AN ABSTRACT OF THE THESIS OF

Vibeke Breinholt for the degree of Doctor of Philosophy

in Toxicology presented on April 21, 1994.

Title: Chlorophyllin Anticarcinogenesis in the Rainbow Trout Model.

Abstract approved by : ___ Redacted for Privacy

George S. Bailey

Chlorophyllin (CHL), a derivative of the green plant pigment chlorophyll, was found to be a potent inhibitor of hepatocarcinogenesis induced by aflatoxin B_1 (AFB_1), the only accepted dietary human carcinogen (IARC Monograph, 1993). At a dietary level of 1400 ppm, a fraction of the chlorophyll content of typical spinach cultivars, CHL provided up to 70% protection against tumorigenesis in the rainbow trout model. Molecular dosimetry analyses revealed that CHL-mediated reduction in target organ AFB_1-DNA adduction completely accounted for the decrease in tumor incidence at lower CHL doses, whereas at the highest level of CHL tested the inhibition of AFB_1-DNA binding was less than 50% of the observed tumor reduction. This result indicates that CHL exhibits other protective mechanisms in addition to inhibiting target organ DNA damage. The nature of these inhibitory properties of CHL is currently under investigation.

Results from a series of short-term in vivo experiments strongly suggested that protection against precarcinogenic DNA adduction was in part exerted via non-covalent complex formation between CHL and AFB_1 in the gut and concomitantly

reduced carcinogen bioavailability. The explanation was supported by *in vitro* complexation studies using AFB_1 fluorescence quenching as an indirect measure of CHL-AFB_1 complex formation, which revealed that CHL formed a relatively strong complex with AFB_1. The dissociation constant (Kd) for the complex was estimated to be 1.4 ($\pm$ 0.4) μM from Scatchard plot analysis. From this analysis it was also concluded that the carcinogen and the inhibitor associated at an approximate one-to-one stoichiometric ratio. Stop-flow fluorometry indicated that the AFB_1-CHL complex formed instantaneously or within the instruments reaction time of 2.5 msec.

As previously mentioned, our tumor study showed anti-initiating mechanisms in addition to those involved in carcinogen-DNA binding. One such protective mechanism may involve mito-suppression, which would function non-specifically toward most carcinogens. This proposed cancer protective mechanism, along with the ability of CHL to protect against carcinogenesis via complex formation, would provide protection against a wide range of both environmental and dietary carcinogens. Because of the abundance of chlorophyll in the human diet, it is suggested that this compound might be an important contributing factor to the observed cancer preventive activities associated with a high intake of vegetables and fruits.

CHLOROPHYLLIN ANTICARCINOGENESIS

IN THE RAINBOW TROUT MODEL

by

Vibeke Breinholt

A THESIS

submitted to

Oregon State University

in partial fulfillment of the requirements

for the degree of Doctor of Philosophy

Completed April 21, 1994

Commencement June 1994

Redacted for Privacy

Professor of Toxicology in charge of major

Redacted for Privacy

Chairman of the Toxicology Program

Redacted for Privacy

Dean of Graduate School

Date thesis is presented   April 21, 1994

Typed by   Vibeke Breinholt

ACKNOWLEDGEMENT

TABLE OF CONTENTS

LIST OF FIGURES

FIGURE NO.

LIST OF TABLES

TABLE NO.

CHLOROPHYLLIN ANTICARCINOGENESIS
IN THE RAINBOW TROUT MODEL

CHAPTER 1

INTRODUCTION

It has been generally accepted that human cancer is a direct reflection of exposure to environmental carcinogens and that the variation in incidence rates of specific cancers is a result of differential exposure to external and internal carcinogens. More recently, however, it has become evident that this theory cannot quantitatively explain the observed occurrence of cancer (Thorling, 1993). The pronounced variation in incidence rates of specific cancers (Henderson, 1992) compared with the fairly moderate variation in overall cancer rates strongly suggests the presence or lack of cancer inhibitory substances in the environment. Differences in the occurrence of these cancer protective factors from one location to the other, and among groups of varying social behaviors and lifestyles, could logically contribute to these observed differences in specific cancer rates among population groups.

Although cancer diagnosis and therapy have advanced in recent years and improved the quality of life for many cancer patients, there has been little or no effect on the mortality rate in the U.S. for most common cancers (Epstein, 1993; Vital Statistics, 1993). Cancer researchers have increasingly come to the realization that cancer prevention may have the greatest impact on cancer mortality rates.

Special attention has been given to the potentially preventive influence of diet on the carcinogenic process. Epidemiological data strongly suggest major associations between a high intake of fruits and vegetables and a marked decrease in cancer incidence and mortality rate of several common human cancer types (Doll, 1990; Hirayama, 1985; Dragsted, 1993). Although this relationship between diet and cancer is widely recognized, there was until recently little information on the specific compounds in the diet, that can be attributed anticarcinogenic properties. Within the past decade more than 600 compounds belonging to 30 different groups of chemicals, have been identified as inhibitors of experimental carcinogenesis (Thorling, 1993; Dragsted, 1993; Wattenberg, 1985). In most experimental settings, however, the inhibitor doses required to exert chemoprotective activities by far exceed what humans could naturally ingest from plant sources.

An exception to this dose requirement may be chlorophyll, the ubiquitous green plant pigment abundantly present in vegetables commonly consumed by humans. In typical spinach cultivars and isolates, chlorophyll constitutes 5% or more of the dry weight of the plant tissue (Khalyfa, 1992; Khachik, 1986), and thus a moderate daily intake of spinach or other leafy green vegetables would contribute a dose of chlorophyll (50,000 ppm) that may be 50-100 times higher than the concentration of most known anticarcinogens present in a well-balanced diet.

The aim of the current research was to evaluate the anticarcinogenic properties of chlorophyllin (CHL), a commercially available derivative of chlorophyll. From the literature it is evident that commercial preparations of CHL consist of more than one pigment; however, no information was available about the purity of the specific CHL

source employed in the present studies. To qualitatively evaluate the potential

chemoprotective activities of CHL, knowledge about the specific pigment content in

the CHL preparation was essential. Quality assessment of the inhibitor source was

thus undertaken by use of preparative thin-layer chromatography and spectral analysis.

The ability of CHL to inhibit precarcinogenic mutations was tested in the Ames test

against the human carcinogen aflatoxin B_1 (AFB_1) and two heterocyclic amines, also

suspected to be involved in human carcinogenesis. The protective properties of CHL

against carcinogen-DNA adduction was evaluated in a series of short term *in vivo* and

in vitro experiments. The rainbow trout, which is extremely sensitive to the

carcinogenic activity of AFB_1, was the experimental model employed in the present

research. Both the *in vivo* and *in vitro* experiments were designed to provide

information about the specific mechanisms responsible for CHL-imposed protection

in addition to assessing the inhibitory potency of the compound. From these

experiments it was strongly suggested that complexation with the carcinogen accounted

for part of the observed inhibitory activity of CHL. The strength and molecular

stoichiometry of the complex was evaluated *in vitro* using AFB_1 fluorescence

quenching as an indirect measure for the complex formed between AFB_1 and CHL.

The actual chemopreventive potential of CHL was assessed in a nine-month tumor

study using multiple doses of both the carcinogen and the inhibitor. The endpoints for

the CHL-associated chemoprotective properties were the effects of CHL on final

tumor incidence and tumor multiplicity evaluated nine months after initial exposure

to AFB_1. Molecular dosimetry analysis was conducted by relating AFB_1-DNA

adduction levels in the target organ, assessed immediately following the initial

carcinogen exposure period with final tumor outcome assessed nine months later. This analysis also provided information about the relative contribution of blocking and post-initiation mechanisms to overall chemoprotection.

The following sections provide an abbreviated introduction to the various biochemical, biological and molecular events involved in cancer development in general, followed by a brief introduction concerning the metabolism and carcinogenicity of AFB_1 in the rainbow trout model. The proposed mechanisms of protective factors present in fruits and vegetables will be presented along with a more detailed introduction to the chemistry, preparation and commercial uses of the anticarcinogen chlorophyllin employed in the current studies.

BIOCHEMICAL, BIOLOGICAL AND MOLECULAR MECHANISMS INVOLVED IN CANCER DEVELOPMENT

Initiation of DNA damage

It is now widely believed that alteration of DNA constitutes the initiating step in chemical carcinogenesis (Miller and Miller, 1984). The damage of DNA imposed by the carcinogen, its metabolites or both involves the intracellular formation of a highly reactive electrophilic species capable of attacking nucleophilic components within the cell. The intracellular nucleophiles most likely to be attacked comprise not only DNA but also RNA, proteins and polysaccharides. DNA distortions induced by chemical carcinogens can occur as a consequence of direct interaction between the carcinogen and the DNA bases and lead to the formation of covalent carcinogen-DNA adducts. Reactive carcinogens exhibiting oxidative, methylating or deaminating activities can exert modifying activities toward specific chemical groups on the DNA bases and give rise to structurally altered DNA bases such as 8-hydroxy 2'deoxy-guanosine (Ames, 1989). Like the DNA adducts these kinds of DNA modifications also have the potential to induce mutagenesis.

Mechanisms of mutagenesis

Modified DNA can induce mutagenesis by various means. The direct modification of the DNA base can change the concomitant coding property of the involved base and incorporate an incorrect base in the opposite DNA strand during replication. The presence of a bulky group covalently bound to the DNA strand can result in premature termination of replication, as the advancement of the polymerase is structurally hindered. Repair activities resulting in apurinic or apyrimidinic sites will often lead to the synthesis of an altered sister strand as the DNA backbone lacking the base constituents does not contain the information required for the polymerase to insert the correct base. When DNA is extensively damaged, the high fidelity error-free repair system of the cell may be overwhelmed and replication of the altered DNA sequence may take place before the DNA damage has been corrected. The increased activity of the low fidelity error-prone repair enzymes in a cell undergoing stress will inevitably result in a high mutagenesis rate, as this repair system non-specifically and randomly substitutes the damaged purines or pyrimidines with any available DNA bases present in the cell at the time of damage.

Point and frameshift mutations

The most simple mutation encountered is the exchange of one base for another. These base pair switches or point mutations can either be transitions, which are base changes within the group of purines or pyrimidines, or transversions, which involves

changes from a purine to a pyrimidine base or the opposite. Mutations at position 3 in the codon rarely affect the cell, as most base substitutions at the third position do not change the coding properties of the triplet codon. Changes in the first and second position in the codon, however, can lead to a permanent change in the encoded protein due to the exchange of one amino acid for another. The more extensive DNA alterations caused by deletions or insertions are signified frameshift mutations and are in contrast with the point mutations in that frameshift mutations are almost exclusively irreversible. The DNA damage induced by this group of mutations not only involves the change of one codon, but gives rise to a frameshift that affects all downstream codons and thus completely disrupts the synthesis of the protein.

Fixation of DNA damage and promotion

If the cell divides before repair mechanisms revert the damaged DNA to its original constellation, the DNA damage will be fixed during subsequent cell division and all daughter cells will inherit a gene with a mutation. Not all mutations, however, necessarily lead to the formation of a cancer cell. For most cancers several mutations in the same cell are required to form a malignant cell population. Promotional environments(e.g. high caloric intake, hormonal stimulation, chronic viral infections) that stimulate high rates of target cell proliferation are believed to markedly elevate the probability that the cells undergo further mutations. This requirement for

sustaining DNA damage via repetitious cycles of replication is believed to explain the long lag phase in humans from carcinogen exposure to metastatic cancer, which can span over several decades for some cancers.

Oncogenes

The extent to which a mutation evokes a cancerous cell population is highly dependent on the specific gene sequence in which the DNA damage takes place. For a mutation to induce the first event toward malignancy, specific genes, gene sequences or single bases need to be mutated (Miller and Miller, 1984). One set of DNA sequences which have been identified as possible sites where the initiating mutation of cancer occurs are termed proto-oncogenes (Weinberg, 1989; Simon et al., 1991). The protein products of the proto-oncogenes function in a normal cell to control cell proliferation and are normally expressed at a very low level. In the ras oncogene family, a single point mutation and a subsequent single amino acid substitution converts the proto-oncogene into an oncogene which transforms the cell into a state of dysregulated, chronic proliferation. Activation of proto-oncogenes also takes place as a result of insertion of a promoter upstream of the coding sequence of the proto-oncogene (c-myc), resulting in highly efficient expression of the gene product. This same oncogene can also undergo activation by translocation from a silent to an actively expressed gene (Weinberg, 1989). It is generally accepted that DNA rearrangements perturb control of the affected cells and increase the tumorigenic potential by sensitizing the cell to undergo further mutations.

Tumor suppressor genes

Other genes thought to be involved at a later stage in carcinogenesis are the tumor-suppressor genes (TSG) (Weinberg, 1991). Opposite the proto-oncogenes the gene products of TSG function as negative regulators of cell growth. In a normal cell the TSG and the proto-oncogenes are in a state of equilibrium functioning to maintain cell growth within normal limits of the given cell types. When both activities are dysregulated the cell loses growth restriction and will continue to grow in an uncontrollable manner.

Progression

This last stage in cancer development is signified by progressive growth of the tumor. Invasion of surrounding tissues and relocation at ectopic sites (metastasis) takes place, facilitated by an increased blood supply (angiogenesis) to the tumor tissue . The presence of metastasis marks the clinical distinction between a benign and a malignant tumor. The proposed events involved in cancer development are depicted in Figure 1.1, taken from a recent review by C.C. Harris (1991).

Carcinogen Exposure   Initiation   Promotion   Conversion   Progression
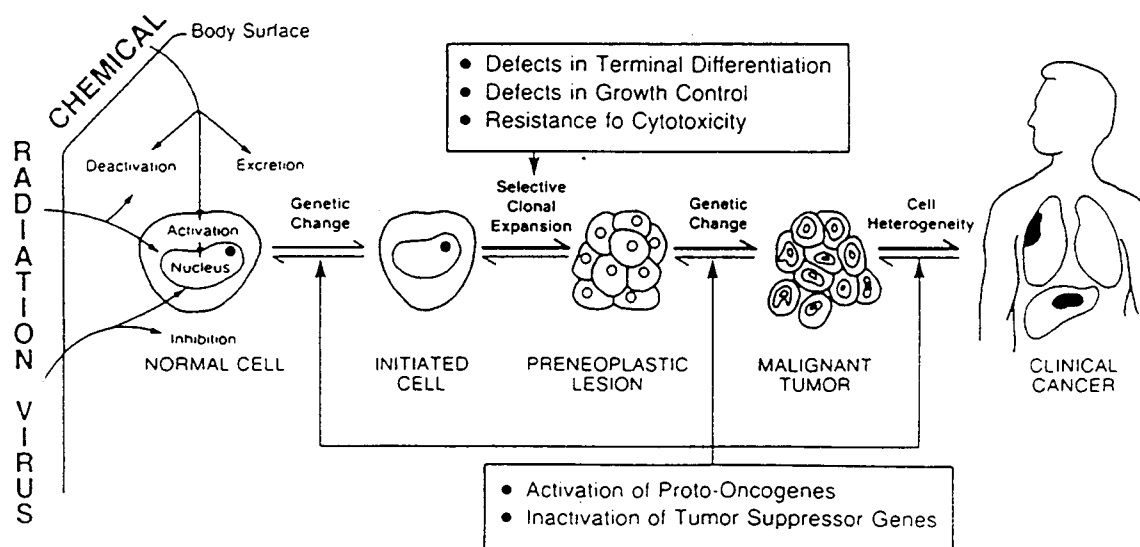
Figure 1.1   Proposed scheme of events from initial carcinogen exposure to formation of malignant tumor tissue (Harris,1991).

AFLATOXIN B_1: METABOLISM AND MOLECULAR BASIS OF CARCINOGENICITY IN RAINBOW TROUT

Occurrence of AFB_1

Aflatoxins are the toxic and carcinogenic secondary metabolites produced by certain strains of *Aspergillus*, particularly *A. flavus* and *A. paraciticus*. The two major genera of aflatoxin-producing fungi can grow in or on a wide variety of foods producing a large array of toxic metabolites. The major naturally occurring aflatoxins are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2) and aflatoxin M_1 (AFM_1) (see Fig. 1.3 for some of these structures). Up to twenty different aflatoxin metabolites, however, have been isolated from various food sources, primarily dried grains, nuts and products produced thereof (Pohland and Wood, 1987). AFB_1 at levels up to 20 ppb have been detected in dried nuts and peanut butter (Schultz, 1981). Among the aflatoxins, AFB_1 is usually produced in greatest quantity and is also the most toxic of the metabolites.

AFB_1 Carcinogenicity

AFB_1 is acutely toxic to a variety of animal species, and is capable of inducing tumors in several experimental animals at doses relevant to human exposure levels, and is one of the most potent hepatocarcinogens known. The primary interest in AFB_1 stems from the widespread human exposure to foodstuffs contaminated with

mycotoxins and the associated concern that AFB_1 may be implicated in the etiology of human liver cancer. The carcinogenicity of aflatoxin in humans was questioned for several years, despite extensive evidence of carcinogenicity in related experimental animals and epidemiological studies indicative of a liver cancer link. Just recently AFB_1 has received the status of being the only accepted human dietary carcinogen according to the International Agency for Cancer Research (IARC Monograph, 1993). The most important indication that AFB_1 is a causative factor in human liver cancer is the demonstration that the metabolic fate and the mechanism of action of AFB_1 at the molecular level are similar in humans and various animal models including the rainbow trout. Of any known group of animals, rainbow trout has been found to be the most sensitive animal species to AFB_1-induced hepatocarcinogenesis.

Metabolism of AFB_1 in rainbow trout

Phase 1 bioactivation reactions: The two principal P450 monooxygenase pathways involved in the metabolism of AFB_1 in rainbow trout are the CYP isozymes 2K1 and 1A1. CYP2K1 is a constitutive trout isozyme that acts upon AFB_1 to form the ultimate carcinogen AFB_1-8-9-epoxide. CYP1A1, inducible by Ah receptor agonists such as dioxin and β-naphthoflavone (BNF), is considered a detoxification enzyme because it catalyzes the hydroxylation of AFB_1 to form the less toxic metabolite AFM_1, which confers 10% the tumorigenic potential of AFB_1 (Bailey et al., unpublished data).

Another CYP-linked enzyme catalyzes the transformation of AFB_1 to aflatoxicol B_1 (AFL), a metabolite with the same carcinogenic potency as AFB_1 (Bailey et al., in press).

AFB_1-DNA adduction: The product formed after the oxidation of AFB_1 at the 8,9 position is a highly unstable electrophile that readily reacts with cellular nucleophiles such as DNA to form covalent adducts (Figure 1.2, page 16). These adducts are thought to elicit the observed mutagenicity, toxicity and carcinogenicity induced by AFB_1. The binding of the AFB_1 epoxide to DNA occurs primarily at the N-7 guanine position of DNA (Figure 1.2) . N-7 guanine adduction also occurs for AFM_1 and probably other AFB_1 phase 1 metabolites possessing an 8,9-double bond, which is potentially capable of forming an epoxide and subsequently binding to DNA. The AFB_1 epoxide, however, is quantitatively of far greater importance than the other aflatoxin epoxides.

Phase 2 detoxification pathways: The products of the initial steps in AFB_1 metabolism can undergo further biotransformation if not involved in adduct formation. The highly reactive AFB_1 epoxide can be neutralized by the action of epoxide hydrolase or spontaneously in the presence of water to form AFB_1-8,9-dihydrodiol, which is a readily excretable metabolite. Another detoxification pathway is the conjugation of the epoxide with cellular glutathione (GSH). This conjugation is catalyzed by one or more hepatic glutathione transferase(s) (GST). The coupling of GSH to the epoxide converts the lipophilic metabolite into more polar species. After additional enzymatic modification in the liver and kidney the highly hydrophilic metabolite is excreted in the bile or urine in the form of mercapturic acids. The activity of GST is primarily

found in the cytosol, with minor activities associated with the mitochondrial and microsomal fraction of the liver. Glutathione conjugation in AFB_1 metabolism in rainbow trout has been found to be insignificant in overall protection against the toxic effects of AFB_1 (Valsta et al, 1988). The lower activity of GST in trout could account for the trout's extreme sensitivity to AFB_1-induced carcinogenesis compared to the high activity of GST in the mouse and the relatively high resistance of this species to AFB_1 toxicity and carcinogenesis.

The major detoxification pathway for AFB_1 in the rainbow trout is the conjugation of the phase 1 metabolites AFL and $AFLM_1$ with uridine-diphosphate-glucuronic acid (UDPGA). The enzyme responsible for this detoxification step is the UDP-glucuronyltransferase (UDPGT), which utilizes UDPGA as the carbohydrate donor and the hydroxylated aflatoxin products formed from the CYP pathway as substrates. The conjugation of AFB_1 to UDPGA results in the formation of highly hydrophilic glucuronides, which are readily eliminated via the bile. The glucuronyl transferases are located in the membrane vesicles of the microsomal fraction. The quantitatively most important AFB_1 metabolites formed by the action of UDPGT in rainbow trout, are $AFLM_1$ glucuronide and AFL glucuronide (Loveland et al., 1984). The $AFLM_1$ glucuronide is predominant when BNF is included in the diet whereas the AFL glucuronidation product is the major biliary metabolite in control trout (Loveland et al, 1984). The proposed metabolic pathways involved in activation and detoxification of AFB_1 in rainbow trout are outlined in Figure 1.3 (page 17).

Molecular basis for carcinogenicity

The rainbow trout has proven to be a good model for studying AFB_1 metabolism and carcinogenesis and much evidence is present that the metabolic scheme of AFB_1 in this species is closely related to the metabolism pathways elucidated for humans. It is also evident that the modification of DNA induced by AFB_1 in the human body occurs in a similar fashion in the trout model. The AFB_1-8,9-epoxide produced by the action of species-specific CYP isozymes has been found to specifically attack nucleophilic sites at guanyl residues in DNA in all species investigated including humans. The primary adducted base 8,9-dihydro-8(N7-guanyl)-9-hydroxy-AFB_1 can be reverted to a normal guanine residue through an error-free DNA repair which removes the adducted base and substitutes it with a new guanine residue. If not repaired, the primary N7-guanyl adduct can undergo spontaneous depurination to leave an abasic site in the DNA-sequence. From *in vitro* studies it has been found that guanine depurination almost exclusively results in G→T transversion mutations because the purine adenine is favored for insertion opposite the apurinic site during DNA replication (Schaaper et al., 1982). The adducted guanine residue can also give rise to formamidopyrimidine adduct isomers which are essentially repair-resistant (Sambamurti et al., 1988: Schaaper et al., 1982). The replication block imposed by the bulky AFB_1 adducts have also been found to induce the low fidelity error-prone repair systems *in vitro* (Schaaper et al., 1983). AFB_1 carcinogenesis in rainbow frequently involves G→T transversion mutations, with the *c-Ki-ras* (Chang et al., 1991) and possibly other *c-ras* proto-oncogenes as the possible target sites. The

involvement of a mutation of the p53 tumor suppressor gene in the trout model as a contributing factor to AFB_1 tumorigenesis have so far not been elucidated.

Striking similarities exist in the metabolic fate of AFB_1 and the ultimate molecular events associated with this mycotoxin in experimental animals and humans. Therefore, the study of inhibiting activities against AFB_1-induced hepatocarcinogenesis in animal models such as the trout, may ultimately be important to humans exposed to the toxin. Data from experimental animal models such as the rainbow trout could thus prove valuable in the establishment of prevention strategies and possibly treatment regimens for subjects at high risk of developing liver cancer as a result of AFB_1 exposure.
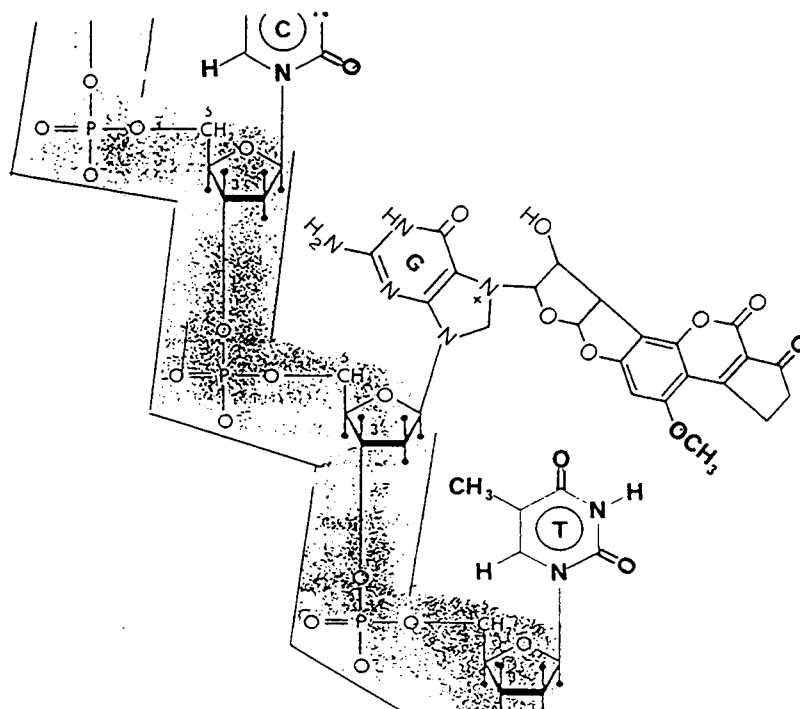
Figure 1.2   Simplified cartoon of the covalent adduction of AFB_1 to a guanine (G) residue in DNA. T: Thymine; C: Cytosine
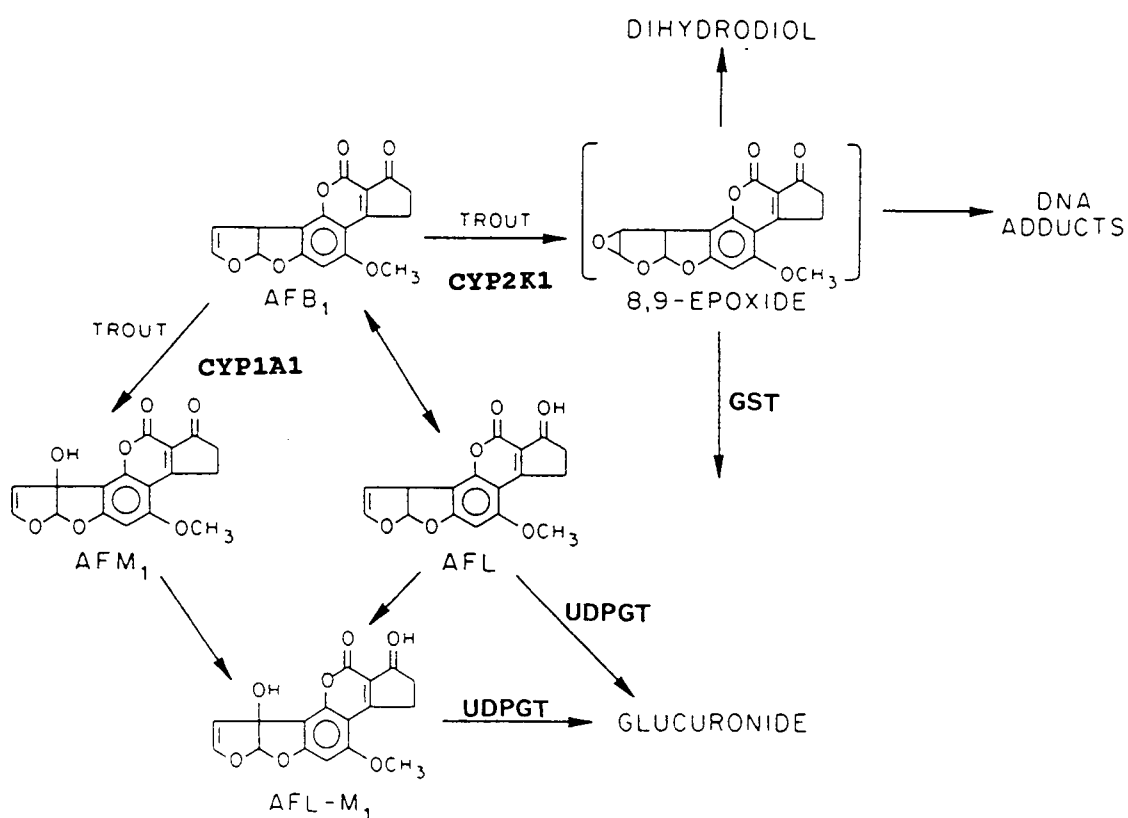
Figure 1.3   Activation and detoxification pathways involved in AFB$_1$ metabolism in rainbow trout. Abbreviations are given in the text.

MECHANISMS OF CANCER PROTECTIVE FACTORS
IN FRUITS AND VEGETABLES

Introduction

As discussed in the previous chapter several chemical and biological processes are closely associated with the risk of developing cancer. Acceleration of bioactivation pathways, enhanced adduction of ultimate carcinogens to cellular target molecules, decreased DNA-repair activity, and increased cell proliferation in the target organ have a definite increased impact on the occurrence of cancer. Substances capable of interfering with one or more of the above activities therefore have the potential to act as anticarcinogens. Numerous compounds present in fruits and vegetables have been identified as modulators of one or more of the processes involved in carcinogenesis (Tables 1.1, 1.2, 1.3, 1.4 and 1.5).

Classification of anticarcinogens

Naturally occurring anticarcinogens exhibit very specialized and diverse activities and for this reason it is difficult to organize them into a cohesive pattern. One approach to categorizing chemoprotective agents, which will be used in this chapter, is to classify the compounds according to the specific step in the carcinogenic process at which they exert their activity (Wattenberg,1985). This chapter will review the properties of some of the most important potential human anticarcinogens, with special emphasis on occurrence and mechanism(s) of action.

Antiinitiators/blocking agents

Antiinitiators or blocking agents act by several distinct mechanisms, all functioning to protect the genome from initial damage by the carcinogen or its metabolites.

Inhibition of carcinogen uptake: Dietary fiber, represented by cellulose and hemicellulose, has been found to inhibit dimethylhydrazine-induced colon cancer (Bingham,1990) and other forms of diet-induced carcinogenesis. A proposed mechanism of action is via adsorption of the carcinogen to the fibers thereby rendering the carcinogen unavailable for absorption and/or activation (Bingham,1990; Hayatsu,1988). We present evidence that a similar mechanism accounts for at least part of the anticarcinogenic property of chlorophyllin, a structural analogue of

chlorophyll, against AFB_1-induced hepatocarcinogenesis (Chapters 5 and 6). Both chlorophyllin and AFB_1 are planar aromatic molecules capable of forming a strong complex *in vitro* (Chapter 5). The complex between the inhibitor and the carcinogen formed in the gut during digestion is speculated to escape absorption due to sterical hindrance and will remain unabsorbed and unmetabolized in the digestive tract until excreted via the feces (Dashwood, 1991; Breinholt, unpublished data). This would result in a decrease in the total carcinogen body burden, which would subsequently reduce the risk of initiating carcinogenesis.

Inhibition of carcinogen formation: Ascorbic acid universally present in vegetables and fruit is capable of preventing the formation of nitrosamines in the gut by its dual actions as a reducing agent and a scavenger. The nitroso carcinogens are readily formed *in vivo* from dietary amino compounds and nitrite, present as a food preservative or formed from ingestion of nitrate. Plant phenols ubiquitous in fruits and vegetables, as well as present in considerable quantities in tea and coffee, have also been found to be effective scavengers of nitrite and to exert a protective effect against nitrosamine-induced carcinogenesis (Stich and Rosin, 1984; Bartsch et al,1988).

Modification of carcinogen metabolism: Biotransformation of procarcinogens into ultimate carcinogens is primarily brought about by the CYP isozymes. Certain indoles and dithiolthiones present in cruciferous vegetables have been identified as modifiers of both phase 1 and phase 2 enzymes. Indole-3-carbinol has been found to exert modifying activities toward phase 1 enzymes whereas the dithiolethiones act

exclusively as inducers of phase 2 detoxification enzymes. The unsubstituted natural compound 1,2-dithiole-3-thione and its synthetic substituted homologue Oltipraz [5-(2-pyrazinyl)-4 methyl-1,2-dithiole-3 thione) have been shown to selectively induce conjugation enzymes such as GST, UDPGT, and epoxide hydrolase thereby facilitating excretion of the carcinogenic species (Kensler et al.,1992). Compounds with these characteristics are considered ideal cancer preventive agents, as they are able to protect against many pro-carcinogens without inducing bioactivating phase 1 pathways.

Oils of garlic and onion and purified sulphur compounds from these sources have also been found to exhibit anticarcinogenic properties against benzo(a)pyrene-induced tumorigenesis (Sparrings et al.,1988). The main protective mechanism is thought to be via induction of GST in the tissues of the stomach lining thereby neutralizing the ultimate carcinogen before DNA-adduction can take place.

Ellagic acid, a polyphenolic compound found in nuts and berries, is a potent inhibitor of both malignant melanoma and lung cancer (Mukhtar et al., 1986) partly by virtue of inducing GST isozymes. At the same time, ellagic acid is capable of inhibiting the activity of certain monooxygenases involved in carcinogen bioactivation (Das et al., 1985; Athar et al.,1989).

Scavenging of electrophiles: Neutralization of direct-acting carcinogens and activated procarcinogens by scavenging is a feature of numerous anticancer agents. Diallylsulphides present in garlic and onions, dithiolthiones and isothiocyanates in cruciferous vegetables and β-carotene from carrots all possess nucleophilic properties which enable them to act as scavengers of electrophilic compounds. Antioxidants such

as ascorbic acid, chlorophyll and chlorophyllin also have the capacity to scavenge reactive carcinogens (Shamberger, 1972; Dashwood, 1991).

Ellagic acid, an effective scavenger of polycyclic aromatic hydrocarbons, neutralizes the electrophilic metabolite in a unique manner by forming a covalent adduct with the activated compound (Wood at al., 1982; Huang et al., 1985; Sayer et al., 1982).

Protection of DNA: Protection of nucleophilic sites on DNA has been suggested as another way of preventing DNA damage. By intercalating with the base constituents of the DNA-strand compounds such as ellagic acid, certain tannins and flavonoids are capable of structurally hindering the adduction of electrophilic species to the genetic material (Tell,1986; Dixit and Gold, 1986; Barch and Fox, 1988) and thereby prevent the precarcinogenic lesions from forming. However, the *in vivo* relevance of such a mechanism is questioned.

Modulation of DNA-repair activity: Mutations induced by the presence of carcinogens adducted to DNA can be corrected by specific DNA-repair mechanisms. These repair mechanisms are capable of recognizing the incorrect base(s) and forming the correct base pair constellation. The fidelity of incorporating the correct base, however, is highly dependent on the specific repair system involved. A potential way of inhibiting mutagenesis and thereby preventing the primary step in the formation of an initiated cancer cell is by inhibiting the enzymes responsible for the error-prone DNA-repair activities, and/or by inducing the error free repair system. It has been proposed that

protease inhibitors, present in beans and cereals, exert part of their antitumorigenic potential by increasing the fidelity of certain DNA-repair activities (Troll et al., 1987). Chlorophyllin is also speculated to affect the activity of DNA-repair enzymes (Whong et al., 1988). However, direct evidence is lacking that this represents an important protective pathway for any anticarcinogens.

Antipromoting agents

The inhibitors in this category act by suppressing the expression of neoplasia in cells that have already undergone certain modifications as a result of previous carcinogen exposure.

Inhibition of cell proliferation: To fix the initial DNA-damage, the cell containing altered genetic material has to replicate to sustain its survival. Several flavonoids such as quercetin and hydrocinnamic acid act in an antipromotive manner by inhibiting replication of the preneoplastic cells (Deschner et al., 1991; Tanaka et al., 1990a). Protease inhibitors also exert antipromotional activity against mammary and skin tumorigenesis by inhibiting cellular proliferation (Troll et al., 1980; Rotstein and Slaga, 1988).

Induction of differentiation: Another mechanism by which an initiated cell can escape promotion is by cellular differentiation. Compounds like b-carotene and the citrus fruit derived compound d-limonene exert an antipromotive effect against various carcinogens by increasing the rate of terminal differentiation. By diverting the neoplastic cell into a new more harmless cell type or by promoting senescence of initiated cells, expansion of the malignant cell type can be suppressed (Suda et al., 1986; Elson et al., 1988).

Antioxidation: Because reactive oxygen species promote cell proliferation, one way to inhibit tumor promotion is by scavenging radicals and active oxygen species. Vitamin E, selenium, vitamin C, β-carotene and several other naturally occurring antioxidants have been found to inhibit tumor promotion in experimental animals by this mechanism (Perchellet et al., 1985; Shamberger, 1972; Smart et al., 1987; Suda et al., 1986). The mechanism of protease inhibitors is speculated to be prevention of excess formation of or release of active oxygen species (Rotstein and Slaga, 1988).

Inhibition of conversion

Additional genetic damage is required for the preneoplastic lesion to be converted into a malignant cancer cell. The molecular processes required for cellular conversion are deletions of genomic material or allelic losses.

Protection against genetic loss or rearrangement: The rearrangement of the genetic material in pre-cancer cells has been found to be counteracted by compounds such as glutathione, β-carotene and d-limonene (Rotstein and Slaga, 1988; Mukherjee et al.,1991; Elson et al.,1988). These agents presumably act by protecting DNA against genotoxic damage and potential deletion of so-called tumor suppressor genes.

Inhibition of progression

Cancer progression is the stage at which the tumor expands in size and possibly develops into a metastatic tumor. Mechanisms that might interfere with this step in cancer development are agents capable of inhibiting cell proliferation and mutagenesis or compounds that act as inhibitors of angiogenesis. Stimulators of the immune system might also have a positive impact.

Inhibition of oncogene expression and enhancement of divergent differentiation: Regression of tumors of the buccal pouch of hamsters have been reported after direct treatment of tumors with β-carotene (Shklar et al., 1989). D-limonene present in citrus fruits has been identified as a potent inhibitor of mammary tumor growth. Also in the same model, metastasis of tumors were strongly inhibited (Ellegbede et al., 1989). The mechanism(s) by which these substances inhibit progression is speculated to be by inhibiting expression of activated oncogenes and by increasing the rate of divergent differentiation of the malignant cells (Abemayor et al., 1990; Okuzumi et al., 1990).

Table 1.1   Antitumorigenicity associated with whole fruits and vegetables
(Dragsted et al., 1993).

Antitumorigenic preparation	Tumor inducing agents	Animal species	Target organ	Decrease in tumor yield[a]	Reference
Orange oil	NMU[b]	Rat	breast	41%	Maltzman et al., 1989
Orange oil	NNK	Mouse	lung	83%	Wattenberg & Coccia, 1991
Lemon oil	NNK	Mouse	lung	81%	Wattenberg & Coccia, 1991
Orange oil	NNK	Mouse	forestomach	100%	Wattenberg & Coccia, 1991
Lemon oil	NNK	Mouse	forestomach	100%	Wattenberg & Coccia, 1991
Brussels sprouts	DMBA	Rat	breast	40%	Stoewsand et al., 1989
Brussels sprouts	DMBA	Rat	breast	34%[c]	Stoewsand et al., 1988
Dried Cabbage	NMU	Rat	breast	38%	Bresnick et al., 1990
Dried Collard	NMU	Rat	breast	0%	Bresnick et al., 1990
Onion oil	DMBA/TPA	Mouse	skin	83%	Belman, 1983
Garlic oil	DMBA/TPA	Mouse	skin	80%	Belman, 1983

a) Total number of tumors reported per animal. The numbers represent the percentage reduction reported in the highest dose group as compared with the control group receiving carcinogen only.
b) NMU nitrosomethylurea; DMBA 7,12-dimethylbenz[a]antracene;
TPA tetradecanoylphorbolacetate; NNK 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
c) Fibroadenomas of the breast were decreased significantly by DMBA + Brussels sprouts fed during initiation.

Table 1.2   Antitumorigenic activity of compounds isolated from fruits (Dragsted et al., 1993).

Anticarcinogenic compound	Source	Tumor inducing treatment	Animal species	Target organ	De-crease in tumor yield[a]	Reference
Ascorbic acid	fruits/ vegetables	UV-light	mouse	skin	90%[b]	Dunham et al., 1982
Ascorbylpalmitate	synthetic (food additive)	DMBA + TPA[c]	mouse	skin	86%	Smart et al., 1987
Ascorbic acid	fruits/ vegetables	DES	hamster	kidney	50%	Liehr & Wheeler, 1983
Ascorbic acid	fruits/ vegetables	DMH/EP	mouse	uterus	44%	Turusov et al., 1991
Limonin 17-ß-D-glucopyranoside	citrus fruits	DMBA	hamster	buccal pouch	55%[d]	Miller et al., 1992
D-limone	citrus fruits	NNK	mouse	lung	70%[e]	Wattenberg & Coccia, 1991
D-limone	citrus fruits	DMBA	rat	breast	31%[e]	Russin et al., 1989
D-limone	citrus fruits	v-Ha-*ras* vector	rat	breast	84%	Moore et al., 1991
Nomilin	citrus fruits	B(a)P	mouse	forestomach	28%	Lam & Hasegawa, 1989

a) Decrease in percentage of tumor-bearing animals, except if otherwise stated.
b) Only tumors larger than 4 mm in diameter considered.
c) DMBA, 7,12-dimethylbenz[a]anthracene; TPA, 12-0-tetradecanoyl-13-acetate; DES, diethylstilbestrol; dimethylhydrazine; EP, estradiol-diproprionate; NNK, 7-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; Benzo[a]pyrene.
d) All animals had multiple tumors. In this study only the percentage decrease in the mass of tumor tis reported.
e) All animals had tumors. Decrease percentage reported in number of tumors per animal.

Table 1.3   Anticarcinogenic potential of compounds from vegetables (Dragsted et al., 1993).

Antitumorigenic compound	Source	Tumor inducing treatment	Animal species	Target organ	Decrease in tumor yield[a]	Reference
Phenethylisothio-cyanate	cruciferous vegetables	NBMA[b]	rat	esophagus	99-100%[c]	Stoner et al., 1991
Phenethylisothio-cyanate	cruciferous vegetables	NNK	rat	lung	47%	Morse et al., 1989
Phenylhexylisothio-cyanate[d]	cruciferous vegetables	NNK	mouse	lung	83-96%[c]	Morse et al., 1992
Phenethylisothio-cyanate	cruciferous vegetables	NNK	mouse	lung	48-79%[c,d]	Morse et al., 1992
B-Sitosterol	several vegetables	MNU	rat	colon	39%	Raicht et al., 1980
Indole-3-carbinol	cruciferous vegetables	DEN	rat	liver	50%	Tanaka et al., 1990b
Oltipraz	cruciferous vegetables[e]	AOM	rat	colon / small intestine	30% / 84%	Rao et al., 1991
Oltipraz	cruciferous vegetables	AFB	rat	liver	100%	Roebuch et al., 1991
Diallyl sulfide	garlic	NMBA	rat	esophagus	100%	Wargowich et al., 1988
Rutin	several vegetables	AOM	mouse	colon	63%	Deschner et al., 1991
Quercetin	several vegetables	AOM	mouse	colon	75%	Deschner et al., 1991
Quercetin	several vegetables	MNU	mouse	skin	67%	Mukhtar et al., 1988
B-carotene	vegetables and fruits	DMBA	hamster	buccal pounch	55%	Suda et al., 1986
B-carotene	vegetables and fruits	DMBA	rat	salivary gland	35%	Alam & Alam, 1987
B-carotene	vegetables and fruits	spon-taneous	rat	liver[f]	75%	Moreno et al., 1991

a) Percent reduction in number of animals with tumors unless otherwise indicated. The numbers represent the percentage reduction reported in the highest dose group as compared with the control group receiving carcinogen only

b) NBMA; N-nitrosobenzylmethylamine. NNK; 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone. MNU; N-Methyl-N-nitrosourea. DEN; Diethylnitrosamine. Oltipraz; 5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione. AOM; Azoxymethane AFB; Aflatoxin B, NMBA; N-nitrosomethylbenzyamine. DMBA; 7,12 Dimethylbenz(a)anthracene.

c) Reduction in number of tumors per animal. In these studies, all animals had multiple tumors, and only the reduction in the number of tumors per animal in the highest dose group of antitumorigen (as a percentage of the control animals) can be reported here.

d) Referred historic values included.

e) Synthetic representative of dithiothioles present in these vegetables

f) Liver nodules (benign).

g) Synthetic analogue of natural isothiocyanates

Table 1.4 Antitumorigenic actions of beverages of plant origin (Dragsted et al., 1993).

Anti-carcinogenic compound	Source	Tumor inducing treatment	Animal species	Target organ	Decrease in tumor yield[a]	Reference
EGC[b]	green tea	DMBA[b,d]	mouse	skin	64%[c]	Fujiki et al., 1990
Menthol	herbal teas	DMBA	rat	breast	25%	Russin et al., 1989
Kahweol	green tea	DMBA	hamster	buccal pounch	35%	Miller et al., 1991
Cafestol	green tea	DMBA	hamster	buccal pounch	35%	Miller et al., 1991
Tannic acid	tea[f]	B(a)P[b,c]/DMBA[b,c]/ MCA[b,c]	mouse	skin	73%/48%/61%	Mukhtar et al., 1988
Tannic acid	tea	B(a)P	mouse	lung	48%	Athar et al., 1989
Tannic acid	tea	B(a)P	mouse	fore-stomach	37%	Athar et al., 1989
Ellagic acid	tea[g]	MCA[b]	mouse	skin	77%[c]	Mukhtar et al., 1984
Ellagic acid	tea	MCA	mouse	skin	0%[c]	Smart et al., 1986
Ellagic acid	tea	B(a)P	mouse	lung	50%[c]	Lesca, 1983
Ellagic acid	tea	B(a)P[b,c]B(a)PDE[b]	mouse	lung	0%/48%	Chang et al., 1985
Ellagic acid	tea	DB(a,h)P[b,c]/ DB(a,h)PDE[b]	mouse	lung	0%/26%	Chang et al., 1985
Caffeine	coffee/tea	DMBA/MNU[b]	rat	mammary gland	50%/0%	Vander Ploeg et al., 1991

a) In these studies, all animals had multiple tumors. Results are therefore reported as the percentage reduction in number of tumors/animal in the treated group compared to number of tumors per animal in control group, receiving carcinogen only.

b) DMBA; 7,12-dimethylbenz[a]anthracene. NMU; N-methyl-N-nitrosourea. B(a)PDE; 7,8-dihydroxy-9,10-epoxy-7,8,9,10 tetrahydrobenzo[a]pyrene. B(a)P; benzo[a]pyrene. MCA; 20-methylcholanthrene. DB(a,h)PDE; 1,2-dihydro-3,4-epoxy-1,2,3,4 tetrahydrobenzo[a,h]pyrene. DB(a,h)P; dibenzo(a,h)pyrene. EGC; (+)-epigallocatechin.

c) Two-stage tumor protocol using 12-0-tetradecanoylphorbol-13-acetate (TPA) as a tumor promotor.

d) Two-stage tumor protocol using teleocidin as a tumor promotor.

e) Percent reduction in number og tumor-bearing animals

f) Tannic acid is also found in many other edible plants.

g) Ellagic acid is a part of hydrolyzable tannins, which are also found in strawberries, grapes, walnuts etc

Table 1.5 Anticarcinogenic activity of compounds isolated from grains, cereals and beans (Dragsted et al., 1993).

Antitumorigenic compound	Source	Tumor-inducing treatment	Animal species	Target organ	Decrease in tumor yield[a]	Reference
Bowman-Birk protease inhibitor	soybeans	DMH[b]	mouse	colon	100%[c]	Weed et al., 1985
Cellulose	all plants	DMH	rat	colon	56%	Freeman et al., 1978
Cellulose	all plants	DMH	rat	colon	56%	Heitman et al., 1989
D-α-tocopherol	whole grain	DMBA/TPA	mouse	skin	35%	Perchellet et al., 1985
DL-α-tocopherol acetate	whole grain	DMBA/high fat	rat	breast	40%	Ip, 1982
DL-α-tocopherol acetate	whole grain	UV-light	mouse	skin	48%	Gensler & Magdaleno, 1991
DL-α-tocopherol acetate	whole grain	DMBA	hamster	buccal pouch	43%	Shklar, 1982
Oleic acid	olive oil	spontaneous	mouse	breast	57%[d]	Nakahara, 1925
Selenite	grain cereals	DMBA	rat	breast	57%	Ip et al., 1991
Selenite	grain cereals	DMBA	rat	breast	90%	Liu et al., 1991

a) Decrease in percentage of tumor bearing animals
b) Abbreviations are: DMH, dimethylhydrazine; DMBA, 7,12-dimethylbenz[a]anthracene; TPA, 12-0-tetradecanoylphorbol-13-acetate.
c) Number of tumors in control group 11/46, in test group 0/24
d) In treated animals, 19/50 had recurrence after radical operation, in controls 22/25.

CHLOROPHYLL DERIVATIVES
CHEMISTRY, PREPARATION AND COMMERCIAL USES

Introduction to chlorophyll

Chlorophyll is the most widely distributed natural pigment and is present in the leaves and other parts of essentially all plants. Chlorophyll is present as a colloidal suspension in plant chloroplasts closely associated with carotenoids, xanthophylls and proteins (Ryberg and Sunquist, 1991). These pigment-protein complexes are members of the two distinct photosystems involved in photosynthesis (Ryberg and Sunquist, 1991). The green coloring matter in plants is a mixture of two chromophores designated chlorophyll a (chl a) and chlorophyll b (chl b). The two pigments are present in a ratio of 3:1 (a:b), which is not substantially affected by external influences (Humprey, 1980; Scheer, 1991). Due to the 3-5 times higher susceptibility of chl a to degradation than chl b, the ratio of the two will inevitably change as a result of processing such as harvesting, drying and solvent extraction (Humprey, 1980). The total chlorophyll content of green plant tissues, however, is greatly influenced by factors such as degree of illumination, fertilizer level, and water deprivation (Humprey, 1980). The concentration of green pigment in spinach for instance, has been reported to range from 1,500 ppm to 60,000 ppm (Khalyfa, 1992; Khachik, 1986).

Chemical structure of chlorophylls and derivatives

The basic ring structure of chlorophylls is composed of four pyrrole groups

interconnected in a complex planar ring structure called porphyrin (Figure 1.4). The

final structure of chl a and b contains a phytyl side chain attached to carbon 7 and a

magnesium atom coordinated with the four nitrogen atoms of the tetrapyrrole ring

(Figure 1.5). The presence of the hydrophobic phytyl moiety confers highly lipophilic

characteristics to the molecule. The two chlorophyll pigments chl a and chl b differ

only in the substitution of a methyl group at position 3 in chl a (Figure 1.5), with a

formyl group in chl b. The presence of a formyl group in chl b moves the red

absorption maximum of chl a from 660 nm to 643 nm, giving rise to a yellow-green

color in solution instead of the blue-green color of chl a (Scheer, 1991).
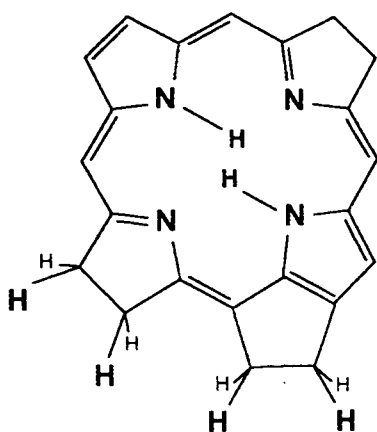
Figure 1.4   Porphyrin
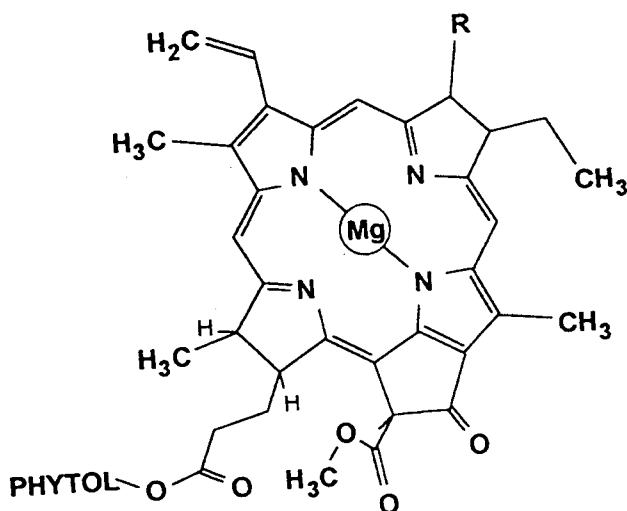
Figure 1.5 Chlorophyll a $(R_{chl\,a} = - CH_3)$
and chlorophyll b $(R_{chl\,b} = -CHO)$.

Chemical modification of chlorophyll

One of the more important characteristics of the chlorophylls is the ease by which the central magnesium and the phytyl side-chain is lost from the molecule. The presence of dilute acids readily replaces the magnesium with two hydrogen atoms or other divalent metals (Kephart, 1955; Hynninen, 1991). Replacement of magnesium with metals such as copper, zinc or iron is easily accomplished chemically as discussed later. The exchange of magnesium with divalent metals present in plant tissue also occurs spontaneously during storage of processed green vegetables (Robinson, 1987). Treatment of chlorophyll with weak acid, yields pheophytin a and b, which are the phytyl-free derivatives of chl a and b respectively (Kephart, 1955; Hynninen, 1991). With stronger acid pheophorbide a and b are formed, which lack both the central metal and the phytyl group. Pheophytin and pheophorbide a and b have been identified as chlorophyll metabolites after ingestion by human subjects (Baxter, 1968). An additional modification of the chlorophylls that takes place in dilute alkali is the hydrolysis of the phytyl ester linkage. Stronger alkali will, in addition to the hydrolysis of the side-chain, cleave the isocyclic ring, and form a compound called chlorin. The general scheme for the above described reactions and the nomenclature and structure of the most common derivatives is shown in Figure 1.6.
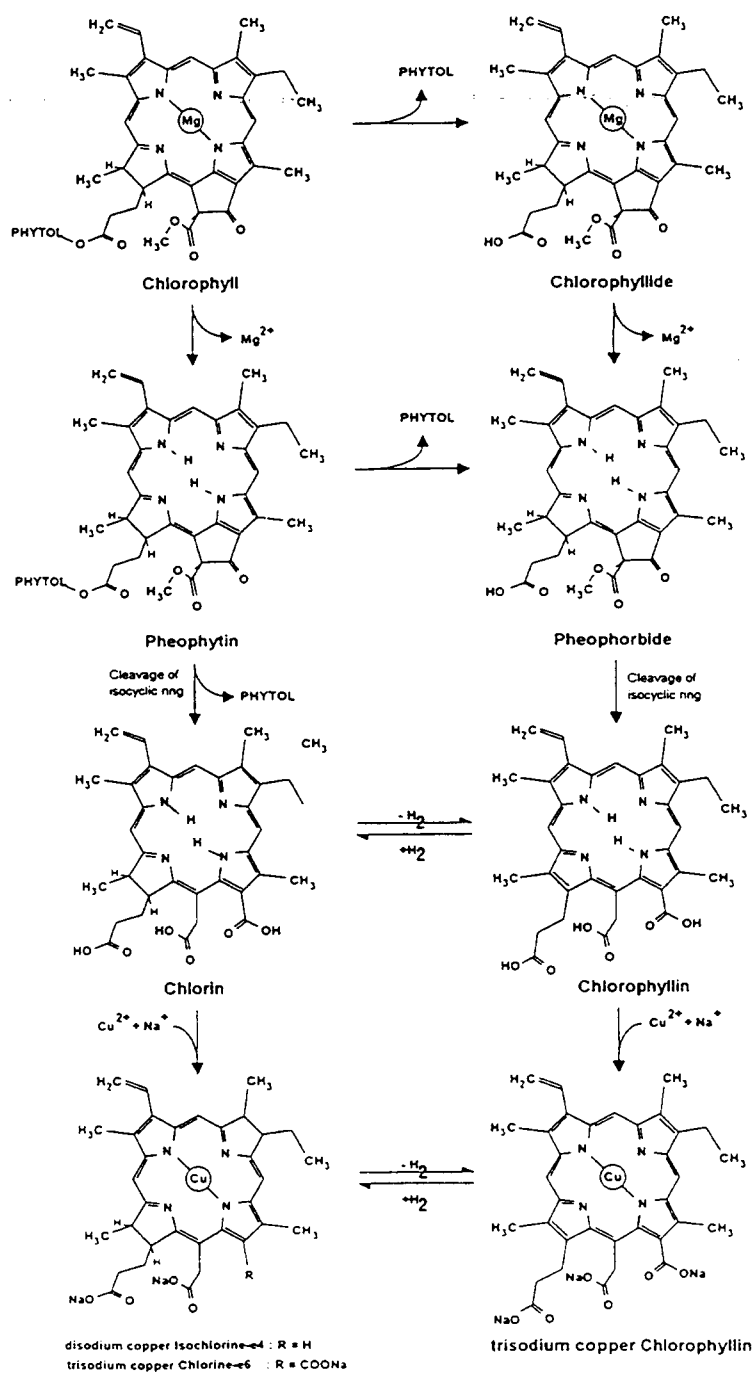
Figure 1.6 General scheme for conversion between chlorophyll, chlorin and chlorophyllin.

Commercial production of chlorophyll derivatives

Although chlorophylls have been employed in the coloring of soaps, waxes and even food for many years (Hendry, 1989), the chlorophyll derivatives, especially the copper salts, have by far exceeded chlorophyll in medicinal and industrial applications (Hendry, 1989; Spikes and Bommer, 1991).

A flow-diagram of the procedures employed in production of commercially available copper-chlorophyllin (CHL) is presented in Figure 1.7. The primary sources of raw material for the production of CHL are alfalfa or grass (Humprey, 1980; Kephart, 1955). After harvesting, the plant material is dried to an approximate moisture content (w/w) of 15%, followed by extraction with acetone or other organic solvent (Porra, 1989; Khachik, 1986). The crude extract is then reacted with methanolic sodium hydroxide. This treatment replaces the phytyl radical and the alkyl radicals of the methoxy groups with sodium and leads to the formation of water-soluble sodium magnesium chlorophyllin. The name for this compound is more correctly chlorin as the initial treatment also causes breakage of the isocyclic ring structure (Hynninen, 1991). After saponification the slurry is reextracted with acetone to extract the unsaponifiable substances. The slurry is then passed through a washing centrifuge and the liquid phase consisting of carotenoids, fats and waxes is discarded. The solid phase is reslurried with 65% actone, 35% water in which waxes, sterols and soaps are insoluble. After centrifugation and removal of the solid fraction, the aqueous phase containing the chlorin is passed through an evaporator, for removal of the acetone. Because of the extremely unstable magnesium chlorin construct, the pigments

are stabilized by the replacement of magnesium with copper or other heavy metals (Kephart, 1955). This exchange is easily accomplished by adding excess copper to the hot aqueous solution of chlorin, followed by acidification. In addition to replacing the magnesium with copper, the sodium ions are exchanged with hydrogen and the water insoluble form of chlorin is produced. After washing and filtration, the chlorin precipitate and the accompanying fatty acids formed from the soaps are dissolved in acetone. The chlorin fraction is then precipitated with hexane, in which the fatty acids are soluble. Following filtration, the precipitated pigment is suspended in water and the hydrogen of the carboxyl groups replaced by the addition of an alkali metal (Na,K), forming the water soluble sodium/potassium copper salt of chlorophyll. Unfortunately, even under the best commercial processes some degradation during harvesting, storage and processing takes place which leads to the formation of several decomposition products of chlorophyll (Kephart, 1955; Hynninen, 1991). During extraction both allomerization and isomerization of chlorophyll and its derivatives take place, and the marketable products are truly the sodium (or potassium) salts of a mixture of copper chlorins (Hynninen, 1991).

Crude plant extract

Acetone extraction

Saponification with
methanolic NaOH

Extraction of unsaponifiable
substances w/ acetone

→ *liquid* → fats, waxes and
carotenoids

↓ **solid**

Solids reslurried with 65%
acetone, 35% water

→ **solid** → soaps, sterols
and waxes

↓ liquid

Cu^{2+} added to hot aqueous
solution: exchange with Mg^{2+}

Acidification: Na^+ exchanges
with H^+

↓ **solid**

Precipitate dissolved in
acetone

Chlorophyllin precipitated
with hexane

→ *liquid* → fatty acids

↓ **solid**

Alkali metal added: H^+
exchanges with Na^+, K^+

**Water soluble copper salt
of Chlorophyll**

Figure 1.7 Flow diagram of the various steps involved in the preparation of commercial chlorophyllin.

Commercial uses of chlorophyll derivatives

The numerous applications of chlorophyll and chlorophyll-derivatives in the cosmetic and food industry will not be further discussed here. Special emphasis will be given to medical applications of the plant pigment, which are of great importance for interpreting and evaluating the proposed mechanisms responsible for the observed antimutagenic and anticarcinogenic properties of CHL.

The copper containing derivatives of chlorophyll, such as the copper chlorins, are particularly resistant to the action of strong acids, due to the strong coordination of copper with the nitrogens of the tetrapyrrole ring structure compared to the highly labile magnesium-chlorophyll(in). The higher stability of the copper derivatives compared to chlorophyll along with their water solubility renders the copper complexes ideal for medical use (Kephart, 1955; Hendry, 1989). Due to the waxy nature of native chlorophyll, its application is extremely limited. Current use of the green plant pigment in medical regimes is therefore exclusively as water soluble chlorins, of which sodium copper chlorin is by far the most commonly employed (Kephart, 1955; Hendry, 1989).

Toxicity of chlorophyll derivatives

The low toxicity of chlorins has been amply demonstrated in studies employing different experimental animals including rabbits, guinea pigs, dogs and mice. Massive doses of water soluble chlorophyll containing chelated magnesium, copper, zinc,

cobalt or nickel were administered by various routes with no obvious toxic effect (Kephart, 1955; Smith, 1944). Although the extremities of some of the animal species turned green, there was a complete absence of toxic effects. During treatment of certain human ailments, doses of up to 1.5 g of copper chlorin per day were administered, often for several weeks, again without noticeable toxic side effects (Kephart, 1955; Young and Beregi, 1980).

Use of chlorophyll derivatives in treatment of anemia

Chlorins have also been applied historically in the treatment of anemia. Intramuscular or oral administration of chlorophyll derivatives has been found by several investigators to shorten the regeneration time of blood constituents after hemorrhage and in general to increase the concentration of hemoglobin in patients with chronic anemia (Patek, 1936; Hughes and Latner, 1936). The positive effect of chlorophyll derivatives may be due to the ability of the pyrrole substances to be used as precursors for the formation of hemoglobin (Patek, 1936; Aoki, 1931).

Stimulation of tissue regeration by chlorophyll derivatives

The water soluble chlorophyll derivatives were also used historically for accelerating healing of wounds and burns in animal models and in human subjects. Topically applied chlorophyll/chlorin solutions appeared to facilitate growth of tissue

granulation and epithelization along with rapid cessation of pus formation (Smith and Sano, 1944; Smith and Livingston, 1945). The mechanisms by which chlorophyll and its derivatives stimulate tissue repair remain unknown.

Antioxidative activities of porphyrin structures

The special structure of chlorophyll and derivatives thereof confers the ability of this pigment, to act as a antioxidant. The basic porphyrin structure is characterized by a very high degree of resonance produced by the conjugation of a large number of double bonds, resulting in a highly delocalized electron system (Scheer, 1991). Electron-rich structures such as these have the potential to trap activated oxygen species, radicals and electrophiles, and could in part explain the positive effect of chlorophyll derivatives in the healing of wounds which are highly concentrated in reactive oxygen compounds.

Use of chlorophyll derivatives in odor control

Another application of chlorophyllins is in the control of offensive body odor, a problem often encountered in geriatric patients and people with colostomies and ileo-stomies (Nahata et al., 1983; Young and Beregi, 1980). The therapeutic effects of the chlorophyll derivatives as an internal deodorizer lies in the ability of the porphyrin molecule to exert antibacteriostatic activity (Smith and Spaulding, 1944). The ability of bacteria, particularly anaerobic species, to grow is severely impaired by the

presence of CHL, whereas the aerobic bacteria of the intestinal flora do not seem to be affected. This specific effect on the anaerobic bacteria reduces formation of bacteria-induced odors. That chlorophyll derivatives display antiseptic potential is seen in the successful treatment of anaerobic bacterial infections (Smith and Spaulding, 1944).

Chlorophyll derivatives in pancreatitis treatment

Therapeutic trials on patients with acute and chronic pancreatitis have revealed that chlorophyll a is a very effective agent in the treatment of this ailment (Oda, 1971). Intravenous administration of chl a resulted in a marked decrease in urinary amylase activity, a biomarker often used in diagnosing pancreatitis. Complications accompanying pancreatitis, such as abdominal pains, nausea and vomiting, disappeared within days of initiating the chl a treatment. The mechanism by which chl a exerts its effect in pancreatitis treatment is thought to be via inhibition of proteases in the pancreas (Oda, 1971). This finding is supported by *in vitro* experiments which indicate that water soluble chlorophyll derivatives containing various chelated metals are capable of inhibiting proteases such as pepsin, trypsin, α-chymotrypsin and kallikrein with an approximate inhibition constant of 90 μM (Oda, 1971). As chl a *in vitro* did not inhibit the proteinase activity, it is speculated that chl a *in vivo* is changed into chlorophyllin by the removal of the phytol group and possible by exchanging the

chelated Mg with other divalent ions (Oda, 1971). The mechanism by which the chlorophyll derivatives exert anti-proteolytic activity is suggested to be via binding to the enzymes, thereby affecting the catalytic properties of the enzyme.

Stimulatory effect of chlorophyll derivatives on the immune system

Intraperitoneal administration of zinc-chlorophyllin, was found to stimulate the production of C3, a key component of the complement system, involved in triggering certain immune factors in the early stage of host defense (Sakamoto and Nishioka, 1992). The induction of C3 increased the host resistance against several bacterial infections, presumably via increased phagocytotic activity (Sakamoto and Nishioka, 1992).

Chlorophyll derivatives as photosensitizers

Another finding discovered during cancer research is the ability of chlorophyll-like compounds to accumulate in growing tissues and tissues undergoing regeneration (Spikes and Bommer, 1991). When porphyrinic compounds are injected into test animals with various forms of carcinomas and sarcomas, the porphyrins specifically accumulate in the tumors. The use of radioactively labeled chlorophyll derivatives could therefore potentially be used in detection and probably therapy of such tumors (Spikes and Bommer, 1991). The therapeutic potential lies in the ability of the porphyrin-structure to undergo photosensitization upon exposure to light. Illumination

of the molecule converts it to a short-lived triplet excited state. Typically, sensitizer molecules in the triplet state can abstract an electron (or hydrogen atom) from, or donate an electron (or hydrogen atom) to, a substrate molecule. The anionic or cationic radicals of the sensitizer, can then react with oxygen and form reactive oxygen species, which rapidly reacts with a number of electron-rich molecules such as unsaturated lipids, amino acids, DNA-bases etc. and hereby interfere with the normal activities of subcellular structures, resulting in the injury and death of cells in close proximity of the sensitizer molecule.

REFERENCES

Abemayor, E.B, Chang, B. and Sidell, N.: Effects of retinoic acid on the in vivo growth of human neuroblastoma cells. Cancer Letters 55:1-5, 1990.

Ames, B.N.: Mutagenesis and carcinogenesis: Endogenous and exogenous factors. Environmental and molecular mutagenesis 14, suppl. 16:66-77. 1989.

Aoki, S.: Experimental studies on effect of chlorophyll upon formation of haemoglobin. N.Y.State Jour. Med. 50:1-14, 1931.

Athar, M., Khan, W.A. and Mukhtar, H.: Effect of dietary tannic acid on epidermal, lung, and forestomach polycyclic aromatic hydrocarbon metabolism and tumorigenicity in sencar mice. Cancer Research 49:5784-5788, 1989.

Barch, D.H. and Fox, C.C.: Selective inhibition of methylbenzylnitrosamine-induced formation of oesophageal O6-methylguanine by dietary ellagic acid in rats. Cancer Research 48:7088-7092, 1988.

Bartsch, H., Ohshima, H., and Pignatelli, B.: Inhibitors of endogenous nitrosation mechanisms and implications in human cancer prevention. Mutation Research 202:307-324, 1988.

Baxter, J.H.: Absorption of chlorophyll phytol in normal man and in patients with Refsum's disease. Journal of Lipid Research 9:636-641, 1968.

Bingham, S.A.: Mechanisms and experimental and epidemiological evidence relating dietary fibre (non-starch polysaccharides) and starch to protection against large bowel cancer. Proceedings of the Nutrition Society, 49:153-171, 1990.

Chang, Y-J., Mathews, C., Mangold, K., Marien, K., Hendricks, J. and Bailey,G.S.: Analysis of ras gene mutations in rainbow trout liver tumors initiated by AFB_1. Molecular Carcinogenesis 4:112-119, 1991.

Das, M., Blickers, D.R. and Mukhtar,H.: Effect of ellagic acid on hepatic and pulmonary xenobiotic metabolism in mice: studies on the mechanism of its anticarcinogenic action. Carcinogenesis 6:1409-1413, 1985.

Deschner, E.E., Ruperto, J., Wong, G. and Newmark, H.L.: Quercetin and rutin as inhibitors of azoxymethanol-induced colonic neoplasia. Carcinogenesis 12:1193-1196, 1991.

Dixit, R. and Gold, B.: Inhibition of N-methyl-N-nitrosurea-induced mutagenicity and DNA-methylation by ellagic acid. Proceedings of the National Academy of Science 83:8039-8043, 1986.

Ellegbede, J.A., Elson, C.E., Tanner, M.A., Qureshi, A. and Gould, M.N.: Regression of rat primary mammary tumors following dietary d-limonene. Journal of the National Cancer Institute 76:323-325, 1986.

Epstein, S.S.: Evaluation of the national cancer program and proposed reforms. International Journal of Health Survey 23(1):15-44.1993.

Harris, C.C.: Chemical and physical carcinogenesis: Advances and perspectives for the 1990s. Cancer Research 51(suppl.):5023s-5044s, 1991.

Hayatsu, H., Arimoto, S. and Negishi T.: Dietary inhibitors of mutagenesis and carcinogenesis. Mutation Research 202:429-446, 1988.

Henderson, M.M.: International differences in diet and cancer incidence. J. Natl. Cancer Inst., 12:59-63. 1992.

Hendry, G.A.F.: Chlorophylls and chlorophyll derivatives. pp 15-34. In Development of food colors vol.3 (Counsell, J.N., Ed.). Elsvier Applied Science, Bracknell, U.K., 1989.

Huang, M-T., Chang, R.L., Wood, A.W., Newmark, H.L., Sayer, J.M., Yagi, H., Jerina, D.M. and Conney, A.H.: Inhibition of the mutagenicity of bay-region diol-epoxides of polycyclic aromatic hydrocarbons by tannic acid and hydroxylated cinnamic acid derivatives. Carcinogenesis 6:237-242, 1985.

Hughes, J.H. and Latner, A.L: Chlorophyll and hemoglobin regeneration after hemorrhage. Jour. Physiol. 86:388-396, 1936.

Hynninen, P.H.: Chemistry of chlorophylls: Modifications. pp.145-209. In Chlorophylls (Scheer, H., Ed.). CRC Press, Boca Raton, Florida, 1991.

International Agency for Cancer Research. IARC Monographs on the evaluation of the carcinogenic risk of chemicals to humans: Aflatoxins. 56:245-396.1993.

Kephart, J.C.: Chlorophyll derivatives, their chemistry, commercial preparation, and uses. Econ. Bot. 9:3-38, 1955.

Khachik, F., Beecher, G.R. and Whittaker, N.F.: Separation, identification and quantification of the major carotenoid and chlorophyll constituents in extracts of several green vegetables by liquid chromatography. J. Ag. Food Chem., 34:603-616. 1986.

Khalyfa, A.; Kermasha, S. and Alli, I.: Extraction, purification, and characterization of chlorophyll from spinach leaves. J. Ag. Food Chem, 40:215-220. 1992.

Kensler, T.W., Groopman, J.D., Eaton, D.L., Curphey, T.J. and Roebuck, B.D.: Potent inhibition of aflatoxin-induced hepatic tumorigenesis by the monofunctional enzyme inducer 1,2-dithiole-3-thione. Carcinogenesis 13:95-100, 1992.

Loveland, P.M., Nixon, J.E. and Bailey, G.S.: Glucuronides in bile of rainbow trout (*Salmo gairdneri*) injected with ^{3}H Aflatoxin B_1 and the effects of dietary β-naphthoflavone. Comp. Biochem. Physiol., 78C:13-19. 198

Motoko, S. and Nishioka, K.: Complement system in nutritional deficiency. World Rew. Nutr. Diet 67:114-139, 1992.

Mukherjee, A., Agarwal, K., Aguilar, M.A. and Sharma, A.: Anticlastogenic activity of β-carotene against cyclophosphamide in mice in vivo. Mutation Research 263:41-46, 1991.

Mukhtar, H., Das, M., Khan, W.A., Wang, Z.Y., Bik, D.P. and Bickers, D.R.: Exceptional activity of tannic acid among naturally occurring plant phenols in protection against 7,12-demethylbenz(a)anthracene-, benzo(a)pyrene, 3-methylcholanthrene, and N-methyl-N-nitrosurea-induced skin tumorigenesis in mice. Cancer Research 48:2361-2365, 1988.

Oda, T., Yokono, O., Yoshida, A., Miyake, K. and Iono, S.: On the successful treatment of pancreatitis. Gastroenterologia Japonoca 6:49-54, 1971.

Okuzumi, J., Nishimo, H., Murakoshi, M., Iwashima, A., Tanaka, Y., Yamane, T., Fujita, Y. and Takahashi, T.: Inhibitory effects of fucoxanthin, a natural carotenoid, on N-myc expression and cell cycle progression in human malignant tumor cells. Cancer Letters 55:75-81, 1990.

Patek, A.J.: Chlorophyll and regeneration of blood: Effect of administration of chlorophyll derivatives to patients with chronic hypochromic anemia. Arch. Int. Med. 57:73-84, 1936.

Perchellet, J-P., Perchellet, E.M. and Belman, S.: Inhibition of DMBA-induced mouse skin tumorigenesis by garlic oil and inhibition of two tumor promotion stages by garlic and onion oils. Nutrition and Cancer 14:183-193, 1990.

Pohland, A.E. and Wood, G.E.: Occurrence of mycotoxins in food, in P.Krogh (Ed.), Mycotoxins in food. Academic Press, London, 35-64. 1987.

Porra, R.J., Thompson, W.A. and Kriedeman, P.E.: Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochem. Biophys. Acta. 975:384-396, 1989.

Robinson, D.S.: Food - biochemistry and nutritional value. pp.414-416. John Wiley & Sons, Inc., New York, 1987.

Rotstein, J.B. and Slaga T.J.: Anticarcinogenesis mechanisms, as evaluated in the multistage mouse skin model. Mutation Research 202;421-427, 1988.

Ryberg, M. and Sundqvist, C.: Structural and functional significance of pigment-protein complexes of chlorophyll precursors. pp.587-625, 1991. In Chlorophylls, Scheer, H., Ed. CRC Press, Boca Raton, Florida, 1991.

Sagher, D.: Insertion of nucleotides opposite apurinic/apyrimidinic sites in deoxyribonucleic acids during in vitro synthesis: Uniqueness of adenine nucleotides. Biochemistry 22:4518-4526. 1983.

Sambamurti, K., Callahan, J., Luo, X., Perkins, C.P., Jacobsen, J.S., Humayun, M.Z.: Mechanisms of mutagenesis by bulky DNA-lesion at the guanine N7-position. Genetics 120:863-873. 1988.

Sayer, J.M., Yagi, H., Wood, A.W., Conney, A.H. and Jerina, D.M.: Extremely facile reaction between the ultimate carcinogen of benzo(a)pyrene-7,8,-diol-9,10-epoxide and ellagic acid. Journal of the American Chem. Society 104:5562-5564, 1982.

Schaaper, R.M., Kunkel, T.A., Loeb, L.A.: Infidelity of DNA synthesis associated with bypass of apurinic sites. Proceedings of the National Academy of Science 80:487-491. 1983.

Schaaper R.M., Glickman, B.W., Loeb, L.A.: Role of depurination in mutagenesis by chemical carcinogens. Cancer Research 42:3480-3485.1982.

Scheer H., 1991.: Structure and occurrence of chlorophylls. In Chlorophylls, Scheer, H., Ed. CRC Press, Boca Raton, Florida, 1991.

Schultz, H.W.: In Food Law Handbook. p.628. Avi Publishing Company, Westport, CT. 1981.

Shamberger, R.J.: Increase of peroxidation in carcinogenesis. Journal of the National Cancer Institute 48:1491-1497, 1972.

Shklar, G., Schwarz, J., Trickler, D. and Reid, S.: Regression of experimental cancer by oral administration of combined a-tocopherol and b-carotene. Nutrition and Cancer 12:107-124, 1989.

Simon, M.I., Strathmann, M.P. and Gautam, N.: Diversity of G-proteins in signal transduction. Science, 252:802-808. 1991.

Smart, R.C., Huang, M.T., Chang, R.L., Sayer, J.M., Jerina, D.M., Wood, A.W. and Conney, A.H.: Effect of ellagic acid and 3-O-decylellagic acid on the formation of benzo(a)pyrene-derived DNA adducts in vivo and on the tumorigenicity of 3-methylchloanthrene in mice. Carcinogenesis 7:1663-1667, 1986.

Smith, L.W.: Chlorophyll: an experimental study of its water-soluble derivatives. Remarks upon history, chemistry, toxicity and antibacterial properties of water-soluble derivatives as therapeutic agents. Am. Jour. Med. Sci. 207:647-664, 1944.

Smith, L.W. and Livingston, A.E.: Wound healing, an experimental study of water soluble chlorophyll derivatives in conjunction with various antibacterial agents. Am. Jour. Surg. 67:30-38, 1945.

Smith, L.W and Sano, M.E.: Chlorophyll: An experimental study of its water soluble derivatives. IV. The effect of water soluble chlorophyll derivatives and other agents upon fibroblasts in tissue culture. Jour. Lab. Clin. Med. 29;241-253, 1944.

Sparrings, L.V., Barany, G. and Wattenberg, L.W.: Effects of organosulphur compounds from garlic and onions on benzo(a)pyrene-induced neoplasia and glutathione-S-transferase activity in the mouse. Carcinogenesis 9:1310134, 1988.

Spikes, J.D. and Bommer, J.C.: Chlorophyll and related pigments as photosensitizers in biology and medicine. pp.1181-1204. In Chlorophylls, Scheer, H., Ed. CRC Press, Boca Raton, Florida, 1991.

Stich, H.F. and Rosin, M.P.: Naturally occurring phenolics as antimutagenic and anticarcinogenic agents. In: Friedman, H. Nutritional and toxicological aspects of food safety. Plenum Press, New York, pp.1, 1984.

Suda, D. Schwartz, J. and Shklar, G.: Inhibition of experimental carcinogenesis by topical β-carotene. Carcinogenesis 7:711-715, 1986.

Tanaka, T., Mori, Y, Morishita, Y., Hara, A., Ohno, T., Kojima, T. and Mori, H.: Inhibitory effect of sinigrin and indole-3-carbinol on diethylnitrosamine-induced hepatocarcinogenesis in male ACI/N rats. Carcinogenesis 11:1403-1406, 1990.

Tell, R.W.: Ellagic acid binding to DNA as a possible mechanism for its antimutagenic and anticarcinogenic action. Cancer letters 30:329-336, 1986.

Troll, W., Wiesner, R., Shellabarger, C.J., Holtzman, S. and Stone, J.P.: Soybean diet lowers breast tumor incidence in irradiated rats. Carcinogenesis 1:469-472, 1980.

Troll, W., Wiesner, R. and Frenkel, K.: Anticarcinogenic action of protease inhibitors. Adv. Cancer Research 49:265-283, 1987.

Valsta, L.M., Hendricks, J.D. and Bailey, G.S.: The significance of glutathione conjugation for aflatoxin B_1 metabolism in rainbow trout and coho salmon. Food Chem. Toxicol. 26:129-135, 1988.

U.S. Bureau of the Census, Statistical Abstract of the United States: 1993 (113th edition). pp 91, 199 and 201. U.S. Government Printing Office, Washington, DC, 1993.

Wattenberg, L.W.: Inhibition of neoplasia by minor dietary constituents. Cancer Research, 43(suppl.):2448-2453, 1983.

Weinberg, R.A.: In Oncogenes and the Molecular Origins of Cancer. Cold Spring Harbor Laboratory Press, New York, 1989.

Weinberg, R.A.: Tumor suppressor genes. Science 254:1138-1146. 1991.

Whong, W., Stewart, J., Brockman, H.E. and Ong, T.: Comparative mutagenicity of chlorophyllin and five other agents against aflatoxin B1-induced reversion in *Salmonella typhimurium* TA98. Teratogenesis, Carcinogenesis and Mutagenesis 8:215-224.1988.

Wood, A.W., Huang, M-T., Chang. R.L., Newmark, H.L., Lehr, R.E., Yagi, H., Sayer, J.M., Jerina, D.M. and conney, A.H.: Inhibition of the mutagenicity of bay-region diolepoxides of polycyclic aromatic hydrocarbons by naturally occurring plant phenols: Exceptional activity of ellagic acid. Proceedings of the National Academy of Science, 79:5513-5517, 1982.

Young, R.W. and Beregi, J.S.: Use of chlorophyllin in the care of geriatric patients. Journal of the American Geriatrics Society.

CHAPTER 2

QUALITY ASSESSMENT OF COMMERCIAL SODIUM-COPPER
CHLOROPHYLLIN BY THIN-LAYER CHROMATOGRAPHY AND
SPECTRAL ANALYSIS

Vibeke Breinholt

and

George S. Bailey

Toxicology Program

Oregon State University

Corvallis, OR 97331

ABSTRACT

Commercial sodium-copper chlorophyllin (CHL) obtained from Sigma Chemical Company was found to resolve into at least 7 distinct chromatographic bands when analyzed by thin-layer chromatography (TLC). The two major bands were tentatively identified as tri-sodium copper chlorin e6 (Ce6) and di-sodium copper isochlorin e4 (Ce4). Addition of water to the pure Ce6 standard gave rise to the formation of several chromophores of which the two major components were identified as Ce6 and Ce4 . Comparison of the copper concentrations and the maximum absorbances of crude solutions of CHL and Ce6 and solutions of individual pigments, with similar Rf values, revealed that commercial CHL consisted of approximately 34% chlorins and 66% inorganic salts.

Further analysis of the extracts of individual bands from preparative TLC revealed a strong correlation between the copper concentration and the absorbance at 406-408 nm. As pure chlorophyllin derivatives are not yet commercial available, determination of the copper concentration or spectral analysis may be important tools to assess the approximate purity of impure commercial preparations of CHL.

INTRODUCTION

Commercially available sodium-copper chlorophyllin (CHL) has been found to consist of several individual chromophores of varying chemical and biochemical properties (Sato et al., 1986; Sato et al., 1984a,b) The two major fractions in commercial CHL have been identified as tri-sodium copper chlorin e6 (Ce6) and di-sodium isochlorin e4 (Ce4) (Sato et al., 1986). The same two pigments were concluded by Sato et al. (1986) to play the principal role in the antioxidative action of CHL, whereas other green chromophores exerted essentially no antioxidant activities.

Because of this great variability in bio-activity of the copper-chlorophyll complexes, and the complete lack of information about the specific copper chlorin content in CHL produced in the U.S., a study was initiated in an attempt to isolate, identify and quantify the major pigment components in Sigma CHL. The purity of the CHL preparation was analyzed by TLC as described by Sato et al. (1986), and the two major pigments were tentatively identified on basis of Rf values and color descriptions obtained from the literature (Sato et al., 1986).

MATERIALS

Chlorophyllin (CHL) was obtained from Sigma Chemical Co, St. Louis, MO. Pure chlorin-e6 (Ce6) was kindly provided by Dr. Hikoya Hayatsu. The Ce6 preparation was analyzed by HPLC and found to be approximately 98% pure. Copper standards, dialysis membranes (MWCO 500) and preparative TLC plates (Silica Gel 60, 200 X 200 X 20 mm) were from VWR Scientific, Philadelphia, PA.

METHODS

The CHL preparation was analyzed by preparative thin-layer chromatography (TLC) using a solvent system consisting of n-butanol:ethanol:ammonium hydroxide (2:1:1). CHL and chlorin-e-6 (internal standard) were dissolved in water (pH 10.0) and applied to the silica plate (200 X 200 X 20 mm) with a glass capillary tube. A total of 5 μl equal to 250 μg was used for each sample and chromatographed in duplicate. Individual bands from the TLC were cut out with a razor blade and extracted with acetone: 50 mM Na_2CO_3 (1:1). After precipitation of the silica by centrifugation at 2,500 rpm in a bench top centrifuge for 5 min, the copper concentration of the supernatant was measured by atomic absorption spectroscopy (AA) and the absorbance at 406-408 nm was determined.

To verify the manufacturer's statement that all copper in CHL is associated with the porphyrin structure, a CHL solution of known concentration was dissolved in distilled water and dialyzed (500 MW cut-off) at 25°C for 72 hours against distilled water. This procedure was followed by AA of the dialate and the dialysis fluid.

RESULTS

The TLC chromatogram revealed that the CHL-sample resolved into a mixture of at least 7 different chlorin chromophores, which was also evident for the "pure" chlorin-e-6 sample (Figure 2.1). The two major fractions were identified as tri-sodium-copper chlorin e6 (Ce6) (band no. 2, bluish green) and di-sodium-copper-isochlorin e4 (Ce4) (band no.5, grass green) based on Rf values and specific color descriptions obtained from the literature (Sato et al., 1986). These two pigments accounted for approximately 80% of the total pigment content. The specific concentration of chlorin in CHL (32.4 $\pm$ 3.2%) based on the actual copper concentration was similar to the purity determined by TLC (33.3 $\pm$ 2.6%) using pure Ce6 as an internal standard. The remaining 66% was stated by the manufacturer to be inorganic salts, primarily sodium salts.

Comparison of the spectral analysis data with the copper determinations of the individual chromophores isolated from the TLC chromatogram revealed that the absorption at 406-408 nm was linearly correlated with the concomitant copper concentration (R=0.98)(Figure 2.2). The concentration of free copper in the preparation was analyzed by dialysis and estimated to less than 0.01 ppm (data not shown).

DISCUSSION

Comparison of the TLC chromatogram of the Sigma CHL with that of a pure preparation of copper chlorin e6 revealed that the specific batch of CHL analyzed, was approximately 35% total chlorins (range 33.5-36.8). This is consistent with the copper content of 3.0 ± 0.2% (Equivalent to 33.6 ± 2.4% total chlorins, MW 722) determined by atomic absorption spectrometry, and the finding that essentially all copper in the preparation is porphyrin associated. The close correlation between pigment absorbance at 406-408 nm and the concomitant copper content (Figure 2.2) indicates that the total chlorin content as well as the fractional contribution of the individual chromophores in a commercial preparation of CHL easily can be assessed by simply measuring the copper content of a solution of the green pigment. This observation is of great importance, in that pure CHL is not currently available on a commercial scale and that the structural and spectral characteristics of several of the detected chromophores have not yet been established. Furthermore, assessing the relative potency of chl a and b from dietary sources on the basis of data using CHL is possible only if the exact pigment content of the given CHL batch is known. That the presumably pure chlorin also resolved into several different chromophores upon addition of water is a typical example of the extreme lability of the chlorins, observed by several investigators (Scheer, 1991).
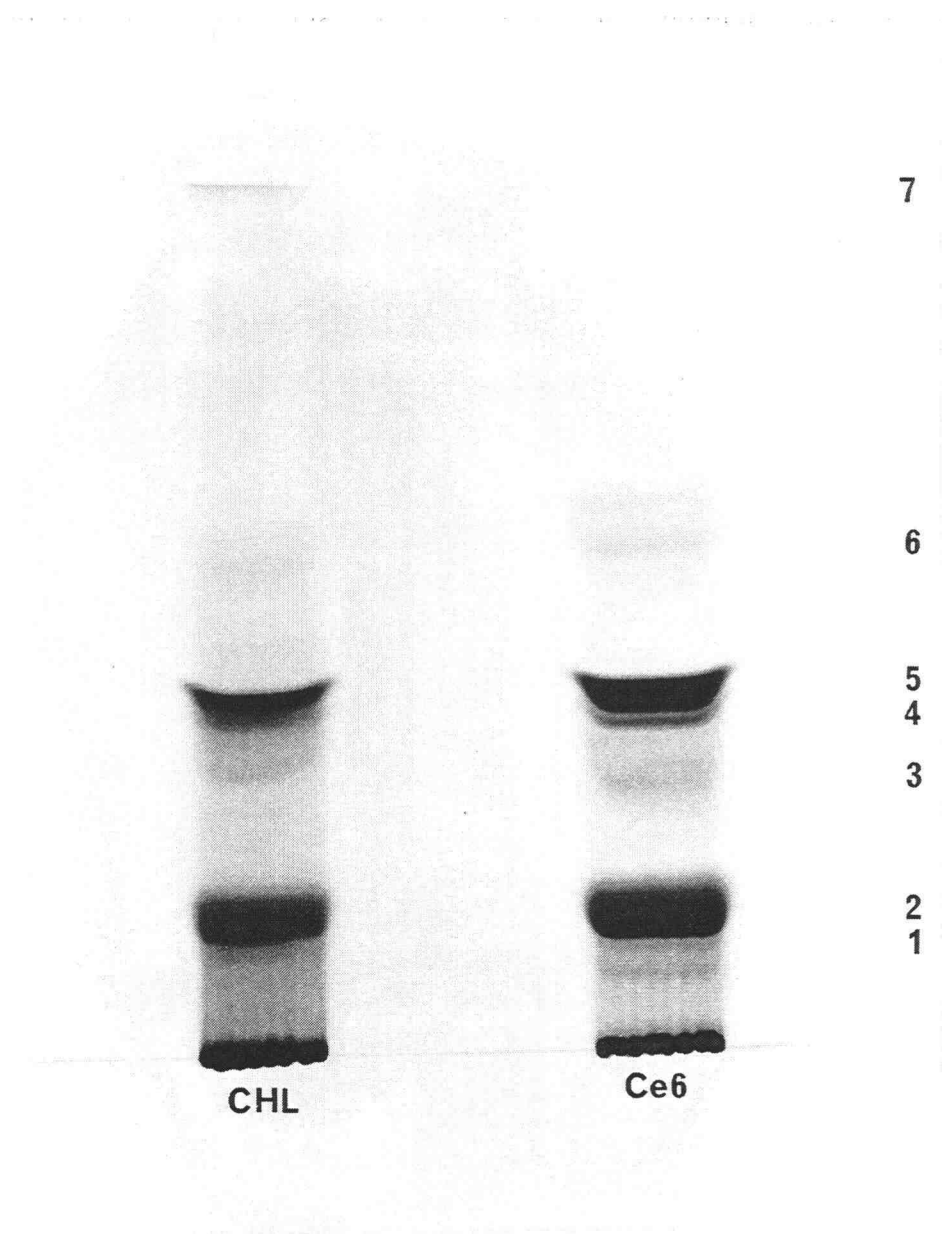
Figure 2.1   TLC chromatographs of chlorophyllin (CHL) and pure chlorin e6 (Ce6). The mobile phase consisted of n-butanol:ethanol:ammonium hydroxide (2:1:1). The samples resolved into several individual bands of which band no. 2 was identified as chlorin e6 and band no. 5 as isochlorin e4.
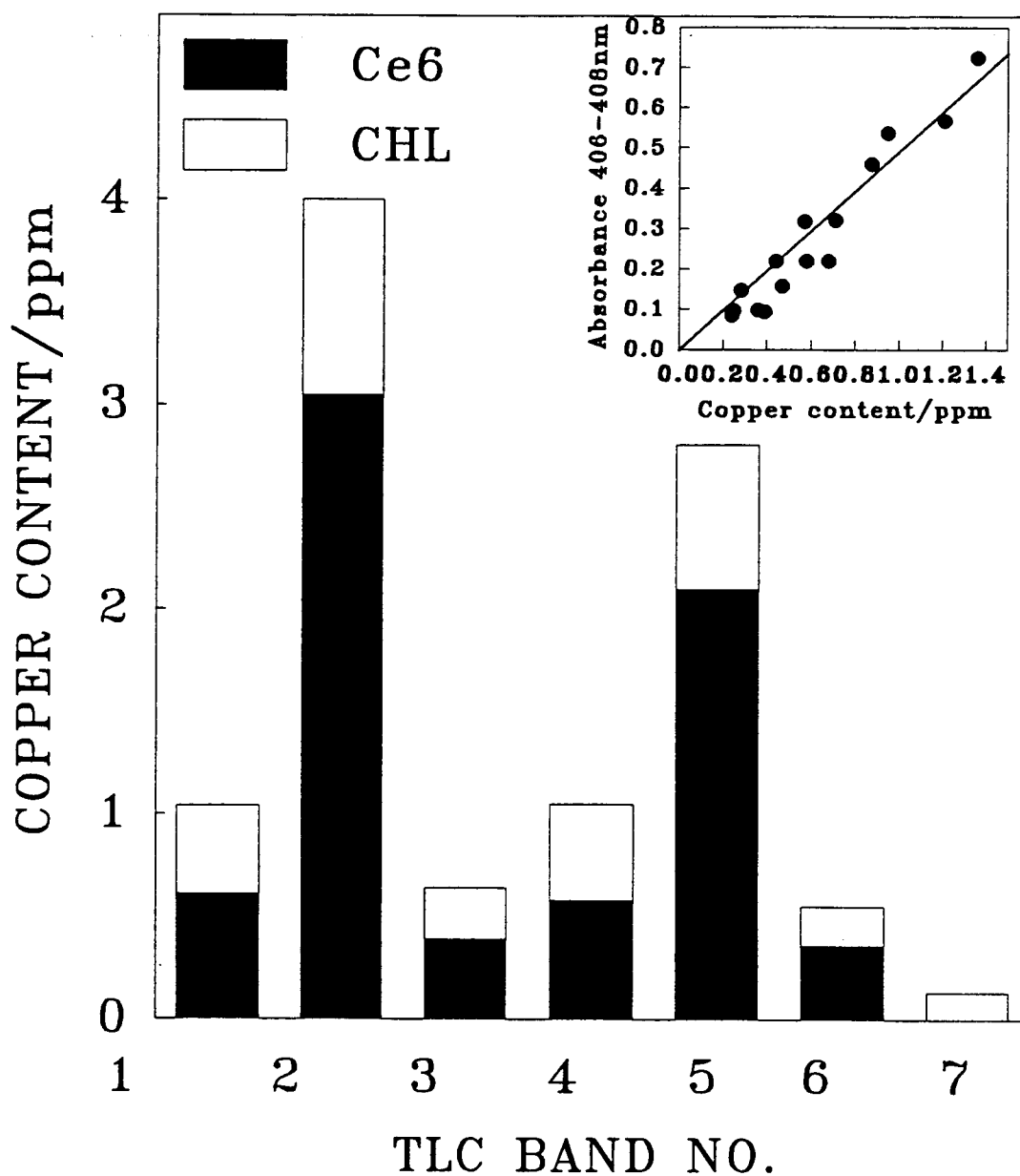
Figure 2.2 Determination of the copper concentration of the major bands from preparative TLC (shown in Figure 2.1). Figure 2.2 inset. Correlation between absorbance at 406-408 nm (< 1.0) and the copper concentration of extracts from individual TLC bands. Data points are from two individual TLC determinations.

REFERENCES

Sato, M., Fujimoto, I., Aimoto, T., Kimura, R. and Murata, T.: Effect of sodium copper chlorophyllin on lipid peroxidation. IX. On the antioxidative components in commercial preparations of sodium copper chlorophyllin. Chem. Pharm. Bull. 34(6):2428-2434, 1986.

Sato, M., Imai, K., Kimura, R. and Murata, T.: Effect of sodium copper chlorophyllin on lipid peroxidation. VI. Effects of its administration on mitochondrial and microsomal lipid peroxidation in rat liver. Chem. Pharm. Bull. 32(2):712-722, 1984a.

Sato, M., Konagai, K., Kuwana, T., Kimura, R and Murata, T.: Effects of sodium copper chlorophyllin on lipid peroxidation. VII. Effects of its administration on the stability of rat liver lysosomes. Chem. Pharm. Bull. 32(7):2855-2858, 1984b.

Scheer H., 1991.: Structure and occurrence of chlorophylls. In Chlorophylls, Scheer, H., Ed. CRC Press, Boca Raton, Florida, 1991.

CHAPTER 3

INHIBITION OF AFLATOXIN B$_1$, AFLATOXIN B$_1$ EPOXIDE AND
HETEROCYCLIC AMINE MUTAGENICITY BY SODIUM-COPPER
CHLOROPHYLLIN

Vibeke Breinholt

and

George Bailey

Toxicology Program

Oregon State University

Corvallis, OR 97331

ABSTRACT

Chlorophyllin (CHL), a sodium/copper derivative of chlorophyll, exhibited potent antimutagenic activity against aflatoxin B_1 (AFB_1) and two heterocyclic amines, 3-amino-1-methyl-5H-pyrido-[4,3-b]indole (Trp-P-2) and 2-amino-3-methylimidazo[4,5-f]-quinoline (IQ), when incubated in the presence of a trout microsomal activation system. CHL was also found to inhibit the direct mutagenic activity of AFB_1-8,9-epoxide, which is the electrophilic species of AFB_1 that adducts DNA *in vivo* and initiates the first event in AFB_1 hepatocarcinogenesis. Kinetic analyses of the antimutagenicity data revealed that complex formation only accounted for part of the protective activity of CHL. CHL did not exert toxic effects on the bacterial tester strains, indicating that the observed decrease in mutagenicity, is truly imposed by CHL on the mutagenic events.

INTRODUCTION

<u>Antimutagenicity of chlorophyll:</u> Chlorophyll (chl), the ubiquitous pigment in green plants has been identified as a dose-responsive inhibitor of mutagenicity induced by a wide range of indirect- and direct-acting mutagens (Lai et al., 1979; Lai, 1980; Kimm et al., 1982a; Kimm et al., 1982b; Barale et al., 1983; Negishi et al., 1989). The chl content of raw juice from a large series of commonly consumed vegetables was found to be correlated with the antimutagenic potency of the extracts (Kimm et al., 1982a; Kimm et al., 1982b). Moreover, chlorophyll was identified as the primary antimutagenic factor when compared to other antimutagenic principles such as the retinoids, tocopherols, ascorbic acid and glutathione. In addition, the mutagenic suppressive activity was still evident after submitting the vegetable extracts to treatments simulating gastric juice conditions (Kimm et al., 1982a; Kimm et al., 1982b).

<u>Antimutagenicity of chlorophyllin:</u> Chlorophyllin (CHL), the structural homologue of chlorophyll, has been subjected to extensive research in the area of antimutagenesis and anticarcinogenesis for almost two decades. The rationale for using CHL and not the true chlorophyll chromophores is primarily the very low cost of CHL compared to the pure chlorophylls. Another more important aspect is the close resemblance of CHL with the chlorophyll metabolite formed upon digestion in humans (Baxter, 1968). In addition, CHL has not been associated with any toxic effects when used chronically in the treatment of certain human ailments (Nahata et al., 1983; Young et al., 1980). Like chl, CHL has been demonstrated to exert profound antimutagenic activities

against both direct- and indirect-acting mutagens as well as complex environmental and dietary mixtures (Whong et al., 1988; Ong et al., 1989; Ong et al., 1986; Arimoto et al., 1980). The use of different tester strains has revealed that CHL is capable of inhibiting both point and frameshift mutations (Romert et al., 1992). Inhibitory activities of CHL have also been obtained from eucaryotic mutagenicity assays employing the yeast *Saccharomyces cerevisiae* (Bronzetti et al., 1983). In these experiments a significant decrease in reverse mutations induced by styrene oxide was observed in the presence of CHL and no cytotoxic effect was evident. As chl consistently has been found to be less effective than CHL in inhibiting mutagenesis (Lai, 1980; Negishi et al., 1989), it is suggested that the chemical changes induced by the mammalian digestive system can convert chl a and b into even more potent suppressors of mutagenesis. The use of CHL rather than unmodified chl, in *in vitro* bioassays where intragastric degradation of chl is excluded, therefore mimics to a much greater extent the potential interaction between mutagens and chlorophyll(in) in the human gastrointestinal tract.

Proposed mechanisms of antimutagenesis: The mechanisms of CHL-induced antimutagenesis are extremely complex, as suggested by the inhibition or complete suppression of the mutagenicity induced by a wide spectrum of mutagens and carcinogens. Several mechanisms of action have been postulated, including complex formation with the parent compound or the ultimate mutagen (Newmark, 1984; Newmark, 1987; Negishi et al., 1989; Arimoto et al., 1989; Arimoto et al., 1980; Dashwood, 1992), activation of the mutagenic species away from nucleophilic sites, (Romert et al., 1992) scavenging of radicals and active oxygen species (Newmark,

1984, Sato et al, 1986b), and interference with metabolic activation of specific cytochrome(s) P450 and other intra- and extracellular drug-metabolizing enzymes (Romert et al., 1992; Imai et al., 1986). In addition, CHL administered intraperitoneally to rats was found to significantly reduce the cytochrome b_5 and total CYP content of hepatic microsomes by interfering with heme synthesis (Imai et al., 1986).

The aim of the current study was to evaluate the potency of CHL to inhibit mutagenesis induced by the known human carcinogen AFB_1 and its ultimate carcinogenic metabolite AFB_1-8,9-epoxide. The ability of trout hepatic enzymes to bioactivate two heterocyclic amines, 3-amino-1-methyl-5H-pyrido-[4,3-*b*]indole (Trp-P-2) and 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), was also evaluated along with the antimutagenic activities of CHL toward these two food-borne heterocyclic amines. The antimutagenic potential of CHL was evaluated using the plate incorporation protocol of the *Salmonella typhimurium* mutagenicity assay (Maron and Ames, 1983).

MATERIALS

The two heterocyclic amines Trp-P-2 and IQ were kindly provided by Dr. Hikoya Hayatsu. AFB_1 was obtained from Sigma Chemical Company, St. Louis, MO. and AFB_1-8,9-epoxide from Dr. Tom Harris. Nutrient Broth (No.2) and Bacteriological Agar (No.1) were from Oxoid Ltd., Basingstoke, England. All other chemicals were of purest grade available. *Salmonella* mutagenicity strains TA98 (detection of frameshift mutations) and TA100 (detection of point mutations) were provided by Dr.Bruce Ames.

METHODS

Ames assay: Two ml soft agar (per 110 ml: 0.6 g Agar No.1, 0.5 g NaCl, 100 ml sterile distilled water and 10 ml His-Bio-mix (0.5 mM d-biotin, 0.5 mM l-histidine)) were added aseptically to presterilized capped borosilicate test tubes (10 ml) and placed in a 45°C waterbath. The inhibitor, contained in 100 μl sterile water, was added to the agar followed by the mutagen dissolved in 50 μl DMSO. Conical flasks (50 ml) containing 10 ml of a nutrient broth solution (0.25 g/10 ml) were inoculated with a single fresh colony of the appropriate tester stain and incubated for 12 hours at 37°C prior to conduction of the test. The incubation was done under continuous mixing (120 rpm) to obtain an approximate bacterial density of 10^9 bacteria per ml. After 12 hours incubation the cultures were placed on ice and allowed to come to room temperature before inclusion in the assay. The tester strains TA100 (AFB$_1$) and TA98 (AFB$_1$-8,9-epoxide, IQ, Trp-P-2) were used in the assay at 200 μl per test tube at the given bacterial density. AFB1 and the heterocyclic amines were activated into mutagenic species by enzymes of the trout hepatic post-mitochondrial fraction S-20 (supernatant after 20,000 g spin, 4°C, 20 min). The bioactivation enzymes were isolated from both control trout and trout fed 500 ppm BNF for 5 days. For activation of AFB$_1$, control S-20 was employed, whereas IQ and Trp-P-2 were activated using BNF induced S-20. S-20 mix (10% S-20, 15 mg protein/ml), 4% Nicotinamide dinucleotid phosphate (78 mg/ml), 5% Glucose-6-phosphate (28.2 mg/ml), 2% Ames salts (1.65 M KCl, 0.4 M MgCl$_2$), 50% Ames Buffer (0.2 M K$_2$PO$_4$,3H$_2$O (pH 7.4)) and 29% distilled water) comparable to rat S-9 mix, was prepared immediately prior

to use. After addition of 200 μl ice-cold S-20 mix and mixing by rapid vortexing for 2 seconds, the content was poured into a sterile 50 ml petri dish containing 20 ml minimal glucose agar (per 500 ml agar: 7.5 g Oxoid No.1 Agar, 440 ml water, 10 ml Vogel-Bonner-salts (VB-salts), 50 ml 20% d-glucose. The VB-salts was made by dissolving the following salts in 125 ml warm distilled water, and allowing each salt to dissolve before addition of the next: 2.5 g $MgPO_4$ X 7H2O, 25.0 g Citric acid X 1 H_2O, 125 g K_2PO_4 (anhydrous, dibasic), 43.8 g $NaNH_4PO_4$. The salt solution was made up to 250 ml with distilled water and autoclaved before use. The agar solution was allowed to cool to 55-60°C before addition of the 20% sterilized glucose and the VB-salts to prevent formation of mutagenic Maillard products).

CHL was dissolved in sterile Ames buffer and a total volume of 10 μl (0.3 or 1.0 μmole/plate) was added to each test tube. Correction for the added volume was made with Ames buffer. Each inhibitor-mutagen combination was tested in triplicate along with triplicate vehicle and inhibitor control plates. The petri plates were incubated for 48 hours at 37°C before scoring the number of revertants. The number of background revertants for the two bacteria strains was within normal range (Maron and Ames, 1983).Another series of mutagenicity assays was conducted using a fixed concentration of Trp-P-2 (0.3 μg/plate) or IQ (3.3 μg/plate) and concentrations of CHL, ranging from 0.1 to 2.0 μmole/plate.

S-20 preparation: The endoplasmic bioactivation enzymes were isolated as described by Coulombe et al. (1982).

RESULTS

CHL was found to be a potent inhibitor of mutagenesis induced by AFB_1 and

AFB_1-8,9-epoxide. At concentrations of 0.3 and 1.0 μmole CHL resulted in a dose-

responsive inhibition of AFB_1 mutagenicity at mutagen doses between 1.3 and 5.2 μg

per plate (Figure 3.1). The presence of 1.0 μM CHL inhibited the mutagenicity by

up to 60%, calculated as described by Ong et al (1986). The direct mutagenicity of

AFB_1-epoxide was also inhibited by co-incubation with CHL, likewise in a dose-

dependent fashion (Figure 3.2). The highest dose of CHL (1.0 μmole) inhibited the

activity of AFB_1-8,9-epoxide by more than 80%.

Assessment of the mutagenic potential of the two heterocyclic amines (HA) and

the antimutagenic activity of CHL toward them was evaluated in the presence of BNF-

induced trout activation system, as HAs in rodent systems are activated by the BNF-

inducible CYP1A2 (Kato, 1986; Mcmanus et al., 1990). The present data from the

Ames mutagenicity assay indicate that HA indeed can be activated by trout

microsomal enzymes but that the potency of the two investigated compounds is

reversed compared to rat-mediated mutagenic activities. With trout sub-cellular

fraction, Trp-P-2 was found to be a more potent mutagen than IQ. Due to the non-

linearity of the mutagenic response over the range of doses employed in the current

study, it is not immediately possible to obtain a specific mutagenicity of the two

compounds. However, comparing the number of revertants obtained from doses of IQ

between 0.3 and 3.3 μg/plate (Figure 3.3) and for Trp-P-2 between 0.1 and 0.3

μg/plate (Figure 3.4), where the mutagenic response is fairly linear, the mutagenic

potential of Trp-P-2 is approximately 10-fold higher than that of IQ at equimolar doses (5000 revertants/μg Trp-P-2 compared to 500 revertants/μg IQ). The mutagenicity of IQ was inhibited by 85% (Figure 3.3) and Trp-P-2 98% (Figure 3.4) at 1.0 μmole CHL/plate. When keeping the concentration of Trp-P-2 or IQ constant and adding increasing concentrations of CHL, complete inhibition of the mutagenic response could be obtained, without toxic effects on the tester strains (Figure 3.5). At doses above 1.5 μmole CHL/plate and 2.0 μmole CHL/plate, at which the mutagenic activity of Trp-p-2 and IQ, respectively, was completely abolished, the number of background revertants started to decrease and the bacteria growth was markedly impaired, visualized by petite revertant colonies. Except for these obvious toxic effects of CHL at relatively high doses, the remaining CHL concentrations employed in this study did not produce toxic effects, such as a decrease in viable cell counts and/or a decrease in the number of spontaneous revertants. This indicates that the observed effects are true antimutagenic activities exerted by CHL.

DISCUSSION

CHL was found to be a potent inhibitor of mutagenicity induced by both AFB_1 and its ultimate carcinogen AFB_1-epoxide. Similar observations have been made by Bronzetti et al. (1990) who found that CHL is capable of interacting directly with other epoxides such as styrene epoxide. Data provided by other investigators suggest that the antimutagenic activity of CHL is brought about by direct interaction between the mutagen and CHL, making the mutagen unavailable for biotransformation (Ong et al., 1986; Whong et al., 1988; Negishi et al., 1989). That mutagenesis of both the parent compound AFB_1 and its activated form the ultimate mutagen/carcinogen AFB_1-epoxide, is strongly inhibited by CHL supports the complexation hypothesis, although an inhibitory effect of CHL on bioactivation pathways cannot be excluded. The antimutagenic properties of CHL have been reported by other investigators using short-term genotoxicity assays such as *Drosophila melanogaster*, *Saccharomyces cerevisiae* and *Neurospora crassa* in addition to *Salmonella typhimurium* (Ong et al., 1986; Whong et al., 1988; Negishi et al., 1989; Bronzetti et al., 1990; Robins, 1986).

Other investigators have demonstrated mutagenic activity of several heterocyclic amines (Sugimura, 1985; Sugimura, 1986) in the *Salmonella* mutagenicity assay when bioactivation was brought about by rat subcellular fraction. The present studies report for the first time that two representative heterocyclic amines can undergo bioactivation by hepatic trout enzymes and induce mutagenesis in the Ames assay. The potency of the two tested heterocyclic amines however was opposite of that observed using a rat activation system (Sugimura, 1985; Sugimura, 1986). Trp-P-2

was found to be ca. 10 times more potent in inducing mutagenicity using trout S-20 mix than IQ, whereas the opposite is evident with rat microsomal activation (Sugimura, 1990). The explanation for this is at present unknown, but likely reflects the relative potential of BNF-induced trout microsomes to catalyze IQ and Trp-P-2 hydroxylation compared to rat microsomes. The mutagenicity of the investigated heterocyclic amines could be completely abolished, without affecting viability of the tester strain. At CHL doses exceeding those required for complete inhibition of mutagenesis, severe toxicity on the background revertants was evident (data not shown).

The ability of the heterocyclic amines to induce mutagenesis to a much greater extent than both AFB_1 and AFB_1-epoxide might suggest that IQ and Trp-P-2 would be good initiators of carcinogenesis in the trout model at doses much lower than those employed in AFB_1-induced tumorigenesis studies. Just recently, however, IQ among several other heterocyclic amines gave at most a weak hepatocarcinogenic response in the trout model at levels 500- to 1000-fold higher than commonly used cancer-inducing doses of AFB_1 (Ashok Reddy, personal communication). The discrepancy between these findings could lie in the high inherent activity of the Ames tester strains to activate promutagens via N-acetylation (Kato, 1986). This enzyme activity, which is required subsequent to hydroxylation of the HA (Pelaran et al., 1987; Turesky et al., 1988) to elicit the mutagenic and the carcinogenic activity, (Kato, 1986) could very well be poorly expressed in the trout, but has yet to be examined.

As mutagenesis of DNA *in vivo* is considered a pre-carcinogenic lesion, inhibition of *in vitro* mutagenicity by CHL suggests that CHL might act as an

anticarcinogenic agent against AFB_1-induced carcinogenesis. The same proposed complex formed in the Ames test between the investigated mutagens and CHL has the potential to form *in vivo* in the gut and afford protection against AFB_1-induced mutagenesis by decreasing total carcinogen burden, as the high molecular weight and possibly charged complex may be less readily absorbed than carcinogen alone.

Accompanying studies will evaluate the effect of CHL on both *in vitro* and *in vivo* DNA-AFB_1 adduction and test the hypothesis that CHL is capable of inhibiting AFB_1-induced tumorigenesis.
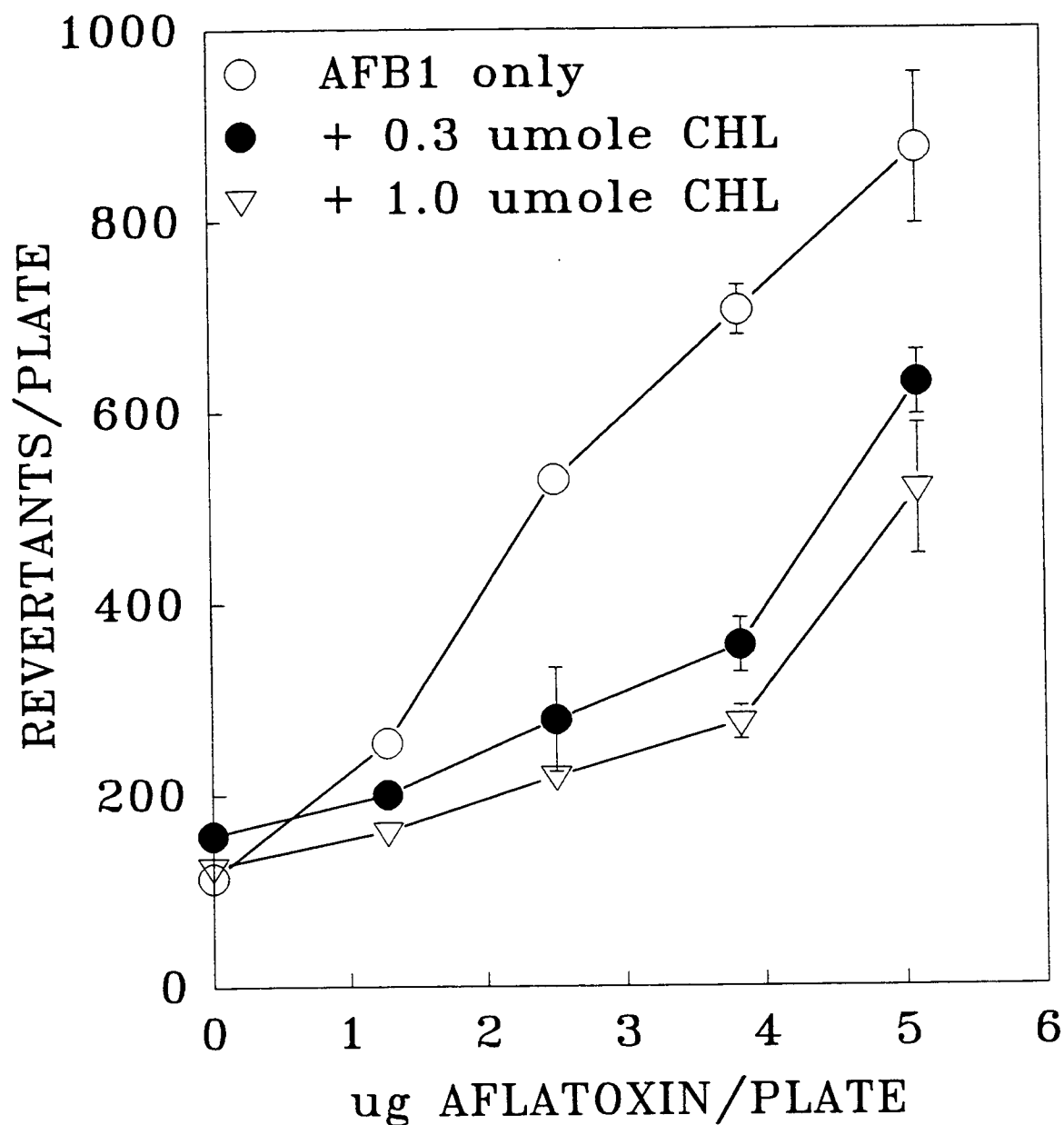
Figure 3.1   Inhibitory effect of CHL on the conversion of AFB_1 to bacterial mutagens by hepatic S-20 from control rainbow trout. Each point represents the mean ± SE of triplicate determinations.
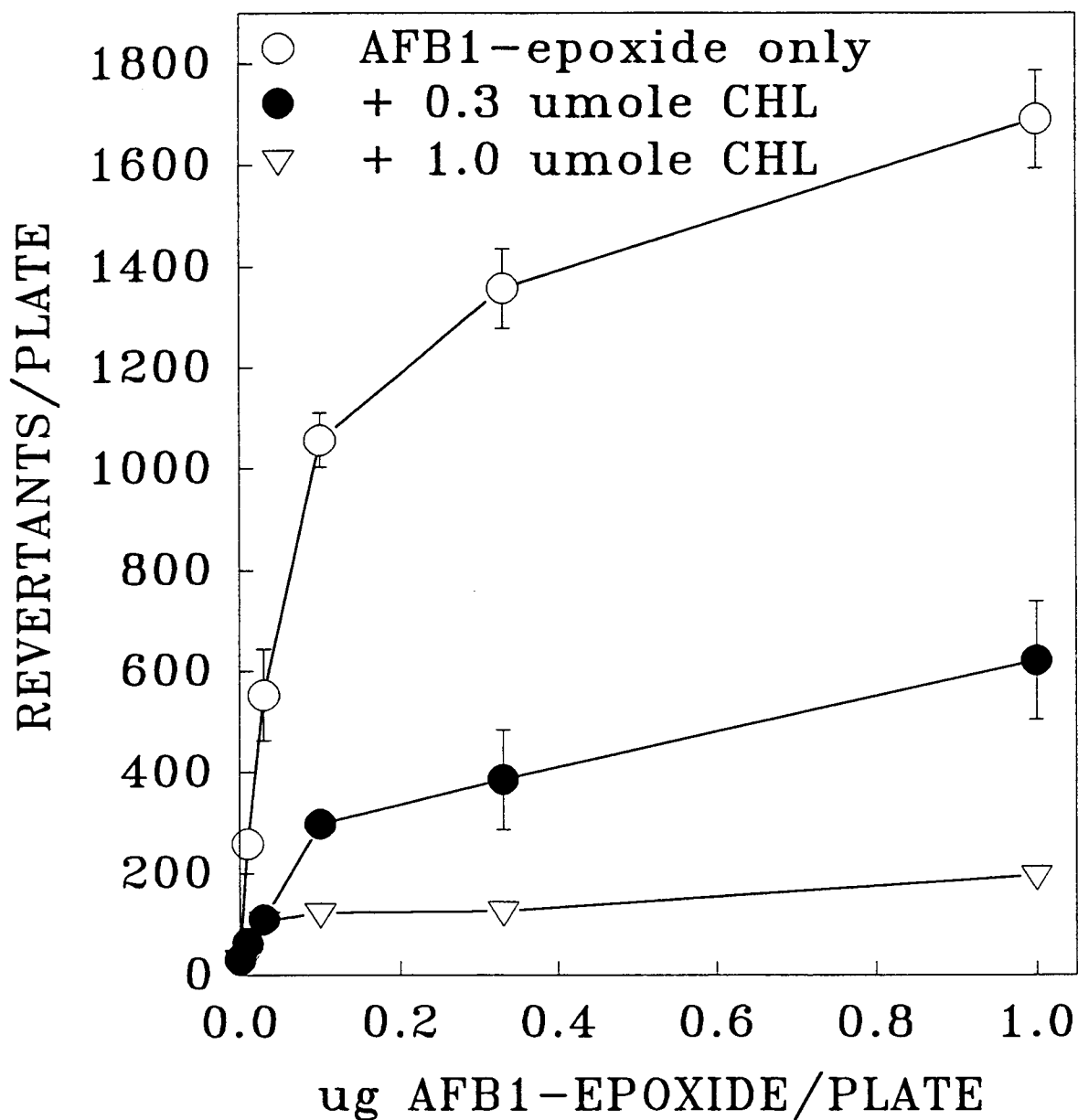
Figure 3.2   Inhibition of the direct mutagenic activity of the ultimate carcinogen AFB$_1$-epoxide by CHL. Each point represents the mean $\pm$ SE of triplicate determinations.
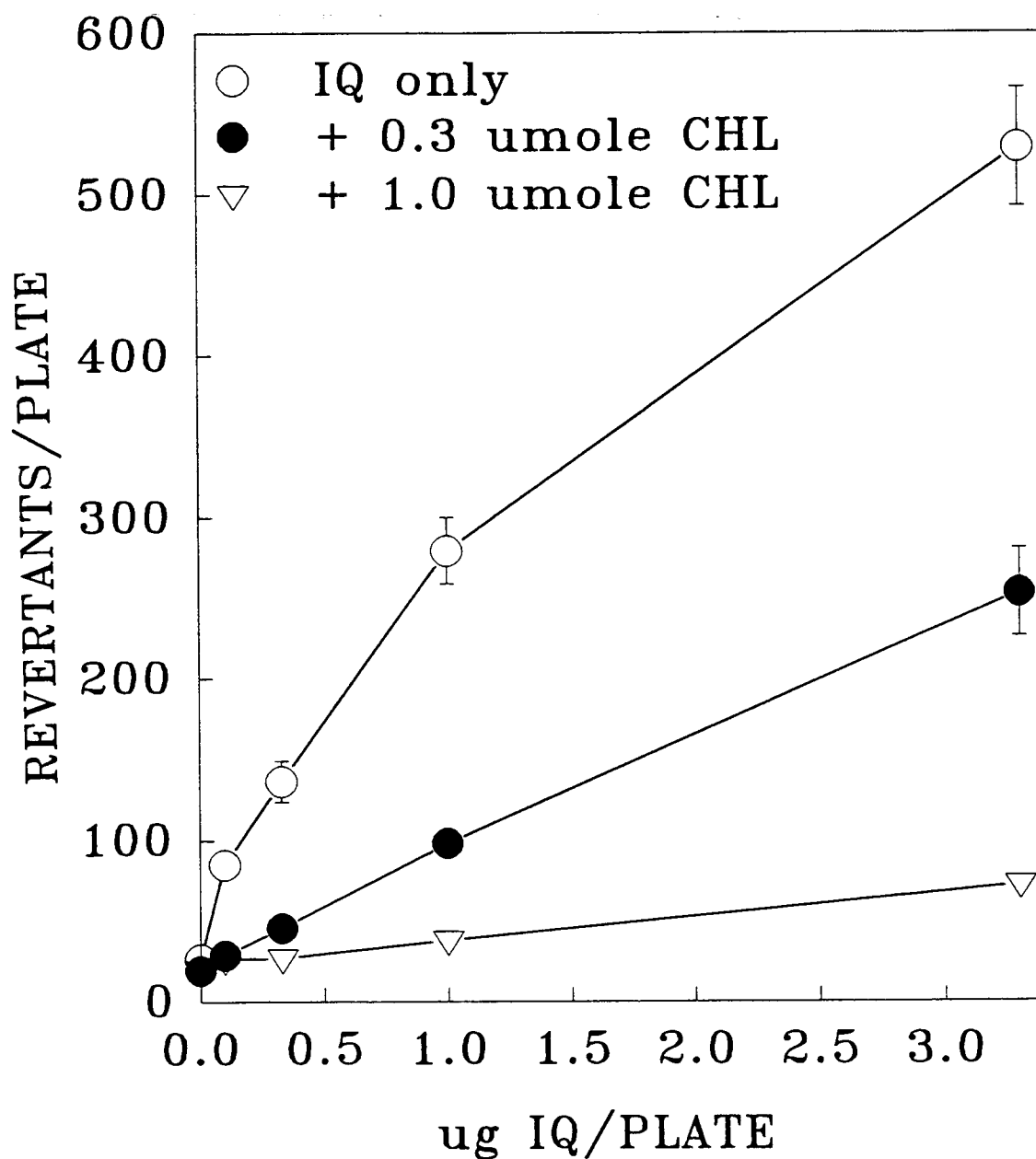
Figure 3.3 Inhibition of IQ mutagenesis by CHL. The mutagenic events were mediated by hepatic S-20, isolated from rainbow trout fed 500 ppm BNF for 5 days. Each point represents the mean ± SE of triplicate determinations.
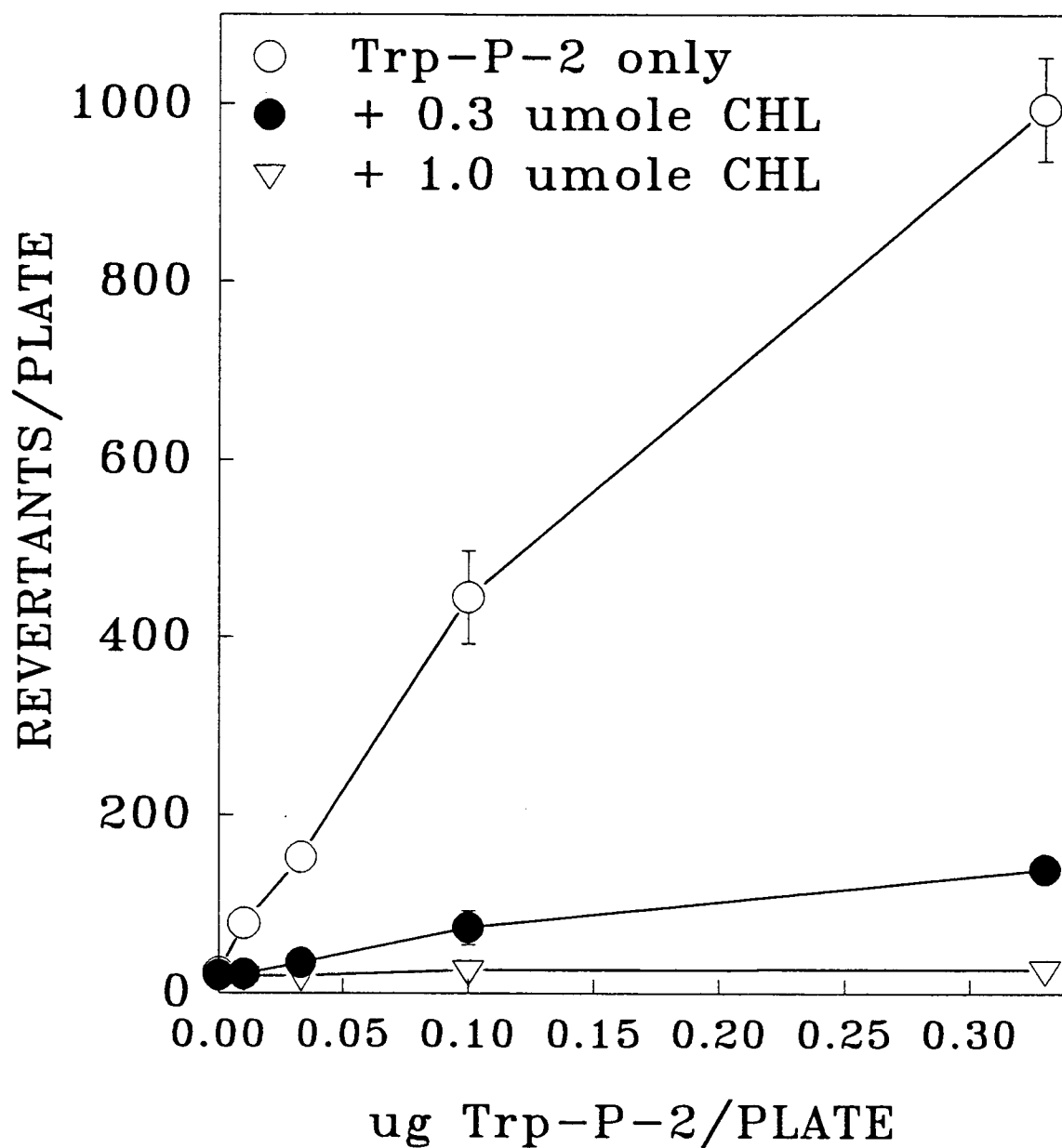
Figure 3.4   Inhibition of Trp-P-2 mutagenesis by CHL. The mutagenic events were mediated by hepatic S-20, isolated from rainbow trout fed 500 ppm BNF for 5 days. Each point represents the mean $\pm$ SE of triplicate determinations.
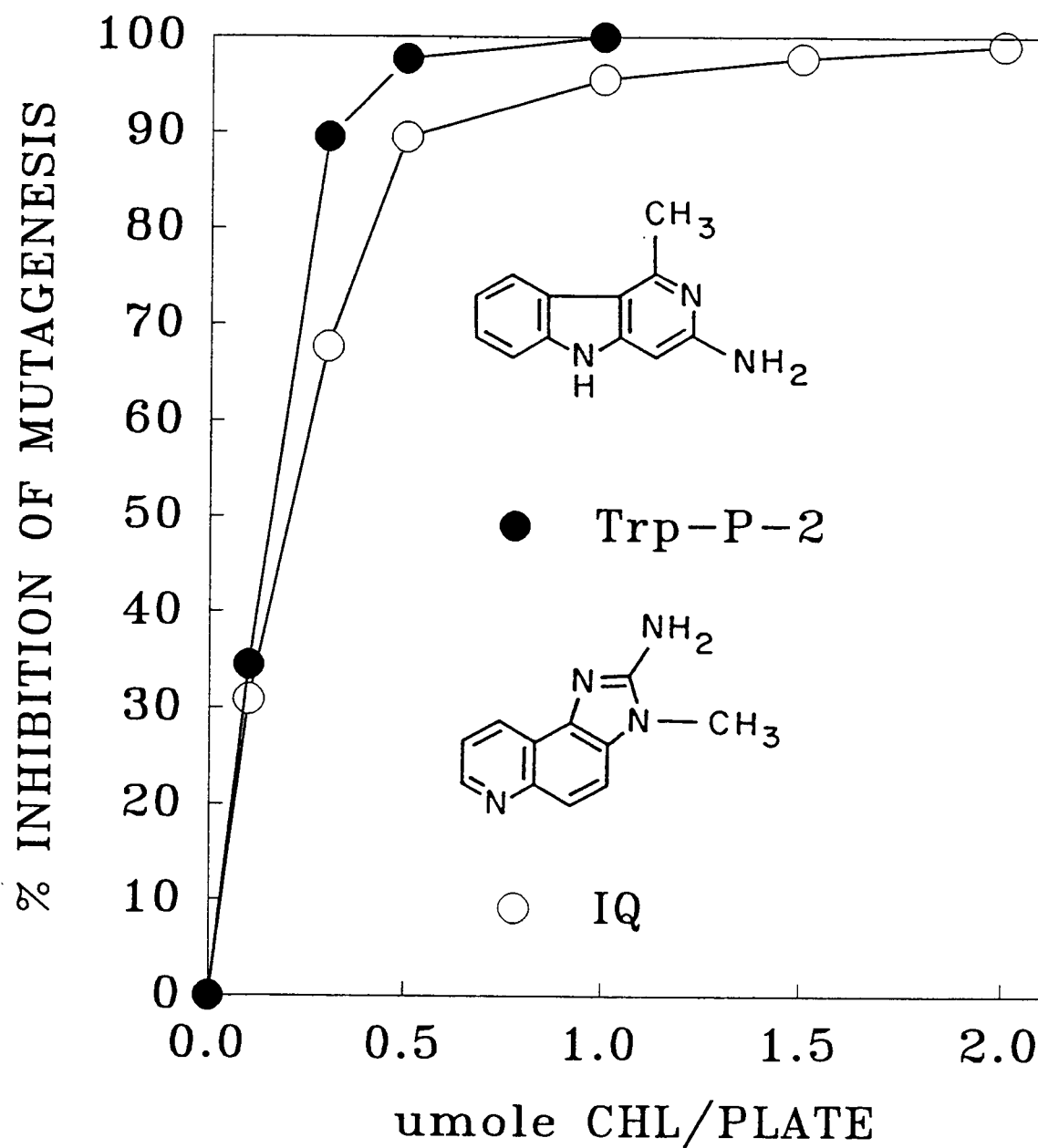
Figure 3.5   Percent inhibition of IQ and Trp-P-2 mutagenesis by CHL. Mutagen concentrations were kept constant at 0.3 μg Trp-P-2/plate or 3.3 μg IQ/plate, and the inhibitor applied at increasing concentrations ranging from 0.1 to 2.0 μmole/plate. Triplicate plates were employed for each mutagen dose.

REFERENCES

Arimoto, S., Negishi, T. and Hayatsu, H.: Inhibitory effect of hemin on the mutagenic activities of carcinogens. Cancer Letters 11:29-33, 1980.

Arimoto, S. and Hayatsu, H.: Role of hemin in the inhibition of mutagenic activity of 3-amino-1methyl-5Hpyrido[4,3-*b*]indole(Trp-P-2) and other aminoazaarenes Mutation Research 213:217-226, 1989.

Barale, R., Zucconi, D., Bertani, R., and Loprieno, N.: Vegetables inhibit, in vivo, the mutagenicity of nitrite combined with nitrosable compounds. Mutation Research 120:145-150, 1983.

Baxter, J.H.: Absorption of chlorophyll phytol in normal man and in patients with Refsum's disease. Journal. of Lipid Research 9:636-641, 1968.

Bronzetti, G., Galli, A., and Della Croce C.: Antimutagenic effects of chlorophyllin. In Kuroda, Y., Shankel, D.M. and Waters, M.D. (eds). Antimutagenesis and anticarcinogenesis mechanisms. Plenum Press, New York, 2:463-468, 1990.

Coulombe, R.A., Shelton, D.W., Sinnhuber, R.O. and Nixon, J.E.: Comparative mutagenicity of aflatoxins using a *Salmonella*/trout hepatic activation system. Carcinogenesis 3:1261-1264, 1982.

Dashwood, R. and Guo, D.: Inhibition of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ)-DNA binding by chlorophyllin: studies of enzyme inhibition and molecular complex formation. Carcinogenesis 13(7):1121-1126, 1992.

Imai, K., Aimoto, T., Sato, M., Watanabe, K., Kimura, R., and Murato, T.: Effects of sodium metallochlorophyllins on the activity and components of the microsomal drug-metabolizing enzyme system in rat liver. Chem. Pharm. Bull 34(10):4287-4293, 1986.

Kato, R.: Metabolic activation of mutagenic heterocyclic aromatic amines from protein pyrolysates. Critical Rew. Tox. 16(4):307-347, 1986.

Kimm, S, Tchai, B., Park, S., and Kang, S.: Antimutagenic activity of chlorophyll to direct and indirect-acting mutagens and its contents in the vegetables. Korean Journal of Biochemistry 14:1-7, 1982.

Kimm, S., and Park,S.: Evidences for the existence of antimutagenic factors in edible plants. Korean Journal of Biochemistry 14:47-59, 1982.

Lai, C.: Chlorophyll: The active factor in wheat sprout extract inhibits the metabolic activation of carcinogens *in vitro*. Nutrition and Cancer 1:19-21, 1980.

Lai, C., Butler, M.A., and Matney T.S.: Antimutagenic activities of common vegetables and their chlorophyll content. Mutation Research 77:245-250, 1979.

Maron, D.M. and Ames B.N. Revised method for the *Salmonella* mutagenicity test. Mutation Research 113:173-215, 1983.

Mcmanus, M.E., Burgess, W.M., Veronese, M.E., Felton, J.S., Knize, M.G., Snyderwine, E.G., Quattrochi, L.C. and Tukey, R.H.: Metabolism of food derived mutagens and 2-acetylaminofluorene by purified rabbit and cDNA expressed human cytochromes P450. Mutation and the Environment Part E:139-147, 1990.

Nahata, M.C., Slencsak, C.A. and Kamp, J.: Effect of chlorophyllin on urinary odor in incontinent geriatric patients. Drug. Intell. Clin. Pharm. 17;732-734, 1983.

Negishi, T., Arimoto, S., Nishizaki, C. and Hayatsu, H.: Inhibitory effect of chlorophyll on the genotoxicity of 3-amino-1 -methyl-5H-pyrido[4,3-*b*]indole (Trp-P-2). Carcinogenesis 10(1):145-149, 1989.

Newmark, H.L.: A hypothesis for dietary components as blocking agents of chemical carcinogenesis: Plant phenolics and pyrrole pigments. Nutrition and Cancer 6 (1):58-70, 1984.

Newmark, H.L.: Plant phenolics as inhibitors of mutational and precarcinogenic events. Canadian Journal of Physiology and Pharmacy 65:461-466, 1987.

Ong, T., Whong, W., Stewart, J and Brockman, H.E.: Chlorophyllin: a potent antimutagen against environmental and dietary complex mixtures. Mutation Research 173:111-115, 1986.

Ong, T., Whong, J.D. and Brockman, H.E.: Comparative antimutagenicity of 5 compounds against 5 mutagenic complex mixtures in *Salmonella typhimurium* strain TA98. Mutation Research 222:19-25, 1989.

Peleran, J.C., Rao, D. and Bories, G.F.: Identification of the cooked food mutagen 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and its N-acetylated and 3-N-demethylated metabolites in rat urine. Toxicology 45:193-199, 1987.

Robins, E.: Inhibition of aflatoxin mutagenicity by chlorophyllin in *Neurospora crassa*. In Shankel, D.M., Hartman, P.E., Kada, T. and Hollaender, A. (eds.). Antimutagenesis and Anticarcinogenesis mechanisms. Plenum Press, New York, pp. 575-576, 1986.

Romert, L., Curvall, M., and Jenssen, D.: Chlorophyllin is both a positive and negative modifier of mutagenicity. Mutagenesis 7(5):349-355, 1992.

Sugimura, T.: Carcinogenicity and mutagenic heterocyclic amines formed during the cooking process. Mutation Research 150:33-41, 1985.

Sugimura, T.: Studies on environmental and chemical carcinogenesis in Japan. Science 233:312-318, 1986.

Turesky, R.J., Aeschbacher, H.U., Wurzner, H.P., Skipper, P.L. and Tannenbaum, S.R.: Major routes of metabolism of the food borne carcinogen 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline in the rat. Carcinogenesis 9:1043-1048, 1988.

Whong, W., Stewart, J., Brockman, H.E. and Ong T.: Comparative mutagenicity of chlorophyllin and five other agents against aflatoxin B_1-induced reversion in *Salmonella typhimurium* TA98. Teratogenesis, Carcinogenesis and Mutagenesis 8:215-224, 1988.

Young, R.W. and Beregi, J.S.: Use of chlorophyllin in the care of geriatric patients. Journal of the American Geriatric Society 28:46-47, 1980.

CHAPTER 4

INHIBITION OF *IN VIVO* AFLATOXIN B$_1$-DNA BINDING BY
CHLOROPHYLLIN; EFFECT OF VARIOUS EXPOSURE PROTOCOLS ON
INHIBITORY EFFECTIVENESS

Vibeke Breinholt

and

George Bailey

Toxicology Program

Oregon State University

Corvallis, OR 97331

ABSTRACT

The ability of chlorophyllin (CHL), the structural homologue of the plant pigment chlorophyll, to inhibit *in vivo* aflatoxin B_1 (AFB_1)-DNA adduction in rainbow trout was investigated using various exposure protocols of both the carcinogen and the inhibitor. Co-administration of AFB_1 and CHL in the diet resulted in substantial decreases in AFB_1-DNA adduction of 34.1, 46.3 and 50.6% at dietary CHL levels of 1000, 4000 and 8000 ppm. A less marked inhibition of the covalent binding of AFB_1 to DNA was evident when AFB_1 was administered intraperitoneally 24 hours post-feeding. Extending the fasting period to 48 hours before the carcinogen challenge resulted in total loss of CHL- imposed protective activities at 1000 and 4000 ppm and only marginal protection against carcinogen adduction at 8000 ppm CHL.

Analysis of the metabolites in the hepatic 600 g postnuclear fraction from the dietary exposed fish revealed a significant decrease ($p < 0.05$) in the relative concentration of AFB_1, AFL and the minor metabolites aflatoxin M_1 (AFM_1), aflatoxicol M_1 ($AFLM_1$) and aflatoxicol M_1 glucuronide ($AFLM_1$-g) in the CHL-AFB_1 co-treated group compared to the control trout receiving AFB_1 only. The only exception was the phase 2 metabolite aflatoxicol-glucuronide (AFL-g) which was not present at a concentration significantly different from the control. The relative percentages of AFB_1 and its individual metabolites in the postnuclear fraction, on the other hand, were strikingly similar for the control and the CHL-treatment, again with the exception of AFL-g which accounted for 10.4% of the total radioactivity recovered in the 600 g supernatant isolated from the CHL-AFB_1 coexposed trout

compared to 5.4% in the AFB_1 control trout (p = 0.001). Intraperitoneal administration of CHL followed by dietary exposure to AFB_1 afforded no protection against the precarcinogenic DNA adduction, despite high concentrations of the inhibitor in the target organ.

The ability of CHL to induce oxidation and conjugation enzymes was investigated as a possible mechanism of protection against carcinogen-DNA adduction. Under the given dietary protocols and duration of the CHL treatment no detectable *in vivo* effect of dietary CHL on the enzymatic activities of the phase 2 enzymes glutathione-transferase (GST) and UDP-glucuronyltransferase (UDPGT) was evident. Analysis of the specific CYP isozymes 2K1 and 1A1, which are involved in biotransformation and detoxification of AFB_1, revealed no changes in the specific protein level of either isozyme after 2 weeks of dietary exposure to 8,000 ppm CHL, whereas very high dietary levels of 24,000 ppm CHL for 2 weeks severely depressed the hepatic protein concentration of the two investigated cytochromes. The lack of an effect on the hepatic CYP isozyme content after 2 weeks dietary exposure to 8000 ppm CHL, which was the highest inhibitor dose employed in the feeding trials, suggests that alteration in metabolizing enzymes do not play a significant role the protective activities of CHL observed in the present studies.

INTRODUCTION

The ability of chlorophyllin (CHL), a derivative of the green plant pigment chlorophyll, to exert antimutagenic activities *in vitro* against a wide range of suspected human carcinogens has been amply demonstrated (Whong et al., 1988; Ong et al., 1989; Ong et al., 1986; Arimoto et al., 1980) whereas the potential protective capacity of CHL on *in vivo* mediated pre-carcinogenic damage has received very little attention. Recent studies (Chapter 3) showed that CHL potently inhibits mutagenesis induced by aflatoxin B_1 (AFB_1), the only accepted human dietary carcinogen (IARC monographs, 1993), as well as its ultimate carcinogenic metabolite aflatoxin B_1 epoxide. The aim of the present study was to investigate the ability of CHL to inhibit AFB_1-DNA adduction, a genomic lesion suspected of initiating hepatic carcinogenesis in humans and experimental animals (Hall and Wild, 1994), including rainbow trout, the experimental model employed in the current experiments. The antimutagenic potency of CHL toward planar molecules with at least partially aromatic structure has been attributed to the property of CHL to form a tight, but non-covalent, complex with the mutagen (Chapter 3; Arimoto et al., 1980). The close interaction between CHL and the mutagenic species has been suggested to impair either the transport of the mutagen to the genomic material or its enzymatic biotransformation. As the proposed complex potentially can form in the diet, the current study was designed to examine the extent to which this complex contributed to overall protection against DNA adduction, compared to other protective mechanisms such as induction of detoxification pathways or inhibition of biotransformation enzymes.

MATERIALS

Unlabeled AFB_1 was purchased from Sigma Chemical Company (St. Louis, MO) and the tritium-labeled form from CalBiochem Inc. (San Diego, CA). The purity was checked by UV-spectrometry and thin-layer chromatography. Chlorophyllin, Proteinase K, RNAse A and T1, glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), α-naphthyl-β-D-glucuronide, Tween-20 and β-naphthoflavone were obtained from Sigma Chemical Company. Ultrapure phenol saturated with Tris was purchased from Amresco (Solon, OH). Octadecyl extraction disks (3M Empore, 47 mm) and all organic chemicals employed were supplied by J.T. Baker (Phillipsburg, NJ). Solvents used for high-performance liquid chromatography (HPLC) of AFB1 metabolites were submitted to filtration on 0.22 μm poresize filterpaper (Millipore, Milford, MA) prior to use. HPLC standards were isolated and purified in our own laboratory. Standards used in the current study were AFB_1, aflatoxin M_1 (AFM_1), aflatoxicol (AFL), aflatoxicol M_1 (AFL-M_1), aflatoxicol-glucuronide (AFL-g), aflatoxin M_1-glucuronide (AFM_1-g) and aflatoxin B_1-glutathione conjugate (AFB1-GS). Secondary antibodies labeled with horseradish peroxidase, Western blotting detection reagents and nitrocellulose paper were purchased from Amersham (Arlington Heights, IL). CYP1A1 and CYP2K1 standards as well as the primary rabbit IgG antibody were kindly provided by Dr. Donald Buhler.

EXPERIMENTAL PROCEDURES

Co-administration of CHL and AFB$_1$ in the diet: Four tanks of 60 rainbow trout of an initial body weight of 5-6 g were administered 0, 1000, 4000 or 8000 ppm CHL at a daily ration equal to 2% of their body weight (dry weight) for 2 weeks. At the end of this period each group of fish was divided in two and placed in separate tanks. Half of the fish in each pretreatment group were administered 80 ppb tritium-labeled AFB$_1$ (15.6 Ci/mmole) via the diet in combination with the same CHL levels as employed in the pre-treatment. The fish were fed for 2 days, and specific tissues sampled at the end of the second day. Livers, blood and bile from five fish were pooled in separate micro-centrifuge tubes, frozen in liquid nitrogen, and stored at -80°C until analysis.

Dietary CHL, intraperitoneal AFB$_1$ administration post-feeding: The remaining fish in each treatment were not fed for 48 hours to allow digestion of the last meal and then administered intraperitoneally 11.1 ng ^{3}HAFB1 (15.6 Ci/mmole) per 10 g body weight contained in 10 μl 95% ethanol. Liver, blood and bile were sampled 24 hours post-injection, also in three pools of five, and stored at -80°C until analysis.

In a separate study, 150 fingerling rainbow trout of ca. 5 g body weight were randomly divided into 5 groups of 30 fish. For a period of 14 days the fish received either 0, 500, 1000, 2000 or 4000 ppm CHL. Twenty four hours after the last meal the fish were administered intraperitoneally 6.9 ng ^{3}HAFB$_1$ (15.2 Ci/mmole) per 10 g body weight in 10 μl 95% ethanol. After an additional 24 hours the livers were

removed and pooled in 3 groups of five fish for each treatment. The livers were quick-frozen in liquid nitrogen and stored at-80°C.

CHL i.p. administration followed by dietary AFB_1: Eighty fingerling trout of 5 g. body weight were randomly assigned to 4 tanks of 20 fish. After three days fasting the fish were injected intraperitoneally with 0, 0.125, 0.250 or 0.500 mg CHL dissolved in 5 μl 0.1 M sodium-phosphate buffer pH 7.4. The fish were fasted for one additional day to allow absorption of CHL from the intraperitoneal cavity followed by three days dietary exposure to 20 ppb AFB_1. It is recognized that this protocol may result in some alteration in hepatic xenobiotic metabolizing, but the presence of a zero CHL control allows evaluation of CHL effects independent of any effect of food deprivation on phase 1 and phase 2 enzymes.

The day after the last dietary regimen of AFB_1, each treatment group was sampled in five groups of four fish. During sampling it was observed that the livers from the trout that received the lowest concentration of CHL attained the color of control fish, whereas the color of the livers from the remaining groups were visibly green in a dose-dependent manner. The concentration of CHL in the microsomal fraction was estimated on the basis of a standard curve constructed from the absorbance at 633 nm of control liver microsomes containing increasing concentrations of CHL (1-15 μM).

<u>Hepatic enzymes from CHL-treated fish</u>: Four tanks of 120 rainbow trout (ca. 25 g body weight) were fed control diet, 500 ppm β-naphthoflavone (BNF) and 8000 or 24,000 ppm CHL for up to two weeks and sampled after 7 and 14 days in three pools of twenty fish. Microsomes and cytosol were isolated from all treatment according to Eisele et al. (1983), and employed for assessment of enzymatic activities and to determine the specific concentration of the two CYP isozymes 1A1 and 2K1.

<u>DNA isolation:</u> DNA from the first two described experiments was isolated from the nuclear fraction obtained after centrifugation of liver homogenate at 600 g for 20 min. The nuclear fraction was resuspended in 3 ml 10 mM Tris buffer, 0.1 M NaCl, 100 μM EDTA (pH 8.0) and subjected to enzymatic treatment and phenol extraction as described below. Hepatic DNA from the second experiment was isolated from whole liver homogenate. The liver samples were homogenized into a fine powder in a mortar facilitated by the addition of small amounts of liquid nitrogen. Half of the sample was transferred to a 15 ml polypropylene test tube and suspended in 3 ml 10 mM Tris buffer, containing 0.1 M NaCl and 100 μM EDTA (pH 8.0). The remaining sample was subjected to microsome isolation for assessment of the CHL concentration in the subcellular fraction. An additional 3 ml of 1% SDS-Tris buffer was added by drops to both the nuclei and the whole liver homogenate samples under continuous vortexing. The samples were then incubated for 3 hours with 60 U RNase A and 1200 U RNase T1 followed by 12 hours incubation with 9 U Proteinase K at 37°C. Enzyme incubations were conducted under continuous mixing in a vertical tube rotator.

DNA was extracted with an equal volume of Tris-saturated phenol (pH 8.0)

by gently inverting the samples 10 times and allowing the two phases to separate (approximately 15 min). The samples were then centrifuged at 2,500 rpm for 10 min. The upper aqueous layer was transferred to another 15 ml tube containing one volume of chloroform. The samples were again mixed by inversion and left for 5 min prior to centrifugation at 2,500 rpm for 5 min. After removal of the organic phase three additional extractions with equal volumes of chloroform: isoamylalcohol (24:1) were performed. After the last extraction step approximately 5.0 ml of the upper aqueous phase was transferred to a 50 ml polypropylene test tube containing 2.5 volumes of isopropanol. The tubes were inverted several times until the DNA was completely precipitated. After 5 min centrifugation at 2,000 rpm, the isopropanol was poured off and the DNA pellets washed twice with absolute ethanol and allowed to dry at room temperature for 15 min. Any remaining ethanol was evaporated off under a stream of nitrogen. The pellet was dissolved in 1 ml 10 mM Tris buffer overnight at -20°C.

The purity was determined by measuring the 260/280 nm absorbance ratio. For all samples the ratio was between 1.8 and 2.0, indicating a pure DNA preparation. The DNA content was measured by the method of Burton (1956) and the specific activity of the DNA determined after duplicate aliquots of hydrolyzed DNA were removed and analyzed by liquid scintillation counting.

HPLC analysis of *in vivo* AFB$_1$ metabolites: The protein in the 600 g post-nuclear fraction was precipitated by addition of four volumes 100% methanol (MeOH). After 1 min high speed vortexing, the protein was centrifuged at 3,000 rpm for 20 min. The supernatant was then transferred to a sonicated pear-shaped flask, and

the MeOH evaporated under vacuum. When a total volume of approximately 10 ml was left in the flask, the solution was adjusted to 10% MeOH with millipore water and subjected to C_{18} purification. C_{18} extraction disks were conditioned with 10 mM potassium acetate (pH 5.0), adjusted to 60% MeOH, followed by 45 ml 100% MeOH, 45 ml 50% MeOH and 45 ml 10% MeOH all added in increments of 15 ml. The flow rate during disk conditioning, obtained by application of a vacuum to the elution apparatus, was approximately 5 ml/min. The sample in 10% MeOH was transferred to the extraction apparatus and passed through the disk at a flow rate not exceeding 2-3 ml/min. Prior to sample elution the disk was washed with 10 ml of 10 mM potassium acetate (pH 5.0) adjusted to 10% MeOH, to remove salts, protein and exchanged tritium. After processing of the sample, air was drawn through the disk for 10 min to remove water. The sample was eluted with 3 X 5 ml 10 mM potassium acetate, pH 5.0, adjusted to 60% MeOH (the flow rate was 1-2 ml/ min). The sample was evaporated down under a stream of nitrogen and the sample adjusted to 15% AMT (acetonitrile: Methanol: tetrahydrofuran (15:20:6)) and 85% 0.02 M potassium acetate, pH 5.0. A total of 150 μl in duplicate were injected per sample.

Analysis of AFB_1 and its metabolites was performed with a Beckman HPLC consisting of a 110B Solvent Delivery Module connected to a 421A Controller unit and a programmable absorbance detector, Spectroflow 783 (Kratos Analytical). Individual metabolites were separated on a 25 cm X 4.7 mm ID Spherex C_{18} column (Phenomenex). A 50 μl precolumn (Upchurch Scientific, Oak Harbor, WA) packed with C_{18} Corasil supported by 0.5 μm frits was used to protect the analytical column and to remove interfering components. AFB_1 and its metabolites were eluted from the

analytical column with 0.02 M potassium acetate (pH 5.0) and AMT (20:15:6) run in

a linear gradient of AMT from 15-46% for 30 min. Between 60 and 100 fractions

were collected per injection (200 μl/fraction). Metabolites were identified on basis of

retention times of external and internal standards.

UDP-glucuronyltransferase: UDP-glucuronyltransferase activity was determined

by the method of Boch and White (1974) with the following modifications. The

reaction was conducted in a final volume of 10 ml using a protein concentration of 1.0

mg microsomal protein/ml. The reaction was initiated by the addition of 10 mg liver

microsomal suspension. Immediately following vortexing of the reaction mixture, 2

aliquots of 0.5 ml (blank values) were removed and transferred to a microcentrifuge

tube containing 1 ml 0.6 M glycine - 0.4 M trichloroacetic acid to terminate the

reaction. Additional aliquots (in duplicates) were removed and the enzymic reaction

stopped after 5, 10, 15, 20, 25, 30, 40 and 60 min incubation. The fluorescence of

α-naphthol-glucuronide (α-NG) was determined in a Photon-Counting

Spectrofluorometer (SLM 8000) using an excitation wavelength of 290 nm and an

emission at 330 nm. The concentration of α-NG formed during incubation was

quantified using standard concentrations of α-NG in the range of 10-100 nmol/ml

subjected to the same extraction procedure as employed for the samples.

Glutathione transferase: Trout enzymatic assays were carried out at 25°C,

which is within the range of optimum incubation temperatures of 25-32°C (Pedersen

et al., 1974) for trout enzymatic activities. The supernatant fraction from the 100,000

g centrifugation (Eisele et al., 1983) was used for assessment of glutathione transferase activity (GST). GST activities was measured by the method of Habig et al. (1974) using 1-chloro-2,4-dinitro-benzene (CDNB) as a model substrate. Protein concentrations of 0.01 mg/ml was used to obtain a linear enzymic response of at least 15 min duration. The CDNB-glutathione conjugate was quantified using an extinction coefficient for the conjugate of 96,000 $M^{-1}cm^{-1}$. The rate of the reaction was estimated by linear regression of the absorbance data obtained on the basis of triplicate samples of 100,000 g supernatant, and assayed in duplicates.

Western blotting: The specific contents of CYP2K1 and CYP1A1 isozymes were assayed by the Western blotting-immunoquantitation method described by Burnette (1981) with the following modifications. Liver samples from trout fed 8,000 and 24,000 ppm CHL were homogenized in 10 mM potassium phosphate buffer, 0.15 M KCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Immediately prior to applying the samples to the gel, the liver homogenate was diluted in 0.5 M Tris (pH 6.6) to obtain a final concentration of 5.0 μg liver protein in a total volume of 2.5 μl. The membrane was blocked in dry milk dissolved in phosphate-buffered saline (pH 7.5) containing 0.1% Tween-20. The nitrocellulose membrane was incubated for 30 min in anti-trout 1A1-IgG or anti-trout 2K1-IgG. The chemiluminescent detection method was conducted according to the protocol provided by Amersham using dilutions of the chemiluminescent secondary antibody of 1:50,000 with an average incubation time of the membrane of 45 min.

Density of bands was determined by scanning laser densitometry using a model SLR504-XL Zeineh Scanning Densitometer (Biomed Instruments, Fullerton, CA). The concentration of specific isozymes was determined by extrapolation from the standard curve.

RESULTS

Effect of CHL on *in vivo* DNA adduction: Two days' coexposure of AFB_1-CHL to rainbow trout pretreated with CHL for 2 weeks resulted in decreases in AFB_1-DNA adduction of 34.1, 46,7 and 50.6% at dietary CHL doses of 1000, 4000 and 8000 ppm, respectively (Figure 4.1). The concentration of AFB_1 and metabolites in blood and bile from the different control and treatment groups was not significantly different as determined by t-test analysis (data not shown). Intraperitoneal administration of AFB_1 following 48 hours fasting only exerted slight inhibitory activity toward carcinogen-DNA binding at the highest CHL dose of 8000 ppm, whereas no inhibition was evident after dietary CHL pretreatments at 1000 and 4000 ppm (Figure 4.1). Also in this experiment no significant differences in total radioactivity in blood and bile among the control and the experimental groups was observed. In a separate study where groups of fish were prefed CHL at 500, 1000, 2000 and 4000 ppm for two weeks followed by i.p. injection of the carcinogen 24 hours post-feeding, protection against DNA binding of 1.5, 7.6, 22.6 and 33.1% at the above mentioned dietary CHL levels was evident (Figure 4.1).

One possible explanation of these results is that dietary CHL is transported to the liver at sufficient concentrations to inhibit metabolism of i.p. injected AFB_1. However, apparent high target organ concentrations of CHL obtained by i.p. administration did not afford protection against DNA adduction when AFB_1 was administered via the diet. When the fish were sampled 4 days after the CHL challenge, which included 3 days of dietary exposure to 20 ppb AFB_1, the livers still

retained a green coloration, not seen after dietary exposure to CHL. The concentration of CHL in liver microsomes from quadruplicate pools of fish receiving 0.250 and 0.500 mg CHL amounted to 2.5 $\pm$ 0.9 and 10.0 $\pm$ 2.3 μM, respectively. No CHL could be detected in the livers of the fish, that received 0.125 mg CHL.

Effect on metabolite concentration and profile: The total concentration of AFB_1 and metabolites (22.7 $\pm$ 3.4 pmole AFB_1 metabolites/gram liver) in the postnuclear fraction isolated from control trout fed 80 ppb AFB_1 was found to be significantly higher (p = 0.05) than the recovered radioactivity from the 80 ppb AFB_1-4000 ppm CHL cofed group (14.4 $\pm$ 4.6 pmol AFB1/gram liver). Determination of the distribution of the radioactive label after Methanol precipitation of the hepatic proteins revealed that significantly more (p = 0.003) radioactivity was bound to the protein pellet in the CHL cofed group (6.9 $\pm$ 0.8%) than in the control group (4.0 $\pm$ 0.4%). The numbers are percentage of total radioactivity in the postnuclear fraction.

Analysis of metabolites in the hepatic postnuclear fraction indicated that the relative concentration (pmole AFB_1 metabolites/gram liver) of all detected metabolites was significantly decreased (p < 0.05), when CHL and AFB_1 were incorporated together in the diet as compared to dietary AFB_1 control (Figure 4.2), with the exception of AFL-g which was similar to the control. The decreases among the individual metabolites were not significantly different as determined by multiple t-test analysis and a mean percentage of the observed decrease was estimated to be 46.0 $\pm$ 11.4%. The only exception was AFL-g which was present at equal concentrations in the control and CHL-treated group. No significant changes in metabolite profile,

determined as percentage of total metabolites recovered, was evident (Table 4.1), again with the exception of AFL-g. The percentage of AFL-g in the postnuclear fraction from CHL-fed trout (10.4 ± 1.4) was approximately two-fold higher than the control group (5.4 ± 1.2)(p=0.001).

UDP-glucuronyltransferase: The enzymic reaction was found to be linear up to an incubation period of at least 30 min (data not shown). The reaction rate was therefore determined on basis of linear regression analysis of the data from time zero to 30 min. The mean activity of UDPGT increased slightly from day 7 to day 14 for both the control and the CHL-treated group. A significant (p = 0.05) increase in liver UDPGT activity was found with the BNF treatment whereas CHL did not affect the activity of this conjugating enzyme (Figure 4.3).

Glutathione transferase: The formation of the CDNB-glutathione conjugate proceeded in a linear fashion for at least 15 min under the given substrate and enzyme concentrations. The levels of GST activity in the current study, estimated from the slopes of the reaction curves, were found to be similar for the control and the experimental groups (Figure 4.4), indicating that neither BNF nor CHL under the given dietary protocols are capable of inducing this detoxification enzyme.

Detection of CYP isozymes: Analysis of the specific CYP isozymes 2K1 and 1A1 by Western blotting and immunoquantification revealed that the levels of both isozymes were markedly decreased in liver homogenate from fish fed 24,000 ppm

CHL for 2 weeks (Figure 4.5). The degree of suppression was about 60% for both isozymes, as determined by scanning densitometry (Figure 4.6). The standard curves were linear within the range of the isozyme concentration of the analyzed samples. The 1.2 pmole 1A1 standard was off-range and therefore not included in the construction of the standard curve (Figure 4.6). CHL at 8000 ppm did not affect the protein concentration of either of the two isozymes (data not shown).

DISCUSSION

In the current study CHL was administered via the diet and AFB_1 intraperitoneally in addition to dietary co-administration of the carcinogen and the inhibitor. This method allowed us to investigate whether the inhibitory potency of CHL might be exclusively due to chemical complexation between AFB_1 and CHL within the diet, thereby hindering the absorption of the carcinogen from the digestive tract (see Chapter 5), or if other mechanisms could contribute to the observed effect on DNA adduction. Bypassing the complex formation in the gastrointestinal tract would thus elucidate whether CHL had additional modifying activities toward biotransformation and detoxification enzymes, events that would result in reductions in DNA adduction levels independent of the route of CHL administration.

The present studies show that co-feeding is not necessary to see inhibition of AFB_1- DNA adduction, but that co-feeding by far provides the best protection against carcinogen-DNA adduction. Inhibition of AFB_1 binding to liver DNA was found to be significantly higher ($p < 0.001$) when AFB_1 was administered in the diet along with 1000, 4000 or 8000 ppm CHL as compared to i.p. administration of AFB_1 48-hours post-feeding, where protection was only evident at the highest CHL dose. A similar experiment, where AFB_1 was i.p. injected 24 hours post-feeding gave a strong dose-responsive inhibition of DNA-adduction, slightly lower than the inhibition obtained with dietary co-feeding. This result clearly shows that AFB_1-CHL co-feeding is not required for inhibition to occur. Two possible mechanisms may be invoked to explain these results. Dietary treatments might provide significant, though not visible,

levels of liver CHL, which could complex with AFB_1 *in situ* to retard its metabolism. Such an effect should increase with CHL dose and decrease with time after last CHL feeding, both of which was observed here. An alternative explanation for this result could be that part of the chemoprotective effect of CHL is exerted on metabolic enzyme activities, but that this effect is only apparent for a relatively short period of time post-feeding. Because the AFB_1-adduction level in the control animals from the i.p. injection experiments and the dietary AFB1 exposures are comparable these results do not reflect inherent differences in the two exposure routes for delivering of AFB_1 to the target organ.

Analysis of the content of the specific CYP isozymes involved in activation (2K1) and detoxification (1A1) of AFB_1 revealed a suppression of the protein levels of both isozymes after dietary CHL administration at 24,000 ppm for two weeks. A CHL-dose of 8000 ppm fed continuously for 2 weeks to rainbow trout, however, did not result in decreased levels of the investigated CYP isozymes. The lack of an effect on the hepatic CYP isozymes, at least up to a dietary level of 8000 ppm CHL, suggests that protection against AFB_1-DNA adduction by this mechanism in the present set of experiments, is of only minor importance. The observed suppressive activity of high doses of CHL (24,000 ppm) on the cytochrome CYP isozymes is in accordance with Imai et al.(1986), who reported similar findings after i.p. administration of CHL to rats. Imai et al. (1986) concluded that the decreased protein levels were brought about by primary actions of CHL on the control mechanisms of microsomal hemeprotein levels, including the cytochrome CYP isozymes. Imai et al. (1986) also observed that inhibition of various CYP-catalyzed enzymatic reactions by

CHL occurred in a non-competitive manner, which is in accordance with an overall reduction in the bioactivation capacity of the liver, rather than CHL-imposed changes in the affinity of the cytochrome for its substrate.

The additional finding that CHL treatment did not alter phase 2 metabolism, including UDPGT, the major AFB_1 detoxification enzyme in trout, suggests that the graded effect of CHL on AFB_1-DNA adduction might be a result of complexation between AFB_1, diffused from the intraperitoneal cavity, and CHL present in the various sections of the digestive tract, rather than a transient effect on metabolic pathways.

After conducting the present experiment we learned that the degree of fasting at the time of carcinogen administration could play an important role in CHL modulation of AFB_1-DNA adduction, because part of the i.p. administered AFB_1 has been found to diffuse from the intraperitoneal cavity to the gastrointestinal tract (Roderick Dashwood, pers. comm.) which will allow complexation with CHL present in the digestive system. After 24 hours fasting it was observed that the bulk of the diet was still present in the upper stomach, whereas at 48 hours most of the diet had passed to the pyloric stomach and the intestines. The observed inhibitory potency against i.p. administered AFB_1, and the decrease in potency with increased fasting, could simply reflect the disappearance of CHL from the digestive tract, which inevitably will result in a decreased probability of i.p. administered AFB_1 encountering CHL molecules in the gut and forming a less absorbable complex. The degree of inhibition would, however, be different from the dietary exposure, which provides optimal conditions for the carcinogen and the inhibitor to interact, because

only part of the i.p. administered AFB_1 is expected to diffuse to the digestive system and form a complex with CHL. The potential for absorbed CHL present in the circulation or in the target organ to form a complex with AFB_1, however, is still present, although the impact is presumed to be relatively minor due to an expected low rate of CHL absorption (Baxter, 1968).

Despite uptake of CHL by the liver after i.p. exposure, no difference in the binding of AFB_1 to hepatic DNA was observed among the control and the CHL treatment groups. This indicates that the physical presence of CHL in the liver cells is not sufficient to protect against AFB_1-DNA adduction. A possible explanations for this observation is that changes in the molecular structure of CHL during digestion are required for CHL to elicit a response, either by changing the activity of the molecule or by altering its pharmacokinetics, or both. Another possibility is that i.p. CHL doses were too low to obtain sufficiently high or homogeneous tissue concentrations for CHL to exert its effect. In rats, however, a fast response in hepatic enzyme activity to i.p administration of CHL has been observed (Imai et al., 1986) at a microsomal CHL concentration of 2.5 μM obtained after administration of 100 mg CHL/kg. In the current study, the concentration of CHL, determined in liver microsomes isolated 4 days post-injection of a 50 mg CHL/kg dose (Imai et al., 1986) was estimated to be 10 μM, with no evident effect on microsomal bioactivation. The concentration of CHL in the rat microsomal fraction, which was 4 times lower than the CHL concentration in the trout microsomal fraction, resulted in significant decreases in total CYP and cytochrome b_5 content and non-competitive inhibition of microsomal aniline hydroxylase, aminopyrine demethylase and NADPH-cytochrome c reductase activities.

The opposing chemical characteristics of the carcinogen and the inhibitor, with AFB_1 possessing a high lipophilicity compared to CHL's highly water soluble characteristics, would evidently favor the distribution of the two compounds in diverse compartments of the liver cells, and this distribution could explain the decreased ability of CHL to protect against AFB_1-DNA binding when the compounds were administered by different routes. The differences in the concentration of CHL in the microsomal fractions from rat and trout, 8-fold higher in the trout at equimolar doses administered intraperitoneally, either suggests that trout absorb CHL to a greater extent than rats, or that the affinity of CHL for the microsomal protein or the systemic persistence of CHL is elevated in trout compared to rats.

Evaluation of the carcinogen metabolite profile in the postnuclear fraction is consistent with the hypothesis that complexation might be the major protective mechanism of dietary CHL against the precarcinogenic lesions induced by dietary AFB_1. The concentration of AFL and AFB_1, which accounted for ca. 90% of the total aflatoxins in the post-nuclear fraction, were both suppressed by approximately 50% when CHL was included in the diet with AFB_1 (Figure 4.2). The phase 2 metabolite AFL-g, however, was not suppressed by dietary CHL and the concentration of the glucuronide was not significantly different from the control treatment. The phase 1 metabolite profile was not altered by dietary CHL (Figure 4.2 inset and Table 4.1), indicating that CHL does not exert inhibiting or inducing activities toward specific CYPs as is evident for BNF and indole-3-carbinol, which specifically induce CYP1A1 in the rainbow trout model (Takahashi, 1994). The percentage of the various metabolites recovered in the post-nuclear fraction from the control and the CHL-

treated fish was not significantly different, with the exception of AFL-g (Table 4.1). The percentage of AFL-g of the total metabolites was almost twice as high in the CHL-treated group as in the control. Assessment of the UDPGT activity in CHL-fed trout from a separate study, however, showed no inducing ability of CHL on UDPGT at dietary levels of CHL up to 24,000 ppm fed continuously for 2 weeks. A possible explanation for this discrepancy between the elevated levels of AFL-g in the hepatic postnuclear fraction from CHL-treated fish and the lack of detection of increased activities of this conjugating enzyme *in vitro* could be that the enzyme assay is not sensitive enough to detect changes in the appropriate UDPGT isozyme or that CHL inhibits bile flow. Glutathione transferase activity which, in contrast to glucuronide conjugation, only plays a minor role in detoxification of AFB_1 in trout, was also found to be unaffected by dietary CHL administration of up to 24,000 ppm for 2 weeks (Figure 4.4). The AFB_1-glutathione conjugate was not detected among the metabolites in the postnuclear fraction in either control or CHL-treated animals, indicating the low impact of this enzyme in overall metabolism of AFB_1 in the rainbow trout.

The present study demonstrates the ability of CHL to inhibit binding of the potential carcinogen AFB_1 to hepatic DNA, thereby reducing the formation of a putative precarcinogenic lesion. The major protective action by dietary CHL in the current study was exerted primarily when the two compounds were brought in direct contact with each other by dietary co-exposure, which is most relevant to human exposure. In the current experimental settings, however, a possible effect on *in vivo* hepatic enzyme activity can not be excluded. If any CHL-induced *in vivo* inhibitory mechanisms are present in addition to carcinogen complexation, they appear to be

largely abolished between 24 and 48 hours following the last feeding. The potential for CHL to suppress the hepatic hemeprotein levels was evident only at very high CHL concentrations, exceeding those at which DNA adduction was investigated. The role for this mechanism in the observed protection against AFB_1-DNA binding is therefore thought to be negligible.

The major potential mechanism for protection against diet-induced human carcinogenesis by chlorophyllin and other dietary porphyrins is thus suspected to be via complex formation with AFB_1 in the digestive system, which presumably maintains the carcinogen in a less absorbable state and decreases the bioavailability of the carcinogen. As the complexation mechanism encounters several other potential human carcinogens in addition to AFB_1, this mechanism might provide some protective activity against dietary induced carcinogenesis in humans.
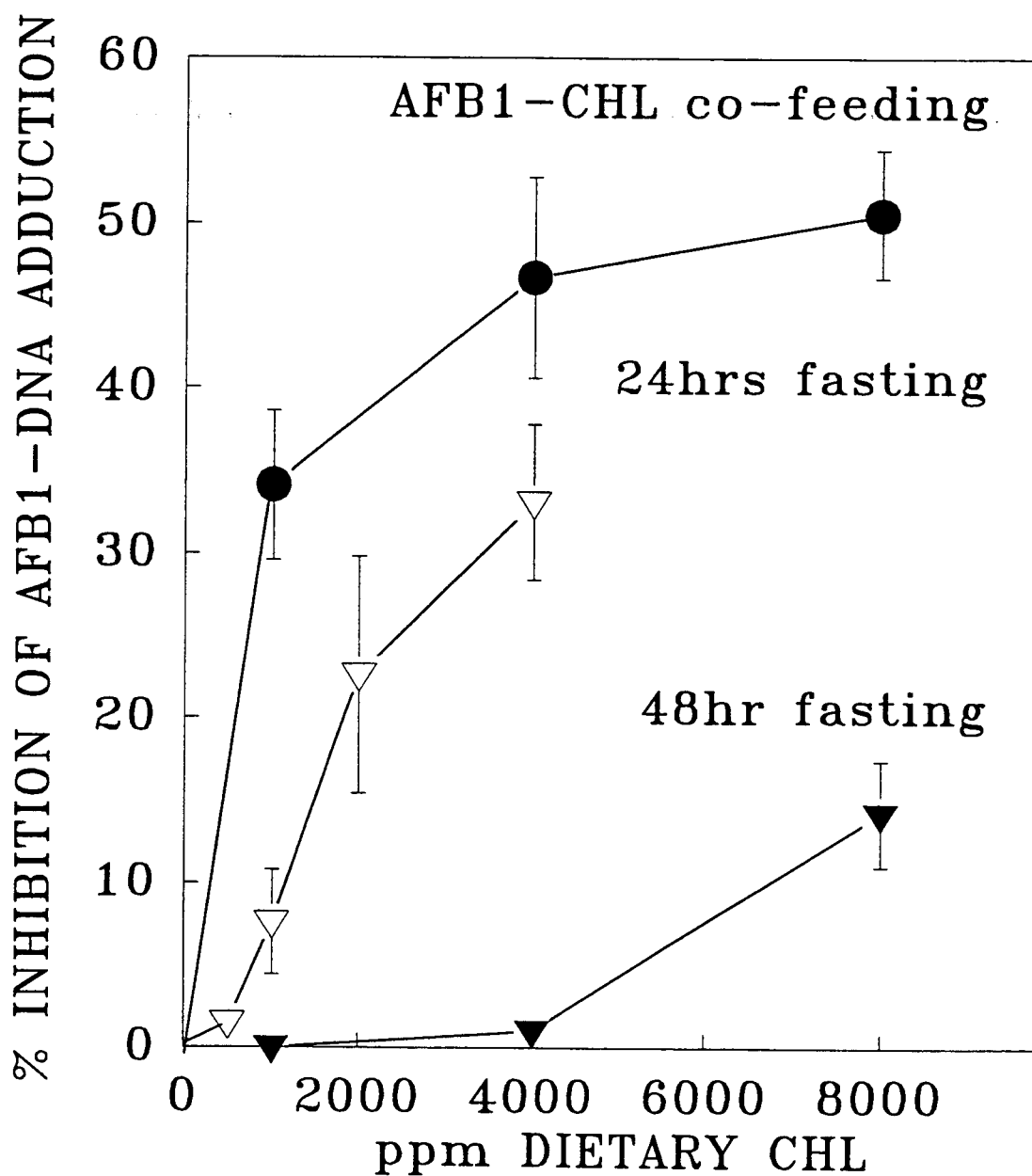
Figure 4.1 Percent inhibition of aflatoxin B_1 (AFB$_1$)-DNA adduction by chlorophyllin (CHL). AFB$_1$-CHL cofeeding (upper curve) illustrates the effect of simultaneous dietary administration of the two compounds to rainbow trout. The two lower curves represent the inhibitory potency of dietary CHL against intraperitoneal administered AFB$_1$, 24 and 48 hours post-feeding. The data points are means $\pm$ SE of triplicate pools of liver.
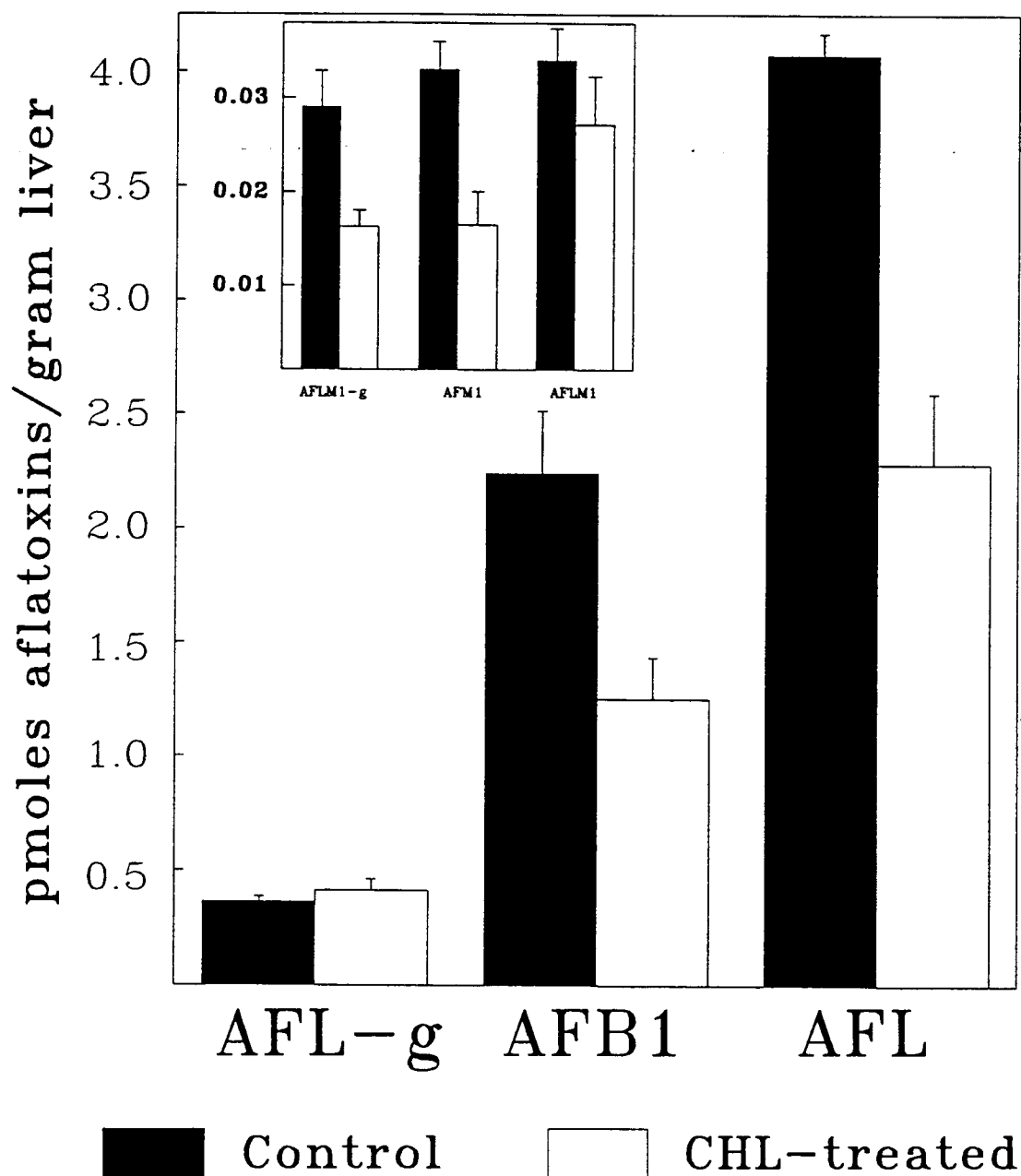
Figure 4.2 Concentration of AFB_1 and metabolites in the 600 g post-nuclear fraction from control and CHL-fed rainbow trout. The data represents the mean ± SD of triplicate tissue samples, injected in duplicates. See text for details on the HPLC conditions. Abbreviations are: AFL-g: Aflatoxicol glucuronide; AFB_1: Aflatoxin B_1; AFL: Aflatoxicol; $AFLM_1$-g: Alatoxicol M_1 glucuronide; AFM_1: Aflatoxin M_1; $AFLM_1$:Aflatoxicol M_1.
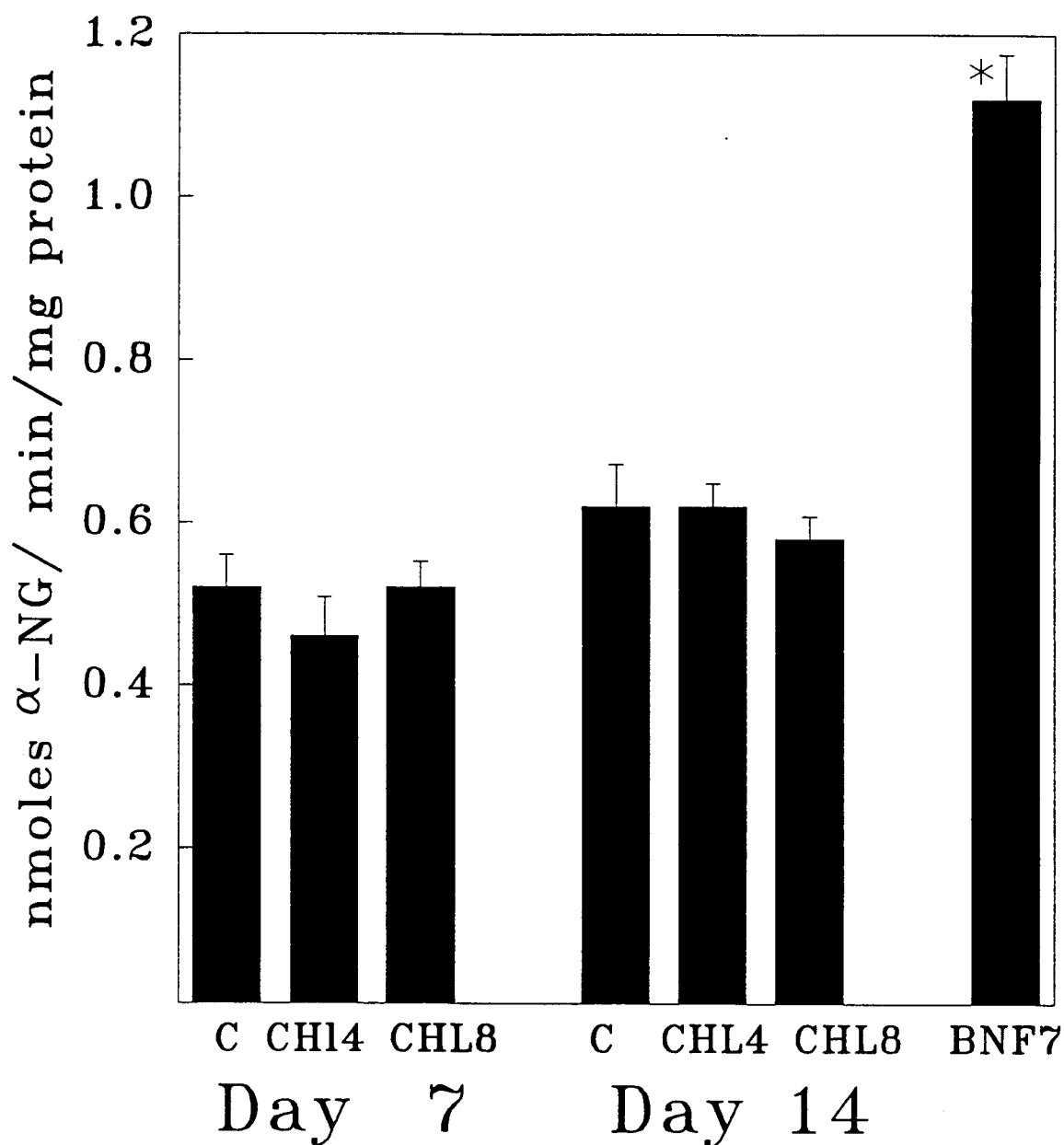
Figure 4.3. Assessment of UDP-glucuronic acid transferase (UDPGT) activity in the microsomal fraction from control and CHL-treated rainbow trout (2 weeks chronic exposure). α-naphthol was used as a model substrate for the enzymatic reaction. α-NG; α-naphthol-glucuronide. Data points are means $\pm$ SD of triplicate incubations. The dietary exposures were C: Control diet; CHL4: 4000 ppm CHL; CHL8: 8000 ppm CHL; BNF7: 7 days BNF treatment.
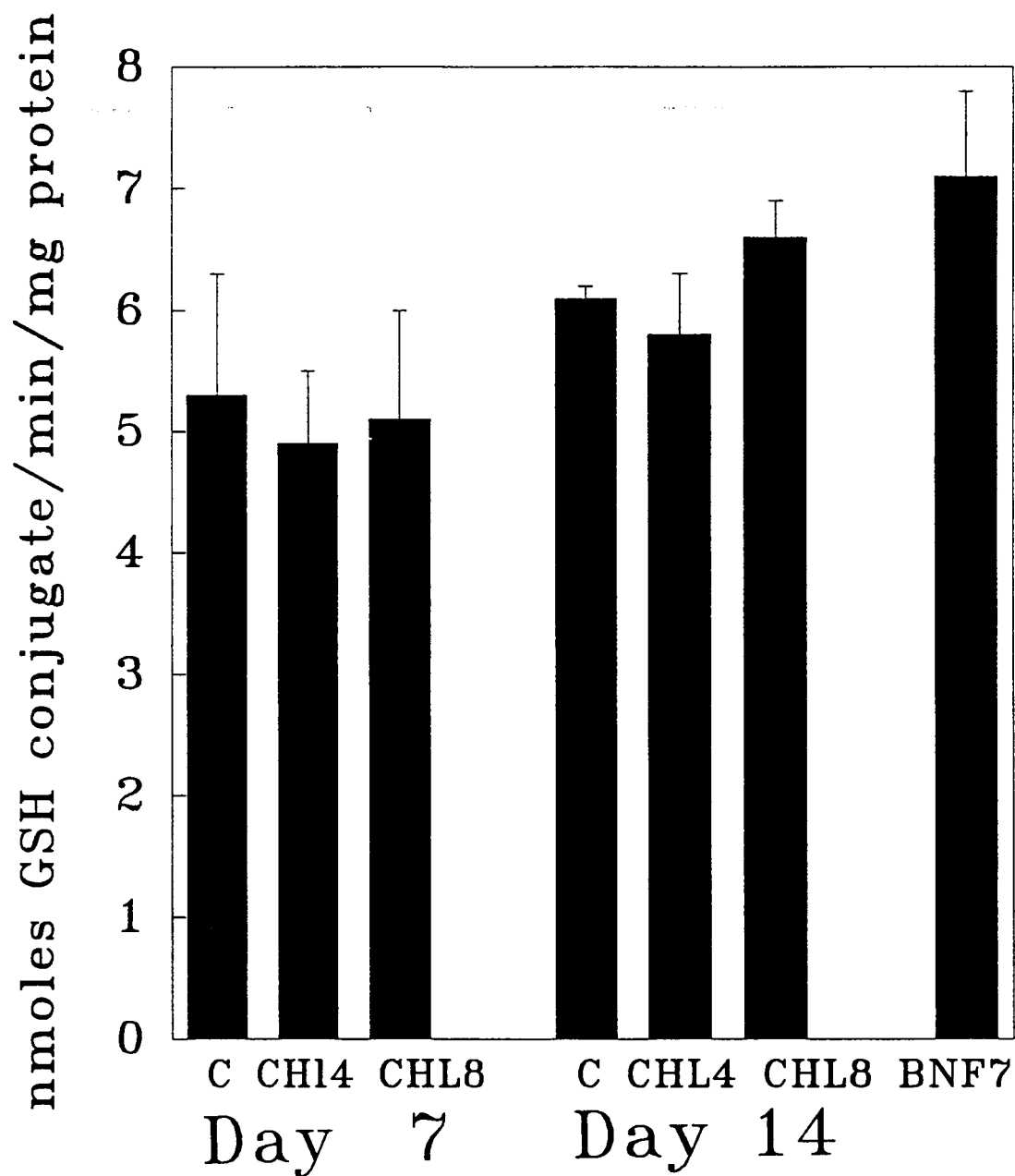
Figure 4.4 Assessment of glutathione transferase activity in cytosol isolated from control and CHL-treated rainbow trout (2 weeks chronic exposure). The substrate used for the reaction was 1-chloro-2,4-dinitrobenzene (CDNB) and the conjugate formed a CDNB-glutathione conjugate. Data points are means ± SD of triplicate incubations. The dietary exposures were C: Control diet; CHL4: 4000 ppm CHL; CHL8: 8000 ppm CHL; BNF7: 7 days BNF treatment.
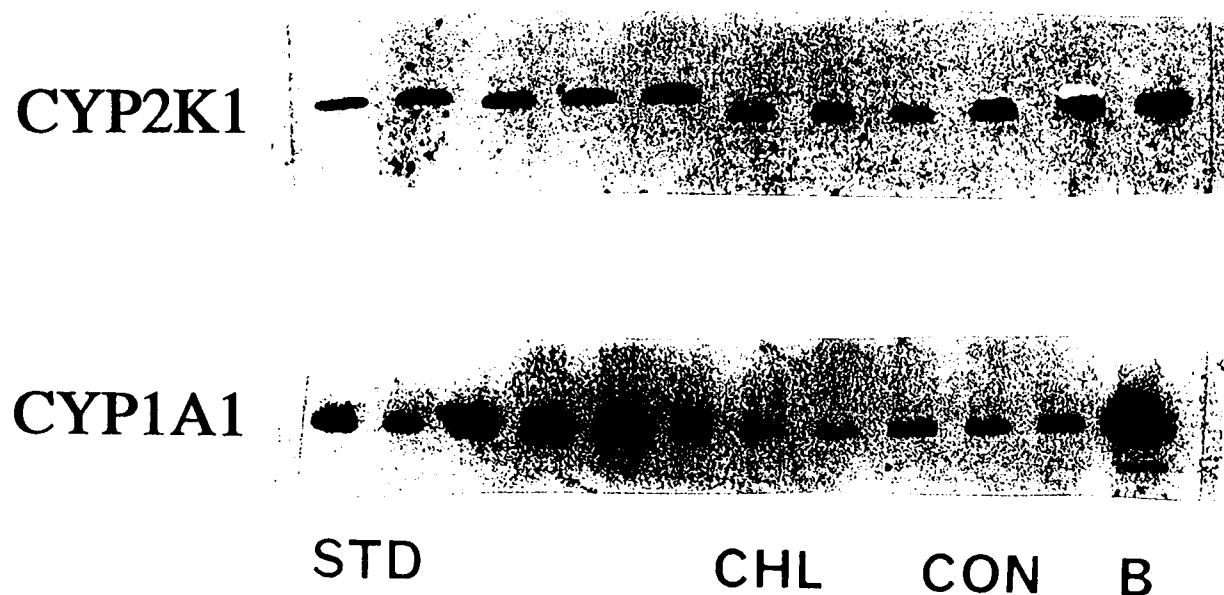
Figure 4.5   Immunoquantification of specific CYP isozymes in liver homogenate isolated from rainbow trout fed 24,000 ppm CHL for two weeks (lanes 6,7 and 8 from left) compared to control (CON) (lanes 9, 10 and 11 from left). The CYP2K1 standards (lanes 1-5, upper blot) were applied at concentrations equivalent to 0.093, 0.186, 0.297, 0.372 and 0.465 pmole protein. The concentrations of the CYP1A1 standards were 0.24, 0.48, 0.72. 0.96 and 1.2 pmole protein (lanes 1-5, lower blot). Five μg liver protein were used in the detection of both 2K1 and LM4b. Lane 13 (lower blot) is a positive BNF control.
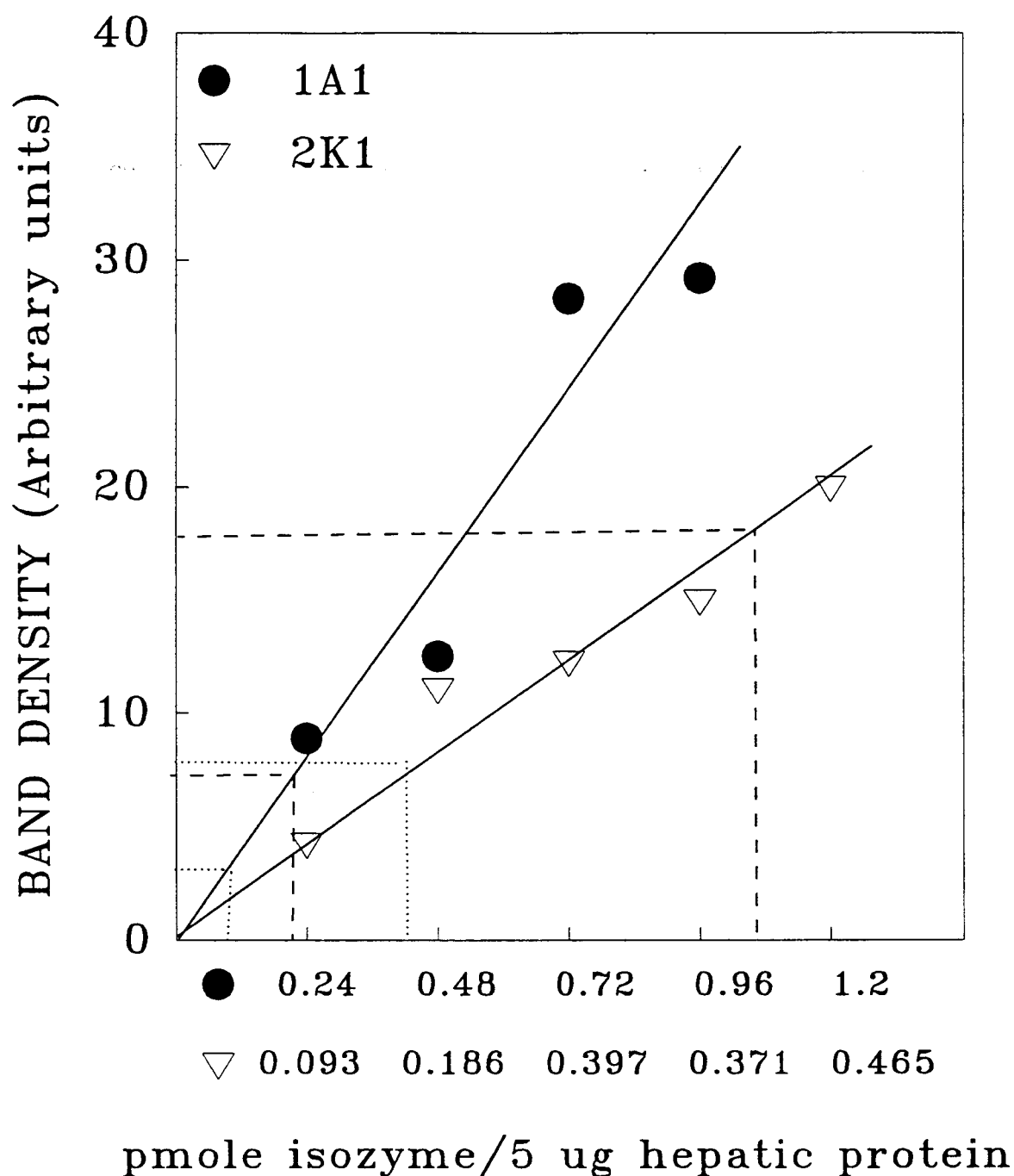
Figure 4.6 Determination of the specific CYP isozyme content in liver homogenate from control and CHL-treated fish by extrapolation to the standard curve. The dotted lines represent the CHL samples and the stippled lines the control. See Figure 4.5 legend and text for further details about diet exposures and Western blotting procedures.

Table 4.1 % AFB_1 and metabolites in the 600 g post-nuclear fraction of liver homogenate from trout fed control diet containing 80 ppb $^3HAFB1$ or 80 ppb $^3HAFB_1$ in combination with 4000 ppm CHL

	CONTROL	4000 ppm CHL
AFL	60.31	57.19
AFB1	33.12	31.10
AFL-g	5.44	10.38*
AFLM-g	0.36	0.32
AFM1	0.33	0.43
AFLM1	0.44	0.58

Data are means of triplicate samples

*: $p < 0.001$

REFERENCES

Arimoto, S., Negishi, T. and Hayatsu, H.: Inhibitory effect of hemin on the mutagenic activities of carcinogens. Cancer Letters 11;29-33. 1980.

Baxter, J.H.: Absorption of chlorophyll phytol in normal man and in patients with Refsum's disease. Journal of Lipid Research 90:636-641. 1968.

Bock, K.W. and White, I.N.H.: UDP-glucuronyltransferase in perfused rat liver and in microsomes: Influence of phenobarbital and 3-methylcholanthrene. Eur. J. Biochem. 46:451-459, 1975.

Burnette, W.N.:Western blotting. Electrophoretic transfer of proteins from sodium dodecyl sulfate-poly-acrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. 112:195-203, 1981.

Burton, K.: A study of the conditions and mechanisms of the di-phenylamine reaction for the colorimetric estimation of deoxyribonucleic acids. Biochem. J. 62:315-322, 1956.

Eisele, T.A., Bailey, G.S. and Nixon, J.E.: The effect of indole-3-carbinol, an aflatoxin B_1 hepatocarcinoma inhibitor, and other indole analogs on the rainbow trout hepatic mixed function oxidase system. Toxicol. Lett. 19:133-138, 1983.

Habig, W.H, Pabst, M.J. and Jacoby, W.B.: Glutathione S-transferases. The first step in mercapturic acid formation. J. Biol. Chem. 249:7130-7139, 1974.

Hall, A.J. and Wild, C.P.: Human carcinogenicity and toxicity. Epidemiology of aflatoxin-related disease. In The toxicology of aflatoxins. Human Health, Veterinary, and agricultural significance, Eaton, D and Gropman, J.D, Ed. Academic Press, Inc., San Diego, CA, 1994.

Imai, K., Aimoto, T., Sato, M., Watanabe, K., Kimura, R., and Murato, T.: Effects of sodium metallochlorophyllins on the activity and components of the microsomal drug-metabolizing enzyme system in rat liver. Chem. Pharm. Bull 34(10):4287-4293, 1986.

Ong, T., Whong, W., Stewart, J and Brockman, H.E.: Chlorophyllin: a potent antimutagen against environmental and dietary complex mixtures. Mutation Research 173:111-115, 1986.

Ong, T., Whong, J.D. and Brockman, H.E.: Comparative antimutagenicity od 5 compounds against 5 mutagenic complex mixtures in *Salmonella typhimurium* strain TA98. Mutation Research 222:19-25, 1989.

Pedersen, M.G., Hershberger, W.K. and Juchau, M.R.: Metabolism of 3,4-benzpyrene in rainbow trout (Salmo gairdneri). Bull. Environ. Contam. Toxicol. 12:481-486, 1974.

Takahashi, N: Ph.D. Thesis, Oregon State University, 1994.

Whong, W., Stewart, J., Brockman, H.E. and Ong T.: Comparative mutagenicity of chlorophyllin and five other agents against aflatoxin B_1-induced reversion in *Salmonella typhimurium* TA98. Teratogenesis, Carcinogenesis and Mutagenesis 8;215-224, 1988.

CHAPTER 5

COMPLEX FORMATION: A POSSIBLE MECHANISM OF PROTECTION AGAINST PRECARCINOGENIC AFLATOXIN B_1-DNA ADDUCTION BY CHLOROPHYLLIN

Vibeke Breinholt

and

George Bailey

Toxicology Program

Oregon State University

Corvallis, OR 97331

CONTRIBUTION OF AUTHORS

Vibeke Breinholt[1], Michael Schimerlik[2], and George Bailey[1]

[1] Department of Food Science and Technology and the Marine/Freshwater Biomedical Sciences Center, Oregon State University, Corvallis, OR 97331

[2] Department of Biochemistry, Oregon State University, Corvallis, OR 97331

ABSTRACT

Chlorophyllin (CHL), a food-grade derivative of the green plant pigment chlorophyll, has recently been shown to be a potent inhibitor *in vivo* of hepatic aflatoxin B_1-DNA adduction (Chapter 4). We report here that CHL forms a strong non-covalent complex with AFB_1 *in vitro*, which may contribute to its protective activity against the precarcinogenic lesions induced by AFB_1. The dissociation constant (Kd) for the AFB_1-CHL complex was estimated by Scatchard analysis to be 1.4 ($\pm$ 0.4) μM, based on an actual copper chlorin content of 34% in the commercial CHL formulation. Kd values for a series of other porphyrinic compounds, including a pure sample of chlorin-e6, were also determined to be in the same order of magnitude as the commercial CHL. Mole ratio analysis provided evidence that all porphyrins examined associate with AFB_1 at an approximate one to one stoichiometric ratio. The commercial preparation of CHL was found to exhibit at least 7 chromatographically resolvable chromophores, the unresolved mixture of which had a complexing ability comparable to that of pure chlorin-e6 at equimolar concentrations. AFB_1 incubated *in vitro* with liver microsomes in the presence of added CHL showed comparable levels of inhibition in the production of several phase 1 metabolites, including the postulated carcinogenic metabolite AFB_1-8,9-epoxide. Kinetic analysis of microsome-catalyzed AFB_1-DNA adduction revealed CHL to behave formally as a non-competitive inhibitor, which would be expected if CHL were capable of interacting with substrate (AFB_1) as well as enzyme-substrate complex. *In vivo*, addition of CHL to concentrated AFB_1 solutions used for gavage resulted in

dose-dependent inhibition of hepatic AFB_1-DNA adduction, whereas the same dosages of AFB_1 and CHL incorporated into a single bolus of trout diet for gavage provided less protection against AFB_1-DNA adduction at all CHL doses. This observation demonstrates that prior or prolonged CHL treatment is not required for its antigenotoxic activity *in vivo*. These findings support a role for CHL-AFB_1 complex formation, and possibly enzyme inhibition, in the potential chemoprotective activities associated with CHL. Since the CHL precursor chlorophyll is present at very high doses in vegetables such as spinach, complex formation between CHL-like compounds and carcinogens with at least partially planar aromatic structure may contribute to the chemopreventive activities associated with a high vegetable intake.

INTRODUCTION

Intense research activity over the past 15 years has led to the discovery of hundreds of chemopreventive agents, belonging to a very diverse class of chemicals, that are capable of interfering with key initiation or post-initiation processes in experimental carcinogenesis (Wattenberg, 1983; Newmark, 1984; Hayatsu, 1988; Dragsted, 1993). Many of these agents are natural constituents of fruit and vegetables, and have exhibited anticarcinogenic activity experimentally against a wide range of chemical carcinogens. Unfortunately, such anticarcinogenic phytochemicals often occur individually in edible plants at relatively low concentrations and the doses required to elicit a chemoprotective effect in experimental animals are often vastly in excess of the concentration humans might encounter in a balanced diet.

A promising exception is chlorophyll, the ubiquitous green pigment abundant in vegetables commonly consumed by humans. Recent studies, described in the previous chapter, have shown that chlorophyllin (CHL), a food-grade derivative and structural analogue of chlorophyll, strongly inhibits aflatoxin B_1 (AFB_1)-DNA damage and therefore has the potential to function as an anticarcinogenic agent.

Since the commercial preparation employed in the DNA-binding studies was only ca. 34% chlorins, the effective chlorin content providing 50% protection against DNA-adduction was less than 1,400 ppm, a fraction of the chlorophyll content of typical spinach cultivars (2.6-5.7% dry weight) (Khallyfa et al, 1992; Khachik et al, 1986). This suggests that a nutritionally realistic intake of chlorophyll has the potential of exerting chemopreventive activities in humans against dietary and environmental

carcinogens. However, the mechanisms responsible for the chemoprotective activities of CHL are not yet known. Recent studies have shown CHL to be capable of forming strong non-covalent complexes *in vitro* with genotoxins that have at least partially planar, aromatic character (Arimoto et al, 1980; Arimoto et al., 1989; Dashwood, 1992; Newmark, 1984; Negishi et al., 1989; Katoh et al., 1983). Complexation between CHL and 2-amino-3-methylimidazo[4,5-*f*]quinoline in the diet or digestive tract has been suggested to provide a less-absorbable complex responsible for reduced carcinogen bioavailability and target organ DNA adduction *in vivo* in rats (Dashwood, 1992). In addition, any CHL taken up systemically may complex *in situ* with procarcinogens to reduce the rate of enzymatic activation, or with their electrophilic metabolites to directly interfere with DNA adduction.

A CHL antigenotoxic mechanism involving strong complex formation with mutagens or carcinogens that are at least partially aromatic and planar, is attractively simple, and should be applicable to many suspected human carcinogens. Among these, AFB_1 is the most potent hepatocarcinogen known and the only naturally occurring compound in the human food chain concluded by the International Agency for Cancer Research (IARC) to be carcinogenic to humans (IARC Monograph, 1993). The purpose of the present study was to examine the strength and stoichiometry of molecular complexation between AFB_1 and CHL *in vitro* and its relevance to CHL inhibition of AFB_1 metabolism and potential inhibition of hepatocarcinogenesis *in vivo*.

MATERIALS

Unlabeled AFB_1 was purchased from Sigma Chemical Company (St. louis, MO) and the tritiated form from Moravek Biochemicals Inc. (Brea, CA). The purity of both preparations was determined by TLC and UV spectrophotometry. Calf thymus DNA, NADPH, Proteinase K, RNAse A and T1 and CHL were obtained from Sigma Chemical Company. The purity of the CHL preparation was analyzed by TLC as described in Chapter 7 and found to consist of 34% chlorins. The remaining constituents of the Sigma CHL sample were, according to the supplier, inorganic salts including NaCl that do not interfere with the fluorometric analysis. Protoporphyrin X (PP) and Zn-protoporphyrin XI (ZnPP) were obtained from Aldrich Chemical Co. (Milwaukee, WI). The purities of these compounds were determined spectrophotometrically, and found to be approximately 94% (PP) and 99% (ZnPP), consistent with the purity claimed by the manufacturer. Solvents used for HPLC analysis of AFB_1 metabolites were of HPLC grade, and were submitted to filtration on 0.22 μm poresize filterpaper (Waters Associates) prior to use. Ultra-pure phenol was from Clontech Laboratories Inc., Palo Alto, CA. HPLC standards were isolated and purified in our own laboratory. Standards used in the current study were aflatoxin B_1 (AFB_1), aflatoxin M_1 (AFM_1), aflatoxicol (AFL), aflatoxicol M_1 ($AFL-M_1$) and aflatoxin B_1-glutathione conjugate (AFB_1-GS).

METHODS

Estimation of AFB$_1$-CHL complex formation Complex formation between CHL and AFB$_1$ was studied fluorometrically by measuring the degree of quenching of the emission spectrum of AFB$_1$ by CHL. A Photon-Counting Spectrofluorometer (SLM 8000) equipped with a 450W xenon lamp was used for the steady state flourescence determinations. AFB$_1$ was excited at 365 nm and the maximum emission peak measured at 440 nm. Experiments were conducted in 1 cm path length quartz cuvettes connected to a thermoelement which maintained a constant temperature of 25°C. The cuvette was equipped with a micro stirring bar to assure adequate mixing. The total volume of buffer (0.1 M Tris HCl, pH 7.4) before titration was 3 ml which included 10 μl AFB$_1$ in 95% ethanol to a final concentration of 10 μM. After scanning the control AFB$_1$ spectrum and noting the total photon count at the given excitation and emission wavelengths, increments of 2 μL CHL stock solution in the appropriate buffer were added to provide 2 μM final concentration increments, followed by remeasuring of the emission peak. Three to five separate titrations were conducted at each pH value up to a total of 50 μM added CHL. All CHL solutions were made up in amber microcentrifuge tubes to protect the compound from light. Corrections for dilution effects during these incremental addition experiments were found to be negligible.

A similar set of analyses was conducted using 50 μM Na$_2$PO$_4$ buffers at pH 1.0, 3.0, 4.5, 7.4 or 8.0 to examine the effect of pH on AFB$_1$-CHL complex formation. These pH values were chosen to encompass the pH of the rainbow trout

digestive tract (stomach at fast: pH 2.5-3.0, stomach containing food: pH 4.5-6.8, pyloric stomach: pH 6.8-7.0, intestine: pH 7.2-7.6, cecum: pH 8.0 (Mark Christensen, pers. comm.)

Determination of complex dissociation constants Dissociation constants (Kd) were established at 25°C for the complex between AFB_1 and four selected porphyrin compounds: CHL, PP, ZnPP and Ce6. The Kd for CHL and AFB_1 was also determined at three additional temperatures; 12°C, 37°C, and 55°C. This set of titrations was conducted as described above, with a constant AFB_1 concentration of 5.5 μM in 3.0 ml of 0.1 M Tris pH 7.4 and successive additions of 5.0 μl aliquots of porphyrin stock solutions. CHL and Ce6 were dissolved in Tris-buffer pH 7.4 and increments of 2 μM were added to the cuvette. Due to the low solubility of PP and ZnPP in solutions compatible with the fluorescence measurements, both compounds were ground into a fine powder and partly dissolved in 100% ethanol. The undissolved particles were sedimented by centrifugation and the supernatant transferred to a new amber micocentrifuge tube. The actual concentrations of PP and ZnPP in the supernatants were determined from absorbances of aliquots diluted into 1 M HCl (PP, $E_{406}=262$ $M^{-1}cm^{-1}$) or pyridine (ZnPP, $E_{425}=146$ $M^{-1}cm^{-1}$). The final concentrations of PP and ZnPP used for each addition were 1.4 and 1.0 μM contained in 2.0 and 2.5 μl respectively. For Scatchard analyses, the concentration of free AFB_1 (AFf) was calculated as:

$$AFf = (AFi)(FCHLx/FAFi)$$

where AFi is the initial concentration of AFB_1 (μM), FAFi the concomitant

fluorescence yield and FCHLx the photon count after adding x μM CHL to the cuvette. The concentration of bound AFB$_1$ (AFb) and of bound CHL (CHLb) was calculated as:

$$AFb = CHLb = AFi - AFf$$

where AFf is the concentration of free substrate. The concentration of free ligand (CHLf) was determined as the difference between the total concentration of CHL added to the cuvette (CHLx) and CHLb, i.e.

$$CHLf = CHLx - CHLb.$$

Plots of (CHLb/CHLf) against CHLb were modeled as linear data sets, with slopes equal to the negative reciprocal dissociation constant (-1/Kd) and the x-intercept equal to the concentration of bound CHL at complex saturation. The value of this intercept divided by the initial concentration of AFB$_1$ provides one measure of the mole ratio of the CHL-AFB$_1$ complex at CHL saturation.

Mole-ratio determinations of the AFB$_1$-CHL complex: The presence of a defined complex mole-ratio between AFB$_1$ and CHL was also evaluated using a similar approach except that the initial AFB$_1$ concentration was 50 μM, and CHL was added in increments of 2 μM. A reverse titration also was conducted in which CHL in excess (50 μM) was titrated with 5 μM aliquots of AFB$_1$ contained in 5 μl dimethylsulfoxide (DMSO). Changes in AFB$_1$ fluorescence were measured at 440 nm. Due to the relatively low water solubility of AFB$_1$ a 33% DMSO-Tris-buffer pH 7.4 was employed for this set of experiments, with the realization that Kd values in this solvent would likely differ from those in an entirely hydrophilic environment.

AFB$_1$ metabolism by microsomes *in vitro* Microsome isolation: Microsomal enzymes were prepared by differential ultracentrifugation as described by Eisele et al. (1983). The microsomal pellet was resuspended in ice cold 0.1 M potassium phosphate (pH 7.5), 30% glycerol and 1 mM EDTA. The protein concentration was determined by the method of Lowry et al. (1951). The microsomal fraction was stored in 0.5 ml aliquots at -80°C until analysis. Mouse cytosol was isolated by the method of Guengerich (1982).

In vitro DNA-adduction: The reaction mixture consisted of 1 mg calf thymus DNA, 0.5 mg microsomal protein, and AFB$_1$ at concentrations between 5 and 50 μM dissolved in 10 μl DMSO (0.86 μCi/sample). Chlorophyllin dissolved in reaction buffer was added to the test tubes to give a final concentration of 0, 10, 25, 50 or 150 μM. Each incubation in triplicate was made up to a total volume of 1.0 ml with a 50 μM Na$_2$PO$_4$ buffer pH 7.4, containing 100 μM EDTA and 1.5 mM MgCl$_2$. After vortexing and 2-3 min preincubation the reaction was initiated with 5 μM NADPH and allowed to proceed for 15 min at 25°C in the dark. During this time period DNA-adduction occurred in a linear fashion (data not shown). The reaction was terminated by addition of 100 μl 10% SDS and 9 U proteinase K dissolved in 50 μl reaction buffer. The samples were then incubated at 55°C for 30 min, allowed to cool to room temperature, and submitted to DNA-isolation and determination of specific AFB$_1$-adduction level.

DNA-extraction: The samples were extracted with an equal volume of Tris-saturated phenol, then once with chloroform and finally twice with chloroform:isoamyl alcohol (1:24). After the last extraction step 500 μl of the aqueous phase was

transferred to a 1.7 ml microcentrifuge tube containing 1.0 ml isopropanol followed by inversion of the tubes several times until the DNA was completely precipitated. The DNA was centrifuged for 5 min at 2,500 rpm, and washed twice with 100% ethanol. The DNA pellet was allowed to dry at room temperature for 5-10 min before dissolution in 1.0 ml reaction buffer. The 260/280 nm absorbance ratio was determined to assess the purity of the DNA, and was found to be between 1.9 and 2.0 for all samples. The DNA concentration was assayed using the method of Burton (1956). To obtain the specific activity of the isolated DNA, the radioactivity of known volumes of hydrolyzed DNA was determined by liquid scintillation counting.

The data sets were modeled by computerized non-linear regression according to Duggleby (1984) using both competitive and non-competitive models.

HPLC-analysis of in vitro AFB₁ metabolites: Metabolism of AFB_1 in vitro was determined as described by Monroe and Eaton (1987) with the following modifications. The incubations were conducted in total volumes of 0.25 ml and only one dose of AFB_1 of 10 μM (5 μCi/sample) was employed, with and without addition of 100 μM CHL to the incubation mixture. The reaction mixture consisted of 6.0 mg microsomal trout protein/ml, 12 mg butylated hydroxyanisole (BHA)-induced mouse cytosolic protein/ml, in a buffer containing 190 mM sucrose, 60 mM potassium phosphate, 80 mM Tris, 15 mM NaCl, 5 mM KCl and 4 mM $MgCl_2$, pH 7.6. The mouse cytosolic fraction was isolated from Swiss-Webster mice fed a AIN-76A semipurified diet containing 0.75% BHA for ten days. CHL and AFB_1 were allowed to interact for approximately 2 min before initiation of the reaction with 5 mM

reduced glutathione (GSH) and 5 μM NADPH. Additional controls were included in this study in order to distinguish between mouse cytosol-dependent metabolism and trout microsome- mediated activities and to verify the formation of the GSH conjugate. The extra controls were: a) no GSH, no mouse cytosol; and b) no microsomes. The reaction was terminated by addition of 1.0 ml 100% methanol followed by high speed vortexing for 30 sec. The denatured protein was centrifuged at 14,000 g for 15 min. The supernatant was transferred to a microcentrifuge vial and frozen at -20°C overnight. The frozen mixture was centrifuged at 14,000 g for 5 min at ambient temperature to allow thawing of the mixture and to precipitate remaining protein. Particulate matter was removed by filtration of the supernatant (0.22 μm syringe filter, Waters Associates) into a 2 ml sonicated glass vial. The methanol was evaporated of under a stream of nitrogen and the sample adjusted to 15% AMT (acetonitrile: methanol: tetrahydrofuran (15:20:6)) and 85% 0.02 M potassium acetate (pH 5.0) immediately prior to injection into the HPLC. A total of 50 μl in duplicate was injected per incubation. Analysis of AFB$_1$ metabolites was performed as described in Chapter 4.

In vivo AFB$_1$-DNA binding by gavage: Ten groups of 9 rainbow trout of 15-20 g body weight were placed in separate tanks and fasted for 7 days, to obtain complete emptying of the digestion system. At the end of this period one group of fish received 6.6 ng (0.3 μCi) tritiated AFB$_1$/15 g body weight by liquid gavage using 10 μl 0.9% NaCl as a carrier. This concentration of AFB$_1$ is equivalent with a 20 ppb daily dietary dose, when the dietary ration (dry weight) is calculated as 2% of the body

weight. Four other groups received the same dose of AFB_1 in combination with CHL concentrations of 0.03, 0.16, 0.66 and 1.31 mg equal to CHL dietary levels of 100, 500, 2000 and 4000 ppm. All solutions were made up in 0.9% NaCl.

The remaining fish received the same doses of AFB_1 and AFB_1 plus CHL but this time the carcinogen and inhibitor were incorporated into a modified Oregon Test Diet mixture consisting of 28% dry ingredients (20% salmon oil, 40% casein, 15% alpha-cellulose, 9% gelatin, 8% dextrin, 4% essential minerals, 2% vitamin mix, 1% choline chloride and vitamin E (660 IU/kg dry ingredients)) and 72% water. The moisture content (72%) of the gavaged diet was slightly increased compared to the water content of 65% of the diets normally used in feeding trails. This dilution of the diet was required in order to increase the fluidity of the diet which greatly facilitated administration of the food bolus. Each fish was given a total of 140 mg diet mixture/15 g body weight. The same solutions of AFB_1 and CHL used in the liquid gavage experiment were used for making up the gavage diet and corrections for the added amount of water were performed accordingly. Forty-eight hours after administration of the carcinogen and the inhibitor, the fish were killed by a blow to the head, the livers removed, and pools of three livers each were frozen in liquid nitrogen and stored at -80°C until analysis.

DNA was isolated and analyzed as described in by Chapter 4. The DNA pellet was dissolved in 1.0 ml 10 mM Tris buffer overnight at 5°C and the purity determined by measuring the 260/280 nm absorbance ratio. For all samples the ratio was between 1.8 and 2.0, indicating a pure DNA-preparation.

RESULTS

<u>CHL quenching of the AFB$_1$ fluorescence spectrum:</u> The ability of CHL and individual porphyrin analogues to quench the fluorescence spectrum of AFB$_1$ was used as a measurement of complex formation with AFB$_1$. Since the fluorescence optimum of CHL is near 700 nm and completely separated from the AFB$_1$ fluorescence peak, AFB$_1$-fluorometric measurements provide a very sensitive and specific, though indirect, index of AFB$_1$-CHL interaction. Stopped-flow analysis revealed that AFB$_1$-CHL association *in vitro* was rapid and complete within the 2.5 msec required to mix the two components in the stopped-flow photometer (data not shown). The optimum fluorescence yield was linear with AFB$_1$ through the concentration range of this study (not shown), which indicates an absence of significant AFB$_1$-AFB$_1$ interaction under these conditions.

During the titration process, AFB$_1$ fluorescence not only decreased but the optimum shifted to higher wavelengths in the presence of CHL (Figure 5.1). This indicates that the fluorescence characteristics of AFB$_1$ change due to the interaction between the two compounds. The degree of spectral shift was approximately 0.3 nm/1 μM CHL at pH 7.4. The non-covalent nature of the complex was verified by dilution of a solution containing AFB$_1$ and CHL at a 1:4 ratio, in which the AFB$_1$ maximum fluorescence peak had been shifted from 440 nm to 462 nm. Serial 1:1 dilutions resulted in successive increases in fluorescence yield despite the gradual

decrease in total AFB_1 concentration in the cuvette. The maximum fluorescence peak was also shifted to lower wavelengths with the resulting reappearance of the characteristic emission peak at 440 nm (data not shown).

Determination of complex dissociation constants: Figure 5.2 depicts Scatchard plots for the AFB_1-porphyrin complexes analyzed. The dissociation constants for complexes between AFB_1 and the four porphyrin compounds CHL, PP, ZnPP and Ce6 were estimated from the slopes to be 1.4 ($\pm$ 0.4), 4.1 ($\pm$ 0.6), 1.6 ($\pm$ 0.3) and 1.9 ($\pm$0.5) μM respectively. This Kd value for CHL is based on the actual concentration of CHL in the preparation (ca. 34%). Without correction the calculated Kd for CHL-AFB1 complex was 11.6 ($\pm$3.3) μM (Figure 5.2). A similar Kd (15.4$\pm$(2.8)) value for AFB_1-CHL complexation was obtained from spectrophotometric rather than fluorescence measurements, conducted as described elsewhere (Dashwood, 1992). The x-intercepts of the Scatchard plots (Figure 5.2) define the concentration of ligand sufficient to saturate complex formation at the particular substrate concentration used, and thus provide a measure of complex stoichiometry. The values for these intercepts were not significantly different by analysis of variance (p > 0.30). The mean value of all replications from the individually repeated titrations was found to be 6.1 ($\pm$1.5) μM. The finding that the x-intercept on the Scatchard plot (6.1 μM) is approximately the same as the concentration of substrate employed in this titration series (5.5 μM), provide evidence that AFB_1 and the porphyrins associate stoichiometrically at an approximate 1:1 ratio.

The stoichiometry between AFB_1 and CHL was also investigated by the titration approach of Christwell and Schilt (1975), using an initial AFB_1 concentration approximately 35-fold higher than the AFB_1-CHL complex Kd, in order to seek an initial linear change in fluorescence. At the sensitivity of the fluorometer, 50 μM AFB_1 was the highest concentration which gave a linear response in AFB_1 fluorescence. The first series of experiments (forward titrations) were conducted by measuring the decrease in AFB_1 fluorescence with increasing CHL concentration (Figure 5.2 inset). The forward titration should ideally result in quenching of the AFB_1 fluorescence spectrum in approximate proportion to the ligand added, until the system becomes saturated. At this point, defining the mole ratio of the associating molecules, all the substrate is maximally complexed and further addition of ligand will not result in changes in fluorescence. The reverse order of reaction (reverse titration) was also examined by determining the ability of a fixed concentration of CHL (in excess over Kd) to bind increasing amounts of AFB_1, such that AFB_1 flourescence emission is suppressed until the stoichiometry of complexation is exceeded.

A fluorescence transition was found to occur with tangents intersecting near a 1:1 mole ratio for the "reverse" titration (Figure 5.2 inset). However, the data display sigmoid rather than linear behavior near the origin, and a sharp break point characteristic of extremely tight complex associations (Christwell and Schilt, 1975) was not evident. Since the commercial CHL when corrected for chromophore content gave a Kd identical to that for the pure Ce6, we assume that the lack of a sharp break point in the titration experiments was not due to the presence of several chlorins in the CHL-preparation of significantly different affinities for forming a complex with

AFB_1. For the "forward" titration of fixed AFB_1 with successive CHL additions, an even less definitive break was observed, and this transitional change occurred in the range more compatible with a stoichiometry of 2 AFB_1:CHL. An additional observation, for which we currently have no satisfactory explanation, is a greater than stoichiometric depression in fluorescence with the first CHL additions in the forward titration. That is, AFB_1 fluorescence decreased by about 40% with the addition of only an 0.2 mole ratio of CHL. The most obvious possibility for these two observations, that complex stoichiometry may vary according to input mole ratio (e.g. that AFB_1:CHL:AFB_1 complexes may be preferred at low CHL input) is not supported by the linear Scatchard plots. However, the latter were conducted at much lower CHL and AFB_1 concentrations, and it is possible that some other form of ligand self-association alters the effective fluorescence suppression of the complex, though no specific mechanism is apparent. Interestingly, the forward and reverse titration curves cross over at a 1:1 mole ratio, though we have devised no theoretical argument that this should provide the best estimate of complex stoichiometry. It should be emphasized that changes in AFB_1 fluorescence do not provide a direct measure of the amount of complex formed, so that this type of experiment can provide only an approximate measure of true complex stoichiometry.

pH effects on AFB_1-CHL complex: Because of our interest in modeling the effects of dietary CHL chemoprevention, it was important to investigate whether pH variations along the digestive tract might have important influence on complex stability or stoichiometry. Based on slopes of Scatchard plots (Figure 5.1 inset) derived from

AFB$_1$ quenching experiments conducted at various pH values, the Kd for the AFB$_1$-CHL complex shows little dependence on pH. There was some evidence for a small (ca. two-fold) effect at pH 4.5, which suggests some role for CHL carboxyl protonation on complex formation, but this was not sustained at lower pH. These results indicate that CHL-AFB$_1$ complex formation and stability in the acid conditions of the stomach are comparable to that expected elsewhere along the digestive tract. Though the Kd is not sensitive to pH change, the apparent intercept values decreased substantially at low pH. A possible explanation to be considered involves the potential for water addition across the AFB$_1$ 8,9 double bond, a vinyl ether structure susceptible to acid-catalyzed hydrolysis (Pohland et al., 1968). We do not believe that the formation of the AFB$_1$ hemi-acetal would be expected to influence Kd since this region is remote from the conjugated, planar portion of the AFB$_1$ molecule and hence is unlikely to be involved in CHL interaction. We do, however, observe a substantial reduction in fluorescent yield when AFB$_1$ is incubated at low pH (not shown), which may be a result of hydrolysis of the double bond with concomitant changes in the fluorescence characteristics of the substrate. That the differences in y-axis intercept from the Scatchard plot are not a result of increased AFB$_1$ self-quenching at low pH, is evidenced by the fact that readjusting the pH of the solution back to pH 7.4, does not result in the expected fluorescence yield at this given pH. Therefore, the data from this experiment support the conclusion that complex Kd is not strongly influenced by pH, but do not provide unambiguous information regarding possible pH effects on complex stoichiometry. Our interpretation is that stoichiometry is not pH dependent, since it is difficult to envision how this could occur without concomitant Kd effects.

CHL effects on DNA adduction and AFB$_1$ metabolism _in vitro:_ CHL was found to act as an apparent competitive inhibitor of microsome-catalyzed AFB$_1$-DNA adduction reactions _in vitro_ (Figure 5.3), which is consistent with complex formation as the sole mechanism of protection against carcinogen adduction. The apparent Michaelis constant derived from non-linear regression modeling (Duggleby, 1984) (Fig.5.3) was estimated to 35.2 ± 8.4 μM and the apparent Vmax was 0.032 ± 0.008 pmol AFB1/mg DNA/mg microsomal protein/min. The inhibition constant Kis was estimated to 20.5 ± 3.7 μM (Figure 5.3 inset). The specific chlorin content of the batch of CHL employed in this study was not measured. However, assuming a chlorin content of 34% a corrected value for Kis of 7.0 μM is obtained.

Co-incubation of AFB$_1$ and CHL in the presence of trout microsomal fraction and subsequent analysis of the metabolites present in the supernatant after protein precipitation revealed significantly lower levels of all metabolites (P < 0.05) and a concurrent significantly higher concentration of unmetabolized parent compound (Table 5.1). The total concentration of metabolites was decreased by approximately 50% from 9.2% in the AFB$_1$ control samples to 4.8% in the CHL-AFB$_1$ co-incubations.

DISCUSSION

Dissociation constant and mole ratio determination: Complex formation has been suggested to be one of the mechanisms responsible for the antimutagenic properties of CHL against various mutagens of planar polycyclic ring structures (Arimoto et al., 1980; Arimoto et al., 1989; Dashwood et al., 1991). Other mechanisms such as enzyme inhibition (Sato et al., 1984; Imai et al., 1986; Oda et al., 1971), electrophile scavenging (Newmark et al.,1984; Sato et al., 1986; Dashwood et al., 1991), inhibition of cell mitosis (Ghosh et al., 1991a; Ghosh et al., 1991b; Robins et al., 1989) and modifying effects on DNA-repair enzymes (Whong et al., 1988) also have been invoked to explain the antimutagenicity of CHL toward non-planar molecules with a non-cyclic chemical structure. Complex formation in the gastrointestinal tract between carcinogens and CHL has recently been suggested to afford protection against carcinogen-DNA adduction in experimental animals *in vivo* (Dashwood et al, 1991; Dashwood, 1992), by reducing absorption of the complex from the gut. In the present study the Kd for the complex formed between AFB_1 and CHL (Kd = 1.4 μM) was found to be two orders of magnitude smaller than the complex between CHL and the heterocyclic amine 2-amino-3-methylimidazo[4,5 - *f*]quinoline (IQ) (Kd = 141 μM) (Dashwood and Guo, 1992). The AFB_1-CHL complex dissociation constant was found to be essentially independent of pH, implying equivalent complex stability along the digestive tract. The Kd values for the various porphyrinic compounds were all in the same range, with PP showing a slightly weaker complexing ability ($p < 0.05$) than CHL, Ce6 and ZnPP.

Analysis of the complex mole ratio by reverse titration of CHL with added AFB$_1$ provided some evidence for a 1:1 CHL:AFB$_1$ complex stoichiometry. By comparison, the fixed concentration of AFB$_1$ for the forward titration was limited by AFB$_1$ solubility, and may have been insufficiently in excess of the Kd (ideally 100 fold) to give a sharp break point indicative of a specific complex stoichiometry. Scatchard plots independently demonstrate a 1:1 stoichiometry between CHL and AFB$_1$ and between AFB$_1$ and PP, ZnPP and Ce6, based on extrapolated intercepts. This is in contrast to the 2:1 stoichiometry reported for CHL complexation with the heterocyclic amine IQ (Dashwood and Guo, 1992), which may reflect the relatively asymmetric planarity of AFB$_1$ and an inability to form preferred symmetrical CHL:AFB$_1$:CHL structures. Another explanation for this discrepancy could be that no correction for the specific chlorin content was performed in the heterocyclic amine studies. If, for example, the CHL preparation used by Dashwood also contained 34% copper chlorins, replotting the mole ratio data gives a stoichiometry of 0.7:1 [CHL:IQ] which is similar to the CHL/AFB$_1$ mole ratio estimated in the current study.

Correction for specific chromophore content: Comparison of the Kd obtained from the pure copper Ce6 with that of the commercial CHL preparation suggested a more than 5-fold difference in the strength of the complexes. The Kd obtained for the pure copper chlorin was 1.9 μM whereas that of the commercial CHL was 11.1 μM (Figure 5.2). However, correction for the specific pigment content in the commercial sample gave a Kd of 1.4 μM, similar to that for the pure chlorin. As pure CHL is

currently unavailable on a commercial scale the finding that the complexing characteristics of the total individual fractions on a molar basis approximates that of a pure chlorin allows correction for the specific pigment content by a simple linear approach, and suggests that the use of pure chlorins for anticarcinogenicity experiments is not essential from a mechanistic point of view.

Complex formation as a mechanism of protection against precarcinogenic lesions:

Complex formation characteristics in pure solution *in vitro* clearly overestimate the protective effect of CHL *in vivo*. As an example, the addition of 4000 ppm CHL (not corrected for actual chlorin content) to diets containing 80 ppb AFB_1 resulted in only 46% inhibition of AFB_1-DNA adduction in the target organ (see Chapter 9). This indicates that ca. 1/2 of the initial AFB_1 dose in this experiment remained effectively bioavailable for absorption and bioactivation, despite a theoretical molar excess of CHL (22,000:1) sufficient to assume 100% complex formation with AFB_1 in solution *in vitro*. Even though complex formation was optimized by dissolving the reactants in a small volume of water and allowing the two compounds to interact prior to incorporation into the diet, the subsequent addition of water (65% of total diet) and dry ingredients may interfere with complex stability; this may occur both because the complex solution becomes diluted and because of the potential binding of CHL to constituents of the diet. The liquid versus the diet gavage experiment also showed that interference with inhibition of AFB_1-DNA adduction is evident when the inhibitor and the carcinogen are incorporated in the diet as compared to administration of the same concentrations AFB_1 and CHL in a highly concentrated form. Liquid gavage of CHL

in combination with AFB_1 afforded consistently higher protection (50-85%) against carcinogen DNA adduction ($p < 0.05$) than CHL and AFB_1 exposure via the dietary route (5-30%) (Figure 5.4).

An alternative mechanism for CHL-mediated reduction of carcinogen-DNA binding may involve CHL inhibition of carcinogen-activating enzymes (Dashwood, 1992). The kinetic data presented here show apparent competitive inhibition by CHL for the enzymatic activation of AFB_1, which would be anticipated from AFB_1:CHL complexation alone. We wish to stress, however, that hepatic concentrations of CHL-derived chromophores have not been systematically investigated and the relevance of enzyme inhibition as a possible inhibitory mechanism for CHL *in vivo* is not understood.

Evaluation of the impact of complex formation as a means of chemoprevention in humans is equally complicated in that CHL, chlorophyll and the degradation products of heme may possess the potential to complex not only with each other but with other dietary components including the aromatic amino acids, vitamins and co-factors exhibiting similar chemical structures. The extent to which the porphyrinic compounds specifically encounter and bind dietary carcinogens like AFB_1 and the heterocyclic amines within the diet or digestive system currently remains unknown. Given that the complex between AFB_1 and CHL is relatively strong, and that CHL-like compounds are abundant in the human diet, complex formation may play an important role in reducing the overall bioavailability of polycyclic planar carcinogens from the human diet, even if the extent of complexation is less than might be predicted by simple solution model measurements.
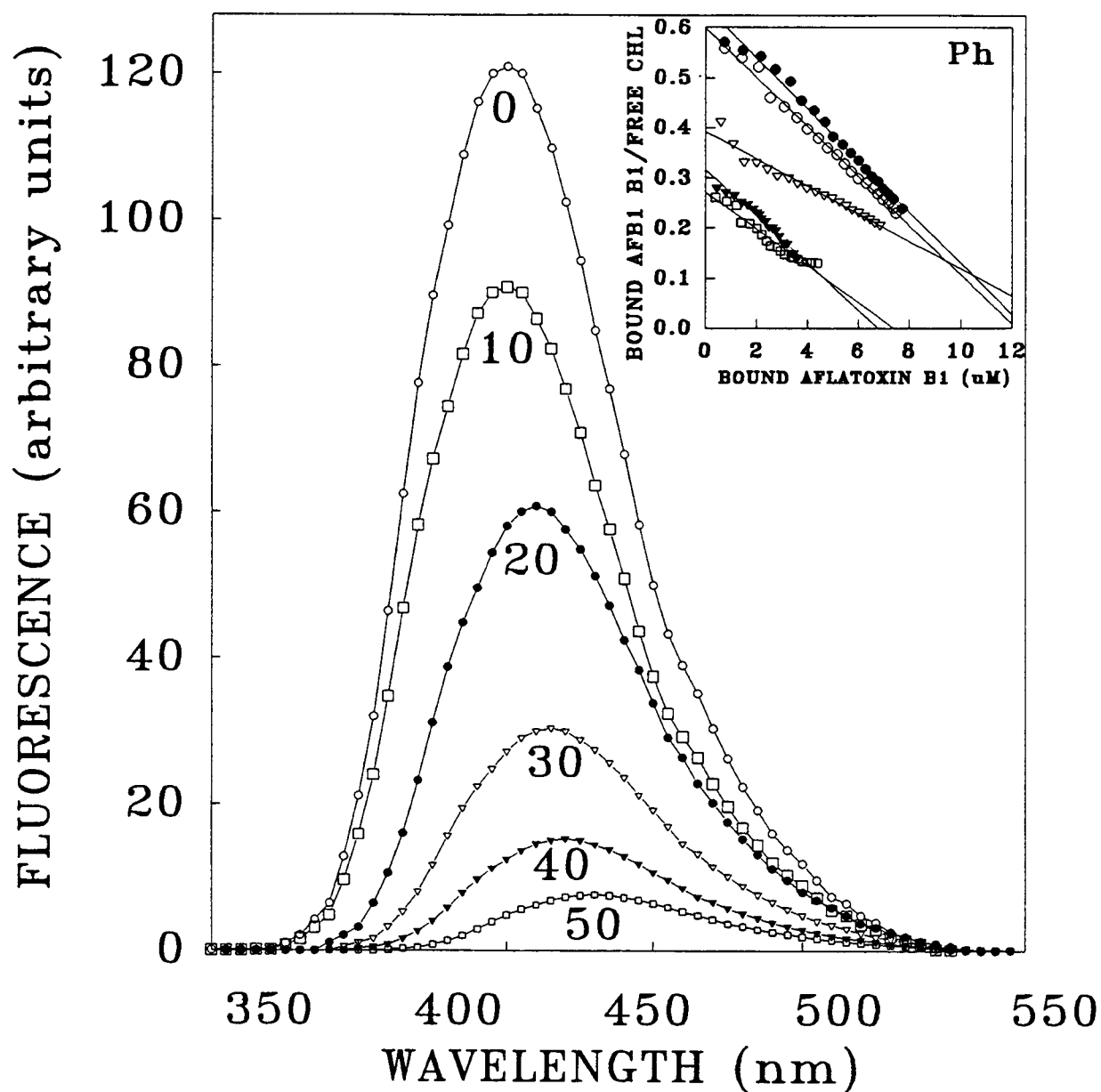
Figure 5.1   Quenching of AFB$_1$ fluorescence spectrum by CHL. AFB$_1$ at a final concentration of 10 μM was titrated with 10 μM aliquots of CHL. The numbers represent the fluorescence yield after addition of 0, 10, 20, 30, 40 and 50 μM CHL. Figure 5.1 inset. Scatchard plots of AFB$_1$-quenching curves at pH 7.4 (filled circles), pH 8.0 (open circles), pH 4.5 (open triangles), pH 3.0 (filled triangles) and pH 1.0 (open squares).
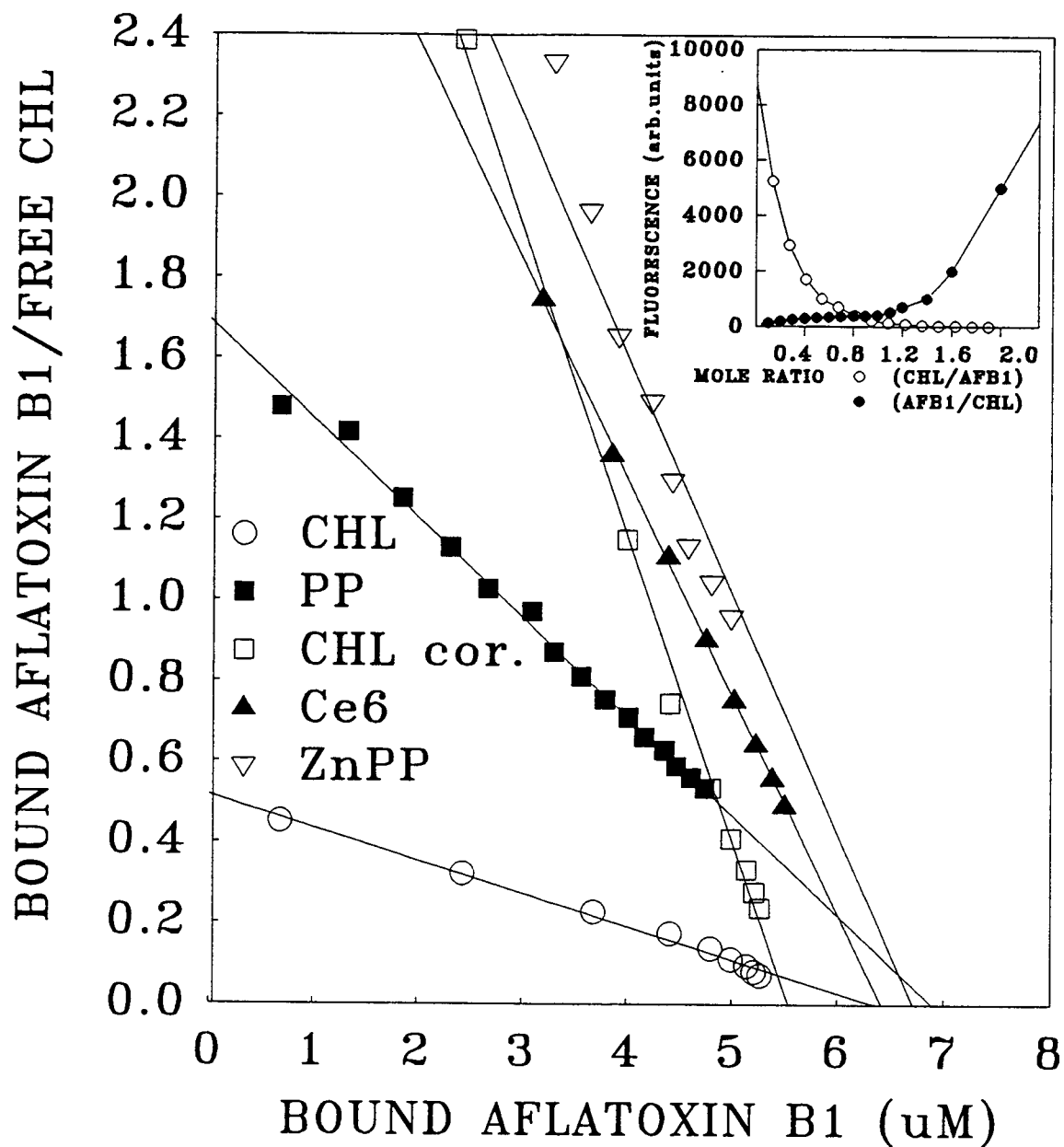
Figure 5.2 Scatchard plot of porphyrin fluorescence quenching data. The data points are mean values of 3-6 individual determinations; error bars are omitted for clarity. CHL-cor.: correction for actual chlorin content included. Figure 5.2 inset. Mole-ratio analysis. The open circles represent the forward titration and the filled circles the reverse titration. See text for details.
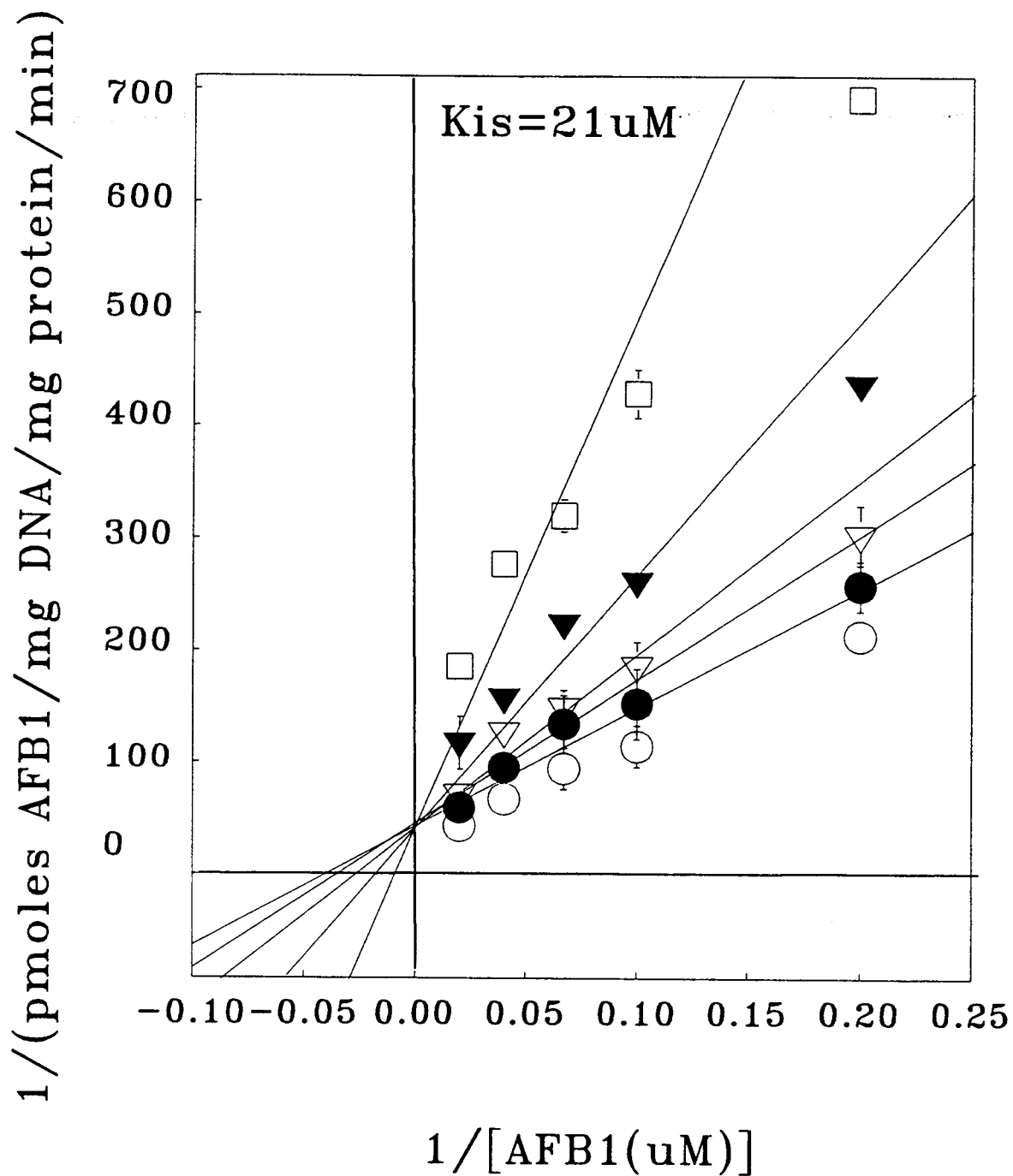
Figure 5.3 Lineweaver-Burk plot of *in vitro* AFB_1-DNA adduction inhibition by CHL. CHL was employed at concentrations ranging from 0-150 μM. The final concentrations of AFB_1 was 5, 10, 15, 25 and 50 μM.
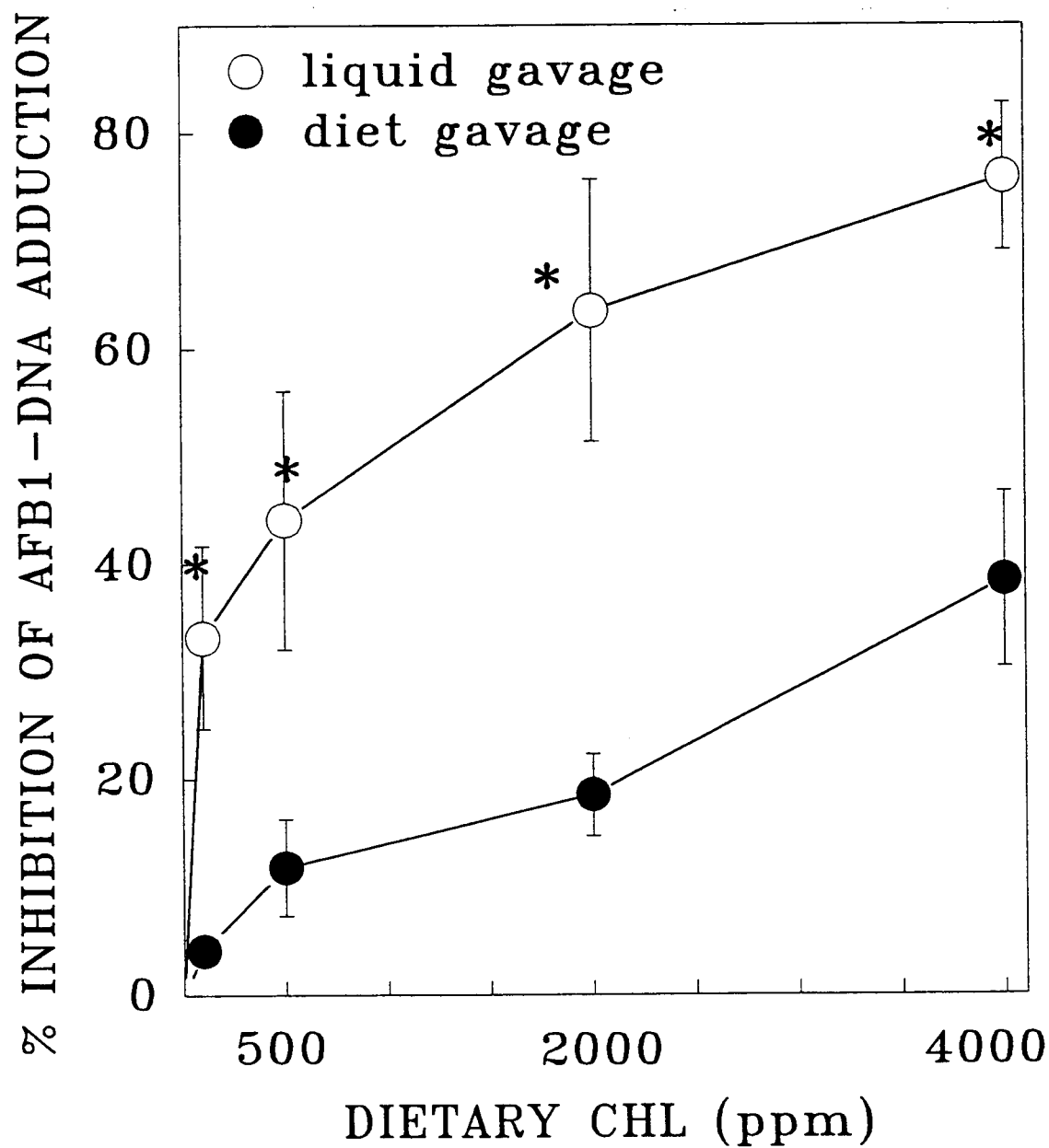
Figure 5.4   Enhanced inhibitory effect of CHL on *in vivo* AFB$_1$-DNA adduction by gavage administration of the carcinogen and inhibitor compared to dietary exposure. Data points represent mean inhibitory potency ± SD. *: $p < 0.05$

Table 5.1   Effect of CHL on the % recovery of AFB_1 and metabolites after *in vitro* activation of AFB_1 with trout microsomal fraction

Metabolite[a]	CONTROL	100 μM CHL
AFB_1	90.8	95.2*
AFM_1	3.1	2.1*
AFB_1-GS	2.3	0.8**
AFL	2.2	1.0*
$AFLM_1$	1.6	0.9*

[a] Abbreviations are: AFB_1: Aflatoxin B_1; AFM_1: Aflatoxin M_1; AFB_1-GS: Aflatoxin B_1-glutathione conjugate; AFL: Aflatoxicol; $AFLM_1$: Aflatoxicol M_1

*: $p < 0.05$

**: $p < 0.01$

REFERENCES

Arimoto, S. and Hayatsu, H.: Role of hemin in the inhibition of mutagenic activity of 3-amino-1methyl-5H-pyrido[4,3-*b*]indole (Trp-P-2) and other aminoazaarenes. Mutation Research 213:217-226, 1989.

Arimoto, S., Negishi, T. and Hayatsu, H.: Inhibitory effect of hemin on the mutagenic activities of carcinogens. Cancer Letters 11:29-33, 1980.

Burton, K.: A study of the conditions and mechanisms of the di-phenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62:315-322, 1956.

Christwell, C.D. and Schilt, A.A.: New and improved techniques for applying the mole ratio method to the identification of weak complexes in solution. Analytical Chemistry 47(9):1623-1629, 1975.

Dashwood, R.H., Breinholt, V. and Bailey, G.S.: Chemoprotective properties of chlorophyllin: inhibition of aflatoxin-B_1 (AFB$_1$)-DNA binding *in vivo* and anti-mutagenic activity against AFB$_1$ and two heterocyclic amines in the salmonella mutagenicity assay. Carcinogenesis 12:939-942 1991.

Dashwood, R.H.: Protection by chlorophyllin against the covalent binding of 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ) to rat liver DNA. Carcinogenesis 13:113-118, 1992.

Dragsted, L.O., Strube, M. and Larsen, J.C.: Cancer-protective factors in fruits and vegetables: Biochemical and biological background. Pharmacology and Toxicology 72(suppl.1):116-135, 1993.

Duggleby, R.G. Comput. Biol. Mod. 14:447-455, 1984

Eisele, T.A., Bailey, G.S. and Nixon, J.E.: The effect of indole-3-carbinol, an aflatoxin B_1 hepatocarcinoma inhibitor, and other indole analogs on the rainbow trout mixed function oxidase system. Toxicol. Lett. 19:133-138, 1983.

Ghosh, A., Sen, S., Sharma, A. and Talukder, G.: Inhibitions of clastogenic effects of cesium chloride in mice *in vivo* by chlorophyllin. Toxicol. Lett. 57:11-17, 1991.

Ghosh, A., Sen, S., Sharma, A. and Talukder, G.: Effect of chlorophyllin on mercury chloride-induced clastogenicity in mice. Food Chem. Tox. 29:777-779, 1991.

Hayatsu, H., Arimoto, S. and Negishi T.: Dietary inhibitors of mutagenesis and carcinogenesis. Mutation Research 202:429-446, 1988.

International Agency for Cancer Research. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans: Aflatoxins. Vol. 56 pp. 245-396. Lyon, France, 1993.

Imai, K., Aimoto, T., Sato, M., Watanabe, K., Kimura, R., and Murato, T.: Effects of sodium metallochlorophyllins on the activity and components of the microsomal drug-metabolizing enzyme system in rat liver. Chem. Pharm. Bull 34(10):4287-4293, 1986.

Katoh, Y., Nemoto, N., Tanaka, M. and Takayama, S.: Inhibition of benzo[a]pyrene-induced mutagenesis in Chinese hamster V79 cells by hemin and related compounds. Mutation Research 121:153-157, 1983.

Khachik, F, Beecher, G.R., and Whittaker, N.F.: Separation, identification and quantification of the major carotenoid and chlorophyll constituents in extracts of several green vegetables by liquid chromatography. Journal of Agricultural and Food Chemistry 34:603-616, 1986.

Khalyfa, A., Kermasha, S., and Alli, I.: Extraction, purification, and characterization of chlorophyll from spinach leaves. Journal of Agricultural and Food Chemistry 40:2150220, 1992.

Monroe, D.H. and Eaton, D.L.: Comparative effects of buthylated hydroxyanisole on hepatic *in vivo* DNA-binding and *in vitro* biotransformation of aflatoxin B_1 in the rat and mouse. Toxicol. Appl. Pharmacol. 90:401-409, 1987.

Newmark, H.L.: A hypothesis for dietary components as blocking agents of chemical carcinogenesis: Plant phenolics and pyrrole pigments. Nutrition and Cancer 6(1):58-70, 1984.

Newmark, H.L.: Plant phenolics as inhibitors of mutational and precarcinogenic events. Canadian Journal of Physiology and Pharmacy 65:461-466, 1987.

Oda, T., Yokono, O., Yoshida, A., Miyake, K. and Iino, S.: On the successful treatment of pancreatitis. Gastroenterologia Japonica 6:49-54, 1971.

Pohland, A.E., Cushmac, M.E. and Andrellos, P.J.: Aflatoxin B_1 hemiacetal. J. Assoc. Offic. Anal. Chemists 51:907-910, 1968.

Robins, E.W. and Nelson, R.L.: Inhibition of 1,2-dimethylhydrazine-induced nuclear damage in rat colonic epithelium by chlorophyllin. Anticancer Research 9:981-986, 1989.

Sato, M., Fujimoto, I., Sakai, T., Aimoto, T., Kimura, R. and Murata, T.: Effect of sodium copper chlorophyllin on lipid peroxidation. IX. On the antioxidative components in commercial preparations of sodium copper chlorophyllin. Chem. Pharm. Bull 34(6):2428-2434, 1986.

Sato, M., Konagai, K., Kuwana, T., Kimura, R. and Murata, T. Effects of sodium copper chlorophyllin on lipid peroxidation. VII. Effects of its administration on the stability of rat liver lysosomes. Chem. Pharm. Bull 32(7):2855-2858.,1984.

Wattenberg, L.W.: Inhibition of neoplasia by minor dietary constituents. Cancer Research 43(suppl.):2448-2453, 1983.

Whong, W., Stewart, J., Brockman, H.E. and Ong T.: Comparative mutagenicity of chlorophyllin and five other agents against aflatoxin B_1-induced reversion in *Salmonella typhimurium* TA98. Teratogenesis, Carcinogenesis and Mutagenesis 8:215-224, 1988.

CHAPTER 6

CHLOROPHYLLIN, A DERIVATIVE OF THE UBIQUITOUS PIGMENT IN
GREEN VEGETABLES, INHIBITS AFLATOXIN B_1 CARCINOGENESIS

Vibeke Breinholt

and

George Bailey

Toxicology Program

Oregon State University

Corvallis, OR 97331

CONTRIBUTION OF AUTHORS

Vibeke Breinholt[1,2], Dan Arbogast[1], Cliff Pereira[3], Jerry Hendricks[1,2] and George Bailey[1,2]

[1] Department of Food Science and Technology, Oregon State University, Corvallis, OR 97331

[2] Marine/Freshwater Biomedical Sciences Center, Oregon State University, Corvallis, OR 97331

[3] Department of Statistics, Oregon State University, Corvallis, OR 97331

ABSTRACT

Chlorophyllin (CHL), a food-grade derivative of the green plant pigment chlorophyll, was found to be an effective inhibitor of aflatoxin B_1 (AFB_1) induced tumorigenesis in the rainbow trout model. The slopes of dose-response curves for AFB_1-DNA adduction in the target organ were progressively decreased with increasing CHL co-treatment, as were the slopes of tumor incidence vs. AFB_1 dose curves determined nine months later. Based on slope ratios, the percentage inhibition of hepatic AFB1-DNA binding was 22% (95% confidence interval (95% C.I.) = 11-32), 30% (95% C.I. = 22-38) and 37% (95% C.I. = 30-44) at dietary levels of 500, 2000 and 4000 ppm, whereas the corresponding inhibition of tumorigenesis was 32% (95% C.I. = 8-50), 36% (95% C.I. = 20-49) and 72% (95% C.I. = 60-80).

The decrease in DNA-adduction did not differ significantly from the decrease in tumor incidence at the two lowest doses of CHL, whereas the percent inhibition of tumorigenesis at 4000 ppm was significantly higher ($p = 0.001$) that the observed inhibition of AFB_1-DNA adduction. A quantitative comparison of CHL effects on AFB_1 TD_{50} values and DNA covalent binding indices, revealed than the decrease in AFB_1-DNA-adduction accounted for about 40% of the observed reduction in tumor incidence at 4000 ppm dietary CHL. This result indicates that the highest CHL dose of 4000 ppm, given during carcinogen exposure, exhibits inhibitory mechanisms amounting to about 60% of the overall protection in addition to blocking of target organ DNA damage.

By contrast, exposure to CHL for nine months following AFB_1 initiation neither suppressed nor promoted tumor response. The proposed blocking mechanism, which involves non-covalent complex formation and reduced carcinogen bioavailability, is expected to be effective toward a wide range of environmental and dietary carcinogens.

INTRODUCTION

Diets rich in fruits and vegetables have been shown consistently to be associated with reduced risk of certain human cancers (Doll, 1990; Farber, 1982). Numerous chemical constituents in fruits and vegetables have been purified and shown to protect against carcinogenesis in experimental animal models (Dragsted et al., 1993, Hayatsu et al., 1988; Wattenberg, 1983), and several candidate chemopreventives are now in clinical trials. The potential anticarcinogenic activity of chlorophyll, the ubiquitous pigment in green plants, is of considerable interest because of its relative abundance in green vegetables widely consumed by humans. Neither chlorophyll nor its food grade derivative chlorophyllin have so far been associated with any human toxicity (Harrison et al., 1954; Siegel, 1960; Young and Beregi, 1980), and both have been demonstrated to exert profound antimutagenic behavior against a wide range of potential human carcinogens (Lai, 1980; Kimm et al., 1982; Barale et al., 1983; Ong et al., 1986; Ong et al., 1989; Sato et al., 1990). Several antimutagenesis mechanisms have been postulated, including tight complex formation with parent mutagens or their activated intermediates (Negishi et al., 1989; Newmark, 1984; Newmark, 1987; Arimoto et al., 1980; Dashwood et al., 1991), premature activation of the mutagenic species away from nucleophilic sites (Romert et al., 1992), scavenging of radicals and active oxygen species (Newmark, 1984; Sato et al., 1986), and suppression or interference with metabolic activation by specific CYP isozymes and other intra- and extracellular drug-metabolizing enzymes (Romert et al., 1992; Imai et al., 1986). Recent studies have confirmed that antimutagenic and

suggested anticarcinogenic properties of chlorophyllin (CHL) also are evident *in vivo* (Chapters 3 and 4). CHL was shown in rodents to efficiently inhibit the pre-carcinogenic DNA-adduction event responsible for initiation of AFB_1- (Dashwood et al., 1991) and IQ- (Dashwood, 1992) induced carcinogenesis. We are aware, however, of no published studies demonstrating inhibition of tumor response by dietary chlorophyll or its derivatives.

The current study was initiated to examine the potential of CHL to inhibit hepatocarcinogenesis induced by AFB_1, a suspect carcinogen present in the human diet (IARC Monographs, 1993). The rainbow trout, a model that we have developed to quantify the relationship between carcinogen dose, anticarcinogen dose, level of target organ DNA-adduction, and final tumor response (Dashwood et al., 1989), was employed in the current study.

MATERIALS

Unlabeled AFB_1 was purchased from Sigma Chemical Company (St.louis, MO) and the tritium-labeled form from Calbiochem Inc. (San Diego,CA). The purity was checked by UV-spectrometry and thin-layer chromatography. Ultra-pure phenol was from Clontech Laboratories Inc. (Palo Alto, CA). All organic chemicals employed were HPLC-grade and supplied by Baker Chemical Company (Phillipsburg, NJ). CHL, Proteinase K, and RNAse A and T1 was obtained from Sigma Chemical Company (St. Louis, MO). The purity of CHL was checked by thin-layer chromatography, as described in Chapter 2. The specific chlorin content was found to be approximately 34%. The dietary CHL levels are given on a (w/w) basis without correction for the actual pigment content.

ANIMALS AND DIETS

Fingerling rainbow trout (9855) of 1.5-2.0 gram body weight were randomly distributed into 73 tanks of 135 fish. The fish were acclimatized for two weeks receiving one daily feeding of control diet (Oregon Test Diet (OTD)). During the following two weeks the fish were fed varying doses of AFB_1 ranging from 10-160 ppb in combination with 0, 500, 2000 or 4000 ppm CHL (Table 6.1). An additional 3 tanks received OTD diet only and 2 tanks per inhibitor dose served as CHL controls and were fed the appropriate CHL-dose only. Another 4 tanks received 20 ppb AFB_1 for 2 weeks followed by 7 days on OTD diet. For the remaining 9 months duplicate groups of animals from this treatment were exposed to either 2000 or 4000 ppm CHL via the diet, to investigate the possible promoting or anti-promoting activity of CHL on AFB_1 hepatocarcinogenesis. All the toxic diets contained 5 ppb radioactive AFB_1 (18.84 mCi/μmole) and varying amounts of unlabeled AFB_1. The diets were prepared by dissolving CHL in 10 ml of water followed by the addition of AFB_1 contained in 50 μl of 95% ethanol. The two compounds were allowed to interact for approximately 5 min before addition of salmon oil, water and drymix (see Chapter 5 for details about the constituents of the trout diet). The final level of radioactive AFB_1 in the diet was determined by digesting 0.2-0.5 g diet samples in 15 ml NCS tissue solubilizer at 50°C, and counting 50 μl aliquots in organic scintillation fluid. All diets were weighed out in individual daily feedings rations and kept frozen at -20°C. Prior to feeding the diets were thawed at 5°C in the dark. The diet ration, fed once a day, was based on 2% (dry weight diet) of the body weight and was estimated immediately

prior to initiating the exposure. During preparation of the diets and feeding, exposure to light was minimized because CHL is highly susceptible to light-degradation.

After 14 days exposure to the various carcinogen and inhibitor combinations 15 fish were randomly selected from each tank. The fish were killed by decapitation, the livers removed, pooled in three groups of five and placed on dry ice prior to storage at -80°C. During the 2 weeks of AFB_1 exposure the fish gained an average 0.53 g and the weight gain was not different among the various treatment groups as determined by analysis of variance ($p > 0.50$). The liver somatic index was likewise not affected by any of the exposure combinations relative to the OTD-control groups ($p > 0.40$).

METHODS

DNA-isolation: DNA was isolated and quantified as described in Chapter 4.

Tumor assessment and classification: One hundred fish or the number of fish less than 100 remaining in each tank nine months after AFB_1 cancer initiation, were anesthetized in MS 222 (1 g/ L) and bled by cutting gill arches. The livers were removed, weighed and inspected with a dissecting microscope to locate tumors present on the surface of the liver, followed by fixation of the livers in Bouins's solution. After fixation the individual livers were hand sliced with razer blades into 1 mm sections to detect additional internal tumors and to retrieve previously seen tumors. Three slides were made per tumor or per non-tumor bearing liver. The tumors were classified according to currently accepted criteria (Hendricks, 1982).

Statistical analysis: The overall tumor response in the present study was somewhat lower than that seen previously with AFB_1 doses in this range (Dashwood et al., 1989). Our study was not designed to accurately estimate the tumor response of 0-2% observed at low AFB_1 doses in this experiment. Graphical and statistical analysis revealed that the data points for these tumor incidences and the corresponding DNA-adduction data were very poorly separated with zero and near-zero tumor responses evident at AFB_1 concentrations of $\leq$ 20 ppb. Accordingly, our statistical treatments were performed on the data obtained from AFB_1 doses of 40 ppb and higher.

The top three doses from each data set were modeled using a iteratively reweighted least squares model based on binomial variance and pooled proportions. Using the assumption of parallel straight lines on the log-logit scale (Dashwood et al., 1989), the relative potencies of CHL to reduce carcinogen-DNA adduction and tumor incidence was compared assuming a constant ratio model.

RESULTS

The covalent binding of dietary AFB_1 to hepatic DNA *in vivo* was examined, and found to increase linearly (R > 0.98) with increasing AFB_1 dietary concentration at the completion of a 14-day initiation exposure (Figure 6.1). Increasing the concentration of CHL in the diet produced a series of curves of decreasing gradient. The slope of each of the curves defined DNA-binding indices (DBI) of 0.168, 0.131, 0.117, 0.105 μmoles AFB_1/mole DNA/ppb AFB_1 for 0, 500, 2000, and 4000 ppm CHL, respectively. The potency of each CHL dose to inhibit AFB_1-DNA adduction is then defined in percentage terms as [100(1 - $DBI_{CHL}/DBI_{control}$)]. Based on this analysis, AFB_1-DNA adduction in liver was inhibited by 22% (95% confidence interval (95% C.I.) = 11-32), 30% (95% C.I. = 22-38) and 37% (95% C.I. = 30-44) at dietary levels of 500, 2000 and 4000 ppm, respectively.

Dietary treatment with CHL during AFB_1 exposure was found to inhibit AFB_1 hepatocarcinogenesis in a dose-dependent manner (Table 6.2). No obvious pathological changes caused by CHL exposure, such as increased mortality rate, altered hepatosomatic index or impaired growth rate, were observed in the present study indicating that CHL toxicity did not contribute to the observed tumor responses (Data not shown). The pathological responses of the various AFB_1/CHL treatment groups are shown in Table 6.3. Since we have never observed an effect of AFB_1 on tumor phenotype distribution, only the highest AFB_1 dose was examined here. The distribution of tumor types seen with 160 ppb AFB_1 (Data not shown) was similar to that reported previously for AFB_1 (Nunez et al., 1990). The lower number of tumors

in the AFB_1-CHL co-fed group tended to skew the distribution of tumors into fewer groups giving higher percentages, but the types of tumors produced still appeared similar that with AFB_1 alone.

The interrelationship between carcinogen dose and final tumor outcome were modeled by two approaches. In the first approach, which was feasible because background tumor incidences were zero and carcinogen-related tumor response did not exceed 50% in this experiment, tumor incidence was found to be linear with AFB_1-dose (correlation coefficient >0.97) when plotted in simple linear coordinants (Figure 6.2). Moreover, each CHL dose successively decreased the number of tumor-bearing animals along the AFB_1 dose-response curve, giving an overall protection against hepatocarcinogenesis of 32% (95% C.I. = 8-50), 36% (95% C.I. = 20-49) and 72% (95% C.I. = 60-80) for CHL doses of 500, 2000 and 4000 ppm, by analysis of slopes as for DNA binding.

We have previously used an alternative and more general analysis based on dose-response TD_{50} values to quantify inhibitor potency (Dashwood et al., 1989). To do this, data from the three highest doses of AFB_1 at each inhibitor level were analyzed on a linearized logit incidence-log dose scale. The curves describing the tumor incidences obtained from the AFB_1-CHL co-fed groups of fish were successfully modeled as straight-line, parallel-offset relationships (Figure 6.2 inset). Each CHL dose displaced the response curve horizontally toward higher AFB_1-doses, as previously seen for indole-3-carbinol anticarcinogenesis (Dashwood et al., 1989). The fact that the response lines were approximately parallel indicates that, over the range of tumor incidences examined (7%-50%), the efficacy of CHL

anticarcinogenesis was independent of carcinogen dose. The AFB_1 doses required for 50% tumor response (TD_{50} values) at each level of CHL were obtained by extrapolation of each data set to 50% incidence, on the basis of constructed equations for the fitted lines. The TD_{50} values for the 0, 500, 2000, and 4000 ppm CHL groups so obtained were 272, 401, 415, and 936 ppb AFB_1, respectively. Percent inhibition of tumorigenesis is then defined as [100 (1 - $TD_{50control}/TD_{50chl}$)] at each dose of CHL tested. The CHL dose-response potency for inhibition of tumorigenesis estimated by this method 32%, 35%, and 71% inhibition at 500, 2000, and 4000 ppm CHL respectively was strikingly similar to the values obtained from the linear regression approach.

We wished to mechanistically determine if CHL-mediated reduction in AFB1-DNA adduction could account totally for CHL inhibition in tumor response. To address this, the correlation between the CHL-induced changes in AFB_1-DNA adduction during initiation and final reduction in tumor outcome at nine months was evaluated by plotting the TD_{50}-ratio ($TD_{50control}/TD_{50chl}$) versus the DBI-ratio ($DBI_{chl}/DBI_{control}$) at each dose of CHL (Figure 6.3). In principal, the data will describe a line of slope unity if CHL inhibition accounted quantitatively for inhibition of tumorigenesis at all doses of CHL tested. However, as seen in Figure 6.3, the data point for the 4000 ppm treatment defining the relationship between TD50-ratios and DBI-ratios lies significantly ($p < 0.0001$) below the theoretical line of unity slope whereas the data point corresponding to the lower CHL doses are not significantly different ($p > 0.40$) from the unity curve. The molecular dosimetry correlation between specific DNA-AFB_1 adduction and tumor response is an alternative means to

address the same issue (inset, Figure 6.3). This illustration also clearly shows that the data points for the control the 500 ppm and 2000 ppm CHL co-fed groups falls along the same line, whereas the data points for the 4000 ppm treatment group are moved somewhat away from the common line.

In addition to these experiments assessing CHL effects on the initiation process, we also examined the effects of CHL treatment after AFB_1 exposure. Chronic post-initiation dietary CHL did not exhibit significant ($p > 0.30$) promotional or anti-promotional activity in this study (AFB_1 only, $3/300 = 1\%$ incidence; 2000 ppm CHL, $1/200 = 0.5\%$ incidence; 4000 ppm CHL, $4/129 = 3.1\%$ incidence).

DISCUSSION

The present study showed for the first time that CHL, a derivative of the abundant dietary constituent chlorophyll, exhibited potent anticarcinogenic properties against the human dietary carcinogen AFB_1. For these present experiments we selected a commercial food-grade CHL preparation such as commonly used in geriatric treatment, which consists of approximately 34% chlorins and 66% salt (Chapter 2). When corrected for salt content, the dose of CHL chlorins required to give an overall protection against AFB_1 carcinogenesis of 70% was less than 1500 ppm. As the concentration of chlorophyll in spinach commonly is in the range of 0.15-6.0% (1,500-60,000 ppm) (Khachik et al., 1986; Khalyfa et al., 1992), the CHL treatments used in this study easily fall well within the range of possible human chlorophyll consumption.

One possible mechanism for CHL blocking of AFB_1 initiation may lie in the ability of CHL to associate with carcinogens having somewhat planar, conjugated characteristics (Negishi et al., 1989; Chapter 5). The formation of such complexes in the diet or in the gut appears to reduce absorption of the carcinogen (Dashwood, 1992; Chapters 4 and 5), thereby lowering the total carcinogen body burden. Analysis of CHL-AFB_1 interaction *in vitro* by fluorescence quenching measurements indeed indicates a relatively strong complex (Kd of 1.4 μM) with a formation that, from stop-flow fluorometry measurements, is very rapid (Chapter 5). This mechanism, however, will account only for the inhibitory component resulting in reduced AFB_1-DNA adduction, which in the current study accounted for the entire protection of

tumorigenesis at dietary doses up to 2000 ppm CHL. Additional mechanisms contributed between 50 and 60% to overall chemoprotection at a dietary CHL-dose of 4000 ppm, visualized by the deviation from the unity line in Figure 6.3. The power of this quantitative anticarcinogenic approach lies in its ability to detect the operation of mechanisms, perhaps unexpected, in addition to or other than simple reduction of carcinogen-DNA damage, and to gain some numerical estimate of their relative importance. The data shown in Figure 6.3 can be taken to indicate a non-linear function (there is no *a priori* reason to assume linearity), suggesting that these secondary inhibitory mechanism(s) gain in relative contribution with CHL dose above 2000 ppm.

Specific properties of CHL elucidated by other researchers which could explain the observed potent non-blocking activity include mitosuppression (Robins and Nelson, 1989; Ghosh et al., 1991) inhibitory activity against several universal proteases (Oda et al., 1971), modulation of DNA-repair processes (Whong et al., 1988), stabilizing effect on lysosomal membranes (Sato et al., 1984) and protection of chromosome damage at fragile sites containing proto-oncogenes (Robins and Nelson, 1989). We currently have no evidence regarding these mechanisms in the trout model.

The lack of promotional activity associated with CHL is in contrast to a recent report of CHL promotional activity in the rat colon carcinogenesis model (Nelson, 1992). The reason for the discrepancy between the two models is not known. It will be important that the reported promotional behavior of CHL be verified and associated mechanisms understood in order to establish the possible human relevance of this observation. We are aware of no reports of tumor promotional activity of natural

dietary chlorophylls. The finding of potent tumor inhibition by doses of CHL comparable to the level of chlorophyll found in green vegetables may have important implications in intervention and dietary management of human cancer risks.
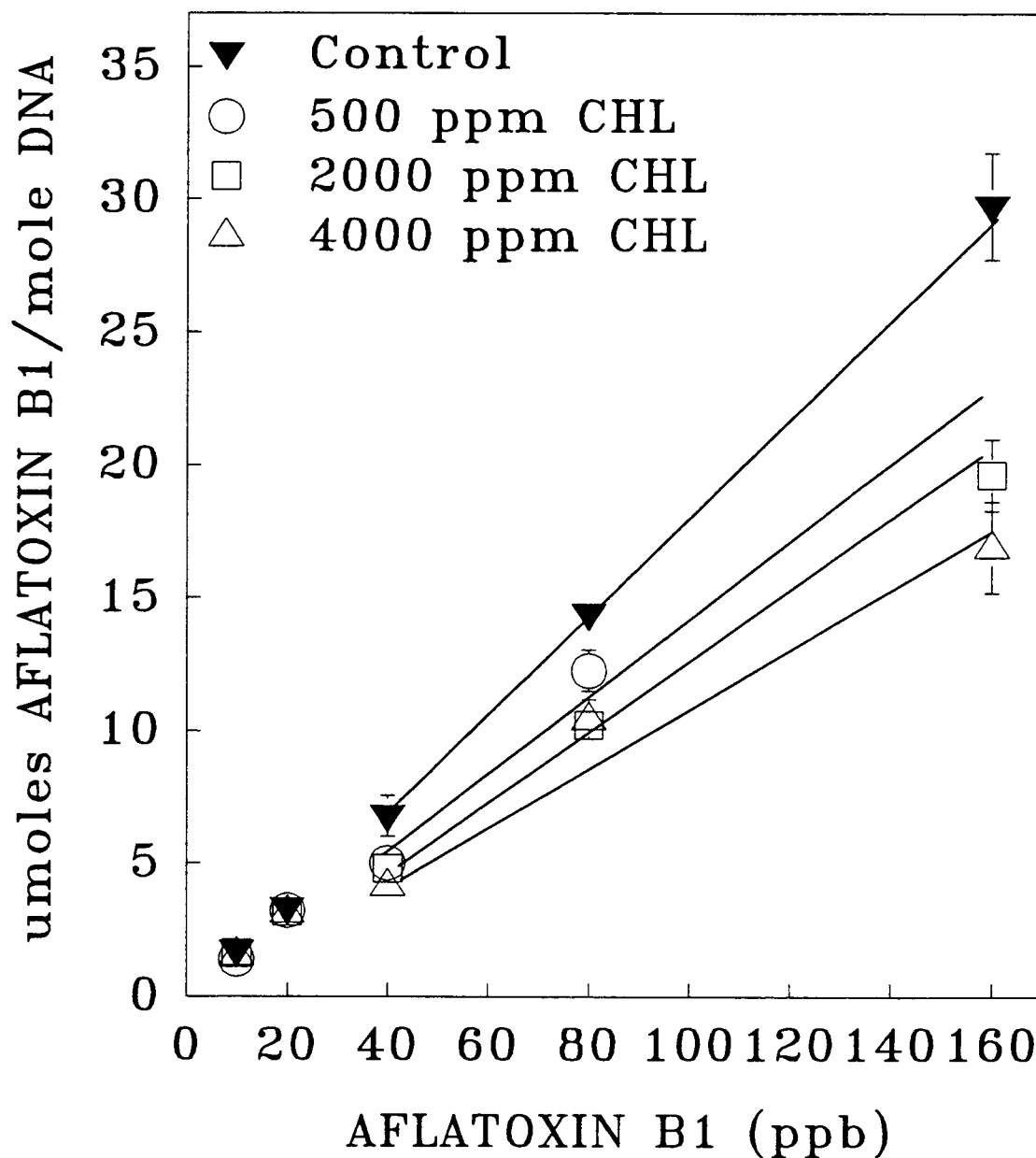
Figure 6.1   Inhibition of AFB_1-DNA adduction by CHL. Data points are geometric means $\pm$ emperical standard error of 6-9 individual determinations.
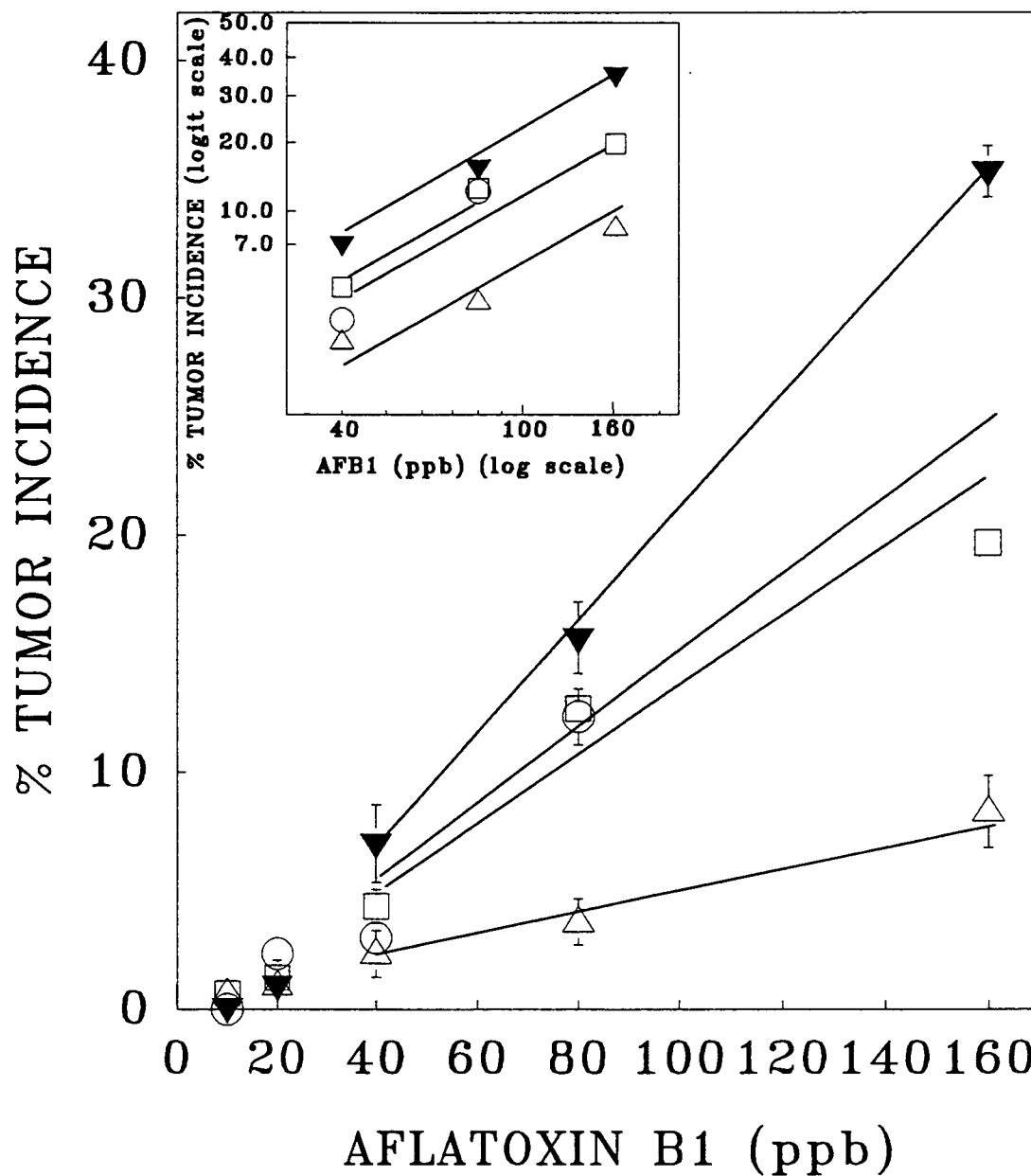
Figure 6.2 Tumor incidence versus AFB₁ dose at varying levels of CHL co-treatment. The points represent pooled proportions with emperical standard errors, obtained from 3 tanks of 100 fish each. The symbols are the same as presented in Fig.6.1. **Figure 6.2 inset** represents the parallel offsets of the inhibitor curves on the log dose-logit tumor incidence scale.
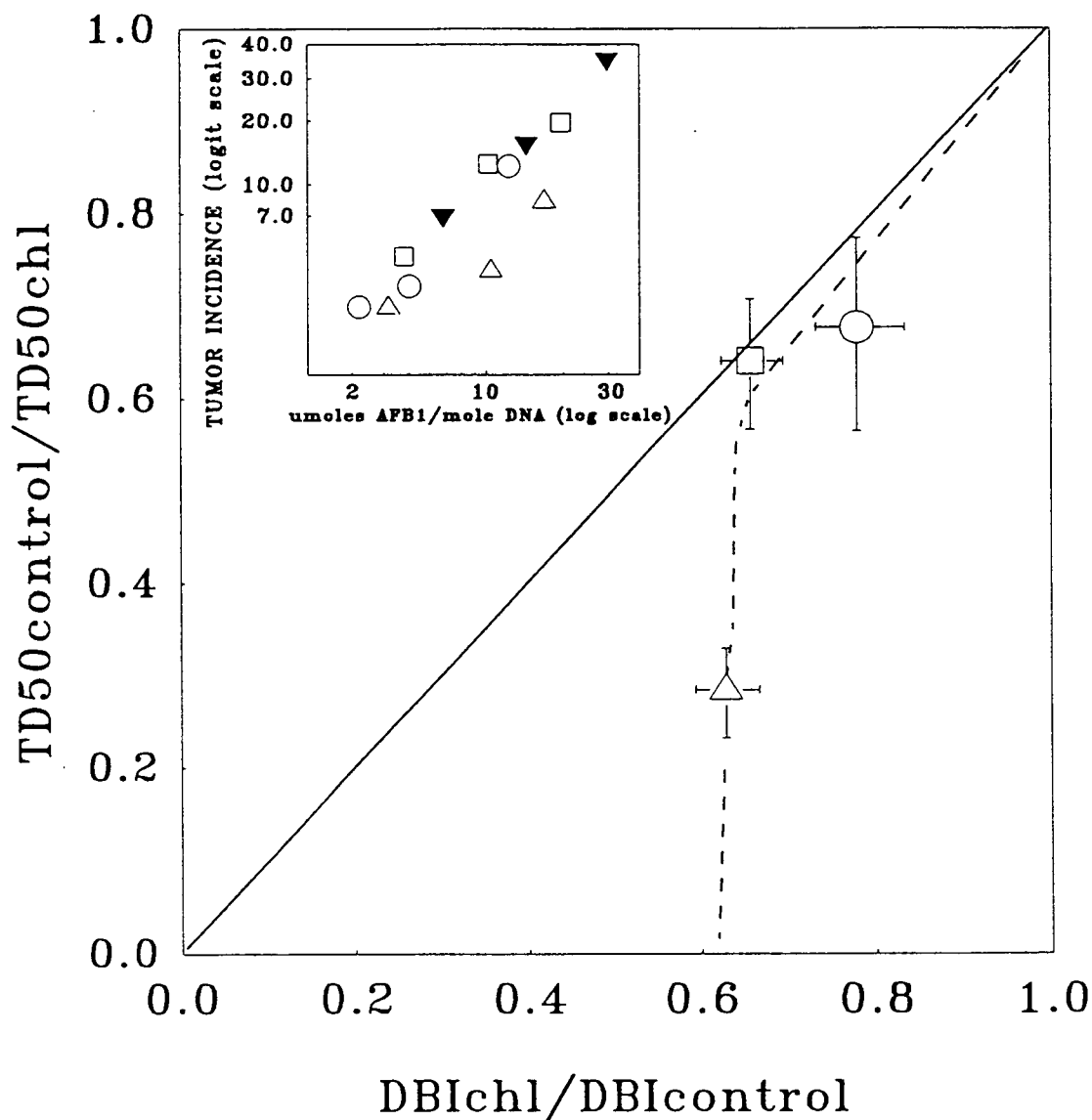
Figure 6.3 Correlation between CHL fractional inhibition of AFB_1-DNA adduction and fractional inhibition of tumorigenesis for various doses of CHL. * : p < 0.0001). See text for definitions. The symbols are the same as presented in Figure 1. Figure 6.3 inset shows the molecular dosimetry between DNA-adduction and tumor outcome.

Table 6.1   Doses of CHL and AFB_1 employed in dose-response studies

Treatment	CHL dose (ppm)	AFB_1 doses (ppb)
1	0	10, 20, 40, 80, 160
2	500	10, 20, 40, 80
3	2000	10, 20, 40, 80, 160
4	4000	10, 20, 40, 80, 160

Table 6.2   Tumor incidence among animals fed varying doses of AFB$_1$ and CHL

AFB$_1$ Dose (ppb)	CHL Dose (ppm)	Tumor incidences (N=100, triplicates)	Average Incidence
0	0	0, 0, 0	
0	2000	0	
0	4000	0	
10	0	1, 1, 0	0.67
20	0	0, 1, 2	1.00
40	0	3, 9, 9	7.00
80	0	12, 17, 18	15.7
160	0	34, 34, 38	35.3
10	500	0, 0, 0	0
20	500	2, 2, 3	2.33
40	500	2, 3, 4	3.00
80	500	10, 12, 15	12.7
160	500	ND	ND
10	2000	0, 1, 1	0.67
20	2000	0, 1, 3	1.33
40	2000	3, 4, 6	4.33
80	2000	12, 13, 13	12.7
160	2000	19, 19, 20	19.7
10	4000	0, 0, 2	0.67
20	4000	0, 1, 2	1.00
40	4000	0, 3, 4	2.33
80	4000	2, 3, 6	3.67
160	4000	6, 7, 12	8.33

REFERENCES

Arimoto, S., Negishi, T. and Hayatsu, H.: Inhibitory effect of hemin on the mutagenic activities of carcinogens. Cancer Letters 11:29-33, 1980.

Barale, R., Zucconi, D., Bertani, R., and Loprieno, N.: Vegetables inhibit, in vivo, the mutagenicity of nitrite combined with nitrsable compounds. Mutation Research 120:145-150, 1983.

Dashwood, R.H., Breinholt, V. and Bailey, G.S.: Chemoprotective properties of chlorophyllin: inhibition of aflatoxin-B_1 (AFB_1)-DNA binding *in vivo* and anti-mutagenic activity against AFB_1 and two heterocyclic amines in the *salmonella* mutagenicity assay. Carcinogenesis 12:939-942, 1991.

Dashwood, R.H.: Protection by chlorophyllin against the covalent binding of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) to rat liver DNA. Carcinogenesis 13;113-118, 1992.

Dashwood, R.H, Arbogast, D.N., Fong. A.T., Pereira, C., Hendricks, J.D. and Bailey.G.S.: Quantitative inter-relationship between aflatoxin B_1 carcinogen dose, indole-3-carbinol anti-carcinogen dose, target organ DNA adduction and final tumor response. Carcinogenesis 10 (1): 175-181, 1989.

Doll, R.: An overview of the epidemiological evidence linking diet and cancer. Proceedings of the Nutrition Society 49:119-131, 1990.

Dragsted, L.O., Strube, M. and Larsen, J.C.: Cancer-preotective factors in fruits and vegetables: Biochemical and biological background. Pharmacology and Toxicology 72(suppl.1):116-135, 1993.

Farber, E.: Chemical carcinogenesis, a biological perspective. American Journal of Pathology 106:271-296, 1982.

Ghosh, A., Sen, S., Sharma, A. and Talukder, G.: Inhibitions of clastogenic effects of cesium chloride in mice *in vivo* by chlorophyllin. Toxicology Letters 57;11-17, 1991a.

Ghosh, A., Sen, S., Sharma, A. and Talukder, G.: Effect oF chlorophyllin on mercury chloride-induced clastogenicity in mice. Food and Chemical Toxicology 29;777-779, 1991b.

Harrison, J.W.E., Levin, S.E. and Trabin, B.: The safety and fate of potassium sodium copper chlorophyllin. Journal of the American Pharmaceutical Association 43:722-737, 1954.

Hayatsu, H., Arimoto, S. and Negishi T.: Dietary inhibitors of mutagenesis and carcinogenesis. Mutation Research 202:429-446, 1988.

Hendricks, J.D.: Chemical carcinogenesis in fish, in Weber, L.J. (ed.), Aquatic Toxicology, Raven Press, New York, pp. 149-211, 1982.

Hirayama, T.: Diet and Cancer: Feasibility and importance of prospective cohort study. In: Eds.: Joossens, J.V., Hill, M.J. and Geboers. Diet and human carcinogenesis. Elsvier Science Publisher B.V., New York.pp 191, 1985.

International Agency for Cancer Research: Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to humans: Aflatoxins, 56:245, 1993.

Imai, K., Aimoto, T., Sato, M., Watanabe, K., Kimura, R., and Murato, T.: Effects of sodium metallochlorophyllins on the activity and components of the microsomal drug-metabolizing enzyme system in rat liver. Chem. Pharm. Bull 34(10):4287-4293, 1986.

Khachik, F., Beecher, G.R. and Whittaker, N.F.: Separation, identification and quantification of the major carotenoid and chlorophyll constituent in extracts of several green vegetables by liquid chromatography. J. Ag. Food Chem. 34:603-616, 1986.

Khalyfa, A., Kermasha, S. and Alli, I.: Extraction, purification and characterization of chlorophyll from spinach leaves. J.Ag. Food Chem. 40:215-220, 1992.

Kimm, S, Tchai, B., Park, S., and Kang, S.: Antimutagenic activity of chlorophyll to direct and indirect-acting mutagens and its contents in the vegetables. Korean Journal of Biochemistry 14:1-7, 1982.

Kimm, S., and Park,S.: Evidences for the existence of antimutagenic factors in edible plants. Korean Journal of Biochemistry 14:47-59, 1982.

Lai, C., Butler, M.A., and Matney T.S.: Antimutagenic activities of common vegetables and their chlorophyll content. Mutation Research 77:245-250, 1980.

Negishi, T., Arimoto, S., Nishizaki, C. and Hayatsu, H.: Inhibitory effect of chlorophyll on the genotoxicity of 3-amino-1 -methyl-5H-pyrido[4,3-b]indole (Trp-P-2). Carcinogenesis 10(1):145-149, 1989.

Nelson, R.L.: Chlorophyllin. an antimutagen. acts as a tumor promoter in the Rat-Dimethylhydrazine Colon Carcinogenesis Model. Anticancer Research 12(3):737-739, 1992.

Newmark, H.L.: A hypothesis for dietary components as blocking agents of chemical carcinogenesis: Plant phenolics and pyrrole pigments. Nutrition and Cancer Vol.6 (1):58-70, 1984.

Newmark, H.L.: Plant phenolics as inhibitors of mutational and precarcinogenic events. Canadian Journal of Physiology and Pharmacy 65;461-466, 1987.

Oda, T., Yokono, O., Yoshida, A., Miyake, K. and Iino, S.: On the successful treatment of pancreatitis. Gastroenterologia Japonica 6:49-54, 1971.

Ong, T., Whong, W., Stewart, J and Brockman, H.E.: Chlorophyllin: a potent antimutagen against environmental and dietary complex mixtures. Mutation Research 173:111-115, 1986.

Ong, T., Whong, J.D. and Brockman, H.E.: Comparative antimutagenicity od 5 compounds against 5 mutagenic complex mixtures in *Salmonella typhimurium* strain TA98. Mutation Research 222:19-25, 1989.

Robins, E.W. and Nelson, R.L.: Inhibition of 1,2-dimethylhydrazine-induced nuclear damage in rat colonic epithelium by chlorophyllin. Anticancer Research 9;981-986, 1989.

Romert, L., Curvall, M., and Jenssen, D.: Chlorophyllin is both a positive and negative modifier of mutagenicity. Mutagenesis 7(5):349-355, 1992.

Sato, T., Ose, Y., Nagese, H. and Kito, H.: Mechanism of antimutagenicity of aquatic plant extracts against benzo[a]pyrene in the *Salmonella* assay. Mutation Research 241;283-290, 1990.

Sato, M., Fujimoto, I., Sakai, T., Aimoto, T., Kimura, R. and Murata, T.: Effect of sodium copper chlorophyllin on lipid peroxidation. IX. On the antioxidative components in commercial preparations of sodium copper chlorophyllin. Chem. Pharm. Bull. 34(6):2428-2434. 1986.

Sato, M., Konagai, K., Kuwana, T., Kimura, R. and Murata, T. Effects of sodium copper chlorophyllin on lipid peroxidation. VII. Effects of its administration on the stability of rat liver lysosomes. Chem. Pharm. Bull. 32(7):2855-2858, 1984.

Siegel, L.H.: The control of ileostomy and colostomy odors. Gastroenterology 38:634-636, 1960.

Wattenberg, L.W.: Inhibition of neoplasia by minor dietary constituents. Cancer Research 43(suppl.):2448-2453, 1983.

Whong, W., Stewart, J., Brockman, H.E. and Ong T.: Comparative mutagenicity of chlorophyllin and five other agents against aflatoxin B_1-induced reversion in *Salmonella typhimurium* TA98. Teratogenesis, Carcinogenesis and Mutagenesis 8;215-224, 1988.

Young, R.W. and Beregi J.S.: Use of chlorophyllin in the care of geriatric patients. Journal of The American Geriatrics Society 28:48-50, 1980.

CHAPTER 7

SUMMARY

Chlorophyllin (CHL), a structural homologue of the green plant pigment chlorophyll, was found to possess potent antimutagenic activities against mutagenesis induced by the dietary carcinogen AFB_1 and two suspected human carcinogens IQ and Trp-P-2. CHL also exhibited inhibitory properties against *in vitro* AFB_1-DNA adduction, mediated by rainbow trout microsomes (Kis = 20.5 μM), in a competitive manner.

Investigation of the ability of CHL to inhibit *in vivo* adduction of AFB_1 to trout liver DNA, which is thought to be the initiating event in AFB_1-induced hepatocarcinogenesis in this experimental model, revealed that CHL afforded substantial protection against carcinogen DNA-damage. By use of specific dietary protocols the potential anticarcinogenic properties of CHL were tested in the rainbow trout model using several doses of both AFB_1 and inhibitor. Comparison of tumor incidences in control and CHL-treated animals revealed that CHL exerted potent antitumorigenic activity when fed with AFB_1, but exhibited neither promotional nor antipromotional behavior when fed chronically after initiation. At a dietary level of 4000 ppm, a fraction of the chlorophyll content in spinach, CHL afforded protection against hepatocarcinogenesis by up to 80% without any detectable toxic side effects.

Molecular dosimetry analysis, comparing AFB_1-DNA binding levels assessed immediately following carcinogen exposure with final tumor outcome 9 months after, suggested that CHL at high dietary doses exhibits protective mechanisms in addition to those responsible for reduction in AFB_1-DNA binding. At lower CHL doses, however, the chemoprotective activities imposed by CHL was completely accounted for by the decreased carcinogen adduction level.

In vitro fluorescence quenching analysis indicated that CHL forms a relatively strong, non-covalent molecular complex with AFB_1 at an approximate one-to-one stoichiometry ($Kd = 1.4 \mu M$). As complex formation in the diet or in the digestive system between CHL and AFB_1 is suspected to decrease the carcinogen bioavailability, this mechanism might account for some of the observed anti-initiating activity associated with CHL dietary administration *in vivo*.

Since the complexation mechanism encounters several other potential human carcinogens it is expected that dietary chlorophyll from spinach and other leafy green vegetables, in particular, might exert protective activities against human cancer. Due to the low toxicity of CHL and chlorophyll, both of these compounds may have important implications in intervention and dietary management of human cancer.