation, apoptosis, internal IP concentrations, calcium concentration and activity of enzymes involved in IP3 generation or signal transduction may provide clues to how dietary PA and its breakdown products may modulate the physiology of the colonic mucosa. Further experiments may also explore interactions of PA and lower IPs with individual SCFA, particularly butyrate on growth and cellular effects of colon cancer cell lines. In addition, it would be of interest to determine the effects of pure PA and lower IP’s on stimulation of intracellular calcium release and calcium transport in a variety of colon cancer cell lines, since these events are key to cell growth, differentiation and apoptosis (Shears, 1998).

The mean percent recovery of PA in the feces ranged from 54% to 87% (Appendix Table 11.6) indicating that some of the PA was broken down and hydrolyzed to myo-inositol (Ogawa, 1999). Some of the PA, both endogenous and exogenous, may have been broken down by intestinal phytase (Nehapetian and Young, 1980) while, some of the endogenous PA in WB may also have been
hydrolyzed within the digestive tract by the native WB phytase (Sandberg and Andersson, 1988), producing myo-inositol. Myo-inositol by itself has been shown to have protective effects in a number of cancer models such as those of the lung (Wattenberg et al, 1996; Wattenberg, 1999), breast (Vucenik et al, 1992, 1993, 1995) and colon (Shamsuddin et al, 1989). Thus, it is possible that some of the effects observed in this experiment are due to the effects of myo-inositol. This is particularly the case for the DWB diet. As described in Experiment 1 (Chapter 4), the DWB was prepared by activation of the phytase naturally present in WB. Thus, rats in the DWB and DWBPA groups consumed approximately 114 mg of myo-inositol (∼1% of the diet) per day from the hydrolyzed PA. Since Shamsuddin et al (1989) have observed a stronger colon cancer protective effect with PA + inositol added to a low fiber diet, it would be expected that the effect of DWBPA, which contains not only 1% PA but also 1% myo-inositol, would be stronger than that of the other groups. However, in this experiment, the effects of the DWBPA group were not significantly lower from those of any of the other treatment diets. It may be that myo-inositol in the presence of high amounts of dietary fiber is not as active as when it is added to a low fiber diet. Thus, the role of myo-inositol on early biomarkers of colon carcinogenesis and colon tumourigenesis in high and low dietary fiber diets warrants further study.

Since the recovery of PA in the feces was not 100%, it is also possible that some of the dietary PA was absorbed in the small intestine. Sakamoto et al (1993) showed that tritiated PA (single bolus given to fasting animals) is absorbed in the upper digestive tract and distributed to different tissues. The absorbed PA was gradually broken down into lower IPs. Although this is probably the means whereby PA can affect non-digestive tract cancers, the absorbed PA or lower inositol phosphates derived from it, may also affect the colonic mucosa from the basolateral or systemic side. Studies in which pure PA is delivered directly to the colon or using extrusion cooked WB, which inhibits phytase activity and PA breakdown, are necessary to differentiate between the actions of absorbed PA and PA present in the colonic milieu.

Another possible mechanism of WB and PA action not explored in this thesis is the effect of WB and PA on insulin levels. WB and PA could potentially affect blood glucose and insulin levels by
binding starch, inhibiting its digestion and absorption and shifting its breakdown to the colon where it can be fermented (Thompson, 1993). Since increased insulin levels may increase ACF growth (Corpet et al, 1997; Koohestani et al, 1998), decreased insulin levels may reduce ACF growth and cell proliferation in the colon. Future studies are necessary to determine to what extent WB and PA diets can affect insulin levels and whether those effects are related to changes in ACF parameters or colon tumourigenesis.

9.1.6 Implications

The results of this study, which showed that WB at the 25% dietary level has protective effects on early biomarkers of colon cancer risk, is further support for the colon cancer protective effects of WB observed in other studies (Appendix Table 11.2). However, for the first time, the results provide evidence that the beneficial effect of WB is in part due to its endogenous PA. In humans, 25% WB is about equivalent to an intake of 2 cups (120 g) WB by an individual on a 2000kcal/day diet. Based on this study, no deleterious effect on food intake, growth, weight or mineral status were observed upon intake of WB at the 25% level.

Concern with high intakes of WB, in both human (Sandstead et al, 1978; Sandstrom et al, 1980; Van Dokkum et al, 1982; Andersson et al, 1983; Spiller et al, 1986; Morris et al, 1988; Jahnen et al, 1992) and animal studies (Ranhotra et al, 1979; Morris and Ellis, 1980; Akhtar et al, 1987; Shah et al, 1990) has been that the dietary fiber and PA may decrease the availability of minerals due to possible mineral binding in the intestines. However, in agreement with a large number of longer term mineral availability studies (Sandstead et al, 1979; Anderson et al, 1980; Stasse-Wolthuis et al, 1980; Obizoba, 1981; Rattan et al, 1981; Zoppi et al, 1982; Sandstrom et al, 1983; Hallfrisch et al, 1987; Mason et al, 1990; Davidsson et al, 1996), this study showed that calcium, iron and zinc status were not negatively affected by a high WB diet. This is significant since dephytinization of WB has been advocated to counteract the mineral binding effects of PA. This study also showed (Chapter 5) that removal of endogenous PA can reduce the effectiveness of WB by increasing colon cell proliferation indices. This is an important biomarker which suggests that endogenous PA plays a protective role along with the
fiber component of WB. Since endogenous PA plays a significant role in reducing early colon cancer biomarkers by WB and since there are no deleterious effects of WB or its PA on mineral availability, it does not appear necessary, in terms of cost and effect, to dephytinize WB for public consumption.

This study has also shown that pure PA in a low fiber diet has protective effects on early biomarkers of colon cancer suggesting that more research on the effects of pure PA is necessary. However, a reduction of serum zinc by pure, exogenous PA (PA group) but not by endogenous PA (WB group) or exogenous PA added to a high fiber diet (DWBPA group) has also been observed (Chapter 8). This suggests that pure exogenous PA added to a low fiber diet, but not endogenous PA within the matrix of WB or exogenous PA in the presence of high dietary fiber, may affect zinc status. Although a number of studies suggest that PA has no negative implications on mineral status (Walker et al, 1948; Ranhotra et al, 1979; Morris et al, 1988) more research is necessary before the use of pure PA can be advocated for colon cancer treatment. This is important since PA pills are readily available and being promoted for this purpose.

This study has also shown that SIM ACF are better markers of colon cancer risk than SUM ACF. This may be of significance in future studies which use ACF as early biomarkers of colon cancer risk. The ability to easily identify on whole mount colons, different populations of ACF based on their extent of internal cellular alterations may allow for both identification of new and further analysis of known dietary modulators of colon cancer risk.

9.1.7 Overall summary

In summary (Table 9.1) this study has shown an inhibiting effect of WB, with its fiber and associated PA as well as pure exogenous PA in the diet on ACF used as early biomarkers of colon cancer risk. These diet components appear to modulate the growth of ACF through a decrease in the rate of cell proliferation, increase in apoptosis and degree of differentiation and do not appear to be related to inhibition of lipid peroxidation. There are indications that these effects may also be related to changes in β-glucuronidase activity and colon content bulk for the WB-containing diets, to increased total colonic production of SCFA for the WB diet and to decrease in zinc availability for the PA diet. The dietary
fiber and PA appear to both be responsible for some of the observed early biomarker protective effects of WB since both components caused a concomitant decrease in ACF parameters and rate of cell proliferation coupled to an increase in differentiation and apoptosis.

Table 9.1: Overall summary of results

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<tr>
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<th>Wheat bran</th>
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<tr>
<td><strong>Effects on early biomarkers of colon cancer risk and cellular processes:</strong></td>
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<tr>
<td>Number of ACF</td>
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<td>Number of SIM ACF</td>
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<tr>
<td>Degree of Luminal Alterations</td>
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<td>Cell proliferation</td>
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<tr>
<td>Rate of Apoptosis</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td>Degree of Differentiation</td>
<td>↑</td>
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<tr>
<td><strong>Effects on:</strong></td>
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<tr>
<td>Mineral Availability</td>
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<td>Ca</td>
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<td>Zn</td>
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<tr>
<td>Lipid Peroxidation</td>
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<td>Colon content bulk</td>
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<td>Colon SCFA</td>
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<tr>
<td>Butyrate</td>
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<tr>
<td>Total</td>
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<tr>
<td>Colon pH</td>
<td>↓</td>
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<tr>
<td>β-glucuronidase activity</td>
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<td>NS</td>
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</table>

NS = no statistically significant differences; ↑ or ↓ = statistically significant increase or decrease
9.2 Overall conclusions

From the results of these experiments, it is concluded that:

a. WB can be effectively dephytinized by incubation of a 12.5% dispersion at 37°C for 18 hours.

b. WB, DWB, DWBPA and PA significantly reduced early biomarkers of colon cancer risk, namely the number of SIM producing ACF, degree of luminal alterations and rate of colonic cell proliferation. WB, with or without endogenous or exogenous PA, along with the exogenous PA added to the low fiber diet, reduced the level of cell proliferation, the proliferative zone, the pH index, crypt cell height and crypt density and increased cell apoptosis and differentiation, suggesting a distinct protective effect.

c. Both the PA and dietary fiber components of WB were responsible for these observed effects. While dephytinization can reduce the effectiveness of WB by increasing cell proliferation indices, it has no effect on ACF parameters, cell apoptosis or differentiation.

d. Endogenous and exogenous PA were equally effective. However, endogenous PA is not the sole active component in WB and exogenous PA is most effective when added to a low fiber diet.

e. ACF have significantly higher rate of cell proliferation than normal crypts and that there are relationships between SIM production in ACF and increased degree of dysplasia and rate of cell proliferation versus SUM ACF. SIM ACF are more altered than SUM ACF and thus may be further advanced types of ACF.

f. These protective effects may be related to decreased pH as well as cell proliferation and increased apoptosis and cell differentiation. They do not appear to be related to an anti-oxidative mechanism, decrease in femur or serum calcium or iron levels, the production of colonic SCFA or butyrate. The effects of the WB-containing diets may be related to decreased β-glucuronidase activity and increased colon content bulk, while those of the WB diet may be related to increased colonic total SCFA and those of PA to decreased serum zinc availability compared to the BD.
11.0 References
10.0 References


Fiala, E.S. (1975) Investigations into the metabolism and mode of action of the colon carcinogen 1,2-dimethylhydrazine. Cancer, 36, 2407-2412.

Fiala, E.S. (1977) Investigations into the metabolism and mode of action of the colon carcinogens 1,2-dimethylhydrazine and azoxymethane. Cancer, 40, 2436-2445.


11.0 Appendices
Table 11.1: Summary of recent human studies on dietary fiber, wheat bran and early risk markers.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects</th>
<th>Endpoint</th>
<th>Amount of WB or wheat fiber</th>
<th>% Fat</th>
<th>% Fiber in control group</th>
<th>Length of feeding period (wk)</th>
<th>RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reddy et al. 1987</td>
<td>Humans with ↑ excretion of fecal mutagen</td>
<td>Fecal mutagens and bile acids</td>
<td>11 g/day of wheat fiber</td>
<td></td>
<td>individual’s regular diet without wheat bran supplement (high fat)</td>
<td>4</td>
<td>↓ fecal mutagenicity</td>
</tr>
<tr>
<td></td>
<td>FAP patients</td>
<td>Polyp recurrence and ratio</td>
<td>22.5 g/day of wheat fiber (+vit. C and E)</td>
<td></td>
<td>individual’s regular diet without wheat bran supplement</td>
<td>208 (4 yrs)</td>
<td>actual intake of fiber negatively correlated with polyp number ratio</td>
</tr>
<tr>
<td>Reddy et al. 1989</td>
<td>Humans with ↑ excretion of fecal mutagen</td>
<td>Fecal mutagens and bile acids</td>
<td>10 g/day</td>
<td></td>
<td>individual’s regular diet without wheat bran supplement (high fat)</td>
<td>5</td>
<td>↓ fecal mutagenicity</td>
</tr>
<tr>
<td>Alberts et al. 1990</td>
<td>high risk</td>
<td>early risk markers</td>
<td>13.5 and 2 g/day</td>
<td></td>
<td>individual’s regular diet without wheat bran supplement</td>
<td>8</td>
<td>↓ LI and cell prol. in 4 out of 8 patients</td>
</tr>
<tr>
<td>Gregoire et al. 1992</td>
<td>male and female</td>
<td>early risk markers</td>
<td>30 g/day</td>
<td></td>
<td>individual’s regular diet without wheat bran supplement</td>
<td>2</td>
<td>ND for pH</td>
</tr>
<tr>
<td>Kashtan et al. 1992</td>
<td>normal and post-polypectomy</td>
<td>early risk markers</td>
<td>16.4 g/day max</td>
<td>37</td>
<td>12.8 of total calories</td>
<td>2</td>
<td>↑ fecal butyrate</td>
</tr>
<tr>
<td>Lampe et al. 1992</td>
<td>male and female</td>
<td>early risk markers</td>
<td>10 or 30 g/day</td>
<td>unspec.</td>
<td>0 added</td>
<td>3</td>
<td>↓ pH</td>
</tr>
<tr>
<td>Reddy et al. 1992</td>
<td>premenstrual females</td>
<td>early risk markers</td>
<td>13-15 g/day</td>
<td></td>
<td>individual’s regular diet without wheat bran supplement</td>
<td>8</td>
<td>↓ neutral sterols</td>
</tr>
<tr>
<td>McKeown-Eyssen et al. 1994</td>
<td>post-polypectomy</td>
<td>Polyp recurrence</td>
<td>50 g/day of total dietary fiber</td>
<td>20%</td>
<td>Normal diet (low fiber); avg. was 16 g/day</td>
<td>52 (1 yr)</td>
<td>ND in polyp recurrence in high versus low dietary fiber intake</td>
</tr>
<tr>
<td>Reddy et al. 1994</td>
<td>females</td>
<td>early risk markers</td>
<td>13-15 g/day</td>
<td></td>
<td>individual’s regular diet without wheat bran supplement</td>
<td>8</td>
<td>↑ total fecal DAG</td>
</tr>
<tr>
<td>Rooney et al. 1994</td>
<td>male and female, high risk</td>
<td>cell proliferation</td>
<td>10.5 g/day (WB fiber)</td>
<td></td>
<td>individual’s regular diet without wheat bran supplement</td>
<td>12</td>
<td>↓ LI of cell proliferation</td>
</tr>
</tbody>
</table>

Continued....
<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects</th>
<th>Endpoint</th>
<th>Amount of WB or wheat fiber</th>
<th>% Fat</th>
<th>% Fiber in control group</th>
<th>Length of feeding period (wk)</th>
<th>RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>MacLennan et al, 1995</td>
<td>post-polypectomy</td>
<td>Polyp recurrence</td>
<td>25 g/day (11 g of fiber)</td>
<td>not &gt; 30%</td>
<td>Normal diet</td>
<td>96 (2 yrs) and 192 (4 yrs)</td>
<td>↓ recurrence of polyps with diameter &gt; 10 mm</td>
</tr>
<tr>
<td>Alberts et al, 1996</td>
<td>Male and female post adenoma resection</td>
<td>2° bile acids</td>
<td>2 or 13.5 g/day (WB fiber)</td>
<td>individual's regular diet without wheat bran supplement</td>
<td>12 &amp; 36</td>
<td>↓ 2° bile acids fecal concentration and excretion rate in high WB group</td>
<td></td>
</tr>
<tr>
<td>Alberts et al, 1997</td>
<td>Male and female post adenoma resection</td>
<td>cell proliferation</td>
<td>2 or 13.5 g/day (WB fiber)</td>
<td>individual's regular diet without wheat bran supplement</td>
<td>12 &amp; 36</td>
<td>ND for LI of cell proliferation</td>
<td></td>
</tr>
<tr>
<td>Earnest et al, 1999</td>
<td>post-polypectomy</td>
<td>Polyp recurrence</td>
<td>13.5 g/day</td>
<td>individual's regular diet without wheat bran supplement</td>
<td>Ongoing</td>
<td>ND for interim results on polyp recurrence</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: 2° - Secondary; DAG - Diacyl Glycerol; LI - Labeling Index of cell proliferation; ND - No significant Difference; WB - Wheat Bran.
Table 11.2: Summary of recent animal studies on wheat bran and colon tumourigenesis.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Animals</th>
<th>Carcinogen</th>
<th>% or amount of WB</th>
<th>% Fat</th>
<th>% Fiber in control group</th>
<th>Length of feeding period(wk)</th>
<th>Carcinogen with diet feeding?</th>
<th>RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wilson et al., 1977</td>
<td>SD rat; male; 11/gp</td>
<td>DMH; 30 mg/Kg; 4 or 8 doses</td>
<td>20</td>
<td>20</td>
<td>0</td>
<td>31</td>
<td>yes</td>
<td>↓ tumour incidence</td>
</tr>
<tr>
<td>Barbolts &amp; Abraham, 1978</td>
<td>SD rat; female; 10/gp</td>
<td>DMH; 30 mg/Kg; 10 doses</td>
<td>20</td>
<td>4</td>
<td>unspecified; non-purified diet</td>
<td>23</td>
<td>yes</td>
<td>↓ tumour incidence / tumour bearing rat</td>
</tr>
<tr>
<td>Chen et al., 1978</td>
<td>CF1 mouse; female; 200/gp</td>
<td>DMH; 20 mg/Kg; 26 doses</td>
<td>40</td>
<td>6</td>
<td>0</td>
<td>27</td>
<td>yes</td>
<td>↓ tumour incidence / incidence of major path. changes in tumours</td>
</tr>
<tr>
<td>Cruse et al., 1978</td>
<td>Wistar rat; female; 20/gp</td>
<td>DMH; 40 mg/Kg; 20 doses</td>
<td>20</td>
<td>unspecified</td>
<td>unspecified; non-purified diet</td>
<td>48</td>
<td>yes</td>
<td>ND tumour incidence / ND mortality</td>
</tr>
<tr>
<td>Fleiszer et al., 1978</td>
<td>Chester Beatty rat; male; 25/gp</td>
<td>DMH; 20 mg/Kg; 20 doses</td>
<td>28</td>
<td>2</td>
<td>unspecified; non-purified diet</td>
<td>28</td>
<td>yes</td>
<td>↓ tumour incidence / ↓ weight gain</td>
</tr>
<tr>
<td>Bauer et al., 1979</td>
<td>SD rat; female; 40/gp</td>
<td>DMH; 15 mg/Kg; 12 doses</td>
<td>20</td>
<td>20</td>
<td>0</td>
<td>15</td>
<td>yes</td>
<td>ND tumour incidence</td>
</tr>
<tr>
<td>Nigro et al., 1979</td>
<td>SD rat; male; 25/gp</td>
<td>AOM; 8 mg/Kg; 21 doses</td>
<td>10</td>
<td>35</td>
<td>0</td>
<td>23</td>
<td>yes</td>
<td>ND tumours / tumour bearing rat</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>20</td>
<td>5-7</td>
<td>0</td>
<td></td>
<td>yes</td>
<td>↓ tumours / tumour bearing rat</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30</td>
<td>5-7</td>
<td>0</td>
<td></td>
<td>yes</td>
<td>↓ tumours / tumour bearing rat</td>
</tr>
</tbody>
</table>

Continued......
<table>
<thead>
<tr>
<th>Reference</th>
<th>Animals</th>
<th>Carcinogen</th>
<th>% or amount of WB</th>
<th>% Fat</th>
<th>% Fiber in control group</th>
<th>Length of feeding period (wk)</th>
<th>Carcinogen with diet feeding?</th>
<th>RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Watanabe et al. 1979</td>
<td>F344 rat; Female; 30/gp</td>
<td>AOM; 8 mg/Kg; MNU; 2 mg/Kg; 6 doses</td>
<td>15 20</td>
<td>0</td>
<td></td>
<td>42</td>
<td>yes</td>
<td>↓ tumour incidence ND tumour incidence tumour bearing rat ND tumour incidence tumour bearing rat</td>
</tr>
<tr>
<td>Abraham et al. 1980</td>
<td>SD rat; female; 10/gp</td>
<td>DMH; 30 mg/Kg; 10 doses</td>
<td>20 4</td>
<td></td>
<td>unspecified; non-purified diet</td>
<td>23</td>
<td>yes</td>
<td>↓ tumour incidence ND tumour incidence tumour bearing rat</td>
</tr>
<tr>
<td>Bar bolt &amp; Abraham, 1980</td>
<td>SD rat; male &amp; female; 10/gp</td>
<td>DMH; 15 mg/Kg or 30 mg/Kg; 10 doses</td>
<td>20 4</td>
<td></td>
<td>unspecified; non-purified diet</td>
<td>23</td>
<td>yes</td>
<td>↓ tumour incidence in females and in low carcinogen dose males</td>
</tr>
<tr>
<td>Fleiszer et al. 1980</td>
<td>Chester Beatty rat; male; Cont: 2/gp, Exp: 19/gp</td>
<td>DMH; 20 mg/Kg; 20 doses</td>
<td>28 Cont: 2 Exp: 15</td>
<td>0</td>
<td></td>
<td>28</td>
<td>yes</td>
<td>↓ tumour incidence ↑ LI</td>
</tr>
<tr>
<td>Reddy &amp; Mori, 1981</td>
<td>F344 rat; male; 50/gp</td>
<td>DMAB; 50 mg/Kg; 20 doses</td>
<td>15 5</td>
<td></td>
<td>5 (cellulose)</td>
<td>42</td>
<td>yes</td>
<td>↓ tumour incidence ↓ tumours / tumour bearing rat</td>
</tr>
<tr>
<td>Reddy et al. 1981</td>
<td>F344 rat; male; 51/gp</td>
<td>AOM; 8 mg/Kg; 10 doses</td>
<td>15 5</td>
<td></td>
<td>5 (cellulose)</td>
<td>32</td>
<td>yes</td>
<td>↓ tumour incidence ↓ no. adenomas ↓ no. carcinomas ↓ tumours / tumour bearing rat</td>
</tr>
<tr>
<td>Barnes et al. 1983</td>
<td>F344 rat; male; 25/gp</td>
<td>DMH; 150 mg/Kg; 2 doses</td>
<td>20 20</td>
<td>0</td>
<td></td>
<td>40</td>
<td>yes</td>
<td>ND tumour incidence No no. tumour incidence</td>
</tr>
<tr>
<td>Jacobs, 1983</td>
<td>SD rat; male; 12/gp</td>
<td>DMH; 20 mg/Kg; 13 doses</td>
<td>20 8</td>
<td>0</td>
<td></td>
<td>31</td>
<td>yes</td>
<td>↑ tumours / tumour bearing rat ↓ no. benign adenoma</td>
</tr>
</tbody>
</table>

Continued......
<table>
<thead>
<tr>
<th>Reference</th>
<th>Animals</th>
<th>Carcinogen</th>
<th>% or amount of WB</th>
<th>% Fat</th>
<th>% Fiber in control group</th>
<th>Length of feeding period (wk)</th>
<th>Carcinogen with diet feeding?</th>
<th>RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clapp et al. 1984</td>
<td>Balb/C mouse; male; 50-70/gp</td>
<td>DMH; 20 mg/Kg; 10 doses</td>
<td>20</td>
<td>5</td>
<td>0</td>
<td>43</td>
<td>yes</td>
<td>↑ tumour incidence for both soft and hard wheat brans</td>
</tr>
<tr>
<td>Calvert et al. 1987</td>
<td>F344 rats; male; 30/gp</td>
<td>DMH; 30 mg/Kg; 5 doses</td>
<td>10</td>
<td>5</td>
<td>0</td>
<td>26</td>
<td>yes</td>
<td>↓ tumour incidence ↓ tumours / tumour bearing rat</td>
</tr>
<tr>
<td>Nigro and Bull, 1987</td>
<td>F344 rats; male</td>
<td>AOM; 8 mg/Kg; 23 doses</td>
<td>10, 20 &amp; 30</td>
<td>5% for the 20 &amp; 30% group; 35 for the 10%</td>
<td>0</td>
<td>23</td>
<td>yes</td>
<td>ND for 10% WB with 35% fat ↓ total no. of tumours with 20 &amp; 30% WB with 5% fat</td>
</tr>
<tr>
<td>Tatsuta et al. 1988</td>
<td>Wistar rat; male; 27-34/gp</td>
<td>AOM; 7.4 mg/Kg; 10 doses</td>
<td>20</td>
<td>9.3</td>
<td>0</td>
<td>45</td>
<td>yes</td>
<td>ND tumour incidence, number or histology</td>
</tr>
<tr>
<td>Iwane, 1989</td>
<td>F344 rat; male; 7/gp</td>
<td>DMH</td>
<td>40</td>
<td>5</td>
<td>0</td>
<td>30</td>
<td>yes</td>
<td>↓ tumour incidence at 26 weeks ND tumour incidence at 30 weeks no protective effects observed</td>
</tr>
<tr>
<td>Sinkeldam et al. 1990</td>
<td>Wistar rat; male; 30/gp</td>
<td>MNNG; 6 mg/Kg; 5 doses</td>
<td>9 &amp; 17</td>
<td>5</td>
<td>0</td>
<td>37</td>
<td>yes</td>
<td>ND tumour incidence no protective effects observed</td>
</tr>
<tr>
<td>Alabaster et al. 1993</td>
<td>F344 rat; male; 12/gp</td>
<td>AOM; 15 mg/Kg; 2 doses</td>
<td>4</td>
<td>20</td>
<td>1 (as wheat bran)</td>
<td>25</td>
<td>yes</td>
<td>↓ tumour incidence (no rats with adenocarcinoma in the WB groups) ↓ tumour incidence ↓ tumour mass ↑ butyrate continued......</td>
</tr>
<tr>
<td>McIntyre et al. 1993</td>
<td>SD rat; male; 16/gp</td>
<td>DMH; 30 mg/Kg; 10 doses</td>
<td>10</td>
<td>20</td>
<td>5-10</td>
<td>33</td>
<td>yes</td>
<td>ND tumour incidence ↓ tumour incidence ↓ tumour mass ↑ butyrate continued......</td>
</tr>
<tr>
<td>Reference</td>
<td>Animals</td>
<td>Carcinogen</td>
<td>% or amount of WB</td>
<td>% Fat</td>
<td>% Fiber in control group</td>
<td>Length of feeding period (wk)</td>
<td>Carcinogen with diet feeding?</td>
<td>RESULTS</td>
</tr>
<tr>
<td>-----------------</td>
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<td>-------------------------------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>Maziya-Dixon et al. 1994</td>
<td>CF1 mice; male; 50/gp</td>
<td>DMH; 20 mg/Kg; 20 doses</td>
<td>45</td>
<td>5</td>
<td>6</td>
<td>20 &amp; 40</td>
<td>yes</td>
<td>↓ tumour incidence with red WB</td>
</tr>
<tr>
<td>Alabaster et al. 1995</td>
<td>F344 rats; male; 20/gp</td>
<td>AOM; 15 mg/Kg; 2 doses</td>
<td>8 (wheat bran fiber)</td>
<td>20</td>
<td>1 (wheat bran fiber)</td>
<td>34</td>
<td>yes</td>
<td>↓ total no. of tumours</td>
</tr>
<tr>
<td>Shivapurkar et al. 1995</td>
<td>F344 rat; male; 25/gp</td>
<td>AOM; 15 mg/Kg; 2 doses</td>
<td>8 (wheat bran fiber)</td>
<td>20</td>
<td>1 (wheat bran fiber)</td>
<td>40 max</td>
<td>yes</td>
<td>ND tumour incidence</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ND tumours / tumour bearing rat</td>
</tr>
<tr>
<td>Nakaji et al. 1996</td>
<td>SD rat; male; 21/gp</td>
<td>DMH; 25 mg/Kg; 19 doses</td>
<td>40</td>
<td>10</td>
<td>9</td>
<td>30</td>
<td>Yes</td>
<td>ND tumour number at 30wks</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓ total no. of tumours between wk 21-24.</td>
</tr>
<tr>
<td>Young et al. 1996</td>
<td>SD rat; male; 30 and 35/gp</td>
<td>DMH; 20 mg/Kg; 10 doses</td>
<td>10 (wheat bran fiber)</td>
<td>10</td>
<td>2.6</td>
<td>31</td>
<td>yes</td>
<td>↓ tumor incidence</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ND tumour number and size</td>
</tr>
<tr>
<td>Alabaster et al. 1997</td>
<td>F344; male; 17/gp</td>
<td>AOM; 15 mg/Kg; 2 doses</td>
<td>4 &amp; 8 WB, All Bran or Bran Flakes</td>
<td>20</td>
<td>1 (WB, All Bran or Bran Flakes)</td>
<td>28</td>
<td>yes</td>
<td>ND in total number of tumours</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓ no. adenocarcinomas in 4 &amp; 8% groups</td>
</tr>
<tr>
<td>Kretchevsky and Klurfeld, 1997</td>
<td>F344; male;</td>
<td>DMH; 20 mg/Kg; 5 doses</td>
<td>10% WB (some grp's calorie restricted)</td>
<td>5</td>
<td>4 (cellulose)</td>
<td>not specified</td>
<td>no</td>
<td>↓ tumor incidence with 0, 10 &amp; 20% calorie restriction</td>
</tr>
<tr>
<td>Zoran et al. 1997a</td>
<td>SD rat; male; 34 &amp; 11/gp</td>
<td>AOM; 15 mg/Kg; 2 doses</td>
<td>12 (6% wheat bran fiber)</td>
<td>5</td>
<td>No control; compared oat bran to WB</td>
<td>38</td>
<td>yes</td>
<td>↑ tumor incidence in oat bran vs. WB</td>
</tr>
</tbody>
</table>

Continued......
<table>
<thead>
<tr>
<th>Reference</th>
<th>Animals</th>
<th>Carcinogen</th>
<th>% or amount of WB</th>
<th>% Fat</th>
<th>% Fiber in control group</th>
<th>Length of feeding period (wk)</th>
<th>Carcinogen with diet feeding?</th>
<th>RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Takahashi et al. 1999</td>
<td>CF1 mice; female; 20 &amp; 5/gp</td>
<td>AOM; 10 mg/Kg; 6 doses</td>
<td>20% WB and microfibril WB</td>
<td>4.2</td>
<td>No control; compared to 20% cellulose</td>
<td>33</td>
<td>yes</td>
<td>ND in tumour incidence ↓ no. adenocarcinomas in WB and microfibril WB groups</td>
</tr>
</tbody>
</table>

Abbreviations: AOM – Azoxymethane; Cont. – Control group; DMH – 1,2-Dimethylhydrazine; DMAB – 3,2-dimethyl-4-aminobiphenyl; Exp. – Experimental group; F344 – Fisher 344 rat; gp – Group; LI – Labeling Index of cell proliferation; MNNG – N-methyl-N’-nitro-N-nitrosoguanidine; MNU – methylnitrosurea; ND – No significant Difference; SD – Sprague Dawley rat; WB – Wheat Bran.
Table 11.3: Summary of recent animal studies on wheat bran and early risk markers of colon carcinogenesis.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Animals</th>
<th>Endpoint</th>
<th>Carcinogen</th>
<th>% or amount of WB</th>
<th>% Fat</th>
<th>% Fiber in control group</th>
<th>Length of feeding period (wk)</th>
<th>Carcinogen with diet feeding?</th>
<th>RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jacobs &amp; White, 1983</td>
<td>SD rat; male; 12/gp</td>
<td>early risk markers</td>
<td>none</td>
<td>20/8</td>
<td>0</td>
<td>4</td>
<td>9</td>
<td>–</td>
<td>↑ large intestine mucosal DNA ↓ cell proliferation ↓ cell migration ND in fecal mutagens or concentration</td>
</tr>
<tr>
<td>Pence et al., 1985</td>
<td>F344 rat; male; 10/gp</td>
<td>early risk markers</td>
<td>DMH; 10 mg/Kg; 16 doses</td>
<td>15 unspecified</td>
<td>0</td>
<td>31</td>
<td>yes</td>
<td>ND in fecal mutagens or concentration</td>
<td></td>
</tr>
<tr>
<td>Robblee et al., 1989</td>
<td>C57-BL/6J mouse; female</td>
<td>early risk markers</td>
<td>none</td>
<td>5 or 10/5.5</td>
<td>0</td>
<td>20</td>
<td>–</td>
<td>ND for LI ND for no. labelled cells ND for mitotic figure</td>
<td></td>
</tr>
<tr>
<td>Floor et al., 1991</td>
<td>F344 rat; male; 15/gp</td>
<td>serum gastrin (trophic effect on colon carcinoma)</td>
<td>none</td>
<td>2.5, 10 &amp; 20/1.5 &amp; 10</td>
<td>0</td>
<td>4</td>
<td>–</td>
<td>ND in serum gastrin levels</td>
<td></td>
</tr>
<tr>
<td>Boffa et al., 1992</td>
<td>SD rat; male; 8/gp</td>
<td>Cell proliferation, &amp; butyrate</td>
<td>none</td>
<td>5, 10, 20/8</td>
<td>0</td>
<td>2</td>
<td>–</td>
<td>↓ cell proliferation &amp; ↑ butyrate in all WB groups</td>
<td></td>
</tr>
<tr>
<td>Lupton &amp; Kurtz, 1993</td>
<td>SD rat; male; 10/gp</td>
<td>early risk markers</td>
<td>none</td>
<td>8/8</td>
<td>0</td>
<td>3</td>
<td>–</td>
<td>↑ cell proliferation ↑ butyrate</td>
<td></td>
</tr>
<tr>
<td>Gestel et al., 1994</td>
<td>SD rat; male; 7/gp</td>
<td>early risk markers</td>
<td>none</td>
<td>10/14 (cont.) 16 (WB)</td>
<td>0</td>
<td>3</td>
<td>–</td>
<td>↑ fecal bile acids ↑ fecal DAPA ↑ fecal enzyme activity ↑ RNA, DNA content Continued……</td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>Animals</td>
<td>Endpoint</td>
<td>Carcinogen</td>
<td>% or amount of WB</td>
<td>% Fat</td>
<td>% Fiber in control group</td>
<td>Length of feeding period (wk)</td>
<td>Carcinogen with diet feeding?</td>
<td>RESULTS</td>
</tr>
<tr>
<td>--------------------</td>
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<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Alabaster et al. 1995</td>
<td>F 344 rats; male; 20/gp</td>
<td>ACF &amp; tumours</td>
<td>AOM; 15 mg/Kg; 2 doses</td>
<td>8 (wheat bran fiber)</td>
<td>20</td>
<td>1 (wheat bran fiber)</td>
<td>34</td>
<td>yes</td>
<td>↓ no. of ACF/rat</td>
</tr>
<tr>
<td>Folino et al. 1995</td>
<td>SD rats; male; 20/gp</td>
<td>early risk markers</td>
<td>none</td>
<td>10</td>
<td>unspec-</td>
<td>0</td>
<td>4</td>
<td>--</td>
<td>↓ cell prol. by coarse and fine wheat bran ↑ butyrate</td>
</tr>
<tr>
<td>Shivapurkar et al. 1995</td>
<td>F344 rats; male; 25/gp</td>
<td>ACF &amp; tumours</td>
<td>AOM; 15 mg/Kg; 2 doses</td>
<td>8 (wheat bran fiber)</td>
<td>20</td>
<td>1 (wheat bran fiber)</td>
<td>40 max</td>
<td>yes</td>
<td>ND no. &amp; mult. ACF</td>
</tr>
<tr>
<td>Ferguson and Harris, 1996</td>
<td>F344 rats; male; 3/gp</td>
<td>ACF</td>
<td>IQ; 50mg/Kg; 3 doses</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>3</td>
<td>yes</td>
<td>↓ no. of ACF/rat</td>
</tr>
<tr>
<td>Ishizuka and Kasai, 1996</td>
<td>Wistar ST rats; male; 4 to 6/gp</td>
<td>ACF</td>
<td>DMH; 20 mg/Kg; 1 dose</td>
<td>5, 10 and 20 unspec-</td>
<td>0</td>
<td>1, 2, 3 &amp; 4</td>
<td>12,3 &amp; 4</td>
<td>yes</td>
<td>↓ no. ACF in 20% WB after 4 wks ↑ ACF multiplicity in 20% WB after 4 wks</td>
</tr>
<tr>
<td>Young et al. 1996</td>
<td>SD rat; male; 30 and 35/gp</td>
<td>ACF &amp; tumours</td>
<td>DMH; 20 mg/Kg; 10 doses</td>
<td>10</td>
<td>unspec-</td>
<td>2.6</td>
<td>17 &amp; 31</td>
<td>yes</td>
<td>ND no. of ACF/rat ↑ cell proliferation (attributed to potato starch and not WB)</td>
</tr>
<tr>
<td>Alabaster et al. 1997</td>
<td>F344 rats; male; 17/gp</td>
<td>ACF &amp; tumours</td>
<td>AOM; 15 mg/Kg; 2 doses</td>
<td>4 &amp; 8 WB, All Bran or Bran Flakes</td>
<td>20</td>
<td>1 (WB, All Bran or Bran Flakes)</td>
<td>8</td>
<td>yes</td>
<td>↓ no. ACF in 4 &amp; 8% diets vs. 1%</td>
</tr>
<tr>
<td>Hioki et al. 1997</td>
<td>APC(delta716) knockout mice</td>
<td>Intestinal and colon polyps</td>
<td>none</td>
<td>20</td>
<td>20 in control; 5 in trt</td>
<td>2.5</td>
<td>7</td>
<td>--</td>
<td>↓ no. intestinal &amp; colon polyps ND is size or distribution of polyps Continued...</td>
</tr>
<tr>
<td>Reference</td>
<td>Animals</td>
<td>Endpoint</td>
<td>Carcinogen</td>
<td>% or amount of WB</td>
<td>% Fat</td>
<td>% Fiber in control group</td>
<td>Length of feeding period (wk)</td>
<td>Carcinogen with diet feeding?</td>
<td>RESULTS</td>
</tr>
<tr>
<td>-----------------</td>
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<td>------------------------------</td>
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<td>---------------------------------------------</td>
</tr>
<tr>
<td>Compher et al, 1999</td>
<td>F344 rats; male; 10 and 20 /gp</td>
<td>ACF</td>
<td>AOM; 20 mg/Kg; 2 doses</td>
<td>20</td>
<td>20</td>
<td>0</td>
<td>1 &amp; 8</td>
<td>yes</td>
<td>↑ rate of apoptosis at 1 week with WB, preserved proliferating zone ↓ no. of ACF after 8 wks</td>
</tr>
</tbody>
</table>

Abbreviations: ACF – Aberrant Crypt Foci; AOM – Azoxy methane; DMH – 1,2-Dimethylhydrazine; F344 – Fisher 344 rat; LI – Labeling Index of cell proliferation; ND – No significant difference; SD – Sprague Dawley.
Table 11.4: Effect of various diets or diet components on β-glucuronidase activities.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Diet or Diet Component</th>
<th>Effect on β-glucuronidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goldin &amp; Gorbach (1976)</td>
<td>High fat, high beef diet</td>
<td>Increase</td>
</tr>
<tr>
<td>Reddy et al (1977)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gorbach and Goldin (1990)</td>
<td>Lactovegetarian and vegan diet</td>
<td>Decrease</td>
</tr>
<tr>
<td>Johansson et al (1990)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ling and Hanninen (1992)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bauer et al (1979)</td>
<td>Pectin</td>
<td>Increase</td>
</tr>
<tr>
<td>Lindop et al (1985)</td>
<td>Pectin</td>
<td>Decrease</td>
</tr>
<tr>
<td>London et al. (1981)</td>
<td>Pectin</td>
<td>Increase</td>
</tr>
<tr>
<td></td>
<td>Oat bran</td>
<td>Decrease</td>
</tr>
<tr>
<td></td>
<td>Soya</td>
<td>Decrease</td>
</tr>
<tr>
<td>Freeman (1986)</td>
<td>Cellulose</td>
<td>Decrease</td>
</tr>
<tr>
<td></td>
<td>Hemicellulose</td>
<td>Decrease</td>
</tr>
<tr>
<td></td>
<td>Pectin</td>
<td>Increase</td>
</tr>
<tr>
<td>Marteau et al (1990)</td>
<td>Dairy product with L. acidophilus and</td>
<td>No difference</td>
</tr>
<tr>
<td></td>
<td>bifidobacterium</td>
<td></td>
</tr>
<tr>
<td>Goldin et al (1980)</td>
<td>L. acidophilus</td>
<td>Decrease</td>
</tr>
<tr>
<td></td>
<td>Oat bran</td>
<td>Decrease</td>
</tr>
<tr>
<td></td>
<td>Corn bran</td>
<td>No difference</td>
</tr>
<tr>
<td>Mallett et al (1988)</td>
<td>Amylase resistant starch</td>
<td>Decrease</td>
</tr>
<tr>
<td>Mallett et al (1985)</td>
<td>Fiber free diet plus saccharin</td>
<td>Decrease</td>
</tr>
<tr>
<td>Jenab and Thompson (1995)</td>
<td>Flaxseed and defatted flaxseed</td>
<td>Increase</td>
</tr>
</tbody>
</table>
Table 11.5: Method of assigning scores for lectin staining (adapted from Chang et al, 1997).

<table>
<thead>
<tr>
<th>Colour stain intensity</th>
<th>0% stained cells</th>
<th>1-25% stained cells</th>
<th>25-50% stained cells</th>
<th>51-75% stained cells</th>
<th>&gt;75% stained cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

Adapted from Chang et al, 1997. The lectin score for each crypt or crypt segment is a based on a combination of the colour stain intensity (from 0-3) and the percent of cells stained. For example, a crypt or crypt segment with an intensity of 2 and 35% of cells stained would receive a score of 2. Intensity of staining was determined by viewing all slides and determining the most and least stained groups of crypts which were then assigned as having a colour intensity of 3 or 0 respectively. All other crypts were ranked and graded for colour stain intensity in comparison to these crypts.

Table 11.6: Average daily consumption of phytic acid and percent recovery in feces.

<table>
<thead>
<tr>
<th>Dietary Groups</th>
<th>PA Consumed mg/day *</th>
<th>Wet Fecal Weight (g)</th>
<th>Fecal PA % †</th>
<th>PA Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD</td>
<td>0</td>
<td>1.26 ± 0.14 c</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>WB</td>
<td>147 ± 4</td>
<td>2.13 ± 0.14 ab</td>
<td>3.67 ± 0.30 b</td>
<td>54.13 ± 6.67 b</td>
</tr>
<tr>
<td>DWB</td>
<td>24 ± 1</td>
<td>2.67 ± 0.22 a</td>
<td>0.74 ± 0.06</td>
<td>87.76 ± 12.05</td>
</tr>
<tr>
<td>DWBPA</td>
<td>140 ± 5</td>
<td>2.40 ± 0.17 a</td>
<td>3.22 ± 0.24 b</td>
<td>54.41 ± 4.38 b</td>
</tr>
<tr>
<td>PA</td>
<td>132 ± 3</td>
<td>1.69 ± 0.10 bc</td>
<td>6.51 ± 0.46 a</td>
<td>83.27 ± 7.34 a</td>
</tr>
</tbody>
</table>

Values are means ± SEM. n=15. There were no statistical differences between the WB, DWBPA and PA diet groups in PA consumed per day. Values with different superscripts within a column are significantly different from each other, p < 0.05. The DWB group was not included in the statistics for the Fecal PA and PA Recovery calculations, since it contains considerably less PA.

* average daily consumption of PA was calculated from the PA content of the various diets and daily diet intake.

† estimated using the AOAC method described in section 4.3.2.1 using 0.5 g of freeze dried fecal sample; corrected for moisture content and expressed on a wet weight basis.