INHIBITION OF CARCINOGEN INDUCED BIOLOGICAL RESPONSES WITH A COFFEE WATER-INSOLUBLE FRACTION AND A MODEL SYSTEM MELANOIDIN

By

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Date **February 11, 1985**
Previous research studies have indicated that coffee brew and water extracts of heated brown food systems such as molasses, beef, prunes and raisins have an inhibitory effect on carcinogen-induced mutagenicity in the Ames Salmonella strains. A hypothesis to explain these observations is that carcinogens are adsorbed onto water-insoluble complexes in coffee brew and melanoidins in heat-treated foods.

The present study was undertaken to characterize the water-insoluble fraction (WIF) from reconstituted instant coffee powder; to examine the genotoxic inhibitory effect of the WIF and browning reaction melanoidins; to determine the degree and type of binding of benzo(a)pyrene (BP) and aflatoxin B1 (AFB1) by the water-insoluble fraction; and to assess the effect of a model system melanoidin (MSM) on the inhibition of BP induction of aryl hydrocarbon hydroxylase (AHH) in the small intestine of rats.

WIF was separated from reconstituted spray dried coffee by precipitation of particulate matter with ethanol at a 90% level and was further purified by resuspension of the precipitate in water and subsequent centrifugal sedimentation. The yield of WIF was about 3% of the instant coffee powder (1.4% moisture). From the non-metallic elemental analysis of WIF, the empirical formula C_{47}H_{79}O_{41}N, was determined. The ratio of C, H, and O atoms suggests the presence of carbohydrates and the nitrogen atom implies the presence of amino acids. The molecular weight of WIF was estimated to be around 200,000 as determined by a column chromatographic technique. A variety of inorganic elements were found in WIF, with potassium in the highest concentration.
Phenolic compounds, reductones and amino acids were found in WIF. Phenolic compounds were detected by a semiquantitative FeCl$_3$ colorimetric method which indicated that these compounds were present at a level of 4 mg of caffeic acid equivalent per 10 mg of WIF. The reductone content of WIF was about 140 mg ascorbic acid equivalent per g. Eleven amino acids were identified in the acid hydrolyzate of WIF. The major amino acids were: aspartic acid, glutamic acid, glycine, valine, isoleucine and histidine.

Dubois et al. (1956) sugar analysis indicated that 56% of WIF consisted of carbohydrates. Analysis of acid-hydrolyzed WIF by paper chromatography, gas liquid chromatography and GLC-mass spectroscopy indicated that mannose, galactose, glucose and arabinose were the major monosaccharides. Gas liquid chromatography-mass spectrometry showed that the carbohydrates in WIF hydrolyzate were mostly simple hexose sugars with trace amounts of deoxy-sugar fragments, but no N-acetyl glucosamines were identified.

The Ames Salmonella test was employed to assess the genotoxic inhibitory effect of coffee WIF and model system melanoidin (MSM) on benzo(a)pyrene (BP), aflatoxin (AFB$_1$) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). The reverse mutation frequencies were reduced in the presence of WIF and MSM over a range of dosages.

Studies were conducted to examine the effect of pH on the binding ability of WIF with BP and AFB$_1$. A maximum binding of about 83% of BP by WIF in buffer at 37°C was achieved at pH 2.0, and approximately 63% binding of BP by WIF occurred at pH values ranging from 4 to 9. The binding of AFB$_1$ by WIF in buffers at pH values between 2 to 9 ranged from 47 to 55% at 37°C. A pH effect on aflatoxin B$_1$ binding to WIF was not apparent.
Binding of BP by WIF in citrate buffer (pH 3.0) was examined by column chromatography using different eluants to try to determine the type of binding. The BP peak coincided with the WIF peak when citrate buffer was used (pH 3.0) with and without added NaCl, urea and mercaptoethanol. With SDS added to the citrate buffer eluant, WIF was broken down into smaller particles yet retaining most of the BP. Hydrophobic bonds are presumably involved in the binding of BP to WIF.

The activities of aryl hydrocarbon hydroxylase (AHH) in the microsomes of the small intestine of rats fed diets with or without BP and with or without MSM were assessed. The AHH activity for rats on a diet containing both BP and MSM was significantly smaller than that for rats on a diet with BP and no MSM. Components of MSM presumably bind BP to the extent that less BP was available for induction of AHH activity.
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GENERAL INTRODUCTION

Cancer Induction and Food

Cancer is a term which encompasses a wide variety of malignant growths of animal and human cells. Three basic types of cells are involved in the formation of tumours: sex specific epithelial cells, other epithelial cells and non-epithelial cells. Carcinomas are tumours which develop by the multiplication of epithelial cells whereas sarcomas, lymphomas and leukemias arise from the proliferation of non-epithelial cells.

Cancer is currently the second leading medical cause of death in Canada and the United States after cardiovascular disease (Stats. Can., 1982). In Canada, the major sites of human cancer are the lung, colon-rectum and prostate for men, and breast, colon-rectum and lung for women. Most cancers are considered to be caused by environmental factors such as occupational chemicals, radiant energy from the sun, tobacco smoke, air pollutants and dietary components (Doll, 1980; Wynder and Gori, 1977; Miller and Miller, 1979; Gori, 1980; Heitanen, 1981). Eighty to ninety percent of all cancer incidences have been attributed to environmental factors and 70% of these have been ascribed to components in the diet (Hirayama, 1981; Higginson, 1979). Weisburger and Williams (1983) have estimated that dietary components may induce 30 to 40% of cancers in men and 50 to 60% in women in the U.S. Table 1 lists proportions of cancer deaths attributable to various factors according to Doll and Peto (1981).

In the Sixties and Seventies, many researchers redirected their investigations on cancer from man-made chemical carcinogens and radiant
Table 1

Proportions of Cancer Cases Attributed to Various Factors

<table>
<thead>
<tr>
<th>Factor or class of factors</th>
<th>England, Birmingham region (based on Higginson and Muir, 1979)</th>
<th>United States (based on Wynder and Gori, 1977)</th>
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<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Tobacco</td>
<td>30</td>
<td>7</td>
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<tr>
<td>Tobacco/alcohol</td>
<td>5</td>
<td>3</td>
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<tr>
<td>Diet</td>
<td>--</td>
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<td>Life-style</td>
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<td>Occupation</td>
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<td>Ionizing radiations</td>
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<tr>
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<tr>
<td>Exogenous hormones</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Congenital</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Unknown</td>
<td>15</td>
<td>11</td>
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\(^1\text{Deduced from histograms. Non-environmental factors equated with congenital and unknown.}

From, Doll and Peto, 1981, p. 1257
energy to naturally occurring chemical compounds, many of which are present in food. Research approaches included: 1) the introduction of foods, food fractions and purified components to experimental animals for assessment of carcinogenic activity, 2) short term testing \textit{in vitro} of foods and food components, and 3) epidemiological studies of human populations.

Since long-term carcinogenicity tests with laboratory animals are expensive and time-consuming, these bioassays must be restricted to a limited number of suspected carcinogens. With thousands of chemical compounds in foods to be assessed as potential risk factors, short-term inexpensive procedures are required. Since the early 1970's, over 100 short term \textit{in vitro} bioassays using a variety of cells have been developed (Hollstein \textit{et al.}, 1979). Test cells include bacteria, yeast and human cells. One of the most popular short-term tests is the Salmonella/Ames assay (Ames \textit{et al.}, 1975). A variety of submammalian and mammalian tests have been developed to determine point mutations of DNA and chromosomal changes (breaks, deletions and translocations). Some tests measure the transmission of mutation to their progeny whereas others measure the preliminary damage to the DNA (Casciamo, 1982). According to Taylor (1982), results of short-term mutagenicity tests for identifying potential carcinogens in foods must be interpreted with caution. The tests serve to identify food components which should be evaluated further by other toxicological techniques. From the standpoint of the Ames test, the percentage carcinogens which were mutagenic ranged from 72 to 90 depending on the types of carcinogens tested and the strain of Salmonella typhimurium used (Devoret, 1979).
Epidemiological data cannot prove causation of a site specific cancer but can provide clues to solve the problem and can be valuable in designing relevant animal experiments (Brown, 1983; Hirayama, 1981). With the epidemiological correlation methodology, food consumption data can be related to cancer incidence data by site. Case control studies deal with dietary history (quantity, frequency) of individuals in relation to site specific cancer. Demographic studies in descriptive epidemiology involve the evaluation of the association of ethnic lifestyles and cancer patterns (Hirayama, 1981).

Several unintentional components in food have been found to be potent carcinogens and mutagens. Fungal metabolites such as aflatoxins which cause cancerous tumours in animal livers were isolated from nuts (Doll, 1980). Nitrosamines, found in some cured meats, induced cancerous growth in the stomach, large intestine and esophagus in experimental animals (Scanlan, 1983). These unintentional components in food have been studied as risk factors in cancer induction by epidemiological methodology. Linsell (1977) showed a significant dose-response correlation between the estimated ingestion of aflatoxin contaminated food and human liver cancer. According to Hill (1979), gastric, colon and urinary cancer in humans may be caused by nitrosamines. In an epidemiological study by Tannenbaum et al., (1978a), an association between nitrosamines and esophageal cancer development was put forth.

Many researchers have suggested that some foods are implicated in the incidence of certain types of cancer. Meat consumption has been strongly correlated with colon cancer incidence and fat consumption has been associated with colon, breast and uterus cancer mortalities (Doll, 1980).
The validity of the meat-colon cancer association has been strengthened by the results of an investigation showing that Seventh-Day Adventists, who are generally ovo-lacto vegetarians have a mortality rate of colon cancer much below that for the general population in California (Phillips, 1975; Phillips and Snowdon, 1983). Weak non-significant associations were observed between cancers of the breast and prostate and meat consumption.

Haenszel et al. (1972) and Haenszel et al. (1976) suggested that geographical differences in cancer incidence could be attributed primarily to dietary factors. When the cancer rates were standardized to 100 for the Japanese population, the relative incidence of stomach cancer for Americans of Japanese descent was 38, close to that for U.S. whites (Haenszel et al., 1972). These investigators also found that the development of cancer of the large intestine was low in Japan in contrast to a high incidence (2.9 times greater) for Japanese descents in the U.S.A. Migrant populations in general attain, after one or two generations, a cancer rate which is similar to that of the population in the adopted country (Brown, 1983). One prominent difference between the lifestyle of the population in the country of origin and that in the U.S.A. is diet.

In Hawaii, a wide range in cancer incidence rates exists among several ethnic groups (Caucasian, Japanese, Chinese, Filipino and Hawaiian). For example, Hawaiians have the highest rate of stomach cancer but the lowest colon cancer rate. Filipino women have the highest incidence of thyroid cancer whereas they have the lowest rate of breast cancer (Kolonel et al., 1983). Differences in dietary patterns have been associated with the incidences of cancer in these ethnic populations.
Action of Carcinogens

Carcinogenesis is a complex biological process which may occur by two mechanisms, namely epigenetic events and genetic alterations. The epigenetic events include hormonal imbalances, irradiation, irritant solids and tissue injury. The genetic alteration events involve the interaction of chemicals or activated forms of chemicals with DNA. The two-stage theory of carcinogenesis includes the initiation process with DNA damage and the promotion process which may be associated with epigenetic events (Trosko and Chang, 1978).

Three basic events are involved in chemically-induced neoplastic transformations in the body: 1. metabolic activation of chemical agents which would otherwise be unreactive, 2. interaction of the agents with macromolecules in target cells, and 3. expression of the neoplastic phenotype.

When absorbed into the body, those chemical agents that are not direct reacting compounds (procarcinogens) are activated metabolically by cytochrome P-450 monooxygenases (mixed function oxidases) which are a mixture of enzymes bound in the membranes of microsomes. Marked qualitative and quantitative differences in monooxygenases in various tissues and species have been reported (Weekes and Bruswick, 1975; Selkirk et al., 1976).

These differences may determine the level of detoxification or activation of carcinogens in different species and the susceptibility to cancer induction. Examples of enzymatic activation of procarcinogen are the conversion of polycyclic aromatic hydrocarbons to diol epoxides (Sims et al., 1974), the aromatic amines to aryl hydroxylamines (Miller and Miller,
1979), and the nitrosamines to \( \alpha \)-hydroxynitrosamines (Magee, 1982). These conversions render the original hydrophobic compounds more water-soluble with the introduction of hydroxy groups.

According to Miller and Miller (1977), all known carcinogens are electrophilic compounds which have the ability to interact with nucleophiles (DNA, RNA, proteins) at sites within cells. The interaction of a carcino­genic electrophile with a nucleophile can be considered as the initiation event.

The types of electrophiles formed from a procarcinogen in the liver are governed by the number of accessible groups which can be attacked by the mixed function oxidases (Dipple, 1983). Benzo(a) pyrene (BP) has several reactive groups for epoxidation and hydroxylation. BP metabolites can be derived from two microsomal mechanisms: 1. epoxide intermediate pathway, and 2. free radical pathway. The epoxide intermediate pathway has been shown to be significant in the creation of an ultimate carcinogen (Selkirk and MacLeod, 1982). 9, 10-, 4, 5-, and 7, 8- epoxides are formed by aryl hydrocarbon hydroxylase action on BP and are either opened because of ring strain to phenols or converted to dihydro-diols by mixed function oxidases. At the labile double bond at the 9,10 position, an epoxide bond is formed. Finally the diol-epoxide is converted to an electrophilic trihydroxy carbonium compound which can interact with DNA.

Dietary fatty acid hydroperoxides may be carcinogenic in the gastro­intestinal tract (Cutler and Schneider, 1974). Peroxidation of polyun­saturated lipids proceeds in animal tissues with low levels of tocopherols
and selenium. Cytochrome P-450 oxidases presumably decompose lipid hydroperoxides in vivo into free radicals (O'Brien, 1981). Free radicals have been considered by some researchers as contributors to cancer induction (Pryor, 1982; Johnson, 1982, 1983). Lipid hydroperoxide may activate some procarcinogens by free radical oxidation. For example, benzo(a) pyrene (BP) can be oxidized to a highly mutagenic compound, 7,8-dihydroxy-9,10-epoxy-7,8,9,10 tetrahydrobenzo(a)pyrene in the presence of 13-hydroperoxy linoleic acid and hematin (Dix and Marnett, 1981).

Carbon tetrachloride is effective in bringing about lipid hydroperoxidation in animals. Presumably homolytic cleavage of carbon tetrachloride by cytochrome P-450 enzymes in the liver creates CCl₂ free radical which initiates the lipid hydroperoxidation (Kornbrust and Mavis, 1980). The decomposition of lipid hydroperoxides leads to active compounds such as the hydroxy free radical, HO*, which can damage DNA (Ames, 1983; Winston and Cederbaum, 1982).

The cytochrome P-450 enzyme system is responsible for the initiation of endogenous lipid peroxidation in microsomal membranes of liver (Gelboin et al., 1980). In vitro studies have shown that this initiation reaction requires NADPH and molecular oxygen as well as a small amount of inorganic iron and ADP. The primary free radicals which initiate lipid hydroperoxidation are superoxide anion O₂⁻ and hydroxy free radical HO* (Fridovitch, 1974).

Cytochrome P-450 reductase and FAD monooxygenase can generate the superoxide anion in a lipid system. The superoxide anion in respiring cells can be eliminated by superoxide dismutase with the production of hydrogen...
peroxide and oxygen (Brawn and Fridovitch, 1980). The reaction is very rapid under physiological pH conditions. Superoxide is a toxic agent in cells by inactivating enzymes, oxidizing polyunsaturated fatty acids and nicking DNA (Brawn and Fridovitch, 1980). With no superoxide dismutases present, cells would be killed. Superoxide in combination with H\textsubscript{2}O\textsubscript{2} can produce a very reactive oxidant: $\text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \text{OH}^- + \text{HO}^- + \text{O}_2\cdot$.

The second phase in the process of chemical transformation involves interaction of electrophiles, either direct acting or activated, with the cellular macromolecules, including RNA, DNA, and proteins. This interaction has been extensively studied as it is presumed to be the critical event in mutagenicity and carcinogenesis (Miller and Miller, 1977). The number of adducts formed with DNA depends on the type of carcinogen. Aflatoxin B\textsubscript{1} produces primarily a single major adduct in DNA (Croy et al., 1978; Hemminki et al., 1979) while other compounds such as benzo(a)pyrene diol epoxide and N-methyl-N'-nitro-nitroso guanidine (MNNG) interact with DNA at multiple sites (King et al., 1975; Singer and Lijinsky, 1976; Eastman and Bresnick, 1979). With MNNG, as many as 15 different DNA reaction products have been identified (Singer and Lijinsky, 1976).

Individuals afflicted with the rare genetic disorder Xeroderma pigmentosum are predisposed to skin cancer when exposed to ultraviolet light. Their cells in culture have been found to be deficient in excision-type repair. This observation lends support to the potential role of altered DNA in the carcinogenic process (Cleaver et al., 1975). A battery of cellular repair mechanisms, dependent on the types of lesions induced by the carcinogens, have been proposed (Regan and Setlow, 1974; Higgins, 1976). DNA repair capacity differs greatly among various cell types.
Although much information has accumulated concerning the active metabolites of various carcinogens and their interaction with DNA, comparatively little is known concerning the steps that lead to the expression of morphological transformation and ultimately to tumorigenicity. Although many structural and functional changes have been described, it has not yet been possible to find an unequivocal marker related to a specific step in neoplastic development. It is evident, however, that the development of neoplastic state is a multistep process both in vitro and in vivo.

Farber (1980) studied the in vivo model of liver carcinogenesis. The preneoplastic phase included the initial induction of molecular alterations, a relatively long period of growth and selection of putative initiated cells forming foci with constitutive enzyme alteration. A premalignant phase follows in which "initiated" cells acquire some autonomy represented by appearance of hyperplastic nodules which invade and ultimately metastasize.

In most cases, the relationship between morphological alterations and other markers of neoplastic transformation is uncertain. Furthermore, most of the properties expressed by transformed cells can be dissociated from their in vivo growth potential. No obligatory step has yet been found in the multistep progressive nature of neoplasia, either in vitro or in vivo (Sivak et al., 1982).

**Nitrosamines**

\[
\begin{array}{c}
R_1 \\
O=N-N-R_2
\end{array}
\]

N-nitroso compounds, \(O=N-N-R_2\), can be classified as chemically-stable nitrosamines with \(R_1\) and \(R_2\) usually as alkyl groups and reactive unstable nitrosamides with \(R_1\) as an alkyl group and \(R_2\) as an acyl group. About 90%
of the more than 300 different N-nitroso compounds tested have been found to be carcinogens (Preussman et al., 1982) and have been shown to produce oxygen radicals (Scanlan, 1983).

N-nitrosodimethylamine and N-nitrosodiethylamine added to the diets of about 30 animal species including subhuman primates cause cancer induction at low dosage levels (Preussman et al., 1982). N-nitroso compounds have induced liver, gastrointestinal, stomach, urinary and bladder cancer in laboratory animals (Andrews et al., 1978; Bartsch and Montesano, 1976; Ziegler and Sheldon, 1978). Epidemiological data has shown a possible correlation between the intake of nitrates and nitrites and human gastric cancer formation.

Interestingly, the East Anglia region of Great Britain has the highest average figures for water nitrates in England and the lowest gastric cancer rates (Cairns, 1981). Gastric cancer is declining throughout the world despite increasing use of nitrate fertilizers and increased consumption of vegetables, which are the most important dietary sources of nitrates.

Many investigations have been carried out to determine the presence of N-nitrosamine compounds in the food supply within many countries and to examine the conditions under which nitrosamines are formed.

Nitroso compounds have been found in a wide variety of commodities such as cured meat products, fried bacon, beer and cheese (Scanlan, 1983; Challis et al., 1981; National Acad. Sci., 1982). Nitrites are formed from ingested nitrate by bacterial enzymatic reduction action in the salivary duct, bladder, and in the hypoacidic stomach (Tannenbaum et al., 1978a, 1978b and Bruce et al., 1979). Beets, celery, lettuce, spinach, radishes and rhubarb all contain about 200 mg of nitrate per 100 g portion (Ames,
1983) and may contribute to nitrite production in the digestive tract of humans if ingested.

Certain compounds, such as vitamins, tannic acid derivatives, lipids, and sorbic acid that may be present in foods, have been found to inhibit the formation of nitrosamines in vitro (Gray and Dugan, 1975; Kurechi and Kikugawa, 1979; and Bogovski et al., 1972). Rooma and Uiba (1983) reported that low fat milk and milk products inhibit nitrosation and the formation of N-nitrosodimethylamine. Stich and Rosin (1983) found that certain phenolic compounds reduced the in vivo formation of mutagenic and carcinogenic N-nitroso compounds.

**Polycyclic Aromatic Hydrocarbons**

Lijinsky and Shubik (1964) were the first to find that benzo(a) pyrene (BP) and related polycyclic aromatic hydrocarbons (PAH) were present in charcoal broiled beef. PAH are generated through incomplete combustion of materials containing carbon and hydrogen. Instead of being completely oxidized to carbon dioxide, hydrocarbon reaction products can interact with one another to build up complex polycyclic structures (Dunn, 1982). Badger (1962) suggested that complex aromatic hydrocarbons are formed at high temperatures from aliphatic hydrocarbons or from simple aromatic hydrocarbons by free radical reaction. Carbon-hydrogen and carbon carbon fission can occur to give "primary" radicals, which combine in various ways to give less hydrogenated, more condensed products.
Some PAH carcinogens, that have been found in foods, are carcinogenic in a variety of animal species (Pierce, 1961; Lo and Sandi, 1978; I.A.R.C., 1973). PAH-containing materials such as shale oil distillate and creosote possess carcinogenic activity as do air pollution particulates, cigarette smoke and coke oven emissions. Some studies have suggested that the ingestion of PAH-containing food is associated with human cancer (Dipple, 1983).

PAH carcinogens are widespread in a variety of commonly-consumed foodstuffs such as vegetables, fruit, grain and some meat and fish products (Dunn, 1982). PAH in foods can arise from either environmental contamination of foods during their primary crop production or by the use of smoking processes (Dipple, 1983). Fish, crustaceans and shellfish may come in contact with PAH-containing water which was contaminated with petroleum products (Dunn and Fee, 1979).

The majority of PAH compounds are detoxified through various metabolic modifications in the liver and subsequently excreted in the urine. The initial metabolic changes in PAH are brought about by the membrane-bound mixed function oxidase system (Nerbert & Gelboin, 1969). Until recently, it appeared that only one form (an inducible microsomal enzyme) of epoxide hydrolase was present within a given tissue, but recent studies have shown that several forms of this enzyme are present (Guenthner, et al., 1981; Guengerich et al., 1979). The oxidative transformation of BP is effectively catalyzed by the Cytochrome P-450 mixed function oxidase enzyme system. Cytochrome P-450 system consists of a family of heme proteins which differ in their physical and enzymic properties and each is apparently under
separate genetic control (Dipple et al., 1983). The relative amounts of the various forms of the mixed function oxidases, epoxide hydrolase and the conjugating enzymes determine the metabolic pathway for PAH. The pathways of BP metabolism are diverse and lead to the conversion of BP to more than 40 different metabolites including phenols, dihydrodiols, quinones, and water-soluble sulphate, glucuronide and glutathione conjugates. In addition, BP is converted to diol epoxides which are considered the major carcinogenic and mutagenic intermediates of BP metabolism (Gelboin et al., 1980; Capdivilla et al., 1980). However, this does not negate the fact that the majority of any administered hydrocarbon is detoxified through various metabolic modifications to water-soluble metabolites which are excreted in the urine.

Figure 1 is a summary of our present knowledge of BP metabolism. BP is converted by the mixed function oxidases to three unstable epoxide intermediates and phenols. The phenols arise largely through epoxide rearrangement but can also be formed by direct oxygenation. The phenols can be changed to quinones. All of these oxygenated intermediates can be further metabolized to water-soluble products by conjugation with either glutathione, sulphate or glucuronic acid. Almost all of these metabolites are detoxification products and exhibit little or no DNA binding, or carcinogenic activity (Dipple, 1983, Capdivilla et al., 1980).

Sims et al. (1974) showed that the DNA-binding metabolites of benzo(a)pyrene are 7,8- dihydrodiol-9,10- epoxide. Unlike other hydrocarbon epoxides, this dihydrodiol-epoxide is a poor substrate for epoxide hydrolase; such a situation may account for the resistance of this compound to further metabolic breakdown.
Figure 1 - Metabolic activation of benzo(a)pyrene. (Capdivella et al., 1980)
Aflatoxins

Aflatoxins are polycyclic mycotoxins produced as metabolites by several species of the Aspergillus molds (A. flavus and A. parasiticus). By definition, mycotoxins are toxic secondary products, resulting from the metabolism of nutrients in molds. These fungi are vigorous competitors with other organisms on stored food products when the temperature is high (30-40°C) and the substrate moisture is low (Aw = 0.85-0.90)(Stoloff, 1976). Mold metabolites such as aflatoxins have been found in grains and peanuts when held under conditions of high humidity and temperature.

Aflatoxins exist in several forms, including aflatoxin B₁, B₂, G₁, G₂, M₁, M₂ and P₁ as shown in Figure 2. The major toxin, aflatoxin B₁, is mutagenic, teratogenic and carcinogenic on the basis of a wide range of test systems and is the most potent hepatocarcinogen known. It is about 100 times more powerful than butter yellow (p-dimethylaminoazo benzene) as a carcinogen in rats (Heathcote and Hibbert, 1974). When a diet containing 5 ppm aflatoxin B₁ (AFB₁) was fed to rats for 9 weeks, liver tumour frequency rose to 100%. After feeding male rats diets with either 0.5 or 0.1 ppm AFB₁ over their lifetime, liver tumour occurrences were 100% and 50%, respectively, of the total number of animals (Butler et al., 1969). Long-term studies on monkeys failed to induce liver tumours at levels of 0.07-1.8 ppm (Cuthbertson et al., 1967) or at 62 μg/kg once per week for 2 years.

Human liver cells contain the enzymes needed to produce the aflatoxin epoxide metabolites that are the most powerful liver carcinogens known for laboratory animals. The metabolic pathway of AFB₁ in animals is shown in Figure 3. The ultimate reactive species of AFB₁ is currently believed to be AFB₁-2,3-oxide although it has never been isolated (Neal and Colley, 1978).
Figure 2 - Forms of aflatoxin.
(Garner et al., 1972)
Studies have shown that the 2,3 double bond in AFB\textsubscript{1} metabolites is required for mutagenic and carcinogenic activities (Butler et al., 1969; Wogan et al., 1971). All other metabolites without a 2,3 double bond are practically devoid of mutagenic activity (Wong and Hsieh, 1976). Other lines of evidence in support of AFB\textsubscript{1}-2,3- oxide as the ultimate reactive metabolite include the formation of covalent adducts of AFB\textsubscript{1} and nucleic acids in vivo (Swenson et al, 1974, 1977). Activated AFB\textsubscript{1} interacts with DNA guanine in vitro to form 2,3- dihydro-2-(N-guanyl)-hydroxy-aflatoxin B\textsubscript{1} (Croy et al., 1978).

All known metabolites of AFB\textsubscript{1} are less mutagenic and carcinogenic than the AFB\textsubscript{1} parental compound. Aflatoxicol, the most potent genotoxic metabolite of AFB\textsubscript{1}, has only 22.8\% of the mutagenicity of AFB\textsubscript{1} as demonstrated by the Salmonella Ames test and is the only metabolite with tumorogenic activity with respect to rainbow trout (Wong and Hsieh, 1976). AFM\textsubscript{1}, the next most potent metabolite of AFB\textsubscript{1}, exhibit only 3.2\% of the mutagenicity and one third of the tumorigenicity compared to AFB\textsubscript{1} in Salmonella/Ames test and for rainbow trout, respectively (Wong and Hsieh, 1976). All known AFB\textsubscript{1} metabolites, like AFB\textsubscript{1} itself, require metabolic activation before their mutagenic effect can be detected in the Salmonella mutagenic test. It is not known whether these metabolites are converted by enzymes back to AFB\textsubscript{1} and then further activated or whether they are activated to reactive species via different route from that of AFB\textsubscript{1}.

Aflatoxins are frequent contaminants of peanuts, maize and cottonseed, and occur in significant quantities of copra, brazil nuts and pistachio nuts. Human exposure to aflatoxins is generally through ingestion of the contaminated products. Stoloff (1976) reported that it is unlikely that
Figure 3 - Known metabolic pathway of aflatoxin B₁ in animal hosts. (Garner et al., 1972)
human exposure to aflatoxins results from the ingestion of meat of animals fed aflatoxin-contaminated feed. Aflatoxin $M_1$, a hydroxylated metabolite has appeared in milk of lactating mammals exposed to aflatoxins (Stoloff, 1976). An aflatoxin $B_1$ level of 30 mg/g in cow feed results in an aflatoxin $M_1$ level of 0.1 mg/ml in the milk from that cow. But although large amounts of milk are consumed, this human exposure to aflatoxins is negligible compared to the direct exposure from ingested plant products such as peanuts and corn.

Epidemiological studies have shown a significant dose response correlation between estimated ingestion of aflatoxin-contaminated food and liver cancer in Thailand, Uganda, Swaziland, Kenya and Mozambique (Popper, 1979). There may be a synergistic effect between hepatitis B and aflatoxin in terms of liver cancer induction. A recent study in the Philippines has shown a presumed synergy between aflatoxin and alcohol (Jayme et al., 1982). There is an extremely high incidence of esophageal cancer in North Honan, China, where Fusaria species have been found in bread. Only Penicilla species have been found in bread in the lower incidence area of China (Doll, 1980).

**Antimutagenic and Anticarcinogenic Components of Foods**

The concept of food components being protective against cancer initiation and promotion has gained considerable attention from cancer researchers and food scientists. Results of epidemiological, animal and short-term assay studies suggest that naturally-occurring antioxidants such as tocopherols, phenolic compounds and ascorbic acid inhibit carcinogenesis and mutagenesis (Rosin and Stich, 1983; Wattenburg, 1983).

Wattenburg (1983) classified inhibitors by sequence points at which they act in the carcinogenic process. Some inhibitors prevent the formation
of carcinogens from procarcinogens, while others, termed blocking agents, prevent carcinogens or mutagens from reaching or reacting with critical target sites. A third group of inhibitors, called suppressing agents, are effective when fed subsequent to administration of carcinogens.

Intake of some foods may contribute to the inhibition of cancer induction. Teas (1983) has provided epidemiological and biological data demonstrating that Laminaria (a brown kelp seaweed) consumption is associated with the relatively low breast cancer rates among Japanese and Eskimo women. Laminaria, a source of non-digestible fibre, changes post hepatic metabolism of sterols and contains an antibiotic substance that may influence fecal microbial ecology. Laminaria also contains β-1,3-glycan (a neutral particulate polyglucose) which can alter enzymatic activity of fecal flora and stimulate the host mediated immune response. Di Luzio et al. (1979) found glycan derived from the cell wall of Saccharomyces is a potent macrophage stimulant and an inhibitor of malignant tumour growth and metastases. Glycan is a major component in oatmeal.

Vitamins A, C and E appear to offer protection against free radicals and promoters in cells (Doll, 1980; Ames, 1983; Rosin and Stich, 1983). In the free radical theory of cancer, a promoter is regarded as a toxic compound or insult that depletes vitamin A and E levels in the tissues; thus the DNA and RNA of the cells may become vulnerable to attack by carcinogens (Johnson, 1982).

Several researchers have shown experimentally that vitamin A and its analogues (retinoic acid and various retinoids) can reduce the risk of chemically-induced cancer (Moon et al. 1983). β-carotene can inhibit auto-oxidation of polyunsaturated fats and phospholipids in membranes.
Calle and Sullivan (1982) found a protective effect of β-carotene against benzo(a)pyrene in rats. Carotenoids (converted to vitamin A in the body) can act as free radical traps and efficient quenchers of singlet oxygen (a reactive form of oxygen that is considered to be mutagenic and cause lipid peroxidation). Sporn and Newton (1981) have suggested this inhibition of carcinogenesis by β-carotene can be attributed to the support of cellular regulatory mechanisms by vitamin A. Heitanen (1981) attributed the reduction of lung cancer in Japan to the daily consumption of green and yellow vegetables, which contribute about 40% of the vitamin A content of the Japanese diet.

Vitamin E can act as an antioxidant for inhibiting the formation of epoxides and peroxides and is a free radical trap in cell membranes (Mergens et al., 1978). Vitamin E was found to reduce the carcinogenicity of the quinones, adriamycin and duanomycin. According to Beckman et al. (1982), tocopherols can act as protective agents against radiation-induced DNA damage and dimethyl hydrazine-induced carcinogenesis.

Some evidence to support the contention that ascorbic acid can act as antimutagenic and anticarcinogenic agents is available. Ascorbic acid has been shown to inhibit in vitro nitrosation (Guttenplan, 1977; Mirvish, 1981). Vitamin C has also been shown to be anticarcinogenic in rodents treated with UV light and with benzo(a)pyrene. Dunham et al. (1982) and Silverman et al. (1983) found no inhibitory effects of ascorbic acid on metastases from transplantable murine tumours. Wattenburg (1979) stated that antioxidants such as ascorbic acid may scavenge the oxygen required to metabolize nitrosamine and related carcinogens such as nitrosamides.
Marshall et al. (1982) hypothesized that the consumption of foods containing large amounts of vitamin A and C (with tobacco and alcohol consumption being constant) reduced the risk of oral cancer induction.

A negative correlation was found between the intake of fresh vegetables and the incidence of human gastrointestinal cancer (Graham et al., 1978; Siquel, 1983). According to Wattenburg (1983), brussel sprouts, cabbage, turnips, cauliflower and broccoli induced benzo(a)pyrene hydroxylase activity in the small intestine of rats and enhanced the metabolic deactivation of benzo(a)pyrene. This induced enzymatic activity appeared to be due to presence of indoles in the plant tissue. When indoles were added to the diet of rats, incidence of mammary tumours was reduced considerably (Wattenburg, 1979). Barale et al. (1983) reported significant inhibiting effects of vegetables such as cauliflower, spinach, and lettuce on formation of dimethyl nitrosamine and nitroso methyl urea in vitro. Chlorophyll and ascorbic acid were found to be suppressors of formation of the above-mentioned nitrosamines. Morita et al. (1978) and Kada et al. (1981) found several vegetable juices possessed strong capacities of inactivating the mutagenicity of tryptophan pyrolysis products.

Stich and Rosin (1983) reported that phenolic compounds inhibited the mutagenic activities carcinogens and procarcinogens in the presence of mixed function oxidases. Certain phenols have been reported to modulate, in vitro, the nitrosation of several amines and amides (Davies et al., 1982; and Pignatelli et al., 1979). The type and extent of such modulation depends upon phenolic structure and on the type of nitrosable substrate.

Coffee is a phenolic-rich beverage (up to 5%) with high levels of chlorogenic and caffeic acid. An average cup of coffee contains 260 mg of chlorogenic acid.
The synthetic antioxidants, butylated hydroxyanisole and butylated hydroxytoluene, can inhibit chemical carcinogen-induced neoplasia (Wattenburg, 1979). More recently, Wattenburg (1980) reported that the phenolic derivatives of cinnamic acid were also able to inhibit benzo(a)pyrene-induced neoplasia in mice.

Dietary fiber is defined as the remnants of plant cell walls that are not hydrolyzed by the digestive enzymes of man (Kritchevsky and Story, 1974). Dietary fiber includes lignins (not degraded by intestinal bacteria), cellulose which is partially degraded (15%) and hemicellulose fraction which are largely degraded (85%). Hemicelluloses are polysaccharides which consist of hexoses, pentoses and uronic acids, and possesses high water-binding capacities (Parrott and Thrall, 1978).

Burkitt (1969) first postulated that diets high in fiber are associated with a decreased risk of colon cancer. The inhibitory effect of fiber was explained by the shortened transit time of food in the intestinal tract and increased fecal bulk with the result of reduced contact of carcinogens with colon mucosa. Smith-Barbaro et al. (1981) found that certain types of dietary fiber bind carcinogens which then are rendered unavailable for the initiation process in the colon mucosa.

Dietary fiber may alter intestinal flora population and concentration of microbial metabolic products. Fiber has also been shown to inhibit enterohepatic absorption of bile acids and thereby influences cholesterol levels (Kritchevsky and Story, 1974).

Browning reaction compounds may be of value as anticarcinogenic agents in the diet of humans. Chan et al. (1982) found that caramelized sucrose
and a heated lysine-fructose solution (Maillard reaction system) possessed compounds with strong antigenotoxic activities. A dose-dependent reduction in the mutagenic activities of aflatoxin B$_1$ (with S-9 treatment) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was experienced when caramelized sucrose or the Maillard reaction system was present in suspensions of Salmonella organisms for the Ames test. Prior to plating, the microorganisms were separated from the carcinogens and browning reaction compounds by centrifugation.

Melanoidins have adsorptive and antioxidant properties which make them candidates as antimutagens and anticarcinogens. Melanoidins from heated sucrose powder were adsorbed to the surfaces of yeast cells and inhibited the activities of yeast invertase and catalase (Schvets and Slysarenko, 1976). According to Horikoski and Gomyo (1976), melanoidin, prepared from a heated glycine-glucose solution, interacted with ovalbumin to form a precipitate. Electrostatic bonding between the macromolecules was responsible for the aggregation process. Melanoidins act as potent synergists for antioxidants such as butylated hydroxyanisole and tocopherol under certain conditions (Kajimoto et al. 1975).

Reactions responsible for non-enzymic browning are commonly designated as (i) Maillard or carbonyl-amine reactions, (ii) caramelization, and (iii) ascorbic acid oxidation. Maillard reactions in aqueous systems involve the degradation of carbonyl compounds, particularly reducing sugars, in the presence of amines, such as amino acids in the free peptide and polypeptide forms (Hodge, 1953; Reynolds, 1963, 1965). With respect to caramelization
in aqueous systems, reducing sugars are decomposed in the absence of amines (Feeney et al., 1975). During the heating of sucrose, hydrolysis occurs with the formation of glucose and fructose. Caramelization reactions can occur in amino acids-containing sugar systems. Several reviews on the non-enzymic browning reactions have been published (Reynolds, 1963, 1965; Feeney et al., 1975).

Most of the initial chemical steps of the Maillard reaction sequence are the same as those for caramelization. However, the rates of the reactions (enolization and dehydration) of reducing sugars are increased considerably when amino acids are present. As the pH of the aqueous solution rises, the reaction rates in Maillard browning and caramelization increase. The pH effect can be attributed, in part, to the rapid conversion of cyclic hemiacetal forms of reducing sugars to acyclic (open-chain) carbonyls which are the initial reactive isomers in the Maillard and caramelization pathways (Hodge, 1953). Furthermore, enolization proceeds more rapidly at pH values above 4, with bases being more effective catalysts than acids.

The pathway of caramelization involves enolization of open-chain reducing sugars, dehydration, fragmentation and polymerization of reaction products to form brown pigments called melanoidins. In neutral and weakly alkaline media, enolization of sugars is rapid and dehydration is slow, whereas in acidic media, enolization is slow and dehydration is rapid. The speed of brown pigment formation is governed by the types of acidic and basic catalysts, the bases being the most effective for browning.

Regardless of pH, the first reaction in caramelization involves the formation of 1,2-enediol by abstraction of hydrogen atom on C-2 of a sugar molecule by a base to produce a hybrid monoanion. The monoanion may produce either a cis- or a trans-enediol depending on the substrate sugar.
The next step is the dehydration of the 1,2-enediols by the elimination of H and OH groups from the α- and β-carbon atoms adjacent to each carbonyl group to form a conjugated, α- and β-unsaturated carbonyl compound called 3-deoxyosone and by the elimination of H and OH groups on C-3 and C-4 to form 3-deoxyosone-3-ene.

In the first step of the Maillard reactions, an amino acid condenses with a reducing sugar to form a Schiff's base (imine) which undergoes cyclization to N-substituted glycosylamine. Then, the glycosylamine is converted to 1,2-eneaminol which is transformed to N-substituted 1-amino-1-deoxy-2-ketose. The transformation of the glycosylamine to the ketoseamine is known as the Amadori rearrangement.

A major pathway of the Maillard reaction leads from the 1,2-eneaminol to 3-deoxyhexosone intermediate. 5-Hydroxymethyl furfural and 5-methyl furfural are formed by the dehydration of 3-deoxyhexosone. Another pathway of the Maillard reaction is the conversion of the 2,3-enediol to methyl-α-dicarbonyl intermediate (1-deoxyhexosone), which undergoes transformation to aldehydes, ketones, furans, pyrroles and thiophenes. Several cyclic flavour compounds, such as maltol and isomaltol, are formed from 2,3-enediol.

Another pathway of the Maillard reaction is the Strecker degradation of amino acids. When α-dicarbonyl compounds are heated with amino acids, the amino groups reacts with a keto group to form a nitrogen-containing carboxy aldehyde. The reaction products include aldehydes from amino acids, sugar fragments, carbon dioxide and pyrazines from the transamination reaction.
Hypothesis and Objectives

In our modern society, thousands of chemical compounds are contaminating our water, air and food. Sources of these compounds include pesticides applied to crops, smoke from factories and automobiles, water from chemical manufacturing plants and metabolites of molds on edible portions of plants. Some of these contaminants are considered to be carcinogenic on the basis of epidemiological and animal studies. In particular, polycyclic aromatic hydrocarbons, aflatoxins and nitrosamines have been examined extensively as potential contributors to the incidence of human cancer.

When data on the cause of death during the past 40 years is examined for Canadians, it is apparent that deaths from stomach cancer for both males and females has declined gradually, and deaths from all other cancers, except lung cancer has remained relatively steady. Such data is surprising in light of the fact that the carcinogen load in our environment presumably has increased with increased industrial activity and chemical pest management.

Within the last 10 years, naturally-occurring inhibitors of genotoxic effects of carcinogens has been explored extensively by researchers. Antioxidants, trace elements, indoles, coumarins, phenolic compounds, fibers and browning reaction products have been shown to be inhibitors of carcinogen-induced genotoxicity and tumorigenicity. Some researchers have speculated that inhibitors of genotoxicity protect human bodies from cancer induction.

Research studies have indicated that coffee brew and other browning reaction systems have a powerful inhibitory effect on carcinogen-induced reversion in the Ames Salmonella strains (Chan et al., 1982; Powrie et al., 1984). A hypothesis to explain these observations is that carcinogens are
adsorbed on the melanoidins and other brown pigments (complexes composed of melanoidins, proteins, polysaccharides and phenolic compounds) in browning reaction systems. Experiments in this study were initiated to test this hypothesis.

The objectives of this study were to: (1) isolate and characterize the water-insoluble fraction from instant coffee brew which was previously found to possess a genotoxic inhibitory effect; (2) determine the extent of inhibition of carcinogen-induced mutagenesis with the coffee water-insoluble fraction and a model system melanoidin; (3) determine the binding of benzo(a)pyrene and aflatoxin B₁ with the coffee water-insoluble fraction and elucidate the type of binding involved; (4) determine the effect of the model system melanoidin on the inhibition of benzo(a)pyrene-induction of aryl hydrocarbon hydroxylase in the large intestine of rats.
CHAPTER 1

CHARACTERIZATION OF THE WATER INSOLUBLE FRACTION IN COFFEE BREW

Introduction

Coffee was first used as a food, the berries being consumed whole or crushed and mixed with fat (Roberts and Barone, 1983). Around 1000 A.D., Arabians were consuming hot coffee brew, but this stimulatory beverage did not reach Europe until the 17th century. An Ontario study (Gilbert, 1974) indicated that the average consumption of coffee by individuals of 15 years of age and above was 2.6 cups per day. It was estimated that about 18% of the population drank 10 or more cups per day. The greatest average amount of coffee brew (about 3.5 cups daily) was consumed by individuals in the 35 to 39 age group. In a 1982 coffee drinking study (I.C.O., 1982), the average number of cups of coffee consumed per person per day in the U.S.A. was 1.9, which is a decrease from 2.35 cups in 1972. Further, 53.9% of coffee drinkers over 10 years of age consumed 3.98 cups per day. Decaffeinated coffee drinkers have increased from 1 out of 25 persons in 1962 to 1 out of 7 in 1982.

Coffee beans are seeds of the berry (cherry) of the coffee tree which belongs to the Madder family, Rubiaceae. The species which are of primary importance in the commercial production of coffee beans are Caffea arabica, C. robusta and C. liberacea (Yamanishi, 1981). Each berry is composed of several seeds embedded in a moist (67% water) cellulosic pulp which is surrounded by a thin layer of epidermal tissue. For harvesting, the berries
must reach a stage of ripeness when the pulp is soft enough to be removed easily from the coffee seeds (Sivetz and Desrosier, 1979).

Pulp is removed commercially either by the washed wet process or by the natural dry process. In the washed process, ripe fruit soaked in water about 1 day are squeezed to remove most of the pulp and the remaining gummy cellulosic residue on the beans is removed with the aid of fermentation and washing. In the natural dry process, fruit is held on the tree for partial drying, then dried to about 12% after harvest and subsequently passed through a hulling machine to remove the dry pulp. Generally, washed coffee beans are superior in aroma when roasted compared to those treated by the natural dry process (Sivetz and Foote, 1963; Sivetz and Desrosier, 1979).

Drying of the green beans may lead to deterioration of quality (Amorim and Amorim, 1978). At drying temperatures in the vicinity of 55°C, polyphenol oxidases can be activated to produce undesirable flavour compounds. Chlorogenic, neochlorogenic and isochlorogenic acids in the coffee bean may be the substrates of the oxidases.

Green coffee beans do not have a pleasant aroma. Acrid and sour are terms used to characterize their odour. When the beans are subjected to temperatures above 180°C, a typical coffee aroma develops during roasting and their colour changes from green to brown, the depth of colour depending upon the temperature and time of the roast (Sivetz and Desrosier, 1979). Free water (10-12%) in green beans is lost very rapidly at the beginning of the roasting period and the temperature rises up to as high as 200°C. Exothermic pyrolytic reactions are accompanied by the puffing of the bean (Feldman et al., 1969). The heat liberated from beans during roasting can be estimated roughly by measuring the amount of CO₂ evolved. The exothermic
heat of pyrolysis for a normal roast of coffee beans is around 100 cal/g (Sivetz and Desrosier, 1979), but for darker roasts, the values may be as high as 167 cal/g.

The average composition of green and roasted coffee beans are presented in Table 2. The major constituents in both the raw and roasted beans are hemicelluloses, cellulose, fat, protein, sucrose, chlorogenic acid and lignin. In the green coffee bean, the fat content varies from 10-17%, the sugar concentration ranges between 5 and 10% and chlorogenic acid values are usually between 5 and 8% (Feldman et al., 1969). Variations in composition will occur within a given species by the region where the plant is grown, by cultural practices and by harvesting, processing and storage conditions (Amorim and Amorim, 1978).

During roasting, the major water-soluble constituents of coffee (proteins, sucrose, chlorogenic acids, trigonelline and ash) as well as some water-insoluble fractions react to produce decomposition products (Table 3), some being water-soluble whereas others are water-insoluble (Clifford, 1975a; 1975b; Sivetz and Desrosier, 1979). The intact coffee bean has been likened to a tiny autoclave where reactive sugars and amino acids can combine during roasting to produce numerous substances. Over 500 components have been identified in the volatile fraction in roasted coffee (Yamanishi, 1981). Baltes et al. (1981) stated that approximately 30% of the non volatile constituents in roasted coffee were unknown. The nature of these volatile compounds in roasted coffee indicates the occurrence of Maillard reaction and in particular the Strecker degradation reactions (Sivetz and Desrosier, 1979).
### Table 2

**Composition of Green and Roasted Coffee Beans**

<table>
<thead>
<tr>
<th>Component</th>
<th>Green (%)</th>
<th>Roasted (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemicelluloses</td>
<td>23.0</td>
<td>24.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>12.7</td>
<td>13.2</td>
</tr>
<tr>
<td>Lignin</td>
<td>5.6</td>
<td>5.8</td>
</tr>
<tr>
<td>Fat</td>
<td>11.4</td>
<td>11.9</td>
</tr>
<tr>
<td>Caffeine</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>Sucrose</td>
<td>7.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Chlorogenic Acid</td>
<td>7.6</td>
<td>3.5</td>
</tr>
<tr>
<td>Protein (based on non alkaloid N)</td>
<td>11.6</td>
<td>3.1</td>
</tr>
<tr>
<td>Trigonelline</td>
<td>1.1</td>
<td>0.7</td>
</tr>
<tr>
<td>Reducing Sugars</td>
<td>0.7</td>
<td>0.5</td>
</tr>
<tr>
<td>Unknown</td>
<td>14.0</td>
<td>31.7</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>100.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

^1 dry weight basis
The roasting of green coffee beans decomposes the carbohydrates, particularly sucrose (Feldman et al., 1969). With the pyrolytic decomposition of carbohydrates, 2, 3, 4 and 5 carbon fragments are created. Recombination of these fragments is probably directly responsible for the formation of aromatic constituents (Gianturco, 1967). Several furan derivatives such as alkyl cyclopentadione, cyclic diketones and maltol have been identified. Their origin can be attributed to the pyrolytic decomposition of the reducing sugars (Reymond, 1978; Yamanishi, 1981). Sucrose, which constitutes about 7% of the green coffee bean, is almost entirely decomposed during roasting. The sucrose is hydrolyzed to reducing sugars which are decomposed at pyrolytic temperatures to brown pigments, volatile organic compounds and carbon dioxide gas (Feldman et al., 1969). Gianturco (1967) showed that, when sucrose was pyrolyzed, compounds were formed with the same structures as those found in roasted coffee. Upon roasting coffee beans, the arabinose and galactose contents decreased markedly and mannose was released from galactomannan.

The protein content of green coffee beans is approximately 12% but roasting decreases it to around 3% (Feldman et al., 1969). Roasting of coffee beans brings about the formation of several low molecular weight products, such as amino acids, ammonia, amines, hydrogen sulphide, methyl mercaptan, dimethyl sulphide and dimethyl disulphide. Some of these react with carbohydrates and their degradation products to form aliphatic aldehydes, furfural methyl sulphide, pyrazines, pyroles and thiophenes (Gianturco, 1967). Roasting denatures and coagulates most of the bean proteins which break down with the formation of cyclic structures such as pyrole and indoles (Feldman et al., 1969). Examination of the free and
Table 3

Carbohydrates in Roasted Coffee Beans

<table>
<thead>
<tr>
<th></th>
<th>Soluble Fraction</th>
<th>Insoluble Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reducing Sugars</td>
<td>1-2</td>
<td>-</td>
</tr>
<tr>
<td>Caramelized Sugar</td>
<td>10-17</td>
<td>0-7</td>
</tr>
<tr>
<td>Hemicelluloses</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>Fiber (hydrolyzable)</td>
<td>-</td>
<td>22</td>
</tr>
</tbody>
</table>

*Sivetz and Desrosier (1979)*
bound amino acids showed that arginine, cysteine, lysine and serine are largely destroyed (Amorim and Amorim, 1978).

Roasting affects the phenolic content of the green coffee bean. Phenolic compounds are produced from thermal degradation of carbohydrates, chlorogenic acid and lignin substances (Yamanishi, 1981; Tressl et al., 1979). Phenolic derivatives are formed by thermal degradation of p-coumaric, ferulic, sinapic or caffeic acids which are found in green coffee beans. Ferulic acid, for example, is decarboxylated to 4-vinyl guiacol which may be transformed into vanillin. With a darker roast, phenol, cresols and 4-ethyl guiacol contents increase considerably and 2, 3-, 2, 5-, and 3, 4- dimethyl phenols appear (Tressl et al., 1979). Phenol, catechol, hydroquinone and pyrogallol are major reaction products of heated quinic acid (Tressl et al., 1979). Thermal degradation of caffeic acid yielded catechol (58%), 4-ethylcatechol (38%), 4-vinylcatechol (0.8%) and 3,4 dihydroxycinnamaldehyde (2%). Chlorogenic acid undergoes hydrolysis to caffeic acid and quinic acid (Tressl et al., 1979) and small amounts of phenol and catechol are formed.

Trigonelline, the inner salt of 3 carboxy-1 methylpyridinium hydroxide is present at the 1% level in green coffee, and is decomposed to pyridines and pyroles upon coffee bean roasting (Reymond, 1978). Monoterpenes such as myrcene, limonene, linalool and linalool oxides probably are formed during roasting by thermal breakdown of higher terpenes which are known to occur in the lipid fraction of green coffee beans (Yamanishi, 1981).

Caffeine for the most part is unaffected by roasting, but may decrease slightly when small quantities become sublimated and accumulate in roaster stacks (Feldman et al., 1969).
High molecular weight brown colored complexes in roasted coffee bean have been termed humic acids (Clifford, 1975b). Humic acids are produced by the interaction of proteins, polysaccharides and chlorogenic acids. Water-dispersible humic acids in coffee brew are a group of complexes formed by the enzymic oxidation of phenolic compounds and by the Maillard and caramelization reactions. According to Clifford (1975b), dried coffee brew (instant) contained 15% humic acids. Humic acids in coffee brew have been isolated by dialysis (Clifford, 1972) and by acetone precipitation (Casier et al., 1978).

According to Casier et al. (1978), a brown precipitate developed when acetone was added to a concentrated coffee brew with added glacial acetic acid. A major brown pigment with a high molecular weight and a minor brown pigment with a much lower molecular weight were separated when the water dispersed acetone precipitate (FI fraction) was chromatographed on Sephadex G-25. When the FI fraction was subjected to chromatography on Sephadex G-200, only one diffuse brown zone was noted. The molecular weight of this major brown pigment of the FI fraction was estimated to be around 200,000. FI fraction from coffee brew contained 2.38% nitrogen, 24.58% sugars, 9.38% ash and 51.19% brown colored pigments. The sugars included arabinose and galactose. The FI polymers were charged negatively in the alkaline pH range and did not move in an electrophoretic unit at acidic pH values.

According to Clifford (1975b), studies have shown that protein-polysaccharide and protein-chlorogenic acid fractions are present in coffee humic acids. Galactose, mannose and arabinose have been found in the hydrolyzate of polysaccharide-containing fractions (Clifford, 1972).
According to Maier et al. (1968), constituents in coffee brew could be separated on a nylon powder column into water-soluble fraction (N₁) and 1% NH₄OH-soluble fraction (N₂). These two fractions were subjected to gel filtration on Sephadex G-25 for further fractionation. Eight brown-colored fractions resulted from the chromatography of coffee brew. Two of the fractions contained Maillard reaction products with molecular weights between 5,000 and 10,000. One of these two brown-colored fractions contained about 19% galactomannan. One of the Maillard reaction fractions contained 13 different amino acids with glutamic acid, aspartic acid and leucine being the predominant acids.

Aurich et al. (1968) separated 17 amino acids from the acid-hydrolyzed humic acid fraction of reconstituted coffee brew powder. The major amino acids were glutamic acid, aspartic acid, glycine, leucine, and proline.

Klocking et al. (1971) separated humic acids from low molecular weight components in reconstituted coffee brew powder by gel filtration on Sephadex G-25. The isolated humic acids were hydrolyzed in an alkaline solution to release phenolic compounds, which included 3,5-dihydroxy benzoic acid, caffeic acid, 3-hydroxy benzoic acid, 4-hydroxybenzoic acid, ferulic acid and vanillic acid. Klocking et al. (1971) assumed that the phenolic compounds in the hydrolysate were similar in structure to those in the unhydrolyzed humic acid. Caffeic acid was considered to be a degradation product of chlorogenic acid.

Maier and Buttle (1973) found that galactomannan, in a brown colored fraction of coffee brew, could be removed from brown pigments by repeated freeze drying of the aqueous dispersion. The brown pigment fraction was considered to be made up of polymer homologs with molecular weights from
5,000 to 50,000. The results of the elemental analysis of their brown pigment fraction were C 39.50%, H 5.15%, N 1.60%, O 50%. When the brown pigment fraction was hydrolyzed with mineral acids and several enzymes, mannose, galactose and arabinose were detected as well as amino acid residues which may be attached to each other in humic acid.

Polyphenol oxidase (O-diphenol oxidase) in green coffee beans is considered to be responsible for the formation of melanin during the drying and storage of beans (Amorim and Amorim, 1978). Diphenols in beans are converted to quinones which polymerize to create high molecular weight brown pigments. So far, no investigations on the level of melanins in roasted coffee beans or the brew have been reported.

Water-dispersible brown pigments from cured Burley tobacco are formed by enzymatic oxidation of chlorogenic acids and rutin (Wright et al., 1964). The non-dialyzable fraction of the brown pigment dispersion contained an iron-protein-chlorogenic acid-rutin complex with a molecular weight in the order of 20,000 to 30,000. Another brown pigment in the same non-dialyzable fraction was associated with a polysaccharide.

The objectives of this research investigation were to isolate the water-insoluble fraction (WIF) from a coffee brew prepared by reconstitution of spray-dried instant coffee and to assess the chemical characteristics of WIF.
MATERIALS AND METHODS

Chemicals

Folin-Ciocalteau reagent and 2,6 dichlorophenolindophenol were purchased from BDH Chemical Co. (Poole, U.K.). Caffeic acid was purchased from Sigma Chemical Co. (St. Louis, Mo.). Sephacryl S-1000 superfine gel was purchased from Pharmacia (Bromma, Sweden). Ampholytes were purchased from LKB (Stockholm, Sweden).

The following molecular weight standards were purchased from Pharmacia: dextran T-500, T-70 and T-40. Blue dextran 2000, dextran T-100 and cytochrome C were purchased from Sigma Chemical Co. All other chemicals were reagent grade.

Preparation of Water Insoluble Fraction of Coffee

Water insoluble fraction (WIF) of coffee was prepared by ethanol precipitation of colloidal particles from reconstituted spray dried coffee (Nescafe) and centrifugation of the water dispersion of the ethanol precipitate as outlined in Figure 4. A 20 g of dry coffee powder (1.4% moisture) was dissolved in 40 mL distilled water, then 360 mL of absolute ethanol was added. After 5 min the mixture was centrifuged in an IEC table top centrifuge (Centra 7 head) at 10,000 X g for 5 min. The supernatant was discarded and the precipitate was dispersed in 80 mL distilled water. The dispersion was centrifuged in a Sorvall RC-5B centrifuge (SS-34 rotor) at 11,200 X g for 1 hr and the residual precipitate dispersed in water was freeze-dried.
Figure 4 - Preparation of the water-insoluble fraction (WIF) from reconstituted spray dried coffee.
Non-metallic Elemental Analysis

Carbon, hydrogen, nitrogen and oxygen in WIF samples were determined with a Carbo Erba model 1106 automatic analyzer. Sulphur was determined by a titrimetric method (Ma and Ritter, 1979). For carbon, hydrogen and nitrogen measurements, each sample was weighed in a tin container and heated in a combustion reactor at 1010°C. The combustion gases were carried by helium gas to a catalytic section of the analyzer for complete oxidation to CO₂, H₂O, N₂ and NₓOᵧ. This gas mixture was passed into a second reactor (645°C) which was filled with copper powder to reduce the nitrogen oxides and to remove oxygen. The N₂, CO₂ and H₂O gases were separated on a column (Porapak Q.S.) and detected with a thermal conductivity detector.

Oxygen in WIF samples was determined as follows: a sample was weighed in a silver container and placed in a pyrolysis reactor (1087°C). After the quantitative conversion of oxygen to carbon monoxide (by means of an activated carbon bed), the pyrolysis gases were passed over a halogen trap for separation of CO which was detected by a thermal conductivity detector.

The sulphur content of WIF was determined as follows: the sample was weighed and wrapped in filter paper (Whatman ashless #42) and combusted in an aerated oxygen flask in a platinum holder. The SO₂ gas was bubbled into a peroxide solution to form sulphate. This sulphate solution was passed through a cation exchange column to remove metals which would interfere with titration endpoint. Titration was performed with barium perchlorate with an end point indicator of thorin (0.2%) and methylene blue (0.1%).
Metallic Elemental Analysis

Mineral analysis of coffee-WIF was carried out with a Jarrell Ash model 975 inductively coupled argon plasma spectrograph (Willard et al., 1974). This instrument was fitted with 35 analytical channels, a spectrum shifter background correction system and a Digital Equipment Corp. PDP 8M computer. The instrument was calibrated before each analytical run.

One g of WIF sample was added to a solution of 100 mL H$_2$O, 5 mL HNO$_3$ and 5 mL HClO$_4$ and the mixture was digested at 400-500°C until fumes evolved.

Flowing argon gas was heated to a plasma by radio frequency radiation passing through an induction coil. When a sample solution aerosol was introduced into this argon plasma, excited atoms were created at 5000 - 8000°K. Electromagnetic energy was emitted at wavelengths characteristic of chemical elements. The electromagnetic radiation was directly proportional to the number of atoms in the sample and monitored by a photomultiplier tube.

Determination of Molecular Weights of WIF Components

The average molecular weight of components in WIF was determined by gel filtration (Andrews, 1970). Sephacryl S-1000 superfine mixed with 0.15 M phosphate buffer (pH 7.0) was packed in a 1.2 x 27 cm glass column. The eluant was 0.15 M phosphate buffer (pH 7.0). Eluant was collected by a Gilford FC80K fraction collector (0.5 mL/tube) and absorbance of the eluant was read at 370 nm with a Perkin Elmer Lambda 3. The following dextran
standards were run at least 5 times each; blue dextran 2000 (mol. wt. $2 \times 10^6$), dextran T-500 (mol. wt. 500,000), dextran T-100 (mol. wt. 123,000), dextran T-70 (mol. wt. 70,000), dextran T-40 (mol. wt. 40,000) and cytochrome C (mol. wt. 12,384). The molecular weight of each WIF fraction was estimated from a linear regression plot of log molecular weight standards against fraction number.

**Reductone Content of WIF**

Reductone content of samples was determined by the colorimetric method using a butanol solution of 2,6-dichlorophenolindophenol (DPI) according to Kato et al. (1968). Weighed samples of WIF were suspended in a mixture of 2% metaphosphoric acid (10 mL) and acetate buffer (2 mL) at pH 3.5. Ten mL of a DPI butanol solution containing 0.3 mg DPI was added to the mixture and shaken for 5 min. Absorbance was measured at 540 nm on a Perkin-Elmer Lambda 3 spectrophotometer. Reductone concentrations of each sample was estimated from a standard curve for ascorbic acid.

**Phenolic Content of WIF**

Total phenolic content of WIF was determined by the method of Singleton (1981). One mL dispersion of WIF (0.5 mg/mL) was added to 0.5 mL Folin-Ciocalteau reagent (diluted 1:2 with distilled water) and 1 mL of 20% Na$_2$CO$_3$ solution. The sample was incubated at 50°C for 5 min and absorbance was read at 775 nm with a Bausch and Lomb model 21 spectrophotometer. Total phenolic content was expressed as mg caffeic acid per g WIF.
Isoelectric Focusing

Isoelectric focusing was utilized for separating the components of WIF. The procedure was similar to that of O'Farrell (1975), but only one dimensional electrophoresis was employed.

Glass tubes (130 x 2.5 mm inner diameter) were soaked in chromic acid, rinsed in water, and then soaked in 95% ethanol containing KOH pellets. This cleaning of the glass tubes facilitated the removal of the acrylamide gel after electrophoresis. Each tube was sealed at one end with parafilm before filling with acrylamide solution.

The 3% acrylamide gel mixture was prepared by mixing the following ingredients: 0.53 mL of 30% acrylamide (28.4% acrylamide and 1.6% bisacrylamide), 3 g urea, 2.0 mL of 10% NP-40, 0.25 mL of LKB ampholytes (pH 3.5/10) and 0.22 mL water. This mixture was swirled until the constituents were dissolved and filtered through a 0.2 μm pore Nalgene disposable filter. This filtrate was added to each gel tube with a syringe to a level 1-2 cm from the top. Air bubbles were tapped out. When a gel formed in each tube after 20 min, 2 mL of 8 M urea was introduced onto the surface. After 1-2 hr the urea solution was removed from the top of each gel and 20 μL of an overlay buffer was applied. The overlay buffer included 9 M urea, 4% NP-40, 5% 2-mercaptoethanol, 1% SDS, and 2% ampholytes. The parafilm on the bottom of each tube was removed. Fine-mesh cheesecloth was placed in both ends of the tube and held by 3 mm sections of rubber tubing. The lower reservoir of the electrophoresis cell was filled with 0.01 M H₃PO₄ and the upper reservoir held degassed 0.02 M NaOH.
Prerun electrophoresis was carried out as follows: 200 volts for 15 min, 300 volts for 30 min and 400 volts for 30 min with the use of a Hoefer Scientific Instruments (San Francisco, California) power supply. The solution in the upper reservoir was removed and the overlay buffer was removed from each tube. Kimwipe was used to remove the last traces of the liquid. The sample was loaded on the gel surface and overlaid with 10 μL of overlay buffer diluted 10X with distilled water. The tubes were carefully filled with 0.02 M NaOH and the samples run at 400 volts for 12-15 hr. The brown color of the pigment was sufficiently intense for visualization of the bands.

**Amino Acid Analysis of WIF**

Proteins in WIF were hydrolyzed with p-toluenesulphonic acid by the method of Liu and Chang (1971). The acid hydrolysis reagent was prepared by adding 1 mL of hot distilled water to 4.5 mg of 2,3-aminoethylindole, dissolving 1.25 g p-toluene sulphonic acid in the solution, and bringing volume up to 2.2 mL. Each hydrolysis tube containing 5 mg of WIF in 1 mL of the hydrolysis reagent was evacuated to 20 - 30 μm Hg and sealed. The mixture was digested for 24 hr at 110°C. After digestion, the hydrolysate was adjusted to pH 2.2 with 2N NaOH, brought up to a volume of 5 mL with distilled water and filtered through a sintered glass filter under suction. The filtrate was used for amino acid analysis. Liberated amino acids were determined with a Beckman model 6300 high performance amino acid analyzer (Beckman Instruments Inc., Palo Alto, CA). A Hewlett Packard 3390A integrator (Hewlett Packard, Avondale, PA) was utilized to calculate peak areas for comparison with the amino acid standards.
Total Carbohydrates Assay

When simple sugars, oligosaccharides and polysaccharides are treated with phenol and concentrated sulphuric acid, an orange-yellow coloration is produced (Dubois et al., 1956). This method was used to estimate the total carbohydrate content of WIF. 2 mL of a suspension of WIF (10-70 µg/mL) was added to 0.05 mL of 80% phenol and thereafter 5 mL of concentrated sulphuric acid (reagent grade) was introduced with mixing. After standing for 10 min, the mixture was shaken again and incubated for 10-20 min in a water bath (30°C). Absorbance readings were recorded at 490 nm with a Perkin-Elmer Lambda 3 spectrophotometer. Total carbohydrate content of WIF was expressed in terms of mannose equivalent.

According to Dische (1962), the breakdown products of sugars and polysaccharides in concentrated H₂SO₄ combine with cysteine at room temperature to form colored products which had absorption peaks between 375 and 410 nm. Pentoses and hexuronic acids produce absorption maxima at 390 nm, and with hexoses, absorption maxima have been found to be between 412 and 414 nm. The Dische (1962) method was also used to estimate the total carbohydrate content of WIF. A 1 mL WIF dispersion (5 - 50 µg/mL) 1 mL of concentrated H₂SO₄ was shaken and allowed to stand for 1 hr at 25°C. Then 0.1 mL of a 3% L-cysteine hydrochloride monohydrate solution was added to the mixture. Within 15-20 min, absorbance at 412 nm was measured with a Perkin-Elmer Lambda 3 spectrophotometer. The total carbohydrate content of WIF was expressed in terms of mannose equivalent.
Hydrolysis of Complex Carbohydrates

For the hydrolysis of complex carbohydrates in WIF, trifluoroacetic acid was used as a catalyst rather than HCl or H₂SO₄ to obviate significant sugar degradation (Albersheim et al., 1975). WIF samples (100 mg) were hydrolyzed in 2 M trifluoroacetic acid (20 mL) at 95°C on a steam bath for 20 hr. The trifluoroacetic acid was removed with rotary evaporator at 40°C under vacuum. Trifluoroacetic acid treatment will completely break down a neutral polysaccharide but will not hydrolyze completely glycuronysyl compounds and aldobiuronic acid (uronic acid bonded to a neutral sugar). To overcome this problem, WIF hydrolysate was treated with methanolic hydrochloric acid (Dutton and Yang, 1973) to cleave most of the glycosidic linkages with the formation of methyl glycosides and methyl esters of uronic acids. The methanolic solution was then treated with NaBH₄ to reduce any uronic acids to corresponding alcohols. The WIF mixture was treated with 2 M TFA to hydrolyze any methyl glycosides to neutral sugars.

The resulting sugar solutions were utilized for paper chromatography (PC) of sugars and for gas liquid chromatography (GLC) and gas liquid chromatography-mass spectroscopy (GLC-MS) after conversion of the sugars to alditol acetates.

Separation of Sugars by Paper Chromatography

Monosaccharides can be separated effectively by paper chromatography without derivatization by employing different solvent systems (Macek, 1963; Block et al., 1958). Paper chromatography can be used for identifying sugars in the hydrolysate of polysaccharides (Robyt and Taniguchi, 1976). Sugars in the WIF hydrolysate were separated by descending paper chromato-
graphy on a 20 x 70 cm Whatman #1 filter paper at about 25°C over a 20 hr period. Two solvent systems were used separately:

A. ethylacetate - pyridine - H₂O (8:2:1)

B. ethylacetate - acetic acid - formic acid - H₂O (18:3:1:4)

The sugars were detected with a spray of ammoniacal silver nitrate reagent (Block et al., 1958).

**Conversion of Sugars to Alditol Acetates for GLC and GLC-MS**

Sugars in the WIF hydrolysate were converted to alditol acetates for GLC separation (Gunner et al., 1961; Jannson et al., 1976). After 5 mL of H₂O was added to the WIF hydrolysate, a few drops of 1% NH₄OH solution were introduced to neutralize any residual trifluoroacetic acid. 100 mg of NaBH₄ was added to the hydrolysate and the mixture was stirred at about 25°C for 45 min. Regenerated IR 120 resin (washed with 2 M HCl) was incorporated into the mixture which was stirred until the solution became acidic (approximately 15 min). This resin treatment brought about the decomposition of excess NaBH₄ and removed sodium ions.

The mixture as well as flask washing water was filtered through glass wool. The dried mixture was evaporated to dryness with a rotary evaporator at 40°C. The mixture was washed 5 times with methanol to remove the borate, which would otherwise complex with alditol and retard acetylation, and subsequently evaporated to dryness with a rotary evaporator at 40°C under vacuum. The alditols were dried further for two hr under vacuum and then acetylated with 1:1 mixture of pyridine and acetic anhydride by refluxing for 1 hr.
Excess acetic anhydride was removed by the extraction of the solution with ethanol for about 20 min. Ethanol and pyridine were removed by rotary evaporation at 40°C under vacuum. The sample was dissolved in about 3 mL of distilled chloroform for GLC injection.

**Gas Liquid Chromatography (GLC) of Alditol Acetates**

Extensive reviews of the applications of GLC for simple carbohydrate analysis have been published by Dutton (1973, 1974).

GLC was performed with a Varian model 3700 gas chromatograph equipped with a flame ionization detector coupled to a Hewlett-Packard model 3385A integrator. Separation of sugars was achieved by the method of Geyer et al. (1982) in a deactivated glass capillary column (0.25 mm x 80 m) coated with non-polar SE-30 (McReynold’s constant $\Sigma_1^5 = 217$) purchased from LKB (Stockholm, Sweden). Hydrogen served as the carrier gas. The injector and detector were held at 260°C and the column temperature was programmed from 110 to 240°C at 2°C/min and held. Samples, in chloroform, were introduced with a moving needle splitless injection system.

**GLC-MS of Alditol Acetates**

Sugars in the WIF hydrolysate were analyzed by mass spectroscopy after conversion to the alditol acetates and separated by GLC. The GLC-MS system consisted of a Hewlett Packard 5840(A)GLC with a Hewlett Packard 5987 GLC/MS. Separation of the sugars was achieved on glass capillary column (0.32 mm inner diameter x 25 m) coated with DB-5. The temperature program was 120 to 260°C at 5°C/min. Ionization potential was set at 70 eV on electron impact mode with a linear scan rate of 0.6 sec over a mass range of 40-500.
RESULTS AND DISCUSSION

Preparation of WIF

For the separation of water-insoluble particulate matter from the water-soluble compounds in coffee brew, ethanol as a precipitating agent was chosen on the basis that it has been used to precipitate high molecular weight polymers such as proteins. By lowering the dielectric constant of water (78.5) with the addition of ethanol (dielectric constant of 67), the degree of protein ionization decreases and the protein molecules aggregate to form a precipitate (Lehninger, 1979). Preliminary experimentation revealed that 70% ethanol brought about a slight turbidity of water reconstituted instant coffee powder. Higher ethanol levels increased, precipitation of particulate matter was evident. Above 85% ethanol in the coffee brew, precipitation of high molecular weight particulate matter was complete. Complete sedimentation of the coagulated particles in a 90% ethanol-coffee brew mix occurred in 5 min at 10,000 X g. The brown-colored supernatant was clear. Apparently, coffee brew contains low molecular weight brown pigments which are soluble in 90% ethanol.

The next step in the isolation of the coffee water-insoluble particles was to disperse the ethanol-precipitated particles in water and centrifuge at a high gravitational force. After WIF sedimentation by centrifugation in distilled water, the precipitate was again dispersed in distilled water prior to freeze drying to inhibit particle aggregation.

The yield of freeze dried brown-colored WIF was about 635 mg per 20 g of instant coffee powder or approximately 3.2%. The WIF moisture content
as determined by the vacuum oven technique (80°C for 6 hr) was $1.45\% \pm 0.04$ as an average. When WIF was added to water, the powder was hydrated immediately upon agitation of the mixture. Within a few minutes, a stable dispersion of WIF particles was formed during agitation.

**Non Metallic Elemental Analysis of WIF**

Elemental analyses of two samples of WIF (dry weight basis) are shown in Table 4. The simplest empirical formula for WIF-II (Table 4) was calculated to be $C_{47}H_{80}O_{41}N$. When the total percentages of $C$, $H$, $O$ and $N$ were considered as 100, the empirical formula for WIF-II was $C_{46}H_{82}O_{40}N$. This formula represents the average composition of many different organic constituents within WIF. The presence of nitrogen in WIF suggests that proteins may be present. With the high hydrogen and oxygen content of WIF, carbohydrates having numerous $OH$ groups would be expected to be major components. According to Maier and Buttle (1973), a brown pigment fraction ($N_1 IA$) of coffee brew had an elemental analysis of $C$ 40.70%, $H$ 6.75%, $N$ 1.65%, $O$ 47.95%. These values are similar to those obtained in the present study (Table 4). Motai and Inoue (1974) analyzed their fractions of air oxidized melanoidins (heated glycine-xylose system) and found that the composition was about $C$ 44-46%, $H$ 5%, $N$ 6.1% and $O$ 42.7-44.7%. The simplest empirical formula was calculated to be $C_8H_{11}O_6N$.

**Metallic Elemental Analysis of WIF**

The ash content of green coffee beans has been reported to be between 3 and 4.5% (Clifford, 1975a; Sivetz and Desrosier, 1979). The variability of the mineral concentration of these beans can be attributed mainly to the
Table 4

Non-metallic Elemental Analysis of WIF Samples

<table>
<thead>
<tr>
<th>Element</th>
<th>% Composition (dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Carbon</td>
<td>39.25</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>5.55</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>0.96</td>
</tr>
<tr>
<td>Oxygen</td>
<td>45.07</td>
</tr>
<tr>
<td>Sulfur</td>
<td>&lt;0.5</td>
</tr>
</tbody>
</table>
profile and concentration of elements in the soil and the cultural practices. Green coffee beans contain a large amount of potassium (1350-1712 mg per 100 g) with smaller levels of magnesium (142-176 mg per 100 g) and calcium (76-120 mg per 100 g) (Clifford, 1975a). During the roasting of green beans, no minerals were lost but a change in mineral solubility occurred, probably through anion alteration (Clifford, 1975a, Sivetz and Foote, 1963). The water solubility of coffee bean minerals rose from about 50% to around 75% during the roasting period (Sivetz and Foote, 1963). During the percolation process, most of the minerals diffuse from the coffee particles into the water phase.

Minerals in a coffee brew presumably exist as hydrated cations and anions and as complexed ions in the colloidal particles. The ash content of a dried coffee brew has been estimated to be about 10% (Sivetz and Derrosier, 1979).

In the present study, the ash content of WIF from reconstituted instant coffee powder was found to be about 3.8% on a dry weight basis. Mineral analysis (Table 5) of the wet ashed WIF revealed that the major metallic element was potassium at a level of 19.6 mg per g. Both calcium and magnesium were present in WIF at a concentration of 2.9 mg per g and iron was at a level of about 104 μg per g. The sodium content of WIF was very low at around 4 μg per g. The sequential order of concentrations of these elements in WIF is similar to those for green and roasted coffee beans, except for sodium and iron which are reversed. The large concentration of potassium is puzzling since only weak bonds are formed between monovalent ions and ligands. Perhaps, potassium salts were trapped in capillary cavities of the WIF particles.
### Table 5

**Mineral Analysis of WIF**

<table>
<thead>
<tr>
<th>Element</th>
<th>WIF (µg/g) dry wt.</th>
<th>% of Total Minerals in WIF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barium</td>
<td>5.12</td>
<td>0.014</td>
</tr>
<tr>
<td>Calcium</td>
<td>2849</td>
<td>7.87</td>
</tr>
<tr>
<td>Copper</td>
<td>4.48</td>
<td>0.012</td>
</tr>
<tr>
<td>Iron</td>
<td>103.5</td>
<td>0.29</td>
</tr>
<tr>
<td>Magnesium</td>
<td>2908</td>
<td>8.03</td>
</tr>
<tr>
<td>Manganese</td>
<td>37.8</td>
<td>0.104</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>10,155</td>
<td>28.05</td>
</tr>
<tr>
<td>Potassium</td>
<td>19,621</td>
<td>54.19</td>
</tr>
<tr>
<td>Silicon</td>
<td>65.47</td>
<td>0.0018</td>
</tr>
<tr>
<td>Sodium</td>
<td>4.19</td>
<td>1.15</td>
</tr>
<tr>
<td>Strontium</td>
<td>19.42</td>
<td>0.053</td>
</tr>
<tr>
<td>Titanium</td>
<td>10.74</td>
<td>0.029</td>
</tr>
<tr>
<td>Zinc</td>
<td>5.58</td>
<td>0.015</td>
</tr>
</tbody>
</table>
Polyion melanoidins are presumably entities of WIF particles. Melanoidins isolated from Maillard reaction model systems possess many exposed carboxyl groups. According to Gomyo and Horikoshi (1976), the number of protons released (mostly from carboxyl groups) during titration from pH 2.5 - 11.0 was about 500 per molecule of melanoidin. Melanoidin was found to bind ferric and cupric ions strongly. From the spectral analysis of Gomyo and Horikoshi (1976), it became clear that melanoidin-metallic ion complexation occurred through the chelation process with carboxyl groups containing electron-donor oxygen atoms. The high concentration of phosphorus suggests that phosphate groups are present in WIF as contributors of negative charges.

**Molecular Weight of WIF**

The molecular weight of the WIF particles was estimated by a gel filtration technique with a chromatographic column of Sephacryl 1000 (Andrews, 1970; Motai, 1974). After a WIF dispersion was applied to the column, a broad brown colored band appeared during elution with a 0.15M phosphate buffer, pH 7.0. The brown colored band was eluted between 22 and 25 mL. At the point of maximum absorbance the elution volume (Ve) for the brown-colored WIF band was about 23 mL (average for 3 runs). Known molecular weight dextran standards were applied to the Sephacryl 1000 column and chromatographed under the same conditions as the WIF samples. The plot of Ve for each standard against the molecular weight is shown in Figure 5. A linear relationship was estimated by linear regression analysis. On the plot in Figure 5, the elution volume of 23 mL for the brown-colored WIF band corresponded to a component with a molecular weight of about 200,000.
Figure 5 - Standard curve plot of elution volume versus log molecular weight with a sephacryl 1000 gel filtration. The average molecular weight of WIF was estimated from this curve.
According to Casler et al. (1978), the molecular weight of the major brown-colored compounds (band FI) of an acetone-precipitated fraction of coffee brew was estimated to be approximately 200,000 when Sephadex G200 filtration was used. In their study, the maximum absorption of FI occurred at an elution volume of 25 mL.

**Reductones Content of WIF**

Reductones are organic compounds which have strong reducing power in acidic solutions (Hodge, 1967). The functional group of a reductone is represented by the skeletal formula (-C≡C-). Ascorbic acid contains this functional group. Kato et al. (1968) reported that reductones were present in nondialyzable melanoidins. The reductone content (as mg of ascorbic acid per g of melanoidin on a dry weight basis) of their melanoidin samples prepared from a variety of heated reducing sugar-amine combinations ranged from 10.4 to 129. In the present study, the reductone content of WIF was determined by a method similar to that used by Kato et al. (1968) and was found to be about 143 mg of ascorbic acid equivalent per g of WIF. Such a high reductone value for WIF is not unexpected since severe heat treatment of coffee beans during roasting enhances the formation in the melanoidin fraction. Kato et al. (1968) found that as the heating time of xylose-glycine solution at 100°C increased up to 4 hr, the reductone content increased progressively.

**Phenolic Composition of WIF**

The major phenolic compounds in green and roasted coffee beans are chlorogenic acids. Green robusta and arabica beans contain between 5.5 and
10% chlorogenic acids. Chlorogenic acids in green beans are mono and diesters of a cinnamic acid and quinic acid. Caffeic and ferulic acids are the main cinnamic acids. 3-caffeoylquinic acid is the dominant chlorogenic acid in green beans at a level of 5.5 to 8% (Feldman et al., 1969). Roasting of green coffee beans brings about a decomposition of mono and dicaffeoylquinic acids. These decomposed chlorogenic acids and some unaltered chlorogenic acids may be bound to nitrogenous compounds to form high molecular weight brown pigments (Clifford, 1975b).

According to Sharma and Johary (1979), the high molecular weight brown polymers in molasses contain a high concentration of phenolic and hydroxyl groups.

In the present study, the addition of 5% ferric chloride solution (pH 5) to a 1% WIF suspension brought about the formation of an olive-green precipitate. This colour change is indicative of the presence of phenolic compounds in WIF. When Casier et al. (1978) added ferric chloride solution to their brown pigment fraction (FI) from coffee brew, a positive coloration indicating the presence of phenolic compounds was observed. The Folin-Ciocalteau reagent was used to determine the total amount of phenolic compounds in WIF according to the method of Singleton (1981). The total phenolic content in WIF was estimated to be 4 mg of caffeic acid equivalent per 10 mg of WIF.

Table 6 shows the phenolic acids in coffee brew and their changes due to roasting. The chlorogenic acid decreased during roasting compared to the green beans. Neochlorogenic acid is believed to increase at the expense of chlorogenic acid.
Table 6

Phenolic acids in green and roasted Columbian coffee beans

<table>
<thead>
<tr>
<th>Acid</th>
<th>Green</th>
<th>Medium</th>
<th>Dark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinic</td>
<td>0.27</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>Ferulic</td>
<td>0.064</td>
<td>0.056</td>
<td></td>
</tr>
<tr>
<td>Caffeic</td>
<td>0.17</td>
<td>0.0041</td>
<td>0.0068</td>
</tr>
<tr>
<td>Cryptochlorogenic</td>
<td>0.26</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>Chlorogenic</td>
<td>3.77</td>
<td>2.02</td>
<td>2.55</td>
</tr>
<tr>
<td>Neochlorogenic</td>
<td>0.60</td>
<td>2.82</td>
<td>3.05</td>
</tr>
</tbody>
</table>

\( ^a \) Feldman et al. (1969)
Isoelectric Focusing Electrophoresis

Coffee WIF was analyzed by electrofocusing polyacrylamide gel electrophoresis with 1% sodium dodecyl sulphate in the buffer. The electrophoretic pattern of the brown bands is shown in Figure 6. Three major brown bands were clearly electrofocused at a pH ranging from 3.01 to 4.56. Two thin brown bands (I and III) were formed at pH 3.01 and 4.56, respectively, and a broad brown band (II) was formed in the region of pH 3.88 to 4.05.

Homma et al. (1982) separated nondialyzable melanoidin into at least 14 bands by electrofocusing electrophoresis at a pH range 2.7 to 3.3 in the absence of 1% sodium dodecyl sulphate. It is apparent from this electrofocusing investigation that the melanoidin in WIF particles were charged negatively in the pH region above pH 4.56 and that the isoelectric points of the three brown pigments were around 4.56, 3.88 to 4.05 and 3.01.

Amino Acid Composition of WIF

Amino acids in the free and combined forms are important in the development of coffee odour and brown colour. Reducing sugars react with amino groups of amino acids through the Maillard browning reaction to produce low molecular weight, volatile odorous compounds and polymers called melanoidins (El Ode et al., 1966; Hodge, 1967).

Studies have shown that amino acids and proteins in model systems are incorporated into melanoidins and can be liberated by acid hydrolysis of isolated melanoidin (Clark and Tannenbaum, 1970; Motai, 1974). The melanoidins in WIF would be expected to contain amino acids.

In the present study, WIF was treated with p-toluenesulfonic acid to hydrolyze the peptide linkages in peptides and proteins and release amino
Figure 6 - Electrophoretic pattern of WIF examined by isoelectric focusing.
acids from the brown pigment structure. Unlike 6N HCl, p-toluene sulfonic acid does not cause the destruction of serine, threonine and tryptophan (Liu and Chang, 1971). Sarwar et al. (1983) found that, with 22 hr of hydrolysis at 110°C with p-toluenesulfonic acid, an optimum yield of serine and threonine was achieved.

Figure 7 shows a typical amino acid chromatographic pattern for hydrolyzed WIF. The amounts of amino acids in WIF hydrolyzate are indicated in Table 7. Relatively large amounts of aspartic and glutamic acids were present in WIF. The carboxyl groups of these acids may be exposed on the surfaces of the melanoidins and provide negative charges. Histidine at a level of about 8 mg per g of WIF may contribute significant positive charges to the melanoidins through the imidazoyl group. Hydrocarbon side chains of glycine, alanine, valine, leucine, isoleucine and phenylalanine may provide loci for hydrophobic bonding. Small amounts of hydroxy amino acids were present in WIF. Methionine, tyrosine, arginine, cysteine and lysine were present in trace amounts. Significant amounts of arginine, cysteine and lysine are decomposed in the coffee bean during roasting (Feldman et al., 1969).

**Total Carbohydrates in WIF**

Carbohydrates comprise 50-60% of the weight of the green coffee bean, which vary from simple sugars to polysaccharides (Feldman et al., 1969). The carbohydrates in roasted coffee are shown in Table 3 as both soluble and insoluble fraction. These include starches in the green coffee bean and dextrins in the roast coffee bean. Long-chain carbohydrates include pentosans which yield mannose and galactose on hydrolysis. Pentosans are
Table 7

Amino Acid Analysis of WIF

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Molecular wt.</th>
<th>Retention time</th>
<th>mg/g WIF 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 aspartic acid</td>
<td>133</td>
<td>7.34</td>
<td>8.02±.21</td>
</tr>
<tr>
<td>2 threonine</td>
<td>119</td>
<td>8.55</td>
<td>1.42±.03</td>
</tr>
<tr>
<td>3 serine</td>
<td>105</td>
<td>9.20</td>
<td>1.78±.05</td>
</tr>
<tr>
<td>4 glutamic acid</td>
<td>147</td>
<td>11.49</td>
<td>15.74±.22</td>
</tr>
<tr>
<td>5 glycine</td>
<td>75</td>
<td>15.97</td>
<td>5.88±.05</td>
</tr>
<tr>
<td>6 alanine</td>
<td>89</td>
<td>17.09</td>
<td>2.49±.04</td>
</tr>
<tr>
<td>7 valine</td>
<td>117</td>
<td>21.66</td>
<td>4.66±.02</td>
</tr>
<tr>
<td>8 isoleucine</td>
<td>131</td>
<td>27.89</td>
<td>4.52±.05</td>
</tr>
<tr>
<td>9 leucine</td>
<td>131</td>
<td>29.04</td>
<td>1.63±.02</td>
</tr>
<tr>
<td>10 phenyl alanine</td>
<td>165</td>
<td>34.18</td>
<td>2.54±.17</td>
</tr>
<tr>
<td>11 histidine</td>
<td>155</td>
<td>39.39</td>
<td>8.21±.17</td>
</tr>
<tr>
<td>12 ammonia</td>
<td>18</td>
<td>43.12</td>
<td>4.83±.09</td>
</tr>
</tbody>
</table>

1average of five runs ± standard deviation on a dry weight basis
Figure 7 - Amino acid chromatographic profile of the hydrolyzed WIF.
part of a larger group of polysaccharides called hemicelluloses which account for up to 24% of the roasted bean.

Some carbohydrates are decomposed upon roasting the coffee beans. The sucrose content of coffee beans decreased from 7.3 to 0.3% during the roasting period (Feldman et al., 1969). Mannan was isolated from roasted coffee probably as galactomannan. The major component of water-soluble polysaccharides in green coffee is arabinogalactan and galactomannan (60-70% mannose).

On roasting, the water-soluble fraction, the arabinose practically disappears, the galactose decreases markedly and the mannose increases from 10 to 70%. Also during roasting, the arabinose and galactose of degradable polysaccharides decrease and the mannan increases proportionately. In the holocellulose fractions, the mannan decreases slightly and galactan remains almost constant during roasting. Two colorimetric methods were used to assess the total amount of carbohydrates in WIF. With the method of Dubois et al. (1956), simple sugars, oligosaccharides and polysaccharides are decomposed in a phenol-sulphuric acid solution to orange-yellow pigments. These pigments are stable and thus variability of results due to reaction time is obviated. Microquantities can be determined colorimetrically by the Dubois et al. (1956) method. In the present study, total carbohydrate in WIF was estimated to be about 56 g per 100 g of WIF on a mannose equivalent basis.

The other method for the estimation of total carbohydrate of WIF was that of Dische (1962). Upon the addition of concentrated H₂SO₄ to a WIF suspension, simple and complex carbohydrates are degraded to furfural and
its homologues. Upon the addition of L-cysteine, a yellow coloration of the dispersion developed. The total carbohydrate was estimated to be about 67 g per 100 g of WIF on a mannose equivalent basis when a wavelength of 412 nm was used. The absorption maxima for hexoses has been found to be between 412 and 414 nm with this method.

**Paper Chromatography of Carbohydrates in WIF**

Paper chromatography is a valuable analytical procedure for sugars which can be separated and identified rapidly, simply and accurately with less than a milligram of material (Hough, 1954). Many sugar diastereomers are separable by paper chromatograph. Separation depends upon differences in the partition coefficients of the sugars between the moving solvent and the water cellulose stationary phase. Enantiomorphs such as DL sugars and anomeric isomers are not resolved on paper chromatograms. Accuracy of ± 5% was reported by Hough and Jones (1962).

In the present study, separation of sugars in the WIF hydrolyzate was achieved with an ethylacetate-pyridine-water (8:2:1) basic solvent system (A) and an ethylacetate-acetic acid-formic acid-water (18:3:1:4) acidic solvent system (B). A silver nitrate-sodium hydroxide spray was used as detected sugar spots on the paper chromatograms. Reducing sugars reacted with silver nitrate to form brown to black pigments within a few min at about 25°C. The chromatograms developed with solvent systems A and B are shown in Figures 9 and 10, respectively. Fructose is heat sensitive under acidic conditions (Hough and Jones, 1962) and could be destroyed during acid hydrolysis of the WIF carbohydrate. Thus WIF was heated in 2 M
Figure 8 - Paper chromatographic separation of reference sugars and sugars in the WIF hydrolyzate with a basic solvent system (ethyl acetate-pyridine-water; 8:2:1).
Figure 9 - Paper chromatographic separation of reference sugars and sugars in the WIF hydrolyzate with an acidic solvent system (ethyl acetate-acetic acid-formic acid-water; 18:3:1:4).
Table 8

Rg Values of Various Sugars in Paper Chromatography Using Two Different Solvent Systems

<table>
<thead>
<tr>
<th></th>
<th>Solvent System A</th>
<th>Solvent System B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rg</td>
<td>Rg</td>
</tr>
<tr>
<td>Arabinose</td>
<td>2.0</td>
<td>1.6</td>
</tr>
<tr>
<td>Fructose</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Ribose</td>
<td>3.7</td>
<td>2.4</td>
</tr>
<tr>
<td>Mannose</td>
<td>1.5</td>
<td>1.3</td>
</tr>
<tr>
<td>Xylose</td>
<td>2.6</td>
<td>1.8</td>
</tr>
</tbody>
</table>

1-ethylacetate:pyridine:water (8:2:1)
2-ethylacetate:acetic acid:formic acid:water (18:3:1:4)

From Hough and Jones (1962)
trifluoroacetic acid at about 100°C for periods of 0.5, 1.0, 2.0 and 3.0 hr to determine if fructose was released and then decomposed. Fructose was not detected in paper chromatograms of each hydrolysate above. From these results, it is assumed that fructose was not present in WIF, was extremely labile, or present below detection limits of this method.

As shown in Figure 8, the spots of reference sugars on the paper chromatogram developed with the basic solvent system (A) were spread out. The Rg values of the reference sugars were similar to those reported in the literature (Table 8) when the Rf for glucose was designated as 1. Glucose and galactose spots overlapped slightly, but the spots were distinct. Further, the fructose and arabinose spots had Rf values close to each other yet could be detected. The sugars in the WIF hydrolysate were separated into 3 spots on the paper chromatogram (Figure 8) developed with the basic solvent system (A). The two spots with Rg values of about 0.8 and 1.0 overlapped slightly. The bottom spot (Rg 0.8) was considered to be galactose. The higher spot (Rg 1.0) with a light brown color was assumed to be glucose at a low concentration. The position of the dark brown spot (Rg 1.5) corresponded to that of the mannose Rf. A faint brown spot with Rg 2.0 was considered to be arabinose at a very low concentration. From these results it is assumed that galactose, glucose, mannose and arabinose were detected. Casier et al. (1978) also found these sugars in their FI fraction of coffee brew.

When the paper chromatogram was developed with an acidic solvent system (B), the reference sugars were not so well resolved as the basic solvent system (Figure 9). Two distinct spots were noted on the chromatogram for
the WIF hydrolysate. The leading spot was considered to be mannose and the next spot with a figure eight shape was considered to be composed of galactose and glucose.

**Gas Liquid Chromatography of Carbohydrates in WIF**

Since multiple hydroxyl groups in sugars render them insufficiently volatile for GLC, heat stable, volatile derivatives of sugars must be prepared. Trimethylsilyl ether derivatives of sugars have been used extensively (Sweeley et al., 1963). However, each monosaccharide may react with this trimethylsilyl ether to form anomeric derivatives leading to several GC peaks. The anomeric centre of a sugar can be removed either by conversion into an oxime or a nitrile by oxidation. Derivatization of sugars to alditol acetates reduces the complexity of the sugar mixture (Bjorndal et al., 1970). An extensive review of the application of GLC to carbohydrate analysis was published by Dutton (1974).

When sugars in the WIF hydrolysate were converted to alditol acetates and thereafter analyzed by GLC with a SE-30 wall-coated capillary column, a chromatograph with distinct, well separated peaks resulted (Figure 10). The chromatographic conditions were identical to those of Geyer et al. (1982) except that the column length in the present study was 80 m. The relative retention times (RRT) for peaks 3, 4 and 5 corresponded to those of mannose (57.06 min), glucose (57.49 min) and galactose (57.92 min), respectively. The RRT of a glucitol hexaacetate standard (alditol acetate of glucose) was 57.5 min and corresponded to the RRT for peak 4. The minor peaks were not identified.
Figure 10 - Gas chromatogram of carbohydrates from WIF.
Mass Spectrometry of Carbohydrates in WIF

The mass spectrometric method has become a reliable technique for characterizing sugars (Lonngren and Svensson, 1974; Dutton, 1974). Stereoisomers of carbohydrate derivatives give similar mass spectra although small differences in peak intensity are sometimes observed. Electron impact ionization of carbohydrate derivatives such as alditol acetates does not produce any appreciable molecular ions.

Systematic studies on the mass spectra of alditol acetates have shown that the mode of fragmentation is simple. The mass spectrum of alditol acetates shows a (M-CH₂-CO₂) species in low abundance. Mass spectra of stereoisomers are almost identical and therefore the mass spectrum of D-glucitol hexaacetate is representative of all peracetylated hexitols (Figure 11). Thus, tetroses, pentoses, hexoses and heptoses give characteristic spectra that can be fully interpreted. Uronic acids as components of polysaccharides can be analyzed, both quantitatively and qualitatively as alditol acetates.

The base peak in the mass spectra of all alditol acetates is the acetylium ion m/e 43 (CH₃-C-0). Primary ions are formed from alditol acetates by elimination of an acetoxy group or by cleavage of the alditol chain as shown in Figure 12 (Lonngren & Svensson, 1974).

The GLC separation of sugars for introduction into the mass spectrometer was carried out on a DB-5 coated capillary column programmed after injection with a temperature increase from 120 to 260°C at 5°C/min and held. Peaks in the GLC chromatograms (Figures 13 to 23) had retention times from 15.8 min to 26.6 min. Scan numbers are presented as abscissa. The scan
Figure 11 - Mass spectra of D-glucitol hexaacetate.
(Hennesian, 1971)
Figure 12 - Primary ions formed from alditol acetates. (Lindberg, 1974)
number as well as the retention time for each peak analyzed by mass spectrometry are located in the upper left-hand corner of the mass spectrum.

Figures 13A and 13B show the mass spectra of the sugar acetates in peaks at scan numbers of 1979 and 1982, respectively. The spectra for peaks 1951 and 1957 (Figures 14A and 14B), respectively, are characteristic of glucitol hexaacetate. The mass spectra for the primary and secondary peaks of the sugar acetate are identical to the characteristic spectra of glucitol hexaacetate. Primary ions are formed from alditol acetates by elimination of an acetoxy group, or by cleavage of the alditol chain. Primary fragments are formed by fission between carbon atoms in the chain. Five primary fragments are formed from D-glucitol hexaacetate which account for m/e 361, m/e 289, m/e 217, m/e 145 and m/e 73. The base peak of all alditol acetates is m/e 43 (CH$_3$C--O) (Lindburg, 1974).

Secondary fragments are formed from primary fragments by single or consecutive loss of acetic acid (m/e 60), ketene (m/e 42), acetic anhydride (m/e 102), methanol (m/e 32) or formaldehyde (m/e 30). The loss of acetic anhydride from the fragments m/e 289, 272, 259, 230, 217, 187, 175, 157 and 145 could account for m/e 187, 170, 157, 128, 115, 85, 73, 55 and 43, respectively. The loss of acetic acid from fragments m/e 289, 159, 145 and 115 could account for m/e 229, 199, 85 and 55, respectively. The loss of ketene from m/e 199, 170, 157, 145 and 85 may account for m/e 157, 128, 115, 103, 55 and 43, respectively. Various other combinations may be found with the elimination of formaldehyde (m/e 30).

The compound at scan number 1965 has a mass spectrum similar to that for glucitol hexaacetate, except for m/e 207 and 60. m/e 60 may be structure I shown in Figure 24. The peak 1924 (Figure 15B) has 8 m/e which
Figure 13 - Mass spectra for peaks (A) 1979 and (B) 1982.
Figure 14 - Mass spectra for peaks (A) 1951 and (B) 1957.

5974 SCANS (466 SCANS, 4.97 MINS)  
MASS RANGE: 39.0, 446.1 TOTAL ABUND: 10362656.

Scan #1951 RET. TIME: 26.26 TOT ABUND: 173232. BASE PK/ABUND: 43.0/61140.

Scan #1957 RET. TIME: 26.26 TOT ABUND: 223441. BASE PK/ABUND: 43.0/65430.
Figure 15 - Mass spectra for peaks (A) 1965 and (B) 1924.

--- A ---
5974 SCANS (63 SCANS, .73 MINS) 
MASS RANGE: 39.0, 446.1 TOTAL ABUND = 10362856.

--- B ---
5974 SCANS (254 SCANS, 2.67 MINS) 
MASS RANGE: 39.0, 446.1 TOTAL ABUND = 18362856.
Figure 16 - Mass spectra for peaks (A) 1914 and (B) 1775.

6974 SCANS (264 SCANS, 2.67 MINS)  
MASS RANGE: 39.0, 446.1 TOTAL ABUND= 10362856.

SCAN #

1914 RET. TIME: 25.80  TOT ABUND= 2264. BASE PK/ABUND: 43.2/394.

B

6974 SCANS (264 SCANS, 2.67 MINS)  
MASS RANGE: 39.0, 446.1 TOTAL ABUND= 10362856.

SCAN #

correspond to glucitol hexaacetate but m/e 281, 210, 186, 168, 139 and 126 do not correspond. Ions m/e 168 and 126 may be structures II and III, respectively, in Figure 24. Peak 1914 (Figure 16A) has many fragments which are probably impurities (m/e 281, 207, 139, 127, 97, 71 and 57). Ions 71 and 97 may be structures IV and V in Figure 24. Peak 1775 (Figure 16B) has the impurities m/e 231, 201, 169, 129, 81 and 69. Ions 69 and 129 may be fragments VI and VII respectively in Figure 24.

Scan numbers 1746 and 1754 (Figure 17A and 17B) have many glucitol hexaacetate fragments with m/e less than 170 except m/e 69 and 127. m/e greater than 170 appear to be impurities, as most fragments have consecutive losses less than 20. Ions 69 may be structure VI in Figure 24.

Scan number 1726 Figure 18B has impurities m/e 207, 129, 97, 71 and 57 which are not characteristic of glucitol hexaacetate. Fragments 71, 97 and 129 may be the structures VI, V and VII, respectively, in Figure 24. Scan numbers 1533 and 1501 (Figure 19A and 19B) have basically the same fragments except 1533 has m/e 69 compared to 1501 with m/e 61. Ions 69 and 175 may be structures VI and VIII, respectively, in Figure 24. Scan number 1501 has most of the characteristic fragments of pentitol pentaacetate (Figure 20) while 1476 (Figure 21B) has most of the characteristic fragments of deoxy hexitol pentaacetate.

Peak 1308 (Figure 21A) has most of the glucitol hexaacetate fragments except m/e 69, 81 and 98. Fragments m/e 69 and 98 may be structures VI and IX, respectively, in Figure 24. Peaks 1245 and 1290 (Figure 22A and 22B), peaks 946 and 982 (Figure 23A and 23B) have some of the characteristic spectra of glucitol hexaacetate, but there are many fragments which appear to be impurities.
Mass spectrometry shows that the carbohydrates separated from the coffee WIF by hydrolysis were, for the most part, simple hexose sugars. Mass spectra for some peaks indicated the presence of some deoxy sugar fragments, but no n-acetyl glucosamines.
Figure 17 - Mass spectra for peaks (A) 1746 and (B) 1754.
Figure 18 - Mass spectra for peaks (A) 1702 and (B) 1726.

**A**

5974 SCANS (254 SCANS, 2.67 MINS)
MASS RANGE: 39.0, 446.1 TOTAL ABUND = 183628E6

SCAN • 1782 RET. TIME: 23.58 TOT ABUND = 1588. BASE PK/ABUND: 43.2/462.

**B**

5974 SCANS (254 SCANS, 2.67 MINS)
MASS RANGE: 39.0, 446.1 TOTAL ABUND = 183628E6

SCAN • 1726 RET. TIME: 23.83 TOT ABUND = 1394. BASE PK/ABUND: 43.2/311.
Figure 19 - Mass spectra for peaks (A) 1501 and (B) 1533.

**A**

E974 SCANS (299 SCANS, 2.48 MINS)  
MASS RANGE: 39.0, 446.1 TOTAL ABUND = 10362856.

**B**

E974 SCANS (239 SCANS, 2.48 MINS)  
MASS RANGE: 39.0, 446.1 TOTAL ABUND = 10362856.
Figure 20 - Mass spectra for pentitol pentaacetate.
Figure 21 - Mass spectra for peaks (A) 1308 and (B) 1476.

**A**

6974 SCANS (1268 SCANS, 12.92 MINS)
Mass Range: 39.0, 446.1 TOTAL ABUND = 10362656.

RET. TIME: 19.66 TOT ABUND = 6045. BASE PK/ABUND: 43.2/2389.

**B**

6974 SCANS (239 SCANS, 2.46 MINS)
Mass Range: 39.0, 446.1 TOTAL ABUND = 10362656.

Figure 22 - Mass spectra for peaks (A) 1245 and (B) 1290.
Figure 23 - Mass spectra for peaks (A) 946 and (B) 982.
Figure 24 - Structure of some specific mass spectra fragments (Henessian, 1971).
A water-insoluble fraction (WIF) was separated from instant coffee brew by first adding ethanol to a level of 90% to coagulate high molecular weight particles and then centrifuging the mixture to sediment the particulate matter. After dispersing the precipitate in water, 11,200 Xg was used to sediment the brown-colored water-insoluble fraction. The yield of WIF was about 3.2 g per 100 g of instant coffee powder.

Elemental analyses of a WIF sample determined the amounts of non-metallic and metallic elements. C, O, H, N analyses revealed a composition of C 39.6%, O 45.9%, H 5.9%, and N 1.0%. The empirical formula for the WIF was C_{46}H_{82}O_{40}N. The formula represents the composition of many different organic compounds such as carbohydrates and proteins. The major metallic elements in WIF were found to be potassium (19.6 mg/g), calcium (2.9 mg/g) magnesium (2.9 mg/g) and phosphorus (10.2 mg/g).

WIF particle size was estimated by introducing a WIF dispersion on a Sephacryl S-1000 chromatographic column and eluting with 0.15 M phosphate buffer (pH 7.0). The molecular weight of the particles passing through the column was estimated to be in the vicinity of 200,000.

Reductone (dihydroxyketone) and hydroxy-containing phenols were detected in WIF. The reductone content was estimated to be about 143 mg of ascorbic acid equivalent per g WIF. The addition of 5% ferric chloride solution to a WIF dispersion gave the formation of an olive-green precipitate indicative of phenolic compounds. The total phenolic content of WIF was calculated to be 400 mg of caffeic acid equivalent per g.
WIF was analyzed by electrofocusing polyacrylamide gel electrophoresis. WIF was dispersed in a buffer containing sodium dodecyl sulphate (SDS). Urea and mercaptoethanol were present in the polyacrylamide gel. Three major brown bands were electrofocused in a pH range of 3.01 to 4.56. The WIF components moving in the gel were charged negatively at pH values above 4.56. It is of interest to note that when WIF was dispersed in a buffer containing SDS, soluble brown pigments were released from WIF. Presumably the brown bands on the column are melanoidins.

WIF was treated with p-toluene sulfonylic acid to hydrolyze the peptide linkages and release amino acids. Eleven amino acids were detected in the hydrolyzate. The major amino acids were found to be glutamic acid, aspartic acid, histidine and glycine.

Two colorimetric methods were used to estimate the total amount of carbohydrates in WIF. With the Dubois et al. (1956) method, a phenol-sulphuric acid solution decomposed carbohydrates to orange-yellow pigments for colorimetric analysis. The total amount of carbohydrate in WIF was determined to be 56 g per 100 g on a mannose equivalent basis. The Dische (1962) method involved carbohydrate degradation to furfural and related compounds under acid conditions and then creation of yellow-coloration upon the addition of cysteine. The carbohydrate content was estimated to be 67 g per 100 g of WIF on a mannose equivalent basis.

The types of sugars in WIF were determined by paper chromatography and gas liquid chromatography--mass spectrometry of the WIF hydrolysate. The hydrolysates contained mannose, galactose, glucose and arabinose. The results from mass spectrometry indicate the presence of some deoxy-sugars but no N-acetyl glucosamines.
Considering the compositional data on WIF, one could presume that WIF consists of complex particles composed of: combined amino acids such as peptides and polypeptides; polysaccharides containing mannose, galactose, glucose and arabinose; melanoidins; and phenolic compounds. Probably the polysaccharides, phenolic compounds and melanoidin portions are complexed with polypeptides which contain electrically-charged groups such as carboxyl (glutamic and aspartic acids) and imidozolyl (histidine). The integrity of the particles may be dependent on the divalent calcium and magnesium ions, the phosphate groups and hydrophobic groups.
CHAPTER 2

Inhibition of Carcinogen-Induced Mutagenesis by Coffee WIF and a Model System Melanoidin

Introduction

Short-term in vitro tests such as the Ames/Salmonella test (Ames et al., 1975) can be used to assess the inhibitory effect of food components and complex food systems on carcinogen-induced mutagenesis (Rosin et al., 1982). In such an assessment Salmonella bacteria are exposed to a solution of a carcinogen acting as a mutagen and a potential antimutagenic agent and then examined for an expression of a change in DNA structure. The level of inhibition of the potential antimutagenic agent on the carcinogen-induced gene alteration in the Salmonella organisms can be estimated by determining the extent of frameshift or base-pair substitution reverse mutations. The Ames test assesses mutagenicity quantitatively on the basis that a mutagen has the ability to revert histidine-requiring auxotrophs of Salmonella typhimurium to form macroscopic colonies. For a compound to be classed as a mutagen, a dose-response curve for the compound must be established when using a short-term test (Ames et al., 1975; McCann et al., 1975).

Several bacterial tester strains of Salmonella typhimurium have been used by researchers in the past few years (Taylor, 1982). Strains TA1535 and TA100 can be used to detect mutagens causing base-pair substitutions in DNA. Strains TA1537, TA1538 and TA98 are used to detect mutagens which bring about frameshift alterations in DNA. Each tester strain possesses a mutation in one of several genes governing the synthesis of histidine as
well as two other mutations which were incorporated to greatly increase its sensitivity to mutagens. One mutation causes the loss of the excision repair system and the other is responsible for a loss of synthesis of lipopolysaccharide that normally coats the surface of bacterial cells. TA98 and TA100 strains have the resistance transfer factor (R factor) plasmids which increases the sensitivity of the strains to reversion of histidine-requiring auxotrophs with many carcinogens including aflatoxin B₁ and benzo(a)pyrene.

Uwaifo and Bababunmi (1979) found that the TA100 strain is a more sensitive tester strain for either aflatoxin B₁ (AFB₁) or aflatoxin M₁ (AFM₁) than TA98 strain, although both strains have R factor plasmids. Since AFB₁ is a procarcinogen and promutagen, it requires metabolic activation before its mutagenic effect can be demonstrated. (Garner et al. 1972; McCann et al. 1975; Wong and Hsieh, 1976; Campbell and Hayes, 1976; Ong, 1975). Nitrosamines are direct-acting mutagens which do not require metabolic activation by the S-9 mix. On the other hand, metabolic activation of benzo (a) pyrene (BP) by the S-9 mix is required for the expression of the carcinogenic potential (Dipple, 1983).

Aeschbacher et al. (1980) noted that both freshly brewed and instant coffee were weakly mutagenic as assessed with the Ames/Salmonella mutagenicity test with strain TA100. The influence of caffeine level, histidine content and pH of the coffee brews on the mutagenicity response was investigated, but none significantly influenced the test system. Coffee brew prepared by a filtration method was found by Nagao et al. (1979) to contain a mutagen(s) which induced 1.4 to 4.6 x 10⁵ revertants per mL, but this effect was totally abolished by the addition of S-9. Aeschbacher et al. (1980) found that the deactivation of coffee brew was not due to protein
binding of the mutagens but rather due to metabolic deactivation. The possibility of such a deactivation was also suggested by the negative results obtained with coffee brew in host mediated assays (Aeschbacher and Wurzner, 1980)

Kasai et al. (1982) found that the major carbonyl compounds from an extract of ground roasted coffee beans were 5-hydroxymethylfurfural, acetol, glyoxal, methylglyoxal and diacetyl. Utilizing TA100 without a S-9 mix, they found that no more than 50% of the total mutagenic activity of coffee could be accounted for by the activity of methyl glyoxal. The addition of S-9 mix abolished its mutagenic activity almost completely. One cup of instant coffee (1g/100 mL) contains 100-150 ug of methyl glyoxal. Presumably the water-dispersible methyl glyoxal would not be present in WIF. Chlorogenic acid, caffeic acid and gallic acid also occur in large quantities in regularly consumed beverages such as coffee and tea. One cup of coffee contains 250-260 mg chlorogenic acid which has been found to be an effective antigenotoxic agent (Rosin and Stich, 1983). The above-mentioned phenolic compounds being water-soluble would not exist in WIF.

The roasting of green coffee beans brings about the formation of numerous Maillard reaction products. According to Baltes et al. (1981) about 30% of the nonvolatile ingredients of roast coffee are unknown. The majority of the unknown components are melanoidins. Melanoidins are resistant to decomposition in the intestinal tract and thus may be classified as a type of fiber (Fujimaki et al., 1979). Melanoidins formed in the Maillard browning reaction have many types of functional groups and thus could bind carcinogens. Melanoidins can bind metals and proteins (Gomyo and Horikoshi,
1976) and are potent antioxidants (Lingnert and Eriksson, 1979).

Chan et al. (1982) found that heating sucrose and an amino acid-reducing sugar system produced browning reaction components which possessed strong antigenotoxic activity. For example, they inhibited induction of reverse point mutations in Salmonella strain TA100 exposed to activated AFB1. The antimutagenic components of the browning reaction products were not identified. Melanoidins in the browning reaction systems may play a role in the antigenotoxic effect. Franzke and Iwainsky (1954) first reported the antioxidant action of melanoidins. Yamaguchi and Okamoto (1978) showed that reductones formed in the Maillard browning reaction from reducing sugars and amino acids were associated with antioxidant activity. Antioxidant potency varied greatly with different combination of sugars and amino acids. Wattenburg (1983) reported that green and roasted coffee beans enhanced the glutathione S transferase activity of liver by 6-fold and of the small bowel mucosa by 7-fold. Kahweol palmitate and cafestol palmitate were isolated from coffee and accounted for 50% of the inducing activity. An inhibitory effect against the occurrence of neoplasia was demonstrated.

Browning reaction products also exhibit weak genotoxic activities by themselves (Mihara and Shibamoto, 1980; Yoshida and Okamoto, 1980; Shinohara et al., 1980). Studies by Bjeldanes and Chew (1979) showed that several 1, 2 dicarbonyl compounds such as maltol and diacetyl displayed weak mutagenic activity with TA100. Both caramelized sucrose and reaction products in Maillard reaction model systems have clastogenic activity in Chinese hamster ovary cells and mutagenic activity with Salmonella strains TA100 and
TA98 (Stich et al. 1981a, 1981c; and Powrie et al. 1981; Rosin et al., 1982).

The objective of this investigation was to determine the extent of inhibition of carcinogen-induced mutagenesis with the coffee water-insoluble fraction and a model system melanoidin.
MATERIALS AND METHODS

Materials

The carcinogen, N-methyl-N'nitro-N-nitrosoquianidine (MNNG), was obtained from the Aldrich Chemical Company (Milwaukee, Wisc.). Procarcinogens benzo(a)pyrene (BP) and aflatoxin B1 (AFB1), were purchased from Sigma Chemical Company (St. Louis, Mo.). The bacterial media was obtained from Difco (Detroit, Mich.). The tester strains TA100 and TA98 Salmonella typhimurium were kindly supplied by Dr. Bruce N. Ames, (University of California, Berkeley). All other chemicals were reagent grade.

Preparation of Model System Melanoidins (MSM)

200 mL of a 0.8 M glucose-0.8 M lysine solution was adjusted to pH 9 prior to autoclaving for 1 hr at 121°C. After cooling, the solution was adjusted to pH 7 with 0.1 N NaOH solution. Absolute ethanol was added to obtain a 90% ethanol concentration in the dispersion. This was centrifuged at 10,800 X g for 10 min to sediment the particulate matter. After the supernatant was removed, the precipitate was resuspended in distilled water and subsequently freeze dried. This freeze dried fraction was termed the model system melanoidin (MSM).

Preparation of WIF from Coffee

Water-insoluble fraction (WIF) of coffee was prepared by ethanol precipitation of macromolecules from reconstituted spray dried coffee (Nescafe) as outlined in Figure 4 in Chapter 1. Twenty g of instant coffee
powder were dissolved in 40 mL of distilled water and subsequently 360 mL of absolute ethanol was added. The mixture was allowed to stand for 5 min and thereafter was centrifuged with an IEC table top centrifuge (Centra 7 head) at 10,000 × g for 5 min. The supernatant was discarded and the precipitate was dispersed in 80 mL of distilled water. The dispersion was centrifuged in a Sorvall RC-5B centrifuge (SS-34 rotor) at 11,200 × g for 1 hr and the residual precipitate was dispersed in distilled water and freeze-dried. For inhibition studies, various amounts of WIF were mixed in distilled water.

**Preparation of Phosphate Buffer Solution (PBS)**

One liter of PBS contained 80 g sodium chloride, 2.0 g potassium chloride, 11.59 g dibasic sodium phosphate and 2.0 g monobasic potassium phosphate.

**Preparation of S-9 Liver Microsomal Mixture**

The S-9 mix was prepared according to the method of Ames et al. (1975) from the livers of male Wistar rats treated with Arochlor 1254. Arochlor 1254, a polychlorinated biphenyl mixture, was diluted in corn oil (200 mg/mL) and injected intraperitoneally in each rat at dosage of 500 mg/kg body weight, 5 days prior to sacrifice. The rats consumed water and Purina laboratory chow ad libitum until 1 day before sacrifice. Their livers were homogenized in 0.15 M KCl (about 1 mL per g of wet liver). The homogenate was centrifuged for 10 min at 9000 × g and the supernatant was stored at -80°C in small glass vials until required.

S-9 mix consisted of 0.1 mL liver supernatant, 0.02 mL 0.4 M MgCl₂,
0.02 mL 1.65 M-KCl, 4.0 mmoles NADP and 10 mmoles glucose-6-phosphate dissolved in 0.2M phosphate buffer (pH 7.4) solution (PBS).

**Salmonella Mutagenicity Assay**

The inhibition of mutagenic activity of AFB₁, BP and MNNG by WIF and model-system melanoidin (MSM) was examined by using the Salmonella mutagenicity assay (Ames et al. 1975). TA100 strain is a histidine-requiring auxotroph which reverts to histidine independence by base-pair substitution. TA100 strain was used as an indicator organism for the mutagenic activity of AFB₁ and BP with S-9 mix treatment. The mutagenic activity of MNNG was assayed by TA100 strain without S-9 treatment. These bacteria were routinely tested for the presence of the ampicillin resistant R factor and with positive mutagenic controls, sodium azide and BP.

Experiments on the inhibition of mutagenicity of BP by MSM and WIF were conducted by using the procedure developed by Ames et al. (1975). Treatment mixtures consisted of the following in the indicated order: 0.1 mL of the bacterial suspension, 0.4 mL of either PBS or S-9 mix, 0.1 mL of MSM or WIF dispersion and 0.1 mL of BP solution (10 mg of BP was dissolved in 1 mL of PBS containing 15% DMSO). The treatment mixtures were incubated at 37°C in a shaking water bath for 20 min. Two mL of molten top agar (47°C) containing a small amount of histidine (0.455 mM solution) was then added to each treatment tube and the mixture was poured over minimum glucose agar in Petri plates (Ames et al. 1975). The small amount of histidine in the top agar allowed bacteria on the plate to undergo several divisions. Following incubation of the plates at 37°C for 2 days, the histidine revertant colonies were counted on an Artek model 880 automatic colony counter (Artek,
Farmingdale, N.Y.). The mutagenic activity was expressed as the number of histidine revertants per plate minus the number of spontaneous revertants.

Inhibition studies with AFB$_1$ and MNNG were carried out by using the suspension procedure of Rosin and Stich (1978), a modification of the method of Ames et al. (1975). This procedure permits an estimation of the survival of microorganisms treated with a carcinogen as well as the incidence of reverse mutations of the bacteria. Logarithmically growing cells were prepared for carcinogen treatment by the following method. 0.1 ml of a cell suspension of Salmonella TA100 grown for 16-18 hr at 37°C was added to 5.0 ml of fresh nutrient broth. The Salmonella cells in nutrient broth were grown with agitation on a rotary wheel in an incubator at 37°C for 4 hr. One ml aliquots of this culture were centrifuged (3000 x g for 5 min) and the resulting bacterial pellets were resuspended in 0.5 ml of treatment media. The treatment media with and without WIF or MSM consisted of either equal volumes of PBS and 5 x 10$^{-6}$ M solution of MNNG or equal volumes of PBS and 5 x 10$^{-6}$ M solution of AFB$_1$ and S$^\text{-9}$ mix. MNNG was dissolved directly into PBS while AFB$_1$ was dissolved in PBS with 15% DMSO. The suspension was incubated for 20 min at 37°C in a waterbath. After the incubation period, the treated bacteria were sedimented by centrifugation (3000 X g for 5 min) and washed by resuspending them in 0.5 ml PBS prior to recentrifugation. Each bacterial pellet was dispersed in PBS to achieve a level of approximately 10$^9$ cells/mL. 0.1 ml aliquot of each cell suspension was added to 2.0 ml molten top agar (0.455 mM histidine solution) which was overlaid on minimal glucose agar in Petri plates (Ames et al. 1975). The plates were incubated for 2 days at 37°C and the histidine revertant colonies were counted with an Artek model 880 automatic colony counter.
Mutagenic activity was expressed as the number of histidine revertants per plate minus the number of spontaneous revertants.

For determining the degree of bacterial cell survival, treated bacterial cells were added to 0.9% sodium chloride solution and introduced into nutrient agar in plates (Ames et al. 1975). The minimal agar plates were incubated for 2 days at 37°C. The number of colonies per plate were determined with an Artek automatic colony counter.
RESULTS AND DISCUSSION

Inhibition of AFB₁ Induced Mutagenesis by WIF and MSM

The WIF was examined for inhibition of AFB₁-induced mutagenesis by using the liquid suspension test modification (Rosin and Stich, 1978). The advantages of using this modification are two fold; 1) both reversion frequencies and survival frequencies can be estimated and 2) the exposure time of tester bacteria to a mutagen can be controlled. Mutagenic activities of the AFB₁ solution and the AFB₁-WIF mixture were expressed as the number of histidine revertant colonies per $10^7$ surviving cells minus the number of revertants from spontaneous mutation in the presence of the appropriate solvent concentrations. The spontaneous mutation rate of strain TA100 was 4.8 revertants per $10^7$ survivors. The strains were routinely tested for histidine requirement, the rfa mutation, ampicillin resistant R factor and uvr B deletion as outlined by Ames et al., (1975). A trace of histidine was added to the top agar to allow all the bacteria on the plate to undergo several divisions, otherwise cell reversion may not occur.

A dramatic inhibition of mutagenicity of AFB₁ (drop from 110 to 20 revertants per $10^7$ survivors) was observed when WIF was added at a level of 3 mg per 1 mL of treatment media containing AFB₁ and S-9 mix. At all levels of WIF from 0 to 50 mg/mL in the absence of AFB₁, insignificant mutagenesis occurred (Figure 25). As shown with the survivor curves in Figure 25, no toxic effects of AFB₁ and S-9 mix or WIF towards Salmonella organisms were experienced.

Aeschbacher et al. (1980) found that a concentration of 25-30 mg/plate of freshly brewed or instant coffee solids induced reversion in Salmonella
Figure 25 - Effect of WIF on cell survival and reversion frequency of AFB$_1$ treated Salmonella (TA100) organisms. (●), AFB$_1$ and S-9 mix treatment. Control samples without AFB$_1$ treatment (△).
TA100 without S-9 mix. This effect was regarded as weak (double the spontaneous reversion rate). The reversion was totally abolished in the presence of the microsomal S-9 mix. Experimentation provided evidence that enzymatic deactivation of the mutagens in coffee occurred when S-9 mix was added. S-9 mix without cofactors was found to have the same detoxification effect on the instant coffee mutagens as did S-9 mix with cofactors. Since the results of the present study showed that WIF did not induce significant reversion of TA100, the weak mutagens in coffee brew must be in the soluble fraction.

Melanoidin utilized in the present study for determining the inhibitory effect on AFB₁ mutagenicity was precipitated from a 90% ethanol dispersion of a heated lysine-glucose model system. A wide variety of high molecular weight species with a brown hue was expected. Increasing concentrations of freeze-dried melanoidin powder (MSM) from 0 to 100 mg/mL of treatment media were used to assess the inhibition of reversion of TA100 organisms treated with AFB₁ and S-9 mix. Figure 26 illustrates a substantial drop in the revertants/10⁷ survivors upon the addition of MSM at a level of 20 mg per 1 mL of treatment media. Further inhibition of AFB₁-induced mutagenesis was experienced with increasing concentrations of MSM from 20 to 80 mg. The inhibition of AFB₁-induced mutagenesis by MSM was not as great and effective as that exhibited by WIF. In the upper % survival graph for the TA100 tester strain (Figure 26), % bacterial survival levels for the control and the AFB₁ treatment media with and without MSM remained around 100.

A reduction in the mutagenicity of AFB₁ in these experiments by WIF and MSM may be explained by the adsorption of AFB₁ and/or the activated AFB₁ (by
enzymes in S-9 mix) to WIF and MSM. The ultimate reactive species of AFB₁ is currently believed to be the 2, 3-oxide although it has never been isolated. Studies have shown that the 2, 3 double bond of AFB₁ is required for activation because AFB₁ is more reactive in its carcinogenic, mutagenic and macromolecular binding activities than AFB₂ which does not have a double bond at the 2, 3 position (Butler et al. 1969; Wogan et al. 1971). Similarly, all other metabolites without a 2, 3 double bond are practically devoid of mutagenic activity (Wong and Hsieh, 1976).

Since the insertion of a 2,3-oxide group into the AFB₁ structure would not be expected to change the hydrophobicity appreciably, both AFB₁ and 2,3-oxide of AFB₁ could be bound to WIF and MSM through hydrophobic bonds.

**Inhibition of MNNG-Induced mutagenesis by WIF and MSM**

The inhibition of MNNG-induced mutagenesis by added WIF or MSM was examined by the liquid suspension test modification of Rosin and Stich (1978) with the use of the Ames/Salmonella TA100 strain test for mutagenicity. As shown in Figure 27, inhibition of MNNG-induced mutagenesis increased markedly after WIF was added to the treatment media at a level of 5 mg per mL. Further increases in the inhibition of mutagenesis were obtained with levels of added WIF to 25 mg/mL. As shown in the upper survival graph of Figure 27, the % survival of Salmonella (TA100) bacteria in the control and MNNG treatment media was around 100 with and without WIF.

Similar results were obtained when MSM replaced WIF as an inhibitor of mutagenicity of MNNG (Figure 29). The mutagenic activity of MNNG was reduced by more than 90% with the inclusion of 25 mg or more of MSM per 1 mL of treatment media. MSM, like WIF, did not induce histidine reversion above
Figure 26 - Effect of MSM on cell survival and reversion frequency of AFB$_1$ treated Salmonella (TA100) organisms (●) AFB$_1$ and S-9 treatment. (▲) Control, no AFB$_1$ treatment.
Figure 27 - Effect of WIF on cell survival and reversion frequency of MNNG-treated Salmonella (TA100) organisms. (•) MNNG treatment (△) Control, no MNNG treatment.
Figure 28 - Decomposition of MNNG.
(Neal and Colley, 1978)
Figure 29 - Effect of MSM on cell survival and reversion frequency of MNNG-treated Salmonella (TA100) organisms. (●) MNNG treatment (△) Control, no MNNG treatment.
the background level of spontaneous mutations. As shown in Figure 29, the % survival of Salmonella organisms was around 100 for increasing amounts of MSM. The degree of inhibition depended upon the concentration of MSM. The proposed decomposition pathway for MNNG at physiological pH values is shown in Figure 28 (Neale and Colley, 1978). Inhibitors act on MNNG by a scavenging action. MNNG is chemically unstable at physiological pH and decomposes spontaneously to a reactive alkylating derivative, a carbonium ion, which is believed to be the mutagenic species (Montesano and Bartsch, 1976; Neale and Colley, 1978). The evolution of the carbonium ion is supported by research showing the transfer of intact deuterated methyl groups to DNA by the treatment of DNA with N-trideuterio-methyl labeled MNNG in vitro (Haerlin et al., 1970; Lingens et al. 1971). Both WIF and MSM appear to prevent MNNG from exerting its mutagenic activity on the critical target of the cells by either interacting with MNNG directly or with some of its degradation products including the carbonium ion. This interaction should reduce the amount of carbonium ions available to cause mutagenesis.

Inhibition of BP-Induced Mutagenesis by WIF and MSM

The inhibition of BP-induced mutagenicity by WIF was examined by using the basic Ames mutagenicity test (Ames et al. 1975). The liquid suspension test modification of Rosin and Stich (1978) was not effective for inducing mutagenesis in Salmonella (TA 100) organisms by activated BP. Presumably the 20 min exposure of organisms to BP and S-9 mix was not sufficient to bring about genetic alterations. In the BP studies, Salmonella organisms were exposed to BP and S-9 mix for 20 min in the treatment mixture as well as 2 days on the agar plate. The results shown in Figure 30 illustrate a
Figure 30 - Effect of WIF on reversion frequency of BP-treated Salmonella (TA100) organisms. (●) BP treatment (△) Control, no BP treatment
gradual inhibition of BP-induced mutagenesis with increasing concentrations of WIF up to 30 mg/mL. Similar results were obtained for added MSM as shown in Figure 31.

According to Dipple (1983) BP is oxygenated by the mixed function oxidases to the 7, 8-epoxide which is stereospecifically converted to the trans-7, 8-diol. This product is in turn further metabolized by the mixed function oxidases to diol epoxide I. This diol epoxide has been shown to be the most mutagenic metabolite of BP (Gelboin et al. 1980). Presumably the epoxide was formed in the treatment mixture and agar plates since S-9 was added to the treatment mixture. WIF and MSM may adsorb this epoxide through hydrophobic bonding and thus prevent the transport of the mutagen into the bacterial cell.
Figure 31 - Effect of MSM on reversion frequency of BP treated Salmonella (TA100) organisms. (●) BP treatment (△) Control, no BP treatment.
SUMMARY

In conclusion, both MSM and the coffee water-insoluble fraction significantly inhibited the carcinogen induced mutagenesis in the Ames test systems studied. Possible explanations for the inhibition of carcinogen-induced mutagenicity by WIF and MSM are:

1. WIF and MSM adsorbed the carcinogens and metabolically activated carcinogens.
2. The cofactors, or enzymes of the monoxygenase system were adsorbed by WIF and MSM.
3. Free radicals formed in the metabolism of procarcinogens were quenched by the antioxidant groups in WIF and MSM.

It is essential to employ caution when using results from the _in vitro_ situation to predict results in _in vivo_ long-term animal studies. However it is of interest to note that several studies have shown that some antioxidants which inhibit mutagenicity in microorganisms have an inhibiting effect of carcinogen-induced neoplasia in animals (Wattenburg, 1979).
CHAPTER 3
BINDING OF AFLATOXIN B₁ AND BENZO(a)PYRENE BY
THE WATER INSOLUBLE FRACTION FROM COFFEE BREW

Introduction

In the previous study (Chapter 2), it was noted that when BP was incubated with an aqueous dispersion of WIF, the mutagenic activity of BP was reduced, the level being dependent on the concentration of WIF. One explanation for this observation is that a certain amount of the BP was bound to the WIF. Hydrophobic bonds would be expected to exist between BP and the hydrophobic groups of WIF.

WIF can be considered to be composed of particles consisting of melanoidins as well as polysaccharide-protein and protein-chlorogenic acid complexes (Clifford, 1975b). The polysaccharides of WIF have not been identified, although the results in Chapter 1 indicate that mannose, glucose, galactose and arabinose are constituents in the acid hydrolysate of WIF. McLaren et al. (1973) found that an 80% ethanol dispersion of molasses contained an insoluble fraction made up of hemicelluloses. Water-insoluble hemicelluloses may be present in WIF. Arabinogalactan has been isolated from instant coffee powder at a level of 5% (Clifford, 1975b) and thus would be expected to be present in WIF.

Polysaccharides have been shown to bind carcinogens. Smith-Barbaro et al. (1981) examined several types of polysaccharide fibres from different plant sources and found that they bound several types of carcinogens. β1-3 glycan is a polymeric carbohydrate found in foods as diverse as oatmeal and seaweed and may act as a protective agent against carcinogens in the diet.
(Teas, 1983; Di Luzio et al., 1979). Normand (1979) found that rice hemicelluloses are very effective binders of bile salts, which are considered to be potential promoters of colon cancer. Story et al. (1979) found a variety of dietary fibers can bind a variety of bile salts. Rubio et al. (1979) found that pectin and cellulose were ineffective in binding nitrosodiethylamine.

Melanoidins are formed during the roasting of the coffee bean and are constituents in the humic fraction. Melanoidins, an end product of the Maillard browning reaction, can be thought of as a type of fiber since they are adsorbed to a limited extent in the intestinal tract (Finot and Magnenat, 1981; Fujimaki et al., 1979). Gomyo and Horikoshi (1976) found melanoidins were very powerful in trapping metallic ions such as Fe$^{3+}$ and Cu$^{2+}$. Horikoshi and Gomyo (1976) found that melanoidins combine with proteins to form a precipitate, which was probably caused by electrostatic interaction. Schvets and Slysarenko (1979) found that melanoidins that had a positive electrokinetic potential were adsorbed on the surface of yeast cells. Somewhat polar carcinogens (such as nitrosamines) may be attached to melanoidins through electrostatic as well as hydrophobic bonds. No studies have been reported which determine the number of hydrophobic groups in melanoidins or the adsorption of hydrophobic carcinogens to these brown pigments. Humus, a mixture of browning reaction products in soil, has been studied extensively from the standpoint of chemical composition and reactivity with pesticides (Bartha, 1980). Physical adsorption and chemical bonding have been used to explain the strong bonding of pesticides to humus particles.
Melanins, the brown colored polymers, formed during the oxidation of phenolic compounds, can bind Aflatoxin B\(_1\) (AFB\(_1\)) (Powrie and Park, 1980). Model system melanin bound AFB\(_1\) rapidly under a wide pH range. A wide variety of drugs can interact with melanin. Larsson and Tjalve (1978) stated that non-electrostatic site on chlorpromazine and chloroquine must be involved in binding to melanin.

The objectives of the study were to examine the extent of binding of BP and of AFB\(_1\) to isolated WIF in aqueous dispersions at various pH levels, by a centrifugation technique, to determine the level of binding of BP by WIF with a coffee brew, to elucidate the type(s) of bonding between BP and WIF and to estimate the number of binding sites for BP on WIF with the aid of saturation plots.
MATERIALS AND METHODS

Chemicals

Procarcinogens, benzo(a)pyrene (BP) and aflatoxin B1 (AFB1), were purchased from Sigma Chemical Co. (St. Louis, Mo.). 3H-benzo(a)pyrene (30 Ci/nmol, 185 MBq) and aqueous counting scintillation cocktail were purchased from Amersham (Arlington Heights, Ill.) Sephacryl S-1000 superfine gel was purchased from Pharmacia (Bromma, Sweden) while Sephadex G-50 was obtained from Sigma Chemical Co.

Centrifugation Method to Assess Carcinogen Binding by WIF

The in vitro binding of two separate carcinogens (AFB1 and BP) and WIF was examined at pH values between 2 and 9. Each test carcinogen (1 x 10^{-9} moles 3H-BP and 1 x 10^{-6} moles of unlabelled BP or 1 x 10^{-6} moles AFB1), was mixed with 5 mL of WIF suspension (3 mg/mL of buffer), vortexed and incubated in a water bath with shaking at 37°C for 2 hr as shown in Figure 32. The following 0.15M specific buffers were employed in the assessment of the pH effect on carcinogen binding by WIF: glutamate buffer, pH 2.0; citrate buffer, pH 3.0; glutamate buffer, pH 4.0; acetate buffer, pH 5.0; citrate buffer, pH 6.0; phosphate buffer, pH 7.0; bicarbonate buffer, pH 8.0 and 9.0. After the 2 hr incubation, the WIF fraction in each carcinogen-WIF dispersion was sedimented by centrifugation for 1 hr at 11,200 X g in a Sorvall RC-5B centrifuge (SS-34 rotor). The unbound carcinogen resided in the supernatant. The BP in each aliquot of supernatant and of the carcinogen-WIF dispersion was determined from the number of disintegrations per min from a Packard tricarb liquid scintillation counter.
Freeze Dried WIF
3mg/mL buffer

100µL 1X10^-5 moles BP+
1X10^-9 moles ^3^H-BP
or 1X10^-6 moles AFB_1

Vortex

Sample 100µL

Incubate 37 °C, 2 Hours
on Rotary Shaker

Sample

Centrifuge 1 Hour 11,200 Xg

Sample

Figure 32 - Flow chart of the separation of unbound BP from bound BP by centrifugation of WIF dispersion.
Disintegrations were counted for 10 min. AFB₁ concentration was determined by measuring the fluorescence with a Turner model 430 spectrofluorometer at 370 nm excitation and 440 nm emission.

**Column Chromatography of BP-WIF Dispersion and BP-coffee Brew Mixture.**

Sephacryl S-1000 and Sephadex G-50 columns (2 x 35 cm) were employed to separate unbound BP from BP bound to WIF dispersed in a 0.15 M citrate buffer (pH 3.0). A Sephadex G-50 column was used for the separation of unbound BP from BP bound to components in coffee brew. The BP-WIF dispersion consisted of $1 \times 10^{-9}$ moles of $^3$H-BP and $1 \times 10^{-6}$ moles of unlabelled BP and 15 mg WIF or 15 mg instant coffee in 5 mL of 0.15 M citrate buffer (pH 3.0). The mixtures were held in a shaking water bath at 37°C for 2 hr. The eluant in all cases was 0.15M citrate buffer (pH 3.0).

For studies on the evaluation of types of bonds involved in binding of BP to WIF, eluants containing compounds which break specific types of bonds were introduced onto a Sephacryl S-1000 column after the BP-WIF dispersion was applied. The BP-WIF dispersion was prepared as indicated above. A 0.15 M citrate buffer (pH 3.0) was used as the eluant which also included the following bond breaking compounds: NaCl (3M), urea (8M), sodium dodecyl sulphate (1.0%), mercaptoethanol (5%) and a combination of sodium dodecyl sulphate (1.0%) and mercaptoethanol (5%).

In all of the above-mentioned experiments, 0.5 mL eluate fractions were collected in test tubes by a Gilford fraction collector. The determination of the scintillations per min for each tube was carried out with a Packard Tricarb liquid scintillation counter. For an estimation of the amount of
brown pigment in each eluate fraction, absorbance at 390 nm was measured with a Perkin-Elmer Lambda 3 spectrophotometer (Nabney and Nesbitt, 1965).

**Saturation Plots**

A procedure similar to that of Rennie *et al.* (1977) was carried out to determine the number of BP binding sites present in 1 mg of WIF. A mixture of unlabelled and tritium labelled BP was added to a dispersion of WIF (1 mg per mL) in 0.15 M citrate buffer at pH 3.0. The concentration of BP in the BP-WIF dispersion ranged from $1 \times 10^{-6}$ to $1 \times 10^{-9}$ M. After vortexing the BP-WIF dispersion, it was incubated at 37°C for 2 hr with shaking. The dispersions were centrifuged at 11,200 X g for 1 hr and an aliquot of each supernatant was removed to determine the disintegrations per min with a Packard tricarb liquid scintillation counter. The results were presented as a direct plot (bound BP vs. free BP) and a Scatchard plot (bound BP/free BP vs. bound BP).
RESULTS AND DISCUSSION

Binding of BP by WIF as Measured by the Centrifugation Method.

The binding of BP by WIF was examined by a centrifugation technique which separated the BP bound to WIF from the water dispersed BP. A 2 hr incubation time was considered reasonable, since transit time of food from ingestion to entrance into small intestine can be as low as 2 hr and since the adsorption of carcinogens to fiber has been found to be almost instantaneous (Rubio et al., 1979). The level of binding of BP by WIF was calculated from the difference in scintillation counts of $^3$H-BP in an aliquot of uncentrifuged WIF dispersion and those in an aliquot of the supernatant obtained after centrifugation of the WIF-BP dispersion. Quenching effect of WIF was minimized by channel ratio correction and the use of a uranium pellet as an external standard. The results in Fig. 33 indicate the level of BP - WIF interaction at various pH values. A binding value of 83% BP was found at the pH 2 level and approximately 63% binding of BP for pH levels between 4 and 9.

To assess if BP was entrapped in sedimented WIF particles, 5 mL samples of incubated BP-WIF dispersions (pH 3.0) were first centrifuged at 11,200 X g for 1 hr and then 2 mL of citrate buffer (pH 3.0) was added to each tube with the sediment and supernatant intact. After each tube was vortexed to disperse the sedimented WIF in the buffer, the dispersion was centrifuged again at 11,200 X g for 1 hr. An aliquot of each supernatant was used to determine the disintegrations per min. The total number of disintegrations per min for the supernatant of the added buffer-recentrifuged sample was approximately the same as the total number of disintegrations per min for
the supernatant of the centrifuged-once sample. These results suggest that no BP was entrapped by WIF sedimented during the first centrifugation period. If BP were entrapped by the sedimented WIF particles, an increase in the scintillation counts for the supernatant produced after the second centrifugation period would be expected particularly when the volume of the mixture was increased from 5 mL to 7 mL and when the diluted mixture was revortexed.

As shown in Fig. 33, the level of adsorption of BP to the WIF is greater at the lower pH values. At low pH values, ionization of carboxyl groups in WIF would be reduced with the possibility of greater exposure and accessibility to hydrophobic groups. Since the pH of the stomach juices is about 2 and the food mixture in the small intestine has a pH of about 8, bioavailability of BP in the presence of WIF should be reduced substantially in the intestinal tract of humans. Concentrations of BP in these in vitro model binding systems were above the values normally found in food.

**Binding of AFB₁ by WIF as Measured by the Centrifugation Method**

The binding capacity of the WIF for aflatoxin B₁ (AFB₁) was examined at pH values between 2 and 9. The experimental conditions were the same as those for the centrifugation technique used in BP binding experiments except that unlabeled AFB₁ was utilized. The concentrations of AFB₁ in the supernatant were determined by a spectrofluorometric technique (Nabney and Nesbitt, 1965).

The results from experimentation to estimate the level of AFB₁ binding to WIF are shown in Figure 34. The binding of AFB₁ by WIF at pH values between 2 and 9 ranged from 47 to 55% and thus pH effect on aflatoxin B₁ binding was not apparent.
Figure 34 - Effect of pH on the binding of AFB₁ by WIF.
Binding of BP by WIF as Assessed by Column Chromatography

The binding of BP with isolated WIF in suspension and with naturally-occurring WIF in reconstituted instant coffee brew was examined by separating the water-dispersed BP from the BP bound to WIF on chromatographic columns. Both Sephacryl S-1000 and Sephadex G-50 columns were used for the BP-isolated WIF suspension whereas Sephadex G-50 column was used for the BP-coffee brew mixture. A pH of 3.0 was selected for the eluant buffer since extensive binding of BP to WIF was experienced at this pH level (See Figure 33).

During the passage of isolated WIF through Sephacryl S-1000 column, three distinct brown bands, one major and two minor, were visible.

As shown in Fig. 35A, one large brown-colored peak (level of brown pigment was measured by absorbance) occurred between elution volumes of 20 and 30 mL during the chromatography of isolated WIF on the Sephacryl S-1000 column. When BP alone was eluted under the same chromatographic conditions, a BP peak (level of BP was measured as disintegrations/min) was experienced between elution volumes 50 and 57 mL (Figure 35A).

If BP were bound to WIF, BP should be eluted in the same elution volume range as that for WIF. The elution pattern (Sephacryl S-1000 column) in Fig. 35B for mixed BP-WIF in citrate buffer (pH 3.0) indicates that, in the elution volume range of 20 to 30 mL, a large BP peak overlaps the WIF peak. A small amount of unbound BP was eluted in the vicinity of 55 mL elution volume. Under the chromatographic conditions used, apparently a BP - WIF complex existed. When the mixed BP-WIF in citrate buffer (pH 3.0) was subjected to gel filtration on a Sephadex G-50 column, the BP peak was superimposed on the WIF peak at an elution volume range of 8 to 12 mL.
Figure 35 - (A) Column chromatogram (Sephacryl S-1000) of WIF alone (---, absorbance 390 nm) and BP alone (---, disintegrations/min) with citrate buffer (pH 3.0) as the eluant. (B) Column chromatogram (Sephacryl S-1000) of mixed WIF (---, absorbance 390 nm) and BP (---, disintegrations/min) with citrate buffer (pH 3.0) as the eluant.
(Figure 36). A small peak of unbound BP appeared in an elution volume range of 40 to 45 mL. These elution volume ranges for WIF and unbound BP are considerably lower than the respective values from the Sephacryl S-1000 column.

The next step in experimentation was to assess the binding of BP by WIF as it exists in a reconstituted instant coffee brew. Feldman et al. (1969) found that when roasted coffee brew was subjected to Sephadex G-25 column chromatography, the first band contained a dark brown high molecular weight pigment. According to Cassiar et al. (1978), who fractionated coffee pigments on a Sephadex G-25 column, one major brown band (FI fraction) was formed. During rechromatography on a Sephadex G-200 column, the FI fraction of the coffee brew passed through the column as one band without further separation.

When the instant coffee brew was subjected to column chromatography on Sephadex G-50, a large peak (brown-colored pigments) with an elution volume range of 10 to 16 mL appeared (Figure 36B). This elution volume range is similar to that for isolated WIF (Figure 36A). Presumably the components in the large peak for coffee brew are similar to those present in isolated WIF and will be considered as naturally-occurring WIF. When a BP-coffee brew mixture was passed through the Sephadex G-50 column, the absorbance peak and the peak for disintegrations per min overlapped in the elution volume range of 10 to 16 mL (Figure 37). From these results, it appears that the BP added to the instant coffee brew was bound by the naturally-occurring WIF in coffee brew.

The next experiments were conducted to try to elucidate the type of binding which occurs between WIF and BP. Several different eluants were
Figure 36 - (A) Column chromatogram (Sephadex G-50) of mixed WIF (---, absorbance at 390 nm) and BP (---, disintegrations/min) with citrate buffer (pH 3.0) as the eluant.
(B) Column chromatogram (G-50) instant coffee brew of alone (---, absorbance at 390 nm) and BP alone (---, disintegrations/min) with citrate buffer (pH 3.0) as the eluant.
Figure 37 - Column chromatogram (G-50) of mixed coffee brew (---, absorbance at 390 nm) and BP (---, disintegrations/min) with citrate buffer (pH 3.0) as the eluant.
used in an attempt to break the bonds between the BP-WIF complex applied to a Sephacryl S-1000 column. The citrate buffer (pH 3.0) eluants consisted of 3 M NaCl, 8 M Urea, 1% SDS, 5% mercaptoethanol, and a combination of 1% SDS and 5% mercaptoethanol.

Figure 38A illustrates the influence of 3 M NaCl-citrate buffer (pH 3.0) as the eluant on the elution pattern of the BP-WIF mixture compared to the BP-WIF elution pattern (Figure 35B) with a 0.15 M citrate buffer (pH 3.0) without NaCl. An increase in the free BP concentration in the eluate would have indicated that the WIF - BP complex was disrupted to some extent by breaking of coulombic bonds. The results from this experiment suggest that coulombic bonds are not the primary bonds involved in binding of BP by WIF. Strong electrostatic bonds would not be expected since BP does not possess electrically-charged groups. However, weak electrostatic bonds, called London dispersion forces, are formed between molecules regardless of their chemical nature (Gribnau, 1982). London dispersion forces are created from the interaction of fluctuating electronic dipoles with induced dipoles in neighbouring molecules. The dispersion forces undoubtedly are involved in the BP-WIF complexation and should not be influenced appreciably with NaCl at the 3M level.

When urea, a hydrogen and hydrophobic bond breaker, was added to the eluant citrate buffer, the majority of the BP added to the WIF dispersion was eluted within the same elution volume range as that for WIF (Figure 38B). If hydrogen bonds were involved in BP-WIF binding, the high concentration of urea in the eluant should release some BP. Since BP has a low hydrophilicity, the role of hydrogen bonding in binding of BP to WIF would not be expected.
Figure 38 - (A) Column chromatogram (Sephacryl S-1000) of mixed WIF (---, absorbance 390 nm) and BP (---, disintegrations/min) with 3 M NaCl in citrate buffer. (B) Column chromatogram (Sephacryl S-1000) of WIF (---, absorbance 390 nm) and BP (---, disintegrations/min) with 8 M urea in citrate buffer (pH 3.0) as the eluant.
With the ability of urea to alter the conformational structure of proteins, any proteins in WIF could possibly be altered structurally by urea treatment. However, such changes should have no influence on BP binding. Since WIF in urea eluant was eluted at about the same elution volume range as WIF in citrate buffer (no urea present), any appreciable alteration in size of WIF particles in an urea environment is unlikely. Levine et al., (1963) found that the denaturation of some proteins does not involve either simple monofunctional hydrogen bonding or a hydrophobic interaction with a denaturing agent, whereas with other proteins, hydrophobic interaction with the denaturing agent appears to play a much more important role. A broad range of H-bonded interactions involving denaturing agents can be expected to occur in proteins and other biopolymers (Vinogradov and Linnel, 1971). Urea exhibits a favorable interaction with non-polar solutes and substituent groups, in spite of its high polarity, but the mechanism of this interaction is not understood. (Roseman and Jencks, 1975).

Figure 39A illustrates the influence of a 5% mercaptoethanol buffer (pH 3.0) eluant on the release of BP from WIF. Mercaptoethanol should break disulphide linkages which may be hydrophobic sites for BP binding. With the formation of more hydrophilic groups, BP binding should be reduced. The results show that difference between elution patterns (Figure 39A) of mixed BP-WIF with the eluant containing 5% mercaptoethanol and the elution pattern of mixed BP-WIF with the citrate buffer (pH 3.0) eluant were not significant. Thus disulfide bonds probably do not participate in the binding of BP to WIF.

The contribution of hydrophobic bonding to the stability of the BP-WIF complex was examined by using an eluant of 1% sodium dodecyl sulphate
Figure 39 - (A) Column chromatogram (Sephacryl S-1000) of mixed WIF (—, absorbance 390 nm) and BP (---, disintegrations/min) with 5% mercaptoethanol in citrate buffer. (B) Column chromatogram (Sephacryl S-1000) of mixed WIF (—, absorbance at 390 nm) and BP (---, disintegrations/min) with 1% SDS in citrate buffer (pH 3.0) as the eluant.
(SDS) in citrate buffer (pH 3.0). Ionic detergents such as SDS are the most potent protein denaturants known (Steinhardt, 1975). SDS brings about an unfolding of proteins because of a difference in binding equivalence. In a 1% SDS solution, about 15 moles of detergent are required to bring about unfolding of one mole of protein. The ratio of urea molecules to protein molecules for unfolding under identical conditions is about 40,000 to 1.

The elution pattern in Figure 39B for a BP-WIF mixture on a Sephacryl S-1000 column with an eluant containing 1% SDS is different from that with a citrate buffer (pH 3.0) eluant as shown in Figure 35B. The WIF appears to be broken down to a brown-colored pigment with a molecular weight less than 10,000. BP was eluted from the column as a peak without the same elution volume range (38 to 43 mL) as that for the peak containing brown-colored pigment. Presumably the BP is bound to the brown-colored pigment in the peak. The brown-colored pigment may be the melanoidin component of WIF and is released from other components of WIF by the breakage of hydrophobic bonds. When WIF was dispersed in a 1% SDS solution, a brown solution containing sedimentable light brown particles was formed. When WIF dispersed in a 1% SDS-citrate buffer, was dialyzed against the buffer in a dialysis tubing (M.W. cut-off of 3,500), brown pigment passed into the dialysate.

When the BP-WIF mixture was heated in the 5% mercaptoethanol and 1% SDS solution (citrate buffer pH 3.0), at 100°C for 1 min to break all but the covalent bonds, an elution pattern shown in Figure 40 was obtained. This pattern was similar to that for 1% SDS eluant. Even with such rigorous treatment of WIF, apparently the BP was bound to the brown-colored pigment originating from WIF.
Treatment of the BP-WIF mixture on a Sephacryl S-1000 column with bond breaking compounds did not release BP from BP-WIF complexes. From these results it may be implied that coulombic, hydrogen and hydrophobic bonds are not involved in the complexation of BP by WIF. Certainly coulombic and hydrogen bonds would not be expected to play a role in BP-WIF binding since BP possesses a high degree of hydrophobicity, and indeed, has electrically-charged or polar groups. It is of interest to note that neither salt nor urea brought about a significant change in the molecular weight of WIF particles. Apparently the majority of bonds between the WIF components are not coulombic or hydrophilic, but rather hydrophobic since SDS decomposed WIF. When SDS molecules came in contact with the BP-WIF complex, BP may have been released from WIF by breakage of hydrophobic bonds. However, the hydrophobic portions of SDS molecules may have formed a bond with the hydrophobic portion of BP and the hydrophilic head of the same SDS molecule may have interacted with the melanoidin portion of WIF. Melanoidins are known to be polyions with positively and negatively changed groups (Gomyo and Horikoshi, 1976). Such an explanation would account for the action of SDS on BP-WIF complex to form a low molecular weight brown-colored pigment with BP presumably attached (Figure 39B).

Hydrophobic bonding is a typical example of a thermodynamic force which exists only in a liquid medium (Gribnau, 1982). The most common type of hydrophobic bond occurs with water as the solvent, since water is a strongly associated liquid with extensive hydrogen bonding. Hydrophobic bonding plays a very important role in the attachment of biopolymers to surfaces and in the establishment of the tertiary structure of proteins. Certainly the possibility of BP interacting with SDS molecules in an aqueous
Figure 40 - Column chromatogram (Sephacryl S-1000) of heat-treated mixed WIF (—, absorbance at 390 nm) and BP (---, disintegrations/min) with 1% SDS and 5% mercaptoethanol in citrate buffer (pH 3.0) as the eluant.
environment through the competition with hydrophobic groups on WIF must be considered.

**Saturation Plots for BP and WIF**

When ligands such as BP and binding sites on particles such as WIF are present in a system, a state of equilibrium occurs between free ligands and bound ligands. The interaction scheme at equilibrium without a breakdown of the ligand is:

\[ S + L \rightleftharpoons SL \]

where \( S \) is the site and \( L \) is the ligand.

The equilibrium equation for this scheme is:

\[ \frac{(S)(L)}{(SL)} = K_d \]

where \( K_d \) is the equilibrium dissociation constant.

As the concentration of the ligand in a system increases, the amount of ligand bound by binding sites increases, or in other words, the level of site saturation increases. The relationship between ligand concentration and site occupancy can be represented graphically by saturation curves (Boeynaems and Dumont, 1980; Klotz, 1982). In the present study, the direct plot (bound BP vs free BP) and the Scatchard plot (bound BP/free BP vs bound BP) were used.

One distinctive characteristic on a direct plot of the concentration of bound ligand vs the concentration of unbound ligand is an inflection point. The presence of an inflection point in a curve introduces a sigmoid shape. A direct plot for the binding of BP by WIF is shown in Figure 41A which has a sigmoid curve with one inflection point. The plot indicates that with ain
increasing concentration of unbound BP, the concentration of bound BP increases markedly and then levels off. Sigmoidicity implies positive cooperativeness which is a positive change in the affinity of the binding sites for the ligand during the process of saturation of binding sites.

The Scatchard plot, a graph of the concentration of bound ligand/concentration of unbound ligand vs the concentration of the bound ligand, is shown in Figure 41B. The right upward convexity pattern of the curve and a maximum indicate increasing affinity of the binding sites on WIF for BP (positive cooperativity). The maximum at about $5 \times 10^{-8}$ moles is evident in Figure 41B. The conditions for obtaining a maximum on the Scatchard plot and a sigmoid direct plot are identical.
Figure 41 - (A) Direct plot, bound BP versus free BP for 1 mg of WIF and increasing concentrations of BP. (B) Scatchard plot bound/free BP versus bound BP for 1 mg of WIF and increasing concentrations of BP.
In summary, WIF was very effective at binding both AFB₁ and BP through a range of pH values in utilizing a centrifuge binding technique. A binding value of 83% BP was found at pH 2 and approximately 63% binding of BP for pH values between 4 and 9. The binding of AFB₁ by WIF at pH values between 2 and 9 ranged from 47 to 55% and thus pH effect on aflatoxin B₁ binding was not apparent. Treatment of the BP-WIF mixture on a Sephacryl S-1000 column with bond breaking compounds did not release BP from the BP-WIF complexes. From these results it may be implied that coulombic, hydrogen and hydrophobic bonds are not involved in the complexation of BP by WIF. The column chromatogram experiments suggest that the majority of bonds between the WIF components are not coulombic or hydrophilic, but rather, hydrophobic since SDS decomposed WIF.

In the 1 mg saturation plots, with increasing concentration of unbound BP versus the concentration of unbound, BP, the concentration of bound BP increases markedly. This implies positive cooperativeness which is an increasing affinity of the binding sites on WIF for BP.
CHAPTER 4

Inhibition of BP Induction of Aryl Hydrocarbon Hydroxylase in the Small Intestine of Rats

Introduction

Two major sources of polycyclic aromatic hydrocarbons (PAH) which contaminate foods are one of the more important compounds in pyrolytic particles in smoke and petroleum products (Dunn, 1982). Smoke particles created by high temperature combustion of petroleum products and wood frequently contain PAH compounds. These pollution particulates can contaminate the surface of growing fruits and vegetables in production areas. Benzo(a)pyrene (BP) has been found in fresh green vegetables at concentrations up to 24 µg/kg. Deliberate exposure of foods to wood smoke in order to enhance flavor or storage characteristics is a source of BP. Further, during charbroiling of meat or fish, fat may drip onto hot coal or metal and become pyrolyzed with the result that additional PAH-containing particulate matter may be deposited on food. Charcoal broiled beef contains BP at levels of up to 50 µg/kg (Lijinsky and Ross, 1967, Lijinsky and Shubik, 1964).

The second major source of contamination of PAH in foodstuffs is petroleum or coal tar products which come in contact with foods. For example, petroleum pollution of the marine environment can contaminate seafoods. Oysters and mussels have been found to contain BP at levels up to 9 µg/kg (Dunn, 1982; Dipple, 1983).

The carcinogenicity of BP in various laboratory animals species has been well established. A single 0.2 mg dose of BP administered intragastric-
cally caused the formation of forestomach tumours in mice (Perce, 1961). Mice fed a diet containing BP at 250 mg/kg developed an increasing number of forestomach tumours as the duration of the experiment was extended (Rigdon and Neal, 1969), and mice exposed to BP at the above level of concentration for 140 days developed lung tumours and leukemia in addition to forestomach tumours (Rigdon and Neal, 1969). In Sprague-Dawley rats, a single dose of 100 mg BP resulted in the induction of mammary tumours (Dipple, 1983). Hamsters, fed a diet with 500 mg/kg of BP for 4 days/week over a duration of up to 14 months, developed esophageal, intestinal and stomach tumours (Chu and Malmgren, 1965).

BP has been shown to be mutagenic to Ames/Salmonella strains (Hollstein et al., 1979; Nagao et al., 1977), to be genotoxic in the hepatocyte primary culture-DNA repair test (Tong et al., 1981) and to induce mutations in the epithelial cells in the liver of rats (Tong et al., 1981). It is not known to what extent these compounds contribute to human cancer.

Metabolism of BP is required for the expression of carcinogenic potential. However, it should be noted that the major portion of BP entering the body is detoxified through various metabolic modifications. Metabolic studies have shown that cultured human colon cells can convert BP to the proximate carcinogen 7,8-dihydroxy BP, which binds to colonic DNA. Human feces and fecal bacteria can also hydrolyze biliary metabolites of BP to dihydrodiols, phenols and quinones (Autrup et al., 1977, Renwick and Drasar, 1976). To bring about an alteration of genetic DNA, PAH must be absorbed in the gastrointestinal tract. In a study with humans, volunteers consumed about 9 µg BP (Hecht et al., 1979). Based on the results with rats, excretion of approximately 0.9 µg of unchanged BP/person was expected. Instead,
BP levels in feces of the volunteers were less than 0.1 ug/person. These results may be explained by differences in metabolism of BP by fecal microflora in the rats and human volunteers and the differences in dose of BP (Hecht et al., 1979).

The oxidative transformation of BP is effectively catalyzed by the microsomal mixed function oxidase enzyme system in which cytochrome P-450 functions as a terminal oxidase. Cytochrome P-450 consists of a family of heme proteins which differ in their physical and enzymatic properties and each is under separate genetic control (Dipple, 1983).

The pathways of BP metabolism are diverse and complex and result in the conversion of BP to more than 40 metabolites including phenols, dihydrodiols, quinones, and water soluble sulphate, glucuronide and glutathione conjugates. In addition, BP is converted to diol epoxides, which are considered to be the major carcinogenic and mutagenic intermediate of BP metabolism (Gelboin et al., 1980, Capdevilla et al., 1980).

Figure 42 presents a summary of the current state of knowledge on BP metabolism. BP is converted by the mixed function oxidase to four unstable intermediates and five phenols, the 1,3,6,7 and 9 phenols. The phenols arise largely through epoxide rearrangement but can also be formed by direct oxygenation. The epoxides can also be enzymatically hydrated to three dihydrodiols by epoxide hydratase. The phenols also give rise to three quinones. All of these oxygenated intermediates can be further metabolized to water soluble products by conjugation with either glutathione sulphate or glucuronic acid. Almost all of these metabolites are detoxified and exhibit little or no mutagenic or carcinogenic activity (Dipple, 1983; Capdevilla et al., 1980).
Figure 42 - Metabolism and activation of BP.
(Capdevilla et al., 1980)
Aril hydrocarbon hydroxylase (AHH) system is present in various tissues of the monkey, hamster and rat (Nerbert and Gelboin, 1969). Administration of a polycyclic aromatic hydrocarbon into the body of an animal induces the hydroxylase to higher activity levels in the liver, lung, gastrointestinal tract and kidney in each of the above-mentioned mammals. The magnitude of aryl hydrocarbon hydroxylase induction varies greatly with the tissue and species from no induction to more than 100 fold increase in enzyme activity. According to Clinton et al. (1978), 1 mg of BP per g of diet brought about a 10 fold increase in the AHH activity in the small intestine of rats.

Induction by dietary components has been suggested as the probable cause for the routine occurrence of aryl hydrocarbon hydroxylase in intestinal mucosa (Stohs et al., 1976). A 15-fold decrease in the hydroxylase activity has been reported to occur in the duodenal mucosa of rats starved for 72 hr or fed a fat free diet (Nerbert & Gelboin, 1969). Clinton et al. (1978) found that when wheat bran was introduced into a rat diet, the induction of AHH by BP was reduced. Presumably this fibre-containing plant component decreased the absorption of BP by physical binding. Smith-Barbaro et al. (1981) hypothesized that certain types of dietary fiber differentially bind colon carcinogens to render them unavailable for absorption with the colon mucosa. Melanoidins may be considered as a type of fiber (Fujimaki et al., 1979) and could possibly bind dietary BP to prevent absorption in the intestinal tract.

The objective of this study was to examine the possibility of reducing the free BP in the small intestine of rats by model system melanoidins (MSM) incorporated in a rat diet containing BP. Powdered rodent laboratory chow was used as the basic feed throughout experimentation. 5% corn oil was
added to increase the lipid content of the diets and to act as a carrier of BP. The bioavailability of dietary BP in the small intestine can be judged by examining the AHH activity in the intestinal wall. The AHH activity can be determined by measuring the conversion of BP to 3-hydroxy BP (Figure 43) by a spectrofluorometric procedure.
Figure 43 - Enzymatic conversion of BP to 3 hydroxy-BP.
(Nerbert and Gelboin, 1969)
**MATERIALS AND METHODS**

**Chemicals and Dietary Components**

Benzo(a)pyrene, bovine serum albumin and trypsin inhibitor were purchased from Sigma Chemical Co. (St. Louis, Mo.). Glucose-6 phosphate was obtained from Calbiochem-Behring Corp (San Diego, California). Heparin was purchased from Harris Labs (Brampton, Ontario). All other chemicals were reagent grade. Corn oil was purchased from Arrowhead Mills (Omaha, Nebraska) while rodent laboratory chow was purchased from Ralston Purina (Woodstock, Ontario).

**Model System Melanoidins (MSM)**

The model system melanoidins (MSM) were prepared as follows: 200 mL of a 0.8 M glucose - 0.8 M lysine solution was adjusted to pH 9 prior to autoclaving for 1 hr at 121°C. After cooling, the solution was adjusted to pH 7 with 0.1 N NaOH. Absolute ethanol was added to obtain a 90% ethanol concentration. This was centrifuged at 10,800 X g for 10 min and the supernatant removed. The precipitate was resuspended in 20 mL of distilled water and subsequently freeze dried. This freeze-dried fraction was called the MSM. The moisture content of the freeze-dried material was about 3%.

**Animal Studies**

Male Wistar rats weighing 155 to 210 g were held individually in stainless steel cages with wire floors and no bedding. Twelve rats were randomly assigned to each of the four experimental diets following a 24 hr fast (Table 9). Diet C was the control diet consisting of powdered rodent
### Table 9

**Experimental Diets in Rat Study**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Rodent Chowder</th>
<th>Corn Oil</th>
<th>Melanoidin</th>
<th>BP</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>+</td>
<td>5%</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>C+M</td>
<td>+</td>
<td>5%</td>
<td>7.5%</td>
<td>--</td>
</tr>
<tr>
<td>BP</td>
<td>+</td>
<td>5%</td>
<td>--</td>
<td>1mg/g</td>
</tr>
<tr>
<td>BP+M</td>
<td>+</td>
<td>5%</td>
<td>7.5%</td>
<td>1mg/g</td>
</tr>
</tbody>
</table>
laboratory chow with 5% corn oil added. Diet C + M was the control diet containing 7.5% MSM powder. Diet BP was composed of the control diet with 1 mg of BP/g of diet. BP was solubilized in the corn oil prior to mixing with the powdered rodent laboratory chow. Diet BP + M was prepared by adding 7.5% melanoidin to the BP diet. Feeding was ad libitum for 48 hr. Feed jars were removed from the cages 3 hr prior to sacrifice.

Aryl Hydrocarbon Hydroxylase (AHH) Determination

The microsomal fraction was removed from the surface of the first 25 cm of the small intestine of rats by a scraping procedure (Stohs et al., 1976). AHH activity was assayed by the method of Nerbert and Gelboins (1969) with the use of a Turner spectrofluorometer (model 430) with excitation at 396 nm and emission at 522 nm. Protein concentration was assayed by the method of Bradford (1976). Crystalline bovine serum albumin was used as the protein reference standard. Analysis of variance was used to assess significant differences (Sokal, 1973).
RESULTS AND DISCUSSION

A dietary component which limits BP entrance into the epithelium of the small intestine should reduce the AHH activity in the microsomes of cells of the intestines. Undigestible components such as dietary fiber and melanoidins may bind carcinogens in vivo to promote their excretion in fecal matter. Clinton et al. (1978) reported that the inclusion of 10% wheat bran to a semi-synthetic diet with added BP (1 mg per g of diet) brought about a reduction in the induction of AHH activity in comparison with the AHH activity induced by BP in the absence of wheat bran in the diet. Since the majority of nondialyzable melanoidins in a heated glucose-glycine model system were found by Fujimaki et al. (1979) to be excreted in the feces of male rats, it was reasoned that melanoidins may bind BP in the intestinal tract, carry the carcinogen into the fecal matter and thus reduce induction of AHH activity in the small intestine.

In the current study, BP was selected as a carcinogen to be added to the rat diet because it is a common contaminant in food, is available as a purified compound and is known to be an effective inducer of aryl hydrocarbon hydroxylase (AHH) activity in the small intestinal mucosa. Clinton et al. (1978) found that 1 mg of BP per g of diet brought about a 10-fold increase in the AHH activity in the small intestine of rats. This concentration level of BP was selected for the experimental diets in this investigation.

Powdered rodent laboratory chow was chosen in this study as the diet to which BP and model-system melanoidin (MSM) were to be added. The chow is readily consumed by rats and thus a reduction in consumption of the chow
with added melanoidin would not be expected. A switch from chow to a semi synthetic diet could lower the intake. Wattenburg (1980) observed that the AHH activity in the gastrointestinal tract of rats was reduced dramatically with a reduction of food intake. Since the rodent laboratory chow used in this investigation contained a small amount of wheat bran, BP in the experimental diet may be bound to the bran as well as to the melanoidin.

Rats on the control and experimental diets had high feed intakes (16.5 to 19.9 g/day) as shown in Table 10. According to the data of Clinton et al. (1978) the intake of semi-synthetic diets with and without BP and wheat bran by rats ranged between 10.8 and 12.5 g per day. In the present study, for a period of 2 days, the average BP intake was 16.5 mg per day for the BP diet, 18 mg per day for the BP melanoidin diet. The weight gains of the rats (Table 10) are in the normal range.

Melanoidins are decomposed and adsorbed to a limited extent in the intestinal tract of rats and thus may act as a type of dietary fiber. Nair et al. (1981) studied the absorption of Maillard reaction products of a $^{14}\text{C}$ glucose-lysine mixture in rats. Absorption of the water-soluble high molecular weight fraction and the water-insoluble fraction was considered to be insignificant since all of the activity of these fractions was found in the feces. These results are in agreement with those of Finot and Magnenat (1981). Homma et al. (1982) found that the growth response and digestibility of protein and lipids in rats fed dietary nondialyzable melanoidin were not significantly different from those of the control. Excretion of melanoidins in the feces was about 76%. When the fecal nitrogen derived from the melanoidin was corrected for, protein digestibility was estimated
to be about 94%. They concluded that the addition of non-dialyzable melanoidin to a diet containing 10% protein level had no effect on protein digestibility. No unusual behavior was observed in the rats fed melanoidin diets throughout a 2-month period. Examination of the digestive tract indicated that no difference existed between the control rats and those on the melanoidin diet.

Horikoshi et al. (1981) examined the role of Maillard reaction products with a glucose-glycine model system to determine possible inhibition or promotion on the growth of intestinal microflora. The number of viable cells of six bacterial groups including enterococci, staphylococci, coliforms, clostridia, and lactobacilli (aerobic and anaerobic) were assessed. The administration of browning products caused an increase in the growth rate of both aerobic and anaerobic lactobacilli in the intestinal tract. The pH at the end of the ileum was found to be lower than expected in the usual digestive juice and may have been favorable for the growth of the lactic group. Melanoidins have been shown to decrease the growth of microorganisms (Leite et al. 1979, Gomyo and Horikoshi, 1976).

The measurement of AHH activity as outlined by Nerbert and Gelboin (1969) is sufficiently sensitive to detect $10^{-12}$ moles/mL of hydroxylated BP. The assay depends upon the enzymatic hydroxylation of BP to phenolic compounds which when added to an alkaline solution become fluorescent (Figure 44). One unit of AHH activity is expressed as the level of catalysis for the breakdown of BP over a 30 min period to hydroxylated BP compounds which have a fluorescence equivalent to 1 nmole of 3-hydroxy BP.

AHH activities for the small intestines of rats fed the different diets are shown in Figure 44. The data of Stohs et al. (1976) indicated
Figure 44 - Specific activity of aryl hydrocarbon hydroxylase in the small intestine of rats fed the experimental diet.
that with rats fed ad libitum on pelleted rat food, the AHH activity for intestinal microsomes was about 2.4 nmoles 3-OH BP/30 min/mg protein. According to Clinton et al. (1978), rats on a semi-synthetic control diet had an AHH activity for intestinal microsomes of 25 nmoles 3-OH BP/30 min/mg protein. In the present study, an intermediate value of around 5.7 nmoles 3-OH BP/30 min/mg protein was determined for intestinal microsomes from rats on a rat laboratory chow. Variations of AHH activity in intestinal microsome of laboratory animals may be attributed in part to dietary and genetic factors (Nerbert and Gelboin, 1969). Sprague-Dawley rats were used in the experiments of Clinton et al. (1978) whereas Wistar rats were used in the present study.

With the addition of 7.5% MSM to the rodent laboratory chow, the AHH activity in the small intestinal microsomes did not increase significantly (around 6.8 nmoles 3-OH BP/30 min/mg protein) above that for rats on the control diet. However, the AHH activity for rats on the BP diet was significantly greater ($p < 0.01$) at around 12.9 nmoles 3-OH BP/30 min/mg protein compared to rats on the BP + M diet, at around 9.7 nmoles 3-OH BP/30 min/mg protein.

These results cannot be attributed to differences in intake of calories, other nutrients or the amount of BP ingested (Table 10). There was no significant difference in wet weights or in total microsomal protein for the first 25 cm of the small intestine (Table 11). These results indicate that the model system melanoidin reduced the exposure of the intestinal epithelium to BP in the free form. The melanoidin may have altered the metabolism of the BP or prevent the BP from contacting the intestinal wall through binding.
Table 10

Food intake, benzo(a)pyrene intake and weight gains in rats on experimental diets

<table>
<thead>
<tr>
<th>Diet</th>
<th>Feed Intake (g/day)</th>
<th>BP Intake (mg/day)</th>
<th>Weight Gain (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>19.9±0.3</td>
<td>—</td>
<td>5.8± 3.2</td>
</tr>
<tr>
<td>C+M</td>
<td>19.7±3.4</td>
<td>—</td>
<td>3.4± 2.0 +</td>
</tr>
<tr>
<td>BP</td>
<td>16.5±1.1</td>
<td>16.5</td>
<td>6.0± 3.3</td>
</tr>
<tr>
<td>BP+M</td>
<td>18.0±2.7</td>
<td>18.0</td>
<td>9.4± 3.3 +</td>
</tr>
</tbody>
</table>
Table 11

Wet weight and total microsomal protein in the first 25cm of the small intestine of rats on the experimental diet

<table>
<thead>
<tr>
<th>Diet</th>
<th>Intestinal Weight (g)</th>
<th>Total Intestinal Microsomal Protein (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>1.8±0.4</td>
<td>8.4±2.7</td>
</tr>
<tr>
<td>C+M</td>
<td>1.7±0.3</td>
<td>10.6±2.7</td>
</tr>
<tr>
<td>BP</td>
<td>2.6±0.2</td>
<td>10.5±2.8</td>
</tr>
<tr>
<td>BP+M</td>
<td>2.2±0.1</td>
<td>9.5±1.6</td>
</tr>
</tbody>
</table>
SUMMARY

In summary, it may be suggested that BP induced AHH activity in intestinal microsomes of rats on different diets was significantly reduced by MSM. This indicates a protective effect of MSM with BP. Components of MSM presumably bind BP to the extent that less BP is available for induction of AHH activity.
CONCLUSION

This study was undertaken to characterize the water-insoluble fraction from reconstituted instant coffee powder; examine the genotoxic inhibitory effect of WIF and MSM; determine the degree and type of binding of BP and AFB₁ by WIF; and assess the effect of MSM on the inhibition of BP induction of aryl hydrocarbon hydroxylase in the small intestine of rats.

The coffee-WIF represents slightly more than 3% of the spray dried instant coffee with an average molecular weight estimated at 200,000. Elemental analysis suggested a structure with high amounts of carbohydrates present and some amino acids. Many different elements were incorporated into the WIF structure, with potassium in the highest concentration. Reductone activity was observed which suggested antioxidant properties. Phenolic compounds were present at a level of 4 mg of caffeic acid equivalent per 10 mg of WIF. Eleven amino acids were identified in the acid hydrolyzate of WIF, the major ones being aspartic acid, glutamic acid, glycine, valine, isoleucine and histidine. Total sugar analysis indicated a carbohydrate content of WIF of approximately 56% by the Dubois et al. (1956) method. Paper chromatography, gas liquid chromatography and GLC-mass spectroscopy indicated that mannose, galactose, glucose and arabinose were the major monosaccharides in acid hydrolyzed WIF. GLC-mass spectroscopy showed that the carbohydrates in the WIF hydrolyzate were mostly simple hexose sugars with trace amounts of deoxy-sugar fragments, but no N-acetyl glucosamine were identified.

The Ames Salmonella test showed that WIF and MSM reduced the genotoxic effect of AFB₁, MNNG and BP over a wide range of dose combinations. The
most likely explanation is that WIF and MSM adsorbed these procarcinogens and/or metabolic activation was inhibited. It may also be possible that the metabolic products, cofactors or enzymes of the monooxygenase system were adsorbed by WIF and MSM. Also free radicals may have been quenched by the antioxidant groups in WIF and MSM.

WIF was very effective at binding both AFB₁ and BP through a range of pH values in various buffers at 37°C. A maximum binding of 83% of BP by WIF was achieved at pH 2.0 and approximately 63% binding of BP occurred at pH values ranging from 4 to 9. The binding of AFB₁ by WIF at pH values between 2 to 9 ranged from 47 to 55%. A pH effect was not observed with the AFB₁ experiments. Binding of BP by WIF was examined by column chromatography using different eluants to try to determine the type of binding. Hydrophobic bonds may be involved in the binding of BP to WIF.

The BP induced AHH activity in intestinal microsomes of rats on different diets indicated that MSM significantly reduced the AHH activity which suggested a protective effect of MSM with BP. Components of MSM presumably bind BP to the extent that less BP is available for induction of AHH activity.

Both WIF and MSM showed an inhibitory effect on carcinogen induced activity in the systems studied. Presumably this protective effect was due to absorption of the procarcinogens onto the water-insoluble complexes in coffee brew and melanoidins in heat treated foods.
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