SAFETY AND EFFICACY OF NOVASIL CLAY AS A DIETARY SUPPLEMENT TO PREVENT AFLATOXICOSIS

A Dissertation

by

EVANS AFRIYIE-GYAWU

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2004

Major Subject: Toxicology

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Approved as to style and content by:

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ABSTRACT

Safety and Efficacy of NovaSil Clay as a Dietary Supplement to Prevent Aflatoxicosis. (December 2004)

Evans Afriyie-Gyawu, B.S., Texas A&M University; MPH, Texas A&M University Systems Health Science Center

Chair of Advisory Committee: Dr. Timothy D. Phillips

It is well documented that aflatoxin contamination in foods presents significant economic and public health burdens worldwide. Aflatoxins, particularly aflatoxin B_1 (AFB_1), have been implicated in the etiology of disease and death in many parts of the world, necessitating research initiatives for intervention strategies designed to diminish biological exposure. Calcium montmorillonite clays (e.g. NovaSil Plus, NSP) have been found to tightly bind and inactivate aflatoxins in the gastrointestinal tract of multiple animal species. In the future, the hypothesis is that this strategy may also be appropriate for humans. Thus, the overall research goal was to investigate NSP suitability for human use through *in vitro* characterization followed by *in vivo* evaluation of NSP-AFB_1 sorption and most importantly, safety of the clay.

The first objective was to characterize the *in vitro* and *in vivo* sorption efficiency of NSP-AFB_1 sorption and determine potential interactions with vitamin A (VA). Isothermal analysis suggested that NSP binds AFB_1 with high

capacity, affinity, and specificity in aqueous solution and further indicated that NSP does not appear to interact with VA. Subsequent short-term studies in Sprague-Dawley (S-D) rats and broiler chicks indicated that dietary inclusion of NSP (0.25%) significantly reduced AFB_1 bioavailability without exerting overt toxicity.

The second objective was to evaluate potential adverse effects of chronic ingestion of dietary NSP using male and female S-D rats in the absence of aflatoxins. Although statistically significant changes to a few parameters were noted, the differences did not appear to be NSP- or dose-dependent, suggesting that NSP at dietary inclusion levels as great as 2.0% (w/w) does not produce overt toxicity. Thus, this information increases the feasibility for using NSP in human trials in populations at high risk for aflatoxicosis.

The third objective was to establish representative baseline data on human exposure to aflatoxins by collecting and quantifying urinary AFM_1 in volunteers living in four separate communities in Ejura district of Ghana. Results revealed that urinary AFM_1 in the study population was substantially high (mean = 1,850.86 ± 274.59 pg/mg creatinine), indicating that this particular population was highly exposed to aflatoxins and could be used for future intervention trials.

DEDICATION

This work is dedicated to my family: To my deceased mother, Grace Amoatemah, for her hard work, consistent prayers, love, and unfailing support for my education before she went to be with the Lord. To my wife, Julie, for her constant prayer support, unconditional love, encouragement, and a heart to serve always. Also, to my brothers for their enormous financial support, love, and admonishment. To Mr. & Mrs. Flippen and Dr. Gary & Mrs. Sorensen for their caring attitudes, love, optimism, and overwhelming support.

ACKNOWLEDGMENTS

First and foremost, I would like to earnestly thank the ALMIGHTY GOD for His amazing grace, mercy, and love in seeing me through to this point of my educational career. There was absolutely no way I could have completed my education without the miraculous provisions by God. Through it all, God has been with me and taught me vital lessons for life for which I am ever grateful.

My sincere gratitude goes to my committee members, Dr. Timothy Phillips, Dr. Kirby Donnelly, Dr. Leon Kubena, and Dr. Ed Price for all their guidance, encouragement, and academic contributions. I thank Dr. Donnelly for being such a great dad, supporting me in every step of the way, and his persistence in making sure that I persevere and finish the degree on time, which tremendously encouraged me to work harder each day. I thank Dr. Kittane Mayura who helped me significantly in learning how to conduct animal studies which proved vital after she retired.

I appreciate all my colleagues in the lab (past and present): Henry Huebner, Melinda Wiles, Bhagirathi Dash, Tracie Phillips, Molly Richardson, and John Taylor who were all instrumental to the success of my research. Had it not been the enormous contributions of these individuals, my research could not have been completed to meet the TAMU deadlines. Finally, I would like to thank Dr. John Mackie for the labor intensive histological analysis; Dr. P. Jolly, Dr. W. Ellis, and Dr. R. Awuah for their incredible help in the urine sample collection in Ghana; and Dr. Jia-Sheng Wang for the serum and hepatic vitamin analysis.

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CHAPTER I

INTRODUCTION

1.1 Definition/Overview of the Aflatoxin Problem

Aflatoxins (AFs), perhaps the most well-known class of mycotoxins, are harmful by-products of mold growth produced primarily by the fungi *Aspergillus flavus*, *A. parasiticus*, and, to lesser extent, *A. nominus* (Kurtzman *et al*., 1987; Lopez *et al.,* 2002). Other common mycotoxins that are of public health, agricultural, and economic importance include ochratoxins, trichothecenes, zearalenone, fumonisins, tremogenic toxins, and ergot alkaloids (Huwig *et al*., 2001; Hussein and Brasel, 2001). Table 1 delineates some of the mycotoxins, the affected commodities, and health implications upon exposure. However, the focus of this research is on the burden of AFs and a clay-based enterosorption strategy, for the prevention of aflatoxicoses in humans.

Although members of the *Aspergillus* species are widely distributed, they thrive in regions of high temperature, high humidity, and relatively low moisture content in the substrate. These conditions make the fungi well-suited to colonize a variety of grain and nut crops including corn, groundnut, cottonseed, and tree nuts (CAST, 2003). The naturally occurring AFs, aflatoxins B_1, B_2, G_1, and G_2 (Figure 1), which are characterized as ubiquitous, can contaminate food/feed

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This dissertation follows the style of *Toxicological Sciences.*

TABLE 1

Health impact of common mycotoxins on humans and animals

Mycotoxin	Affected Commodity	Health Impact
Aflatoxins (B_1, B_2, G_1, G_2, M_1, M_2)	Peanuts and peanut products, corn, wheat, rice, cottonseed, nuts, eggs, various foods, dairy products, figs	Hepatotoxicity, bile duct hyperplasia, hemorrhage of intestinal tract and kidneys, carcinogenesis (liver tumors)
Ergot alkaliods	Rye, flour, forage grasses	Neurotoxic, paralysis, convulsions, necrosis, gangrene, decreased serum prolactin levels
Fumonisins	Corn	Leukoencephalomalacia, porcine pulmonary edema, linked to human esophageal cancer
Ochratoxin A	Cereal grains (wheat, barley, oats, corn), dry beans, moldy peanuts, cheese, coffee, raisins, grapes, dried fruits, wine	Tubular necrosis of kidney, porcine nephropathy, liver damage, teratogenesis, kidney tumors
Patulin	Apples, apple juice, wheat, moldy feed	Brain and lung edema, lung hemorrhage, paralysis of motor nerves, antibiotic, convulsions, carcinogenesis
Trichothecenes (T-2, deoxynevalenol, diacetoxyscirpenol)	Corn, wheat	Digestive disorders, oral lesions, hemorrhage of stomach, heart, intestines, lungs, bladder, kidney edema
Zearalenone	Corn, hay	Estrogenic effects (e.g. edema of vulva, uterine enlargement), testicular atrophy, enlargement of mammary glands, abortion

Adapted from the CAST report (2003)

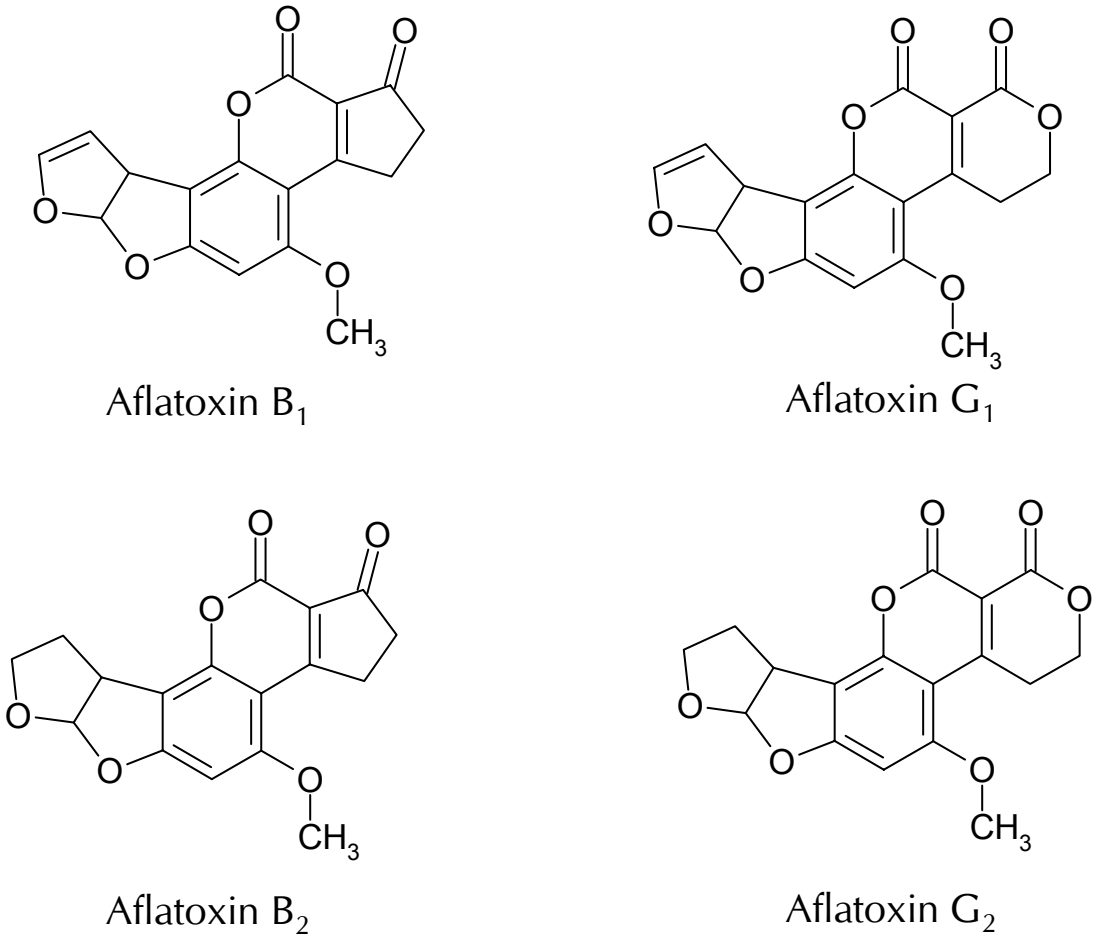

Figure 1. Chemical structures of the four naturally occurring aflatoxins B_1, B_2, G_1 and G_2. The aflatoxins are produced primarily by *Aspergillus flavus* and *Aspergillus parasiticus* fungi, and their series nomenclature denotes a characteristic fluorescence emmission under UV light, that is, (B) blue and (G) green fluorescence.

sources either concurrently or separately (CAST, 1989). Among these metabolites, aflatoxin B_1 (AFB_1) is usually the most predominant in foods/feeds, the most toxic, as well as the most potent hepatocarcinogen known in humans (Busby and Wogan, 1984; Flaherty and Payne, 1997; Lopez *et al*., 2002).

AF contamination in foods/feeds has been longstanding and difficult to control even with good manufacturing practices because AFs are invisible to the naked eye, heat stable, and are not neutralized by common food processing techniques including drying, roasting, canning, cooking, etc (CAST, 1989; Phillips, 1999; Phillips *et al.,* 2002). The compounds are reportedly carcinogenic (Busby and Wogan, 1984; Wild et al., 1992; WHO-IARC, 1993), genotoxic (Wang et al., 1999; Smela et al., 2001), and immunotoxic (Hinton et al., 2003; Turner et al., 2003). In addition, the AFs have been associated with inhibition of protein, enzyme, and clotting factor synthesis (Abdulrazzaq et al., 2004). The toxins can also depress glucose metabolism and fatty acid and phospholipid synthesis. All these effects may lead to teratogenic effects (Di Paolo, 1967) and mutagenicity (Ong, 1975).

Furthermore, the presence of AFs has been found to significantly affect some nutrients such as vitamins A and D (Glahn et al., 1991; Pimpukdee et al., 2004), zinc and selenium (Kalorey et al., 1996; Mocchegiani et al., 1998), and iron status (Dimri et al., 1994). While this may not seriously affect many developed countries where an abundant and safe food supply allows tainted grain to simply be discarded, AF contamination remains a serious threat in many

parts of the world where this grain is either not properly detoxified or is consumed for survival due to limited food supplies (McAlpin *et al.,* 2002). Thus, strategies to limit the occurrence of these compounds in grain or eliminate their presence in the food supply are highly desirable. Also, intervention methods to diminish or prevent biological exposure of humans and animals to the AFs constitute a great need.

1.2 Historical Background

Aspergillus flavus was of little concern prior to the 1960s, although it was reported to be the cause of ear mold in corn as early as 1920 (Taubenhaus, 1920; CAST, 2003). However, in 1960 ingestion of contaminated peanut meal used in formulating livestock feeds on farms near London, England, led to a condition termed Turkey "X" disease that was linked to the deaths of more than 100,000 turkey poults (Blount, 1961). Symptoms included loss of appetite, followed by affects to the animal's gait and ability to stand and, finally, death (Wannop, 1960). Histopathological analysis revealed the most severely affected organ was the liver, but the kidneys, heart, and duodenum were also affected (Siller and Ostler, 1961).

Subsequently, investigations mounted to determine the cause of this unprecedented disease led to the discovery, separation, and conformation of various AFs and their major metabolites (Newberne *et al.,* 1966). The two main series of naturally occurring AFs were categorized as B and G, identified first by

their blue and green fluorescent emissions, respectively (Nesbitt *et al*, 1962). Since this discovery, numerous AF metabolites have also been characterized and reported, including: aflatoxins M_1, M_2, M_{2a}, H_1, P_1, P_1, Q_1, parasiticol, aflatoxicol I, aflatoxicol II, and aflatoxin B_1-8,9-dihydrodiol (Phillips, 1999) (Table 2).

1.3 Physical and Chemical Characteristics

Structurally, AFs are multi-heterocyclic compounds that contain a coumarin structure fused to a dihydrofurofuran (Figure 1) (Wogan, 1969). In the B, M, and Q series, an additional cyclopentenone ring system is adjacent to the coumarin ring, whereas the G series contain a δ-lactone ring adjacent to the coumarin ring. In their nomenclature, the subscript 1 denotes a vinyl ether intact in the terminal furan ring, while subscript 2 indicates a tetrahydrofuran ring (Grant, 1998). The AF analogues with an M notation have a hydroxyl group attached to the carbon between the furan rings.

AFs are heat-stable and invisible to the naked eye (Phillips *et al*., 2002). They are intensely fluorescent in ultraviolet light. Of particular interest, AFB_1 is a white crystalline solid with a melting point of 268-269 °C that emits a pungent smoke and irritating fumes upon decomposition. The compound has a molecular formula of $C_{17}H_{12}O_6$, a molecular weight of 312 g/mol, and is slightly soluble in aqueous solution where the peak absorbance in ultraviolet (UV) light occurs at 362 nm. Also, AFs are soluble in methanol, acetone, and chloroform

TABLE 2

Chemical characteristics of aflatoxin metabolites

Aflatoxin Metabolites	Molecular Formula	Molecular Weight (g/mol)	Melting Point (^{o}C)	Molar Absorptivity (λ/nm)
B_1	$C_{17}H_{12}O_6$	312.06	268-269	21800 (362)
B_2	$C_{17}H_{14}O_6$	314.08	287-289	24000 (363)
B_{2a}	$C_{17}H_{14}O_7$	330.08	240-DEC	20400 (363)
G_1	$C_{17}H_{14}O_7$	328.06	244-246	16100 (362)
G_2	$C_{17}H_{14}O_7$	330.07	230	19300 (365)
G_{2a}	$C_{17}H_{14}O_8$	346.07	190-DEC	18000 (365)
GM_1	$C_{17}H_{12}O_8$	344.28	276	12000 (358)
GM_2	$C_{17}H_{14}O_8$	346.07	270-272	13020 (357)
GM_{2a}	$C_{17}H_{14}O_9$	362.30	195-DEC	19100 (357)
H_1	$C_{17}H_{14}O_7$	330.07	-	-
M_1	$C_{17}H_{12}O_7$	328.06	299	19000 (357)
M_2	$C_{17}H_{14}O_7$	330.07	293-DEC	21000 (357)
M_{2a}	$C_{17}H_{14}O_8$	346.08	240	19300 (358)
MP_1	$C_{16}H_{10}O_7$	314.08	-	-
P_1	$C_{16}H_{10}O_6$	298.05	>320	15400 (362)
Q_1	$C_{17}H_{12}O_7$	328.06	295	18800 (365)
TI	$C_{17}H_{16}O_6$	314.08	234	14100 (332)
TII	$C_{17}H_{16}O_6$	314.08	233-DEC	14100 (325)
8,9-dihydrodiol	$C_{17}H_{14}O_8$	346.07	-	-
parasiticol	$C_{16}H_{14}O_6$	302.08	233-234	9700 (325)

DEC = Decomposed
Adapted from Heathcote and Hilbert (1978), Cole and Cox (1981), and Grant (1998).

but slightly soluble in hydrocarbon solvents (HSDB, 2003). Chemical characteristics of known AF metabolites are listed in Table 2. Additionally, AFs can be degraded by ultraviolet light (Phillips et al., 1994).

1.4 Occurrence of Aflatoxins

1.4.1 Contamination of Aflatoxins in Crops

As noted previously, aflatoxins are produced primarily by the fungi *Aspergillus flavus*, *A. parasiticus*, and, to lesser extent, *A. nominus* (Kurtzman *et al.*, 1987; Lopez *et al.,* 2002). Although members of this genus are distributed worldwide, the fungi are most abundant between latitudes 26° to 35° north or south of the equator (Klich *et al*, 1994). On the basis of these latitudes, it is evident that AFs occur mostly in tropical areas with hot and humid climates, prolonged drought stress, and insect infestation, all conditions that are favorable to the growth and development of molds (Schroeder, 1969; Bhatnagar *et al.,* 2002).

A. flavus and *A. parasiticus* reportedly have overlapping ecological niches and, under favorable pre-harvest conditions, they can produce AFs in developing seeds of corn, peanuts, cotton, almond, pistachio, and other tree nuts (Bhatnagar *et al.,* 2002; CAST, 2003). However, the growth of the *Aspergillus* mold itself does not predicate the presence of the toxin, since production of AFs depends upon the complex interaction of many environmental and nutritional variables, including moisture, temperature, substrates, aeration,

pH, the quantity of both carbon and nitrogen sources, lipids, certain metal salts, and specific nutrient requirements (Ominski *et al*., 1994; Cary *et al*. 2000a; CAST, 2003). For instance, the pronounced stimulatory effect of AF production has been linked to the level of zinc in host species, and has been cited as a contributing factor for the resistance of soybeans (with low levels of zinc) to AF contamination (Gupta and Venkitasubramanian, 1975). In addition, some of these factors may affect gene expression throughout the AF biosynthesis pathway (Bhatnagar et al, 2003).

The process of AF contamination by *Aspergillus flavus* is best characterized in corn. *A. flavus* is a ubiquitous soil inhabitant that reproduces asexually by conidia (Payne, 1998). When the conidia are conveyed to corn silks by wind or insects, they can grow into the corn ear shortly after pollination and colonize the kernel surfaces (Widstrom, 1996; Payne, 1998). Under favorable environmental conditions, the fungus may directly penetrate the seeds and cobs or it may enter through wounds created by insects. In either scenario, significant AF production and contamination do not occur until the moisture content of the kernel is below 32% (Payne, 1998) and continues until the grain moisture reaches 15% (Payne *et al*., 1988). The optimal temperature for the growth of these fungi ranges from 36 to 38 °C (Klich *et al.*, 1994). In field studies, AF contamination in peanuts by *A. flavus* were maximized at 30.5 °C (Cole *et al*., 1995).

1.4.2 Aflatoxin M_1 (AFM_1) in Milk and Urine

In addition to AF contamination in crops, AFM_1, a hydroxylated metabolite of AFB_1 is commonly detected in milk of humans and animals and in urine. Under favorable physiological conditions, cytochrome P450 enzyme, particularly CYP1A2 isoform, is reportedly responsible for oxidizing the parent compound (AFB_1) into the AFM_1 metabolite (Eaton et al., 1994; Wang et al., 1999) (Figure 2). In humans, AFM_1 has been reported to be excreted through breast milk from nursing mothers in tropical countries in Africa and Asia (Somogyi and Beck, 1993) and enhance AF-induced morbidity particularly in children with Kwashiorkor (Adhikari, 1994). In dairy animals, consumption of AFB_1-contaminated rations results in dose-dependent excretion of AFM_1 in milk (Neal, 1998; Chopra *et al*., 1999). Subsequently, AFM_1-contamination may occur in dairy products such as cheese, yogurt, and ice cream (Jones, 1995). Therefore, the U.S. FDA has set a limit of 0.5 ppb for AFM_1 in milk; whereas, the limit for AFB_1, the most toxic and carcinogenic of all the AFs, is 20 ppb for foods intended for human consumption (CAST, 2003). This discrepancy in action limits is due to the fact that milk is consumed as a major source of nutrition in young children.

In addition, several reports have indicated that AFM_1 is dose-dependently excreted in urine following dietary AFB_1 ingestion (Groopman *et al*., 1992a; Hsieh and Wong, 1994; Wang et al., 1999). Other studies have also found that urinary AFM_1 has a dose-dependent association with increased

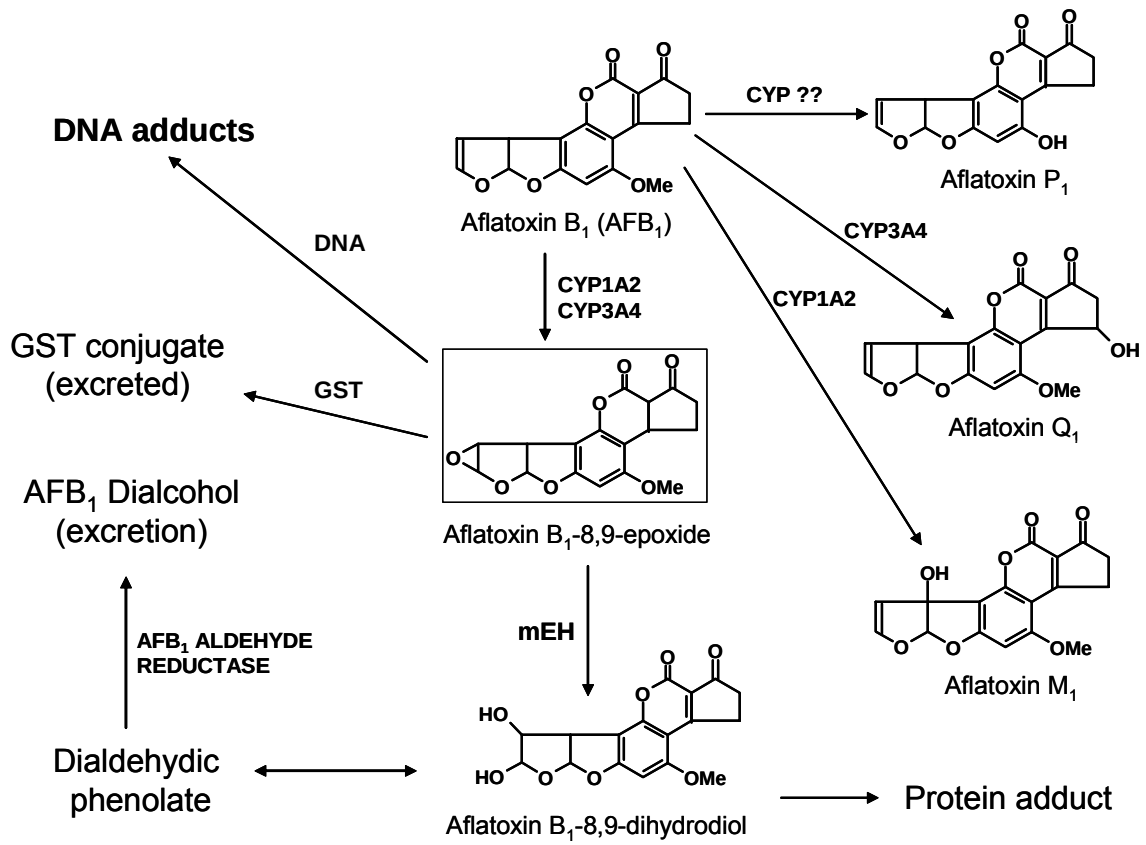

Figure 2. Aflatoxin B_1 (AFB_1) metabolism and the enzymes involved. AFB_1 is activated to the reactive *exo*-8,9-epoxide form by cytochrome P450 (mainly CYP1A2 and 3A4 isoforms) in the liver. The epoxide can react at the N^7 position of guanine in DNA to form the primary AFB_1-N^7-Gua adduct (DNA adduct). Other metabolites such as aflatoxins M_1, Q_1, and P_1 can be formed from different P450 isoforms (Eaton et al., 1994; Groopman, 1994).

relative risk of developing hepatocellular carcinoma (HCC) (Qian et al., 1994; Yu et al., 1997). Thus, monitoring urinary AFM_1 provides for a non-invasive method of AFB_1 exposure quantification.

1.5 Exposure to Aflatoxins

Exposure to AFs occurs predominantly through ingestion of contaminated food/feed, although the compounds can also be absorbed through the lungs and skin (Leeson *et al*., 1995; Coulombe, 1994). In the occupational setting, exposure to AFs in humans is mainly via the respiratory tract upon inhalation of AF-contaminated dust (Coulombe, 1994). In fact, several studies have reported that AFB_1 is virtually always present at higher levels in grain dust compared to those found in contaminated food/feed (Baxter et al., 1981; Sorenson et al., 1981; 1984). Generally, in many parts of Africa and the developing world protective gears are usually not available to farmers and communities that routinely process foods/feeds that may be contaminated by the AFs. In this situation, not only do the people get exposed through dietary ingestion of the toxins, they can also be exposed through dermal and inhalation routes.

1.6 Biotransformation of Aflatoxins

AF biotransformation involves several biochemical processes including oxidation (most importantly, epoxidation) (Essigmann et al., 1977; Groopman, 1994), reduction (Wong and Hsieh, 1978), hydroxylation (Moss and Neal, 1985), and

conjugation (Holeski et al., 1987), and excretion of the parent compound (particularly AFB_1) and its metabolites. Because of the fairly lipophilic nature of the compounds, absorption across the membranes of the gastrointestinal (GI) tract is the most likely entry point for the AFs into humans and animals. From the intestines, the toxins enter the liver through the hepatic portal blood supply (Wilson *et al*., 1985) and are then distributed to most soft tissues and fat deposits, however the majority of the toxins accumulate in the liver and kidneys where biotransformation occurs (Leeson *et al*., 1995).

Specific processes in AF metabolism are best characterized using AFB_1 as a model compound. Figures 2 and 3 represent some relevant biotransformation pathways of AFB_1 as presented by Eaton et al. (1994). In the liver, AFB_1 undergoes metabolism by phase I metabolizing enzymes, primarily cytochrome P450, resulting in the formation of the AFB_1-8, 9-epoxide and hydroxylated metabolites such as AFM_1 and AFQ_1 (Ramsdell and Eaton, 1990), and the O-demethylated AFP_1 (Wong and Hsieh, 1980). Two possible epoxides,

AFB_1-exo-8,9-epoxide and AFB_1-endo-8,9-epoxide, are formed when the 8,9 vinyl group is transformed. The AFB_1-exo-8,9-epoxide has been reported to be three orders of magnitude more genotoxic than the AFB_1-endo-8,9-epoxide (Ueng *et al*., 1995). Cytochrome P450 3A4 is responsible for producing the exo-epoxide form, whereas the 1A2 isoform may produce either the endo- or exo-epoxides. A high concentration of the epoxide may lead to protein- and DNA-adduct formation in the liver (Figure 3).

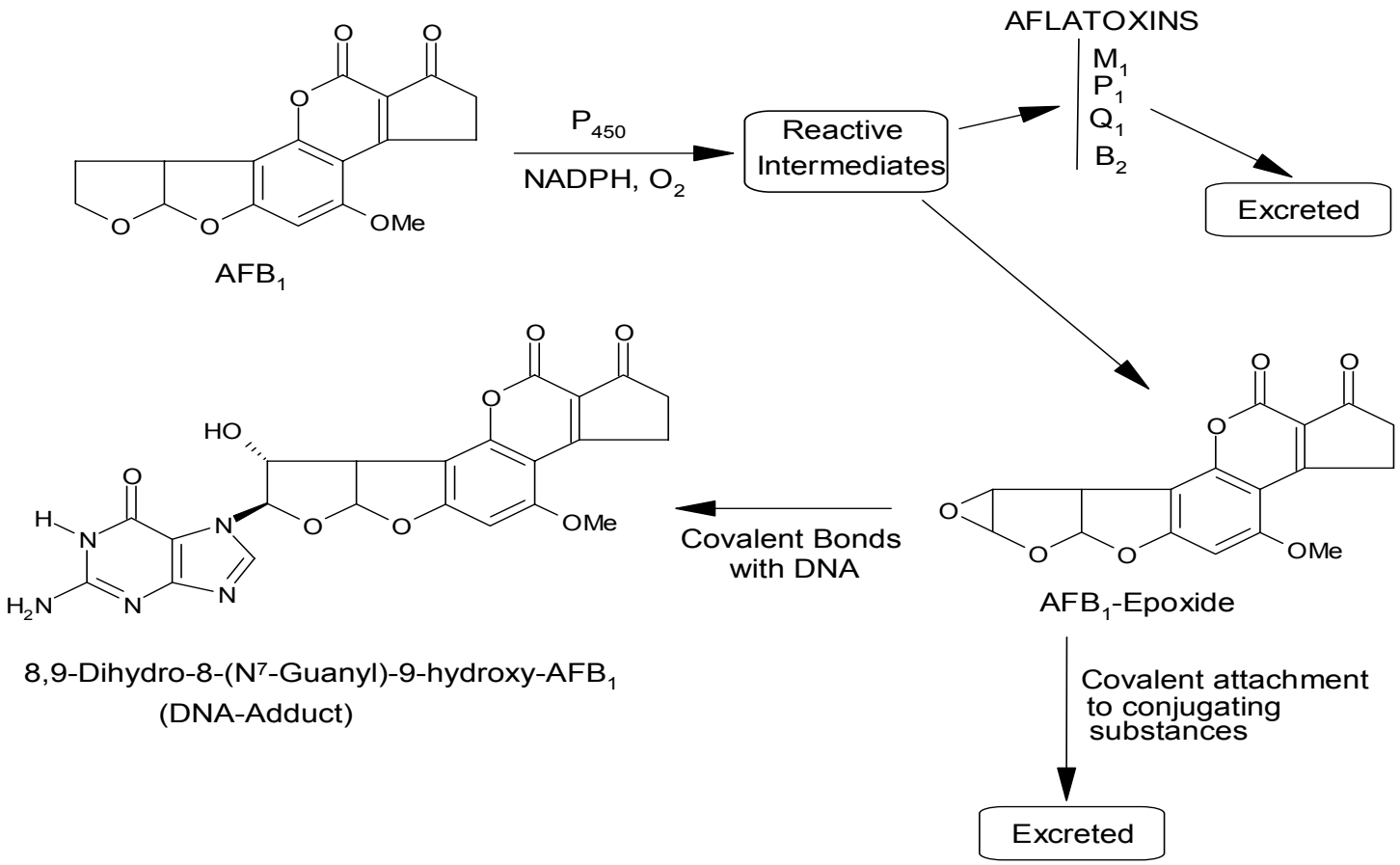

Figure 3. Cytochrome P450 activation of aflatoxin B_1 and interaction with DNA.

Excretion of AFB_1 and its metabolites occurs primarily through the biliary pathway, followed by the urinary pathway. Phase II metabolizing enzymes such as glutathione-s-transferase (GST) and epoxide hydrolase (EH) may convert the epoxide into GSH conjugates or dihydrodiols, respectively. These products may then be excreted (Eaton *et al*., 1994), although a small portion may remain in the body tissues as residues (Leeson *et al*., 1995).

1.7 Toxicity of Aflatoxins

An abundance of scientific evidence implicates AFs, particularly AFB_1, in the etiology of disease and death in both animals and humans (IARC, 1987). In 1993, WHO-International Agency for Research on Cancer evaluated the carcinogenic potential of certain diverse mycotoxins and identified the naturally occurring AFs as human carcinogens (Group I) (WHO-IARC, 1993). *In vitro* evidence indicates that all the naturally occurring AFs (B_1, B_2, G_1, and G_2), as well as AFM_1, produce some form of injury to DNA (Cullen and Newberne, 1994). For instance, AFB_1 reportedly produces chromosomal aberrations and strand breaks, micronuclei, sister chromatid exchange, unscheduled DNA synthesis, and forms DNA-adducts in rodent and human cells used for *in vitro* experiments (IARC, 1987).

Additionally, *In vivo* studies have shown that the liver is the predominant target organ for both AFB_1 toxicity and carcinogenicity (Miller and Wilson, 1994). However, disposition and effects of this AF have been reported in several organ

systems, including the pulmonary, renal, gastrointestinal (especially in the colon), nervous, reproductive, and immune systems in both animals and humans (Coulombe, 1994; Kolars et al., 1994; Larsson and Tjalve, 1995). Specific AF-related adverse effects in animal species and humans are detailed below.

1.7.1 Animal Toxicity

Since the incidence of Turkey X disease (characterized by massive hepatotoxicity in turkeys) in England in 1960, a number of acute dosing studies have been conducted to determine LD_{50} values for a wide range of animal species (CAST, 1989). Reports generated from those studies delineated that the effects of AFs are species-dependent, primarily due to the dependence on metabolic rates, type and duration of exposure, age, dosimetry, and dietary quality or nutritional status (Newberne, 1986; Miller and Wilson, 1994). For instance, duckling, rabbit, rainbow trout, and cats are highly sensitive to AFB_1 (Muller et al., 1970) while a variety of mouse strains were relatively resistant to AFB_1 in carcinogenicity studies (Newberne et al., 1982). In general, young animals are more susceptible than older ones to the toxic effects of AFs (CAST, 2003). However, Newberne (1986) reported that weanling rats are more sensitive than newborn and older animals (1 yr or more) to toxic effects of AFB_1. Also, male rodents are more sensitive to the toxic and carcinogenic effects of AFB_1 than females (Cullen and Newberne, 1994).

AFs have been associated with acute and chronic toxic effects, particularly hepatitis and hepatic cancer, in multiple animal species. Contamination of livestock feed with AFs often results in poor growth and feed conversion efficiency, increased disease susceptibility, and increased mortality rates (Smith and Hamilton, 1970; Jones et al., 1982; Huff et al., 1986). For instance, feeder pigs can suffer weight loss, rough hair coat, anorexia, ataxia, tremors, coma, and death from dietary AF exposure (Coppeck et al., 1989). In dairy cattle, ingestion of contaminated feed has been associated with decreased milk output carrying a metabolite AFM_1 (Hsieh, 1985), tachycardia, and death (Cockcroft, 1995). In turkeys, acute necrosis and bile duct proliferation have been reportedly associated with ingestion of dietary AFs (Lancaster et al, 1961). In dogs, acute aflatoxicosis is clinically manifested by jaundice, abdominal pain, edema, anorexia, GI bleeding, and death (Ngindu et al., 1982). Also, several reports have indicated that, hepatic and renal injuries, GI tumors, urogenital system toxicity, fetotoxity, and liver cancer (i.e. HCC) are linked to dietary AFB_1 exposure in rats that ingested contaminated feed (Clifford and Rees, 1967; Goerttler et al., 1980; Butler and Hempsall, 1981).

Generally, acute and chronic exposure to large doses of AFB_1 can cause liver failure and ultimately, death of the animal, while with low doses, necrosis occurs (Roebuck and Maxuitenko, 1994). Sublethal doses of AFs may lead to chronic toxicity and low levels of chronic exposure can result in cancer (Wogan and Newberne, 1967; Sinnhuber et al., 1977), primarily liver cancer in certain

species (Busby and Wogan, 1984). Other effects of AFs (mainly AFB_1) in multiple animal species include severe hypoproteinemia, hemorrhagic anemia and lesions, impaired blood coagulation, fatty liver, immunosuppression, unscheduled pathologic changes to organs, and teratogenic effects (Doerr et al., 1974; Hayes, 1981; Cysewski et al., 1978; Robens and Richard, 1992; CAST, 2003).

1.7.2 Human Toxicity

1.7.2.1 Acute Toxicity

In developed countries, the levels of AF contamination in foods are typically too low to cause severe aflatoxicosis, however, in less developed countries human vulnerability can vary with age, health, quantity, and duration of AF exposure (U.S. FDA, 2003). It is estimated that ingestion of 2-6 mg AF/day for one month can lead to acute hepatitis and death in humans (Patten, 1981). In humans, AF exposure is manifested primarily as an acute hepatitis and is usually associated with ingestion of highly contaminated foodstuffs, particularly corn and peanuts (Krishnamachari *et al.,* 1975a,b; Shank, 1977; Ngindu *et al.,* 1982). Patients with acute aflatoxicosis typically experience symptoms including jaundice, low-grade fever, GI bleeding, edema, depression, anorexia, diarrhea, fatty liver, ascites, and abdominal pain, and possibly, liver failure, and death (Ngindu *et al.,* 1982; CAST, 2003).

A severe outbreak of acute hepatitis due to dietary AFs occurred in India in 1974. In the outbreak, death occurred in about 25% of the patients that had consumed AF-contaminated corn at levels of 0.25-15 mg/kg (Krishnamachari *et al.,* 1975a,b). Liver tissues obtained from patients that died contained detectable AFB_1 levels, which confirmed their exposure to the contaminated food (CAST, 2003). Another incident, which solidified the evidence of acute toxicity in India, was a fatal hepatitis outbreak in Kenya that was attributed to ingestion of AF-contaminated maize (Ngindu et al., 1982). Additionally, the most severe outbreak of AF poisoning occurred recently in Kenya and was linked to consumption of meals prepared from locally grown and poorly stored maize contaminated with AFs. This outbreak was blamed for 125 deaths, which was 39% of the 317 cases reported from early May to July 20, 2004. Analysis of 31 food samples including maize and maize products from households in the affected communities revealed AFB_1 contamination levels as high as 8,000 ppb (CDC, 2004).

Acute exposure to AFs via contaminated diets has also been linked to Kwashiorkor and Reye's syndrome, both of which are of undefined etiology. Geographically, Kwashiorkor has been associated with the seasonal occurrence and distribution of AFs in foods (Hendrickse et al, 1983). The link was shown to be credible because Hendrickse (1985) evaluated liver tissues taken at autopsy from 36 children with Kwashiorkor and found AFs to be the cause of this human disease. Also, animals given dietary AFs experienced hypoalbuminemia, fatty

liver, and immunosuppression, which are some of the attributes of Kwashiorkor (CAST, 2003). The link of Reye's syndrome (RS) to dietary AFs, however, remains controversial (CAST, 2003). Some manifestations of RS have been associated with AF because the toxin has been found in RS patients in some countries including Thailand, New Zealand, and the United States (Shank et al., 1971; Becroft and Webster, 1972; Ryan et al., 1979). However, cause-effect relationship of AFs with RS has not been adequately established (CAST, 2003).

1.7.2.2 Chronic Toxicity

The most commonly associated effect of chronic AFB_1 exposure is the increased incidence of hepatocellular carcinoma (HCC) (Shupe and Sell, 2004), although lung cancer is also a risk among workers handling contaminated grains (Kelly et al., 1997). HCC is reportedly the fifth most common cancer and the third most common cause of cancer deaths worldwide (Pisani *et al*., 2002). About 80% of the HCC cases occur in developing countries (Wild and Hall, 2000). Internationally, there are geographic variations; a low incidence occurring in the Western world including the US and countries in Western Europe (4 cases per 100,000 people); and a particularly high incidence of up to 150 per 100,000 people in parts of sub-Saharan Africa, China, and South East Asia (Manns and Kubicka, 1997).

Epidemiological studies conducted in Africa and Asia reveal an association between HCC and dietary AF content (Jaimez *et al*., 2000).

Moreover, the combination of AF with hepatitis B and C, which is prevalent in Asia and sub-Saharan Africa, is reportedly synergistic, increasing the relative risk of developing liver cancer to more than 10-fold compared with either exposure (Qian *et al.,* 1994; Jackson and Groopman, 1999; Turner et al., 2000). In addition to the relative risk, the potency of AF in individuals infected with HBV (HBsAg+) is about 30-fold higher compared to that of non-infected (HBsAg-) individuals (Henry *et al*., 2002).

Other adverse effects that have been linked to AF exposure and toxicity include genetic mutation (GC to TA transversions) at codon 249 of tumor suppressor gene p53, DNA damage, AF interaction with RNA and intercellular proteins, and teratogenicity (Wang *et al.,* 1999; Smela *et al.,* 2001; Eaton *et al.,* 1994). In addition, AFs are also associated with stunting growth in children (Gong et al., (2002). It is also reported that even at low levels of exposure, AFs can suppress the immune system (cellular and humoral) and subsequently increase animal susceptibility to bacterial, viral, parasitic, and other fungal infections (Peska and Bondy, 1994; Turner *et al.,* 2003; Miller and Wilson, 1994).

1.8 Molecular Mechanism of Action

1.8.1 Mutagenicity

AFB_1 is reportedly a direct acting mutagen that covalently interacts with DNA (Essigmann, 1977). It is metabolized in the animal liver by the action of

phase I metabolizing enzymes, primarily cytochrome P450, resulting in the formation of the AFB_1-8, 9-epoxide and other metabolites (Figure 2). The cytochrome P450 enzymes are mostly concentrated in the liver but are also found at lower concentrations in nearly every other tissue in the body, including lungs and kidneys (Guengerich and Mason, 1979). The most important metabolite is the AFB_1-*exo*-8,9-epoxide, which is highly reactive and is required in order to damage DNA (Miller, 1978). The *exo*-8,9-epoxide covalently reacts with DNA at the guanine residues of specific sites to form a number of adducts (Essigmann et al., 1983). For instance, the C8 position of AFB_1-exo-8,9-epoxide intercalates into DNA at the N7 position of guanine forming 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy-AFB_1 adduct (Gopalakrishnan *et al*., 1990) (Figure 3).

This adduct can break down into two secondary DNA lesions (i.e. the apurinic site lesion and a more stable AFB_1-formamidopyrimidine (FAPY) adduct) and collectively, may lead to heritable genetic alterations that can enhance malignant transformation of the affected cells (Smela et al., 2001). Although structural variations exist among the AFB_1-induced DNA adducts formed in treated cells, the mutational profile of AFB_1 is dominated by one genetic change: GC to TA transversion. To date, studies in biological systems indicate that GC to TA transversion is the most frequently observed mutation induced by the chemically reactive forms of AFB_1 (e.g. AFB_1-epoxide or other electrophilic derivatives) (Smela et al., 2001). For instance, investigations on AFB_1 mutagenicity involving bacterial mutagenesis using an endogenous *lacI*

gene in an *E. coli* strain (Foster et al., 1983), *ras* gene of rainbow trout liver (Lawrence et al., 1990), *lacI* gene in transgenic C57BL/6 mice and Fisher 344 rats (Gentil et al., 1992), and human liver tumors (Hsu et al., 1991; Bressac et al., 1991) have demonstrated the GC to TA transversion.

1.8.2 Genotoxicity

Laboratory evidence reveals that DNA damage (AFB_1-N^7-Gua adduct formation) in hepatocytes, albumin-adduct formation (Abdoulaye *et al*., 1999), decreased specific antibody production, covalent bonding between nucleic acids and AFB_1, and cell death, occur in humans and animals upon acute or chronic exposure to AFB_1 (Aguilar *et al*., 1993; Roebuck and Maxuitenko, 1994). One of the most important effects of AFB_1 is its mutagenic and genotoxic effect on tumor suppressor gene p53. For instance, codon 249 (at the third position) of p53 gene is a hotspot for AFB_1 modification and AFB_1-induced mutation, specifically AG<u>G</u>C to AG<u>T</u>C (Smela et al., 2001). In regions where AF exposure is high – namely, China, India, South Africa, The Gambia, Mozambique, and Senegal, this mutation has been detected in 44% of the total HCC cases examined showed a predominance of GC to TA mutations (Katiyar et al., 2000; Kirk et al., 2000; Yang et al., 1997; Shimizu et al., 1999) compared to 1% of the HCC cases in regions with low AF exposure – namely, Australia, Europe, Japan, and the US (Shimizu et al., 1999; Katiyar et al., 2000; Vautier et al., 1999; Boix-Ferrero, 1999). Further, a dose-dependent association between AFB_1 ingestion

and codon 249 of p53 mutations has been observed in human HCC from Asia, Africa and North America (Hsu et al., 1991).

In addition to the DNA damage, AFB_1 has been associated with depletion of RNA and intercellular proteins and involvement in teratogenicity in both humans and animals (Wang *et al.,* 1999; Smela *et al.,* 2001). Possible interactions with the latter involve the formation of AFB_1-8,9-dihydrodiol which can react with protein amino groups (e.g. lysine and tyrosine) to form a Schiff base that can destroy the functionality of the protein (Eaton *et al*., 1994). For instance, several studies have reported AFB_1-albumin adduct in the urine and blood of both human and animal studies exposed to AFB_1 (Sabbioni et al., 1987; Wang et al., 1999; 2001). This product is particularly important in that it reveals exposures to AFs over a long period of time in humans and animals (Groopman, 1994).

1.8.3 Tumorigenicity

AFB_1 has also been shown to affect genes involved in tumor growth. Upon administration of AFB_1, hepatocellular tumors in male Fisher rats contained an activated form of a c-Ki-*ras* family proto-oncogene and high expression of the proto-oncogenes c-H-*ras* and c-*myc* (McMahon *et al*., 1986; 1987). Proto-oncogenes stimulate growth in the normal cell, but when mutated, loss of function results in rapid growth characteristic of carcinogenic cells. Tumor suppressor genes generally antagonize this system of uncontrollable cell

growth. Unfortunately, AFB_1-*exo*-8,9-epoxide binds to guanine and causes GC to TA transversions in the p53 tumor suppressor gene (TSG) in human hepatocytes. Thus, this mutation causes the p53 TSG to lose its physiological functionality, thereby making some humans and animals biologically susceptible to AFB_1-induced liver tumorigenesis (Aguilar *et al*, 1993). For instance, Herrold (1969) observed small intestinal tumors in 6 of 10 Syrian hamsters dosed 0.2 mg AFB_1 ip weekly for up to 8½ months. Also, in studying AFB_1 metabolism and activation, Authrup et al. (1980) used cultured colon tissues from rats and humans and found out that these tissues were capable of activating AFB_1 to form DNA adducts. Similar to hepatic and pulmonary system, the main adduct formed in the cultured colon was AFB_1-N7-Gua (Authrup et al., 1979).

1.8.4 Carcinogenicity

Chronic exposure is the most efficient method to produce AF-induced hepatocellular carcinoma in both humans and animals (Roebuck and Maxuitenko, 1994). Thus, AFs have been identified as human carcinogens (Group I) by IARC with AFB_1 included as a primary carcinogenic compound and AFM_1 as a secondary carcinogenic compound (WHO-IARC, 1993). In particular, AFB_1 is the most potent hepatocarcinogen known in humans (Busby and Wogan, 1984; Flaherty and Payne, 1997; Lopez *et al*., 2002). Reports have indicated that the carcinogenic potential of AFB_1 is mediated in part by the mutagenic properties of one or more of the AFB_1-DNA adducts. This effect is

even more severe when the AFB_1 exposure victim is also positive for hepatitis B virus (HBV) due to the synergistic association between these two carcinogens (Kew, 2003). For instance, in evaluating AF exposure in HCC cases in Taiwan, Lunn et al. (1997) reported that people who were exposed to AFB_1 alone had a relative risk (RR) of 17.4 (95% CL = 3.4, 90.3) of developing HCC compared to RR of 67.6 (95% CL = 12.2, 373.2) for those infected with HBV and chronically exposed to AFB_1.

As presented above, the AFB_1-DNA adduct formation (DNA damage), mutation and activation of proto-oncogenes, and especially the mutation of p53 TSG (GC to TA transversion) enhances carcinogenic activities in the affected cells or tissues. It is reported that this mutation abrogates the normal functions of p53, including those in cell cycle control, DNA repair and apoptosis, thereby contributing to the multistep process of hepatocarcinogenesis (Kew, 2003).

1.8.5 Immunosupression Related to Aflatoxin Exposure

Besides their potent toxicity and carcinogenic character, reports indicate that AFs are also immunotoxic in various animal species and possibly humans (Marasas and Nelson, 1987; Wild and Turner, 2002; Hinton *et al*., 2003; Turner *et al*., 2003). Several studies have indicated that even at relatively low exposures, AFs can suppress the immune system (cellular and humoral) and subsequently enhance animal susceptibility to bacterial, viral, parasitic, and other fungal infections (Miller and Wilson, 1994; Peska and Bondy, 1994; Turner

et al., 2003). Most information on AF-related immunotoxicity is generated from studies of farm or laboratory animals.

The effects of AFs on the immune system can be divided into two main categories – 1) effects of cellular responses and 2) effects on humoral factors. AF effects on cellular (cell-mediated) responses include reductions of a) phagocytosis by macrophages, b) delayed cutaneous hypersensitivity, and c) lymphoblastogenesis. The toxin effects on humoral factors include reductions of a) concentrations of serum immunoglobulins (IgG and IgA), b) complement activity, and c) bactericidal activity in serum (CAST, 2003). Suppression of the immune system via cell-mediated responses is particularly sensitive to AF attack as evidenced by reduction of thymus weight and lower numbers of peripheral T-lymphocytes in chickens fed AFB_1 (Ghosh et al., 1990), lower numbers of T-lyphocytes in cattle (Bodine *et al*., 1984; Brown *et al*., 1981), and swine (Liu *et al*., 2002; Mocchegiani et al., 1998).

Specifically, AFs have been shown to: impair immunoglobulins synthesis in chicken and mice; reduce complement levels in guinea pigs (Pier et al., 1989), chickens, and pigs (Kadian et al., 1988); impair phagocytosis and reduce locomotion and bactericidal activity of neutrophils in chickens; decrease monocyte locomotion and phagocytosis in rats, chickens, and rabbits; and reduce several factors involved in cell-mediated immunity in chickens (Kadian et al., 1988), guinea pigs (Pier et al., 1989), turkeys, cows, and mice (Reddy et al., 1987). In a separate study, the number of splenic CD4 (helper T) cells and

interkeukin 2 production significantly decreased in mice upon treatment with AFB1 at a dose of 0.75 mg/kg (Hatori et al., 1991).

In humans, however, evidence of immunosuppression by AFs is limited and uncertain. In a study by Turner et al. (2003), 93% of Gambian children that participated tested positive for AF-albumin adducts in their sera at a range of 5-456 pg/mg. In that study, a multivariable analysis revealed that secretory immunoglobulin A (sIgA) was markedly lower in children with detectable AF-albumin adduct compared to those with nondetectable levels. This adduct, which is reflective of exposure to AFs over a long period of time, and all the animal studies presented above suggest that immunity in humans could also be impaired upon acute or chronic exposure to the AFs.

It should be noted that immunosuppression by a toxicant (e.g. AFB_1) can be enhanced by various mechanisms such as decreased protein and/or DNA synthesis (e.g. AFB_1-DNA adduct or AFB_1-albumin adduct), changes or loss in enzymatic activity, and changes in metabolism or cell cycles, which may result in apoptosis or necrosis. Toxic effects on T-lymphocytes (Dugyala and Sharma, 1996) and/or other lymphoid cells such as the cytotoxic T-cells and natural killer cells (Methenitou *et al.,* 2001), which impair the function of direct or indirect killing of tumor cells, can have pronounced and devastating effects, especially during the progression phase of tumorigenesis (Raisuddin *et al*., 1991; Hinton et al., 2003).

1.9 Impact of Aflatoxins on Selected Nutrients

Chronic AF exposure has been shown to affect nutritional status in multiple animal species. In chickens, AFs have been shown to significantly reduce nutrients including vitamin A (VA) in liver (Pimpukdee *et al.,* 2004) and vitamin D in plasma (Glahn *et al.,* 1991). VA deficiencies have been linked to compromised immune responses and increased disease susceptibility in poultry (Aye et al., 2000 a,b; Dalloul et al., 2002). Vitamin A is vital for vision, controlling the differentiation program of epithelial cells in the digestive tract and respiratory system, skin, bone, the nervous system, the immune system, and for hematopoiesis (Gursu et al., 2002). Because the nutrient induces lymphoproliferation to stimulate the immune system, deficiency has been shown to decrease specific antibody production, the number of circulating lymphocytes, and lymphocyte proliferation (West et al., 1991). VA deficiency is the leading cause of childhood blindness in developing countries, and is a major contributor to childhood morbidity and mortality resulting from common infections (Fieldler et al., 2000).

Iron (Fe) is reportedly essential in the body as a cofactor in several enzymatic and metabolic functions (Lehninger et al., 1997a). Fe is required for the synthesis of the heme proteins including hemoglobin and myoglobin, metalloenzymes, and cytochromes (Nicholls, 1996; George-Gay and Parker, 2003). It is reported that Fe deficiency (FeD) is one of the most prevalent nutritional deficiency problems in the world. FeD has been associated with

anemia, impaired cognitive development, and fitness and aerobic work capacity in humans (Rodgers et al., 2002). In animals, it is reported that AFs have been shown to affect the physiological concentrations of Fe in the serum (Dimri et al., 1994; Harvey et al., 1988), however its potential effects have not been evaluated in humans. Further studies are warranted to evaluate the impact of AFs on Fe levels in humans.

Zinc (Zn) is essential in the body as a cofactor in many enzymatic and metabolic functions (Lehninger et al., 1997a). It promotes health via its role in immunity, cognitive development, and human capacity (Mendez and Adair, 1999). The WHO estimates that Zn deficiency (ZnD) affects about a third of the world's population, although severe ZnD is rare (Rodgers et al., 2002). ZnD can lead to reduced growth rate, skin lesions, impaired immune response, low sperm count, fetal abnormalities, and cognitive impairment (Chesters, 1996; Prasad et al., 2001). The role of AFs in ZnD is not certain in humans but animal data suggest that AFs may promote the extent of ZnD, particularly during early childhood (Kalorey et al., 1996; Mocchegiani et al., 2001). Further studies are needed to determine the extent of AF effect on Zn levels in humans since the impact of ZnD is very significant especially in developing countries.

1.10 Regulatory and Economic Impact

As stated above, consumption of AFB_1-contaminated rations results in dose-dependent excretion of AFM_1 in milk of dairy animals (Neal, 1998; Chopra

et al., 1999). This hydroxylated metabolite of AFB_1, AFM_1, has been identified as a secondary carcinogenic compound (IARC, 1993), although it is two orders of magnitude less toxic than AFB_1 (Hsieh, 1985). Therefore, AFs are strictly regulated in many countries of the world with known standards ranging from 4 – 50 ug/kg (ppb) food intended for human consumption. For instance, Denmark and Germany have a regulatory limit of total AFs set to 4 ppb in human foods whereas the allowed limit is 50 ppb in Taiwan (CAST, 2003). Also, the U.S. FDA has set a limit of 0.5 ppb for AFM_1 in milk while that of AFB_1, the most toxic and carcinogenic of all the AFs, is set to 20 ppb of AF for foods intended for human consumption (CAST, 2003). This means that large amounts of tainted food or food products and milk or milk products may be discarded in developed countries causing substantial economic losses to farmers.

Contamination of foods and feeds with mycotoxins is a significant worldwide problem. It is estimated that about 20 - 25% of the world's food crops, mainly of plant origin, are affected annually in some way by mycotoxins (Fink-Gremmels, 1999; Gilber and Anklam, 2003). Since moldy grain often cannot be sold, farmers usually feed it to their livestock. Consequently, the AF contamination in poor quality grain reduces animal productivity and decreases the food supply in developing nations. The economic losses from lower animal production can lead to even greater poverty, which further enhances the conditions culpable for poor human health (Miller and Marasas, 2002).

Although effort to precisely calculate the economic losses due to AF contamination is usually a difficult task, some estimates have been made in several countries. For instance, a comprehensive economic assessment undertaken by Lubulwa and Davis (1994) revealed that the annual social costs of AFs in Indonesia, Thailand, and the Philippines amounted to almost 1 billion U.S. dollars. This estimate included losses to human and animal health and market impacts. In a recent World Bank study (Otsuki et al., 2001), it was reported that compliance with an AF standard of 2 ppb could lead to an estimated economic loss of about $720 million (in food export only) in African nations. However, in the context of the sub-Saharan regions of Africa, where AF levels are comparable to or worse than the situation in the three Asian nations mentioned above, health losses are likely to present a more severe AF-related impact than the economic aspect because corn is a staple in the human diets (Lubulwa and Davis, 1994).

In the U.S., the potential estimated cost of mycotoxin contamination of crops ranges from $418 million to $1.66 billion, with the mean estimated cost of about $932 million. Additionally, the estimated costs to manage mycotoxins in the United States, including research and testing, are in the tens of millions of dollars. Further, livestock losses and mitigation expenses could increase the mean estimated cost by an additional $6 million and $466 million, respectively (U.S. FDA, 2000; CAST, 2003).

1.11 Intervention Strategies for Aflatoxins

1.11.1 Pre-harvest Intervention

In historical context, it is important to note that the significance of pre-harvest contamination of corn by the aflatoxigenic fungi was largely discounted until 1971, since AF contamination was only associated with poor post-harvest storage facilities and conditions (Eddins, 1930; Butler, 1947; CAST, 2003). It is now known that under favorable conditions, the *Aspergillus* fungi can grow and produce AFs in developing seeds of crops in the field, particularly corn, peanut, and cottonseed. The fungi have been found to be most abundant between latitudes 26° to 35° north or south of the equator (CAST, 2003), primarily in tropical areas including the sub-Saharan Africa, Asia, and South America. As noted above, several studies indicate that the AF biosynthetic pathway is very sensitive to certain environmental, biochemical, and/or genetic influences (Gupta and Venkitasubramanian, 1975; Ominski *et al*., 1994; Cary *et al*. 2000a; Bhatnagar et al, 2003; CAST, 2003). Results of these studies suggest that field strategies could be devised to perturb or disrupt the AF biosynthetic pathway, particularly during the interaction between AF-producing fungi and plants (Bhatnagar et al., 2003).

AF production can be dramatically influenced by environmental factors through direct effects of the fungi or indirect effects on the crop and crop-associated insects and microflora. The factors, which include temperature, humidity, pH, and specific salts or acids common in foods/feeds, can diminish or

prevent AF production through direct effects on *A. flavus*. For instance, at temperatures above 40 ^{o}C, AF production is prevented, although high temperature and favorable associated conditions can enhance the fungal colonization of the crop (Bhatnagar et al., 1994). The direct and indirect environmental influences of fungal contamination on the host also include high temperatures and prolonged drought stress.

During drought and high temperatures, wounded surfaces of corn kernels can be excellent sites for *A. flavus* colonization and possible AF production (Diener et al., 1987). Also, these conditions can reduce the natural defenses of groundnuts by shutting down phytoalexin production, making the pods more susceptible to fungal attack (Hill et al., 1985; Cole et al., 1989). Although producers have no control over these environmental conditions, proper irrigation schemes can help reduce or alleviate this problem if it is economically feasible (CAST, 1989). For instance, a study by Cole et al. (1989) found that AF concentration in edible grade groundnuts grown under drought stress was high whereas edible grade kernels from irrigated groundnuts contained practically no AFs, suggesting that drought stress is a significant contributor of AF contamination in foods/feeds.

Insect infestation also creates an environmental stress to crops, thus making them susceptible to fungal colonization and AF production. Methods to reduce growth of *Aspergillus* fungi and production of these AFs in the field include the application of fungicides and insecticides. Unfortunately, the

fungicidal approach has generally not been effective while crop treatment with insecticides during fungal contamination has shown some promising results in slowing the growth of the fungi (Lisker and Lillehoj, 1991).

Another pre-harvest control measure against fungal colonization and AF production is to select naturally resistant plant species or breeding hybrids, which have desired genetic traits and may impede fungal growth and toxin production (Lisker and Lillehoj, 1991). Conventional plant breeding techniques have made this possible by identifying a variety of corn strains (Gorman and Kang, 1991; Lisker and Lillehoj, 1991) and groundnuts (Mehan et al., 1986) with reduced susceptibility to inoculation and/or AF contamination by aflatoxigenic fungi. Efforts have been made and research continues to develop AF resistant crops, via modifying plant genes to inhibit AF biosynthesis and withstand extreme temperature and moisture conditions (Minto and Townsend, 1997; Ehrlich *et al.,* 1999b; Bhatnagar *et al.,* 2003).

1.11.2 Post-harvest Storage Conditions

Post-harvest contamination occurs mainly when crop drying is delayed and storage conditions favor fungal growth and production of the toxins (CAST, 2003). Conditions that maximize AF production and subsequent contamination of stored grain include a relative humidity greater than 85%, air temperature ranging from 24 to 35 °C (optimum temperature being 30.5 °C), and a grain moisture content of 14-32% (Cole *et al*., 1995; Payne, 1998; Shephard, 2003). It

must be noted that slowly growing fungi may up-regulate the water content of the grain through metabolic mechanisms when the grain moisture falls below 14% (Wilson and Payne, 1994). Therefore, it is essential to allow the grain to mature and dry in the field. However, if conditions do not permit the drying process, the grain can be dried after harvest before storage (Williams, 1991).

Temperature also affects the moisture content in food/feed products. When temperatures are warm, it almost invariably increases the humidity of the storage facility, thereby enhancing fungal contamination. Prevention of the effects of humidity can be achieved when grain is stored in buildings that allow proper aeration. Usually, low and flat buildings with dimensions of the width approximately half of its height are recommended to enhance adequate air flow, keeping temperatures and moisture content in the facility relatively low (Williams, 1991).

Another problem that faces producers is infestation by insects, rodents, and/or birds which consume the stored foods and inflict damage to the remaining grains, making them highly susceptible to fungal attack and toxin production. Control of these pests may be achieved by using storage facilities made with steel or concrete and elevated from the ground. Pesticide application may also be used, but has not been completely effective. In addition, pesticide residues have the potential to adversely affect animal and human health upon exposure (Williams, 1991). As presented above, pre-harvest and post-harvest storage conditions are difficult to control or manage, making AF contamination

unavoidable in foods/feeds (Phillips, 2002). Thus, it is imperative that effective, yet economically feasible decontamination or intervention strategies be devised for the protection of both animal and human health against the harmful effects of these toxic substances. Several methods have been reviewed, all of which are geared towards minimizing contamination of foods/feeds and diminishing biological exposure of humans and animals to the AFs. Below are some of the preventive strategies that are applied or under extensive scientific investigation.

1.11.3 Detoxification Methods for Aflatoxins

1.11.3.1 Physical Methods

The first option of the AF reduction strategies is the physical removal of fungal-damaged kernel/seed/nut from the intact and uncontaminated product (Park, 2002). This includes non-invasive methods such as cleaning, sorting, and handpicking (Dickens and Whitaker, 1975) that do not significantly affect the integrity or texture of the product. In addition, floatation and density segregation methods have been useful in separating the AF-contaminated corn and peanut which are generally less dense compared to the undamaged grain (Cole, 1989). Dry-milling is useful because fungal contamination is usually concentrated on the outside of the kernel so that the highest AF concentrations are found in the germ and hull fractions. Separation of these fractions from the rest of the grain allows for the removal of the majority of AFs (Trenholm *et al*., 1991; Park, 2002). Although these methods effectively reduce AF levels by 40-80%, and are

relatively cost-effective processes, physical removal is not effective to achieve complete removal of the contaminated product (Park, 2002).

Although AFs are relatively heat-stable, thermal inactivation has been used to reduce toxin levels in foods and feeds. The toxins are not completely destroyed when boiled in aqueous solution, autoclaved, and processed utilizing a variety of food/feed processing techniques (Christensen, 1977). Several studies have indicated that roasting is a good method used to reduce AF levels in certain commodities, including oil and dry-roasted groundnuts (Peers and Lindsell, 1975), microwave roasted groundnuts (Luter et al., 1982), and corn and coffee (Conway *et al*., 1978; Levi, 1980). For instance, Conway and co-workers reported that roasting corn at temperatures ranging from 145 to 165 ^{o}C reduced AFB_1 levels by 40-80%. In a recent study, the process of toasting AF-contaminated maize samples at 285 ^{o}C reportedly reduced AF levels by a maximum of 81% (Mendez-Albores et al., 2004).

1.11.3.2 Chemical Inactivation of Aflatoxins

Several chemical methods of AF detoxification have also been used and include ammoniation, ozonation, and nixtamalization. Ammoniation involves subjecting contaminated food products (e.g,. corn, peanuts, or cottonseed) to ammonium hydroxide or gaseous ammonia and heat to hydrolyze the lactone ring (Park and Price, 2001). Several ammonia-based procedures have been developed and studied but the high pressure/high temperature method utilizing

ammonia (0.5-2.0%) under controlled conditions of moisture (12-16%), pressure (45-55 psi), and temperature (80-100 ^{o}C) for 20-60 min has been shown to be the most efficient and produces a relatively safer product (Park, 2002). If ammoniation is allowed to proceed for a sufficient time period decarboxylation will occur and the process will form less toxic compounds (Park et al., 1988). Whether these compounds can exhibit toxicity in humans or animals however has not yet been clearly established. However, it is reported that the ammoniation process subjects AF molecules to chemical modification into compounds possessing reduced or non-detectable toxic or mutagenic potentials. The same report indicated that the presence of identified AF/ammonia reaction products, including aflatoxin D_1 and a compound with molecular weight of 206 g/mol, in animal feeds exert no health impact on animals (Park, 2002).

Nixtamalization, the alkaline treatment of corn as part of the process of making tortillas, has also been reported to significantly reduce AF levels (Ulloa and Herrera, 1970), although AFB_1 may be reformed upon acidification (Price and Jorgensen, 1985), making this process inadvisable. Ozonation may also reduce AF contamination (McKenzie *et al*., 1997). In one study, ozonation of contaminated corn effectively eliminated AF toxicity in turkey poults without affecting the nutritional value of the feed (McKenzie *et al*., 1998).

Dietary inclusion of specific chemicals or nutrients has also been shown effective in the reduction of AF toxicity. As an example, inclusion of vitamin A has been shown to inhibit the formation of AFB_1-adduct and phenobarbitol may

increase the rate of natural detoxification processes (Leeson *et al*., 1995). In addition, vitamin E has also been shown to ameliorate AF-induced alterations in steroidogenesis in mice. Further, it has been suggested that vitamin E may reduce AF toxicity by the reduction of both oxidative damage and bioavailability of the toxin (Verma and Nair, 2002). In a separate study, vitamin E was found to possess a high affinity for AF and it acts by forming a stable association with it, thus reducing the bioavailability of the toxin (Odin, 1997). Finally, dietary inclusion of clay minerals, which is of particular interest in this research, has proved effective for binding AF in the gastrointestinal tract of animals, thus reducing toxin bioavailability and distribution in the blood and target organs (Phillips et al., 1988; Phillips, 1999). Since this detoxification method was discovered (Phillips et al., 1988), many scientific studies have focused on finding clay minerals and other sorbents for research on mycotoxin decontamination in foods/feeds.

1.12 Clay Research

1.12.1 Clay Mineral Structures and Chemistry

Solid particles of soil are grouped into three categories, including sand, silt, and clay, based on their sizes. Generally, the particle size of clay minerals is less than 2 μm, while those of silt and sand range from 0.002 – 0.05 mm and 0.05 – 2 mm, respectively (Sylvia et al., 1997). Minerals are divided into classes based on the chemical identity of the dominant anion or anionic group. Classes of

major mineral groups include: sulfides, sulfosalts, oxides and hydroxides, halides, carbonates, nitrates, borates, phosphates, sulfates, tungstates and silicates (Hurlbut and Klein, 1977). Within these classes, minerals are further subdivided depending upon structural similarities (Schulze, 1989). On the basis of both the number of mineral types formed and their overwhelming contribution to total mass of the earth's crust, silicates are the largest class of minerals identified (Schulze, 1989).

The basic structural unit for the silicates is a SiO_4 tetrahedron in which Si^{4+} is located at the center and four O^{2-} are positioned at the apices. The tetrahedral structures can be linked together by sharing four O^{2-} ions and together can form a variety of more complex structures including rings (cyclosilicates), chains (inosiliates), sheets (phyllosilicates) and three-dimensional arrangements (tectosilicates) (Schulze, 1989). It should be noted that tetrahedra (e.g. SiO_4) and octahedra (e.g. Al_2O_3) (Figure 4) are commonly found as structural components in many mineral structures (Schulze, 1989).

1.12.2 Phyllosilicate Clay Minerals

The phyllosilicate clay minerals contain both tetrahedral (Figure 5) and octahedral sheets (Figure 6). The tetrahedral sheets are composed of SiO_4 tetrahedra are arranged such that three O^{2-} ions of each tetrahedron are shared with the three adjacent tetrahedral components. This O^{2-} ion arrangement

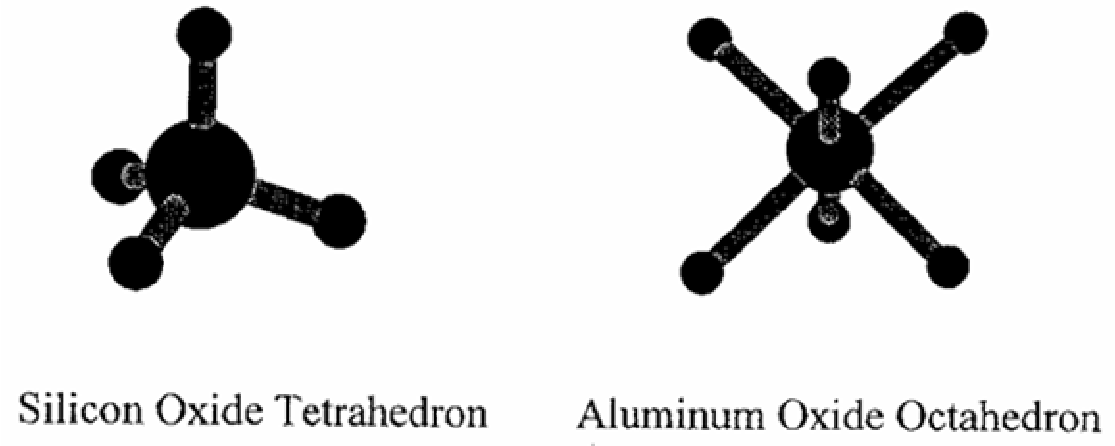

Figure 4. Structural representation of the arrangements of silicon oxide tetrahedral and aluminum oxide octahedral basic units of phyllosilicate clay minerals. The three dimensional structures were drawn using HyperChem v 7.0 software based on AM1 energy minimized molecules. Adapted from Schulze (1989).

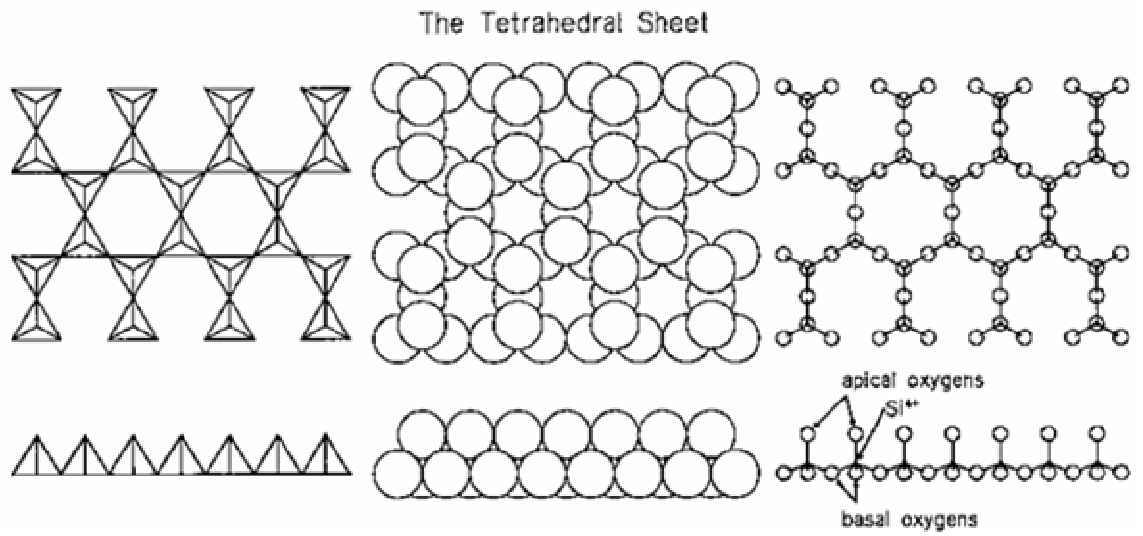

Figure 5. Structural representation of tetrahedral sheets found in phyllosilicate clay minerals. Silicon oxide basic units are arranged in such a way that they form the tetrahedral layer. Adapted from Schulze (1989).

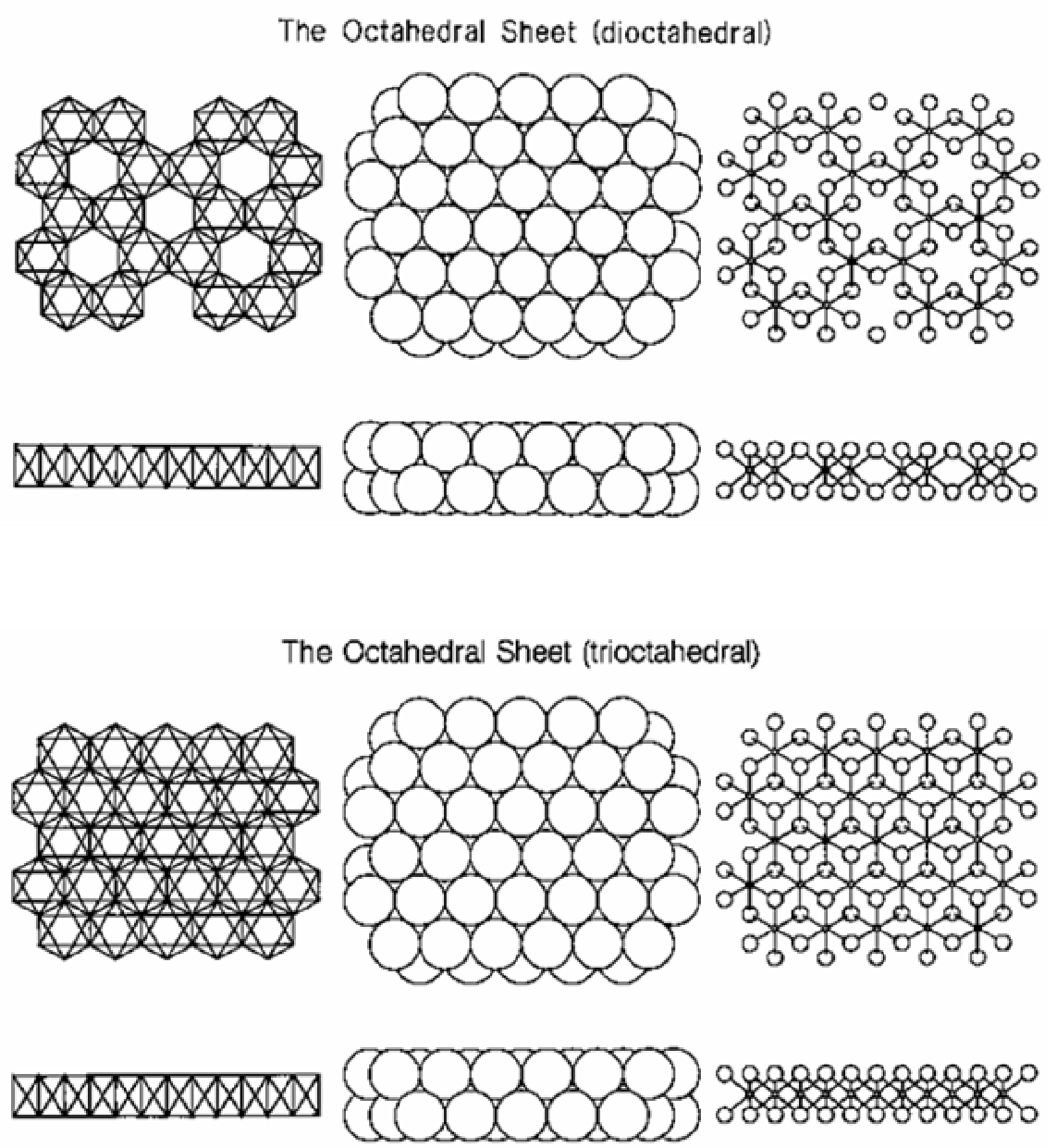

Figure 6. Structural representation of octahedral sheets found in phyllosilicate clay minerals. Metal oxide basic units are arranged to form the dioctahedral layer (top), in which 2 out of every 3 octahedral sites are filled by a trivalent cation (e.g. Al^{3+}); and trioctahedral layer (bottom), in which 3 of every 3 octahedral sites are occupied by a divalent cation (e.g. Mg^{2+}). Adapted from Schulze (1989).

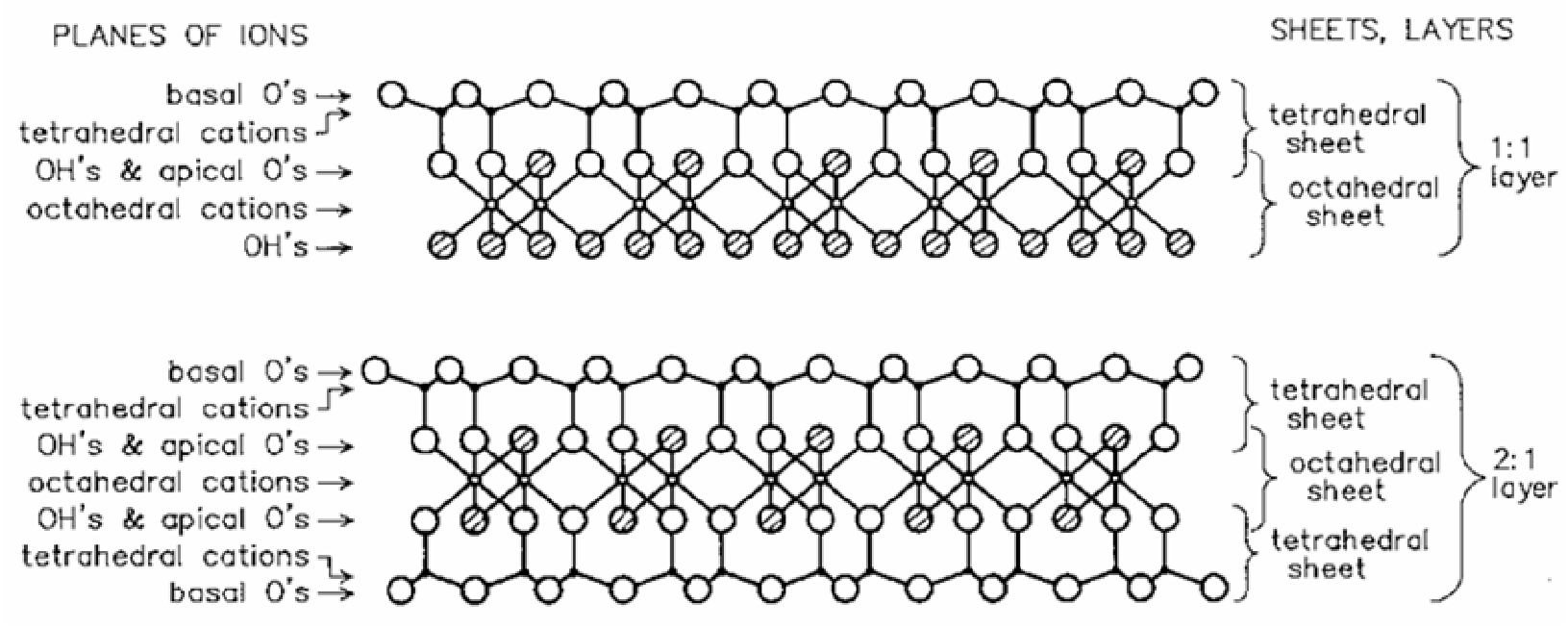

Figure 7. Structural configuration of the linkage between silicon oxide tetrahedral and aluminum oxide octahedral sheets forming either 1:1 or 2:1 layer lattice structures in phyllosilicate clay minerals. Adapted from Schulze (1989).

extends in all directions at the same plane, and the oxygens involved are referred to as basal oxygens. The fourth O^{2-} ion of each tetrahedron is not shared with another SiO_4 tetrahedron, and is referred to as the apical oxygen and is free to bond to other elements. Structures in which all the apical oxygens of a single sheet point to the same direction are the most common but structures can also occur in which these oxygens point to alternate directions. Minerals that contain this sheet-like structural arrangement of the SiO_4 tetrahedra are categorized as the phylosilicates or sheet silicates (Schulze, 1989).

The octahedral sheet consists of two planes of hydroxyl (OH^-) groups that form a hexagonal closest packing arrangement. In order to neutralize the negative charge of this structure, cations are located in the octahedral sites between the layers. There are two possible ways of filling the octahedral sites depending upon the valence of the cation involved. First, a divalent cation (e.g., Mg^{2+}) must fill every available octahedral space to produce a trioctahedral arrangement (Figure 6). In this case three of every three octahedral sites are filled, giving a formula of $Mg_3(OH)_6$ or $Mg(OH)_2$, thereby keeping the sheet electrically neutral since charge is balanced within the sheet. A second possibility is when a trivalent cation (e.g., Al^{3+}) fills only two of every three possible octahedral sites to produce a dioctahedral arrangement (Figure 6). To balance the charge and keep the sheet electrically neutral, each Al^{3+} ion is effectively surrounded by three OH^- ions to give a formula of $Al(OH)_3$.

In phyllosilicate minerals, the apical oxygens from the SiO_4 tetrahedral layer can replace OH^- groups from the octahedral layer and coordinate with the metal cation of the octahedral layer, thus linking the octahedral and tetrahedral layers. The tetrahedral-octahedral bonding arrangement commonly occurs in one of two ways depending upon the number of tetrahedral and octahedral sheets involved in the layer structure. The 1:1 layer structure occurs when one tetrahedral layer is bonded to one octahedral layer, and the 2:1 layer structure also occurs when an octahedral layer is bonded to two tetrahedral layers, one on each side of the octahedral layer. Figure 7 depicts the structural configuration of tetrahedral and octahedral sheets in the phyllosilicate clay minerals. Examples of the 1:1 layer structure type phyllosilicates include kaolinites and halloysite, while the 2:1 type includes talc, pyrophyllites, micas, vermiculites, smectites, and chlorites. Figure 8 is an illustration of structures of selected phyllosilicate minerals (Schulze, 1989).

The phyllosilicate mineral series are grouped by the amount of substitution and therefore, charge per layer. It is reported that among the 2:1 phyllosilicates, talc and pyrophyllite have almost no substitution and therefore very little charge; smectites have a layer charge per formula unit of 0.25 to 0.6; vermiculites have 0.6 to 0.9; and micas have complete substitution, resulting in a charge of 1 per formula unit (Bohn *et al.*, 1979). Selected mineralogical properties for several phyllosilicate minerals are presented in Table 3. The kaolinites have 1:1 layer structure and are electrically neutral. Adjacent layers

TABLE 3

Summary of some properties of selected silicate minerals and organic matter

Mineral Group	Layer type	General Formula	CEC [1] (cmol/kg)	Surface area (m^2/g)	Expansible	Colloidal activity
Kaolinite	1:1	$Al_2Si_2O_5(OH)_4$	1 - 10	10 - 20	No	Low
Montmorillonite	2:1	$Na_x\,[(Al_{2-x}\,Mg_x)Si_4O_{10}(OH)_2]$	80 - 120	600 - 800	Yes	High
Vermiculite	2:1	$Na_x\,[(Mg_3)(Si_{4-x}\,Al_x)O_{10}(OH)_2]$	120 - 150	600 - 800	Yes	High
Mica	2:1	$K_x\,[(Al_4)(Si_{4-x}\,Al)O_{10}(OH)_2]$	20 - 40	70 - 120	No	Medium
Organic Matter [2]	--	--	100 - 300	800 - 900	--	Medium

Note. Data presented were summarized from Bohn *et al.* (1979).
[1] CEC denotes the cation exchange capacity range.
[2] Properties for organic matter are shown for comparative purposes

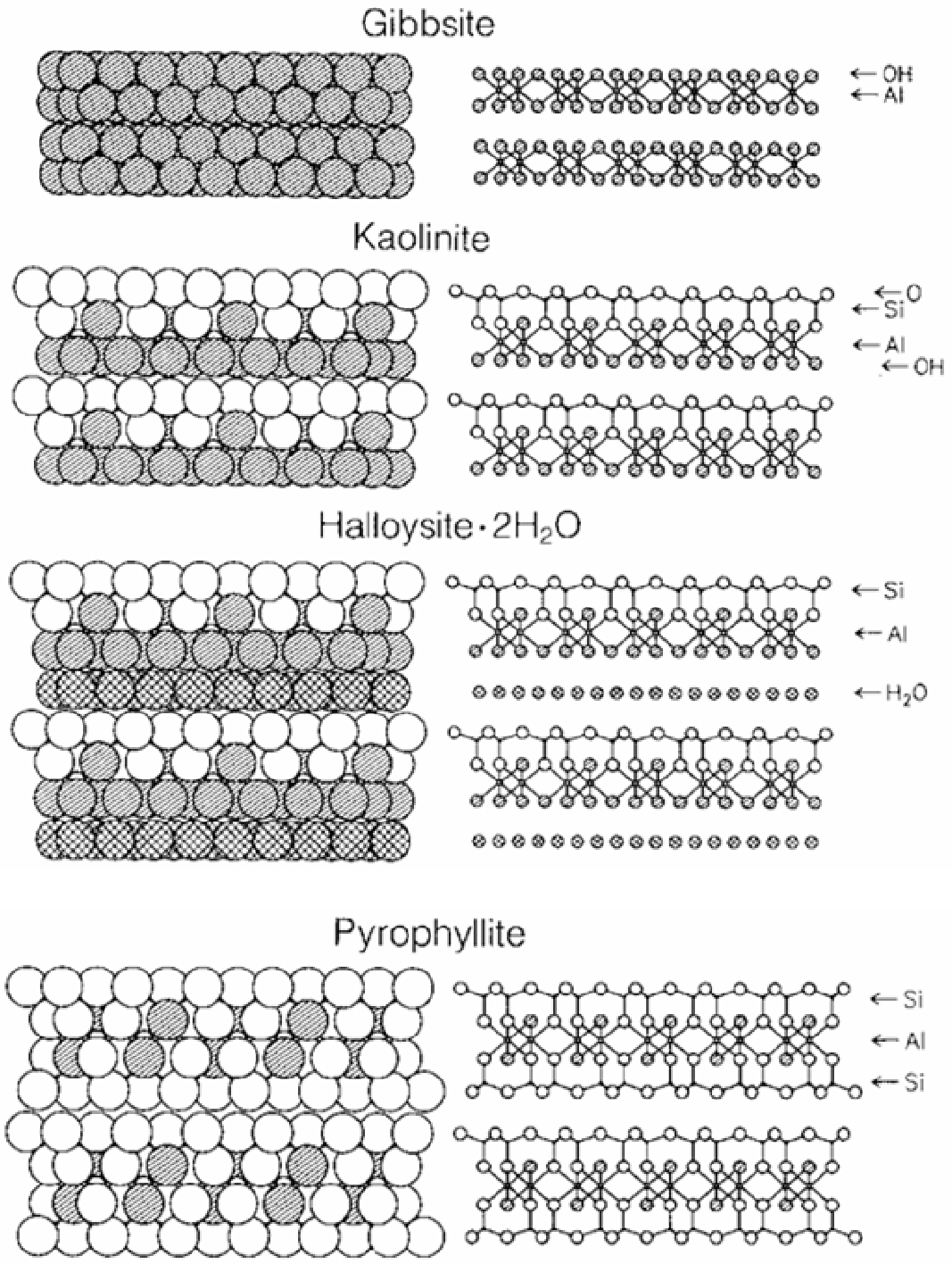

Figure 8. Structural representation of common phyllosilicate minerals. Adapted from Schulze (1989).

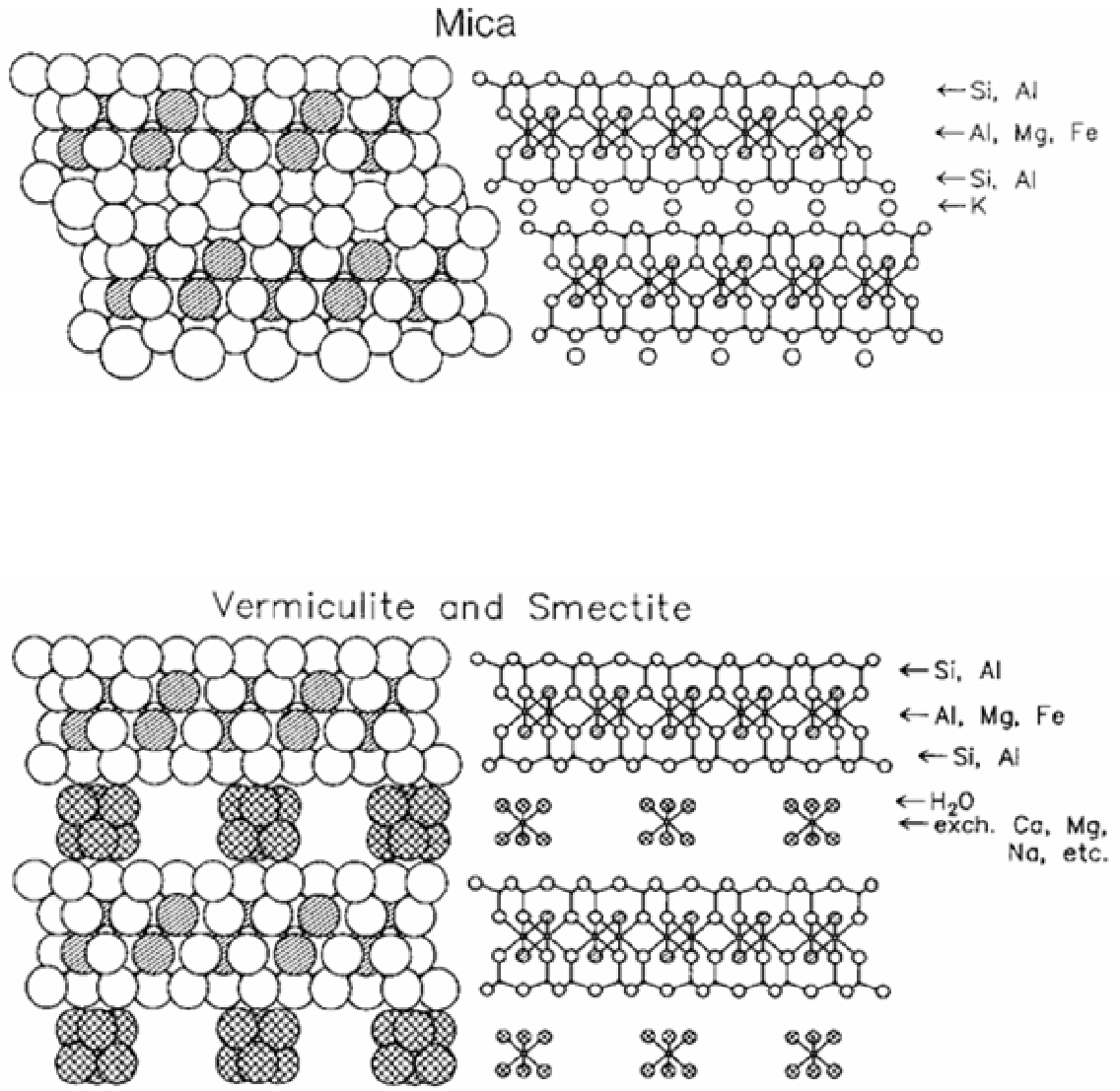

Figure 8 (*Continued*).

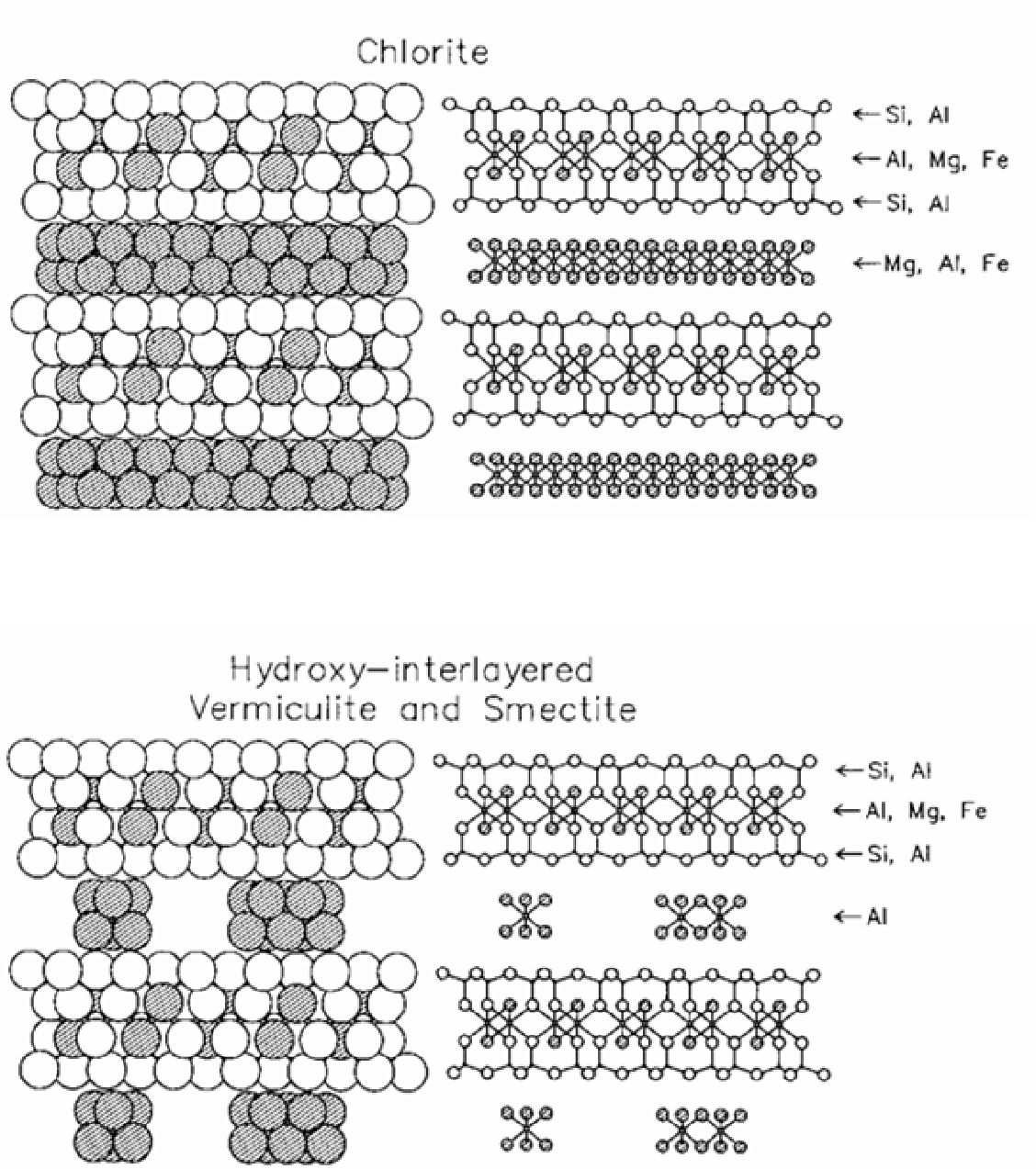

Figure 8 (*Continued*).

are held together by electrostatic bonding between the basal oxygens of the tetrahedral sheet and the OH^- ions of the octahedral sheet. Kaolinite is a common mineral in the soil and in fact, is the most common member of the 1:1 type phyllosilicates. These minerals have very little isomorphous substitution in either the tetrahedral or the octahedral sheets, thereby making their cationic exchange capacity and surface areas very low. The dioctahedral smectites are the most common in soils among the smetitic group of minerals. These smectites are very important minerals in soils found in temperate regions due to their high surface area and adsorptive capabilities. One property that is relevant is the fact that smectites swell upon wetting and shrink upon drying. Of particular significance to this research, it must be noted that the optimum swelling properties smectites are primarily due to the interlayer charge after substitution. Other phylosilicate minerals include chlorites, palygorskite, and sepiolite, which are also 2:1 layer structures (Schulze, 1989).

By virtue of industrial applications, the phyllosilicate clays are unique in that they have been integrated into various production processes. Reports have indicated that kaolinites are widely used in ceramic industry and serves as a filler and coating for paper. Similarly, vermiculite, which becomes very light and porous when heated, is used as filler for concrete in building and also serves as a thermal and sound insulator. Montmorillonites have been employed in industry as decolorizing agents and for filtration of water and food products (Millot, 1979).

The functionality of this class of minerals is a result of the distinctive structural and chemical properties of silicate layers.

NovaSil Plus (NSP), a calcium montmorillinite (CM) clay, is a 2:1 layer-lattice phyllosilicate clay. These 2:1 types of clay consist of layers containing one octahedral alumina sheet between two tetrahedral silica sheets. Structural morphology of the 2:1 layer-lattice dioctahedral montmorillonite clays shows that SiO_4 tetrahedra are linked together, with each tetrahedron sharing three O^{2-} ions with three adjacent tetrahedra. This arrangement extends in all directions to form a plane of basal oxygens. Al^{3+} ions are octahedrally coordinated with SiO_4 sheets, in the center of clay platelets, a surface characteristic that limits the bioavailability of Al (Phillips *et al*., 2002). The montmorillonites are a subclass of smectites that contain isomorphic substitution in the octahedral sheets, resulting in a general formula of $Na_x[(Al_{2-x}Mg_x)Si_4O_{10}(OH)_2]$. These clays are naturally abundant and have surface areas as high as 800 m^2/g, making them ideal sorbent materials (Borchardt, 1989) (Table 3).

The clay minerals possess a characteristic negative surface charge on their platelets. This phenomenon results from either isomorphic substitution of cations within the platelet surface, or dissociation of H^+ ions from the hydroxyl groups attached to Si and Al atoms at the broken edges of clay platelets. The negative charge is attributed to isomorphic substitution results from the naturally occurring replacement of a Si or Al atom by a cation of similar geometry, but of lower charge (e.g. Mg^{2+} for Al^{3+} or Al^{3+} for Si^{4+}). This phenomenon produces a

net negative charge, which is distributed across the platelet. Overall, the negative charge on 2:1 clays results primarily from the isomorphic substitution and, to a lesser extent, dissociation of H^+ ions from the hydroxyl groups. The negative charge is generally neutralized by cations (e.g., Na^+ and Ca^{2+}) attracted into the interlayer regions (Huebner *et al*, 1999). Interlayer cations are subject to formation of layers of hydration, thus water enters the interlayer regions of the clay and causes it to swell (Bohn *et al*., 1979). The interlayer region and other surface characteristics have been established to be especially important in the binding efficacy of NS for AFs (Grant and Phillips, 1998).

1.12.3 Sorption of Ligands onto Clay Surfaces

A systematic, quantitative, and qualitative means of investigating adsorption is very essential when analyzing sorption processes and comparing effectiveness of sorbent materials. One simple approach is the single-concentration sorption method, a procedure that measures the sorption of one concentration of sorbate (or ligand) by one concentration of sorbent in aqueous solution that allows for rapid screening of large sample numbers (Phillips *et al*., 1988). More importantly, complete isothermal analysis reportedly measures surface adsorption using a fixed concentration of sorbent, coupled with a range of initial ligand concentrations at a constant temperature (Kinniburgh, 1986).

The isothermal procedure was originally developed for the purpose of examining adsorption of a gas to a homogenous solid surface, with the

assumption that the sorbent surface contains a finite number of sorption sites (Langmuir, 1916). The mathematical equation introduced by Langmuir relates the amount of ligand bound to that unbound at equilibrium and incorporates capacity (Q_{max}) and affinity (K_d) parameters. The Q_{max} provides an estimated maximum amount of ligand that could be adsorbed by a given weight of the sorbent under optimum experimental conditions, while the K_d is an equilibrium constant which depicts the sorbent affinity for the ligand. Several mathematical equations have been developed to model isothermal data after the introduction of the Langmuir equation (LM). These equations include Freundlich, Langmuir-Freundlich, generalized Freundlich, Multi-Langmuir, and Toth (Kinniburgh, 1986). For instance, the Freundlich equation utilizes a generalized *K* to establish a computational relationship between the amount bound and that left in solution at equilibrium, and it incorporates an *n* variable to evaluate the deviation of an isothermal curve from a linear sorption isotherm. The LM equation is most applicable to a single ligand adsorbing to a single type of site on a particular sorbent, whereas the Langmuir-Freundlich, Freundlich, generalized Freundlich, and Toth equations are applicable to heterogeneous solid surfaces. Multi-Langmuir equation has also been used to model heterogeneous adsorption by the addition of Langmuir equations to delineate different sites (Langmuir, 1916).

The shapes of isotherm curves are generally categorized into four main types, designated H (high affinity), L (Langmuir), S (sigmoidal), and C (constant partion), representing different adsorption mechanisms (Giles et al., 1960,

1974a,b; Kinniburgh, 1986). Both the H and L isothermal shapes are characterized by initial steep concave slopes. The shape of the H type isotherm curve is generally shows a much sharper initially elevation at low concentrations of the ligand and it plateaus quickly, an indicative of a high initial affinity and rapid saturation of the binding site(s). However, the shape of the L type isotherm curve demonstrates a more gradual initial rise before plateaus, which also indicates saturation of binding site(s). The S isotherm shape is exemplified by very little initial ligand binding, followed by a steep concave elevation and then a plateau. Basically, this shape suggests that the adsorption process is contingent upon the ligand concentration, thus, the presence of initially bound ligand enhances the affinity of other molecules to bind as well. When saturation point is reached, the isotherm shape begins to plateau. Lastly, the shape of the C type isotherm denotes a constant partition process. In this case, the isotherm shape demonstrates linearity in the sorbent-ligand interactions and hence the ligand concentration sorbed increases continuously as its concentration in the aqueous phase also increases. Analysis of these isotherm shapes compared to standard isothermal curves supplies a great deal of information to be able to predict the adsorption mechanism involved in the binding process (Grant and Phillips, 1998; Lemke, 2000).

Thermodynamic parameters, such as Q_{max} and K_d, may also be used to estimate the effectiveness and affinity of a sorbent and allow comparison between sorbent materials (Grant and Phillips, 1998). From these isotherm

equations, K_d values can be used to calculate the Gibbs standard free energy change of adsorption (ΔG^o_{ads}) and the enthalpy of adsorption (ΔH_{ads}) (Fischer and Peters, 1970; Stumm *et al*., 1992), giving information about the adsorption mechanism, particularly, physisorption or chemisorption. Physisorption involves weak associations which include van der Waals, dipole-dipole, induced dipole, and hydrogen bonding. Chemisorption implies a chemical reaction or sharing of electrons between the adsorbent and the adsorbate. Physisorption is described as having an enthalpy of <20 kJ/mol, while chemisorption is generally >20 kJ/mol (Gu *et al*., 1994).

1.13 Geophagy in the Historical Context

The use of clay by humans and animals is not a recent phenomenon. Geophagy, the deliberate consumption of soil, in particular clay minerals, alone or with food, has been recorded from traditional human societies on all continents for centuries, especially among pregnant women (Johns and Duquette, 1991; Abrahams and Parsons, 1996; Geissler *et al*., 1999; Carretero et al., 2002). This practice is also widespread throughout the animal kingdom including mammals, birds, and reptiles. (Diamond, 1999; Gilardi et al., 1999; Wilson, 2003).

Although critics view it as a maladaptive or compulsive behavior driven by cravings, consumption of certain clays is reported to have medicinal values and can be beneficial to both humans and animals (Johns and Duquette, 1991;

Krishnamani and Mahaney, 2000; Mahaney and Krishnamani, 2003). The potential health benefits of geophagy (although ill-defined in the scientific literature) may include the enterosorption and decreased bioavailability of toxic chemicals and hazardous microbes from contaminated food and water. Possible explanations, regarding geophagy, include the belief that the practice may provide essential minerals (Johns and Duquette, 1991) and/or may detoxify the body of poisonous substances (Gilardi *et al*., 1999). Specifically, Peruvian macaws consume clay after ingestion of seeds and unripe fruits high in alkaloids (Diamond, 1999). In humans, clays have been used therapeutically as laxatives and antidiarrhetics and consumption has been associated with the sorption of toxins, bacteria, and viruses (Carretero *et al*., 2002).

Although many types of clay and zeolitic minerals are routinely added to animal feeds for various purposes, it is important to note that not all clays are beneficial. In particular, inclusion of clinoptilolite in an AFB_1-contaminated diet resulted in severe liver pathology in pregnant rats that was not observed in the presence of AFB_1 alone, suggesting the sorbent may be involved in either nutrient sequestration or metabolism of the AFB_1 on catalytic surfaces of the clay (Mayura *et al.,* 1998). Nonselective or versatile sorbents may bind enzymes and critical nutrients, and may release sorbed contaminants, making prolonged use inadvisable. This presents a great challenge particularly during prenatal development because mothers and fetuses are highly prone to nutritional deficiencies. For instance, a previous research found that the

hemoglobin, hematocrit, and red blood cell levels in rats fed high levels (20%) of kaolin in the diet throughout the gestation period were significantly reduced compared to controls, indicative of kaolin-related maternal anemia, and pups borne to these rats had lower birth weights (Patterson & Staszak, 1977). Therefore, it is essential to completely evaluate the safety of any potential additive prior to routine inclusion in animal feeds or humans diets.

1.14 Detoxification by Clay-Based Enterosorption Method

The use of non-nutritive materials to adsorb or covalently bind AFs has been a method of current interest for AF decontamination. In particular, the use of calcium montmorillonite clays (e.g. NovaSil) to tightly sorb and inactivate AFB_1 in the gastrointestinal tract of various animal species has shown significant promise in the prevention of aflatoxicoses. Several studies have demonstrated that inclusion of NovaSil (NS), an anti-caking agent previously used in animal feeds, protected multiple animal species from the adverse effects of AFs including: rodents (Mayura et al., 1998), chickens (Phillips et al., 1988; Kubena et al., 1990a), turkeys (Kubena et al., 1991), dogs (Bingham, 2003), swine (Lindermann et al., 1993), lambs (Harvey et al., 1991a), goats (Smith et al., 1994), and dairy cattle (Harvey et al., 1991b) (Table 4). In addition, the clay (at 1.0% level in feed) reduced AFM_1 levels in milk without altering the nutritional quality or causing overt toxicity itself (Ellis *et al*., 1990; Harvey *et al*., 1991) (Table 4).

TABLE 4

***In vivo* studies with dietary NovaSil (NS) or NSP[1] for mycotoxin prevention: Efficacy and selectivity**

Animals	Mycotoxin in Feed	% NS/NSP in Feed (duration)	NS/NSP Toxicity	Major Effects of NS/NSP Reported	References
chickens	AFs	0.5 (28 d)	None	Growth inhibition diminished; gross hepatic changes prevented; decreased mortality	Phillips et al., 1988
chickens	AFs/OTA	0.5 (21 d)	None	Decreased growth inhibitory effects; no effect against ochratoxin A	Huff et al., 1992
chickens	AFs/TTC	0.25/0.37/0.8 (21 d)	None	Diminished growth inhibition; no effect against trichothecenes (e.g. T-2 toxin)	Kubena et al., 1990b
chickens	None	0.5/1.0 (14 d)	None	NS did not impair utilization of riboflavin, vitamin A, Mn; slight reduction of Zn	Chung et al., 1990
Turkeys	AFs	0.5 (21 d)	None	Decreased mortality rates	Kubena et al., 1991
Rats	AFs	0.5 (21 d)	None	Significant prevention of maternal and developmental toxicity	Mayura et al., 1998
Pigs	AFs	0.5 (42 d)	None	Diminished growth inhibition	Lindermann et al., 1989
Pigs	AFs	0.5/2.0 (28 d)	None	Decreased growth inhibition; prevented serum effects and hepatic lesions	Harvey et al., 1989
Dogs	AFs	0.5 (48 hr)	None	Significantly reduced bioavailability of AFs and excretion of AFM_1 in urine.	Bingham, 2003
Lambs	AFs	2.0 (42 d)	None	Diminished growth inhibition and immunosuppresion	Harvey et al., 1991a
Diary Cows	AFs	0.5/1.0 (28 d)	None	Reduced the levels of AFM_1 in milk	Harvey et al., 1991b

Note. [1]NSP = NovaSil Plus ; AFs = Aflatoxins; TTC = Trichothecenes ; OTA = Ochratoxin A

NovaSil, at an inclusion level as low as 0.5% in the AF-contaminated diet of sensitive animal species (i.e., young chickens, turkeys, swine, lambs, and rats), effectively reduced the health manifestations of aflatoxicosis (Phillips et al., 1995). In a previous research, NS effectively reduced the acute effects of AFB_1, as evidenced by the reduction of AFM_1 (a dose-dependent biomarker of dietary AFB_1 exposure) levels in urines of AF-exposed rats (Sarr et al., 1995). It is hypothesized that the edge sites, basal surfaces, and mainly the interlayer surfaces of NS interact with the β-dicarbonyl system of AFB_1 in a chemisorption process (Phillips *et al*., 1995). With this arrangement, the toxin can remain tightly sequestered as substantiated by previously reported thermodynamic results in which NS-AFB_1 binding interaction yielded estimated ΔH_{ads} and $\Delta G°_{ads}$ values of –40 KJ/mol and –29 KJ/mol, respectively (Grant, 1998). Specificity of NS for AFs has been well-characterized in several animal studies. For instance, NS was shown to be ineffective for sorption of diacetoscirpenol (DAS) (Kubena *et al*., 1993), ochratoxin A (OA) (Huff *et al*., 1992), or T-2 toxin (Kubena *et al*., 1990b) in chicks, or of deoxynivalenol (DON) in pigs (Patterson *et al*., 1993). However, when a combination of each of these mycotoxins with AF was tested in these same studies, NS effectively protected against aflatoxicosis, suggesting specificity of this clay for AFs.

NovaSil Plus (NSP), a new calcium montmorillonite (CM) clay, which is structurally similar to NS, and currently available as an anti-caking agent in livestock feeds, has demonstrated high efficacy for AF binding. In a previous

study, male Fisher-344 rats (fed diets containing 0.5% NSP) were administered NSP equivalent to 0.5% of the estimated maximum daily intake (30 g) of feed concomitantly with AFB_1 (dissolved in corn oil) as previously described (Mayura et al., 1998). Results in the study indicated that NSP significantly protected the rats as evidenced by a 95% reduction of urinary metabolite aflatoxin M_1 (AFM_1) levels compared to rats dosed without NSP (Bingham, 2003). In a separate study using dogs, Bingham (2003) found that dietary inclusion of 0.5% NSP also protected the animals by reducing the AFM_1 levels an average of 48.4% compared to dogs fed diet containing no NSP. In another study, concentrations as high as 2% NSP in the diet throughout pregnancy produced neither maternal nor fetal toxicity in Sprague-Dawley (S-D) rats, and did not affect metal bioavailability in a variety of tissues (Wiles et al., 2004). Therefore, NSP clay may be a good candidate that can be considered for a carefully designed, short-term feasibility studies in humans. However, further *in vitro* and *in vivo* (short-term and long-term) analytical studies must be conducted to evaluate and predict potential adverse effects of the clay itself in animal models, which is the overriding basis of this work.

1.15 Research Objectives

AF contamination in foods/feeds has been longstanding and difficult to control even with good manufacturing practices because AFs are invisible to the naked eye, heat stable, and are not neutralized by common food processing

techniques (CAST, 1989; Phillips, 1999; Phillips *et al.,* 2002). While this problem may not pose significant threats to most developed countries owing to their extensive commodity screening, regulations, and monitoring programs, AF contamination in food and feed products remains a serious burden in many parts of the world especially where insufficiency of food supplies presents a major and persistent impediment to improvements in food safety (McAlpin *et al.,* 2002; Shephard, 2003). Thus, intervention strategies to diminish or prevent biological exposure of humans and animals to the AFs have warranted a highly desirable pursuit. However, any intervention method must satisfy the following conditions for it to be successful particularly in developing nations: it must be practical, safe, economically feasible, culturally acceptable, environmentally benign, and sustainable in order to be successful in the prevention of health problems associated with AF contamination. For instance, concerns have been raised as to whether or not NSP has the ability, or lack thereof, to interact with nutrients such as vitamin A (VA) *in vitro* or *in vivo* in the presence or absence of AFB_1. Currently, the NSP, a new CM clay, has demonstrated high efficacy for AF binding in animals and shows promise for application to the human diet.

Therefore, the objectives of this research were to:

1) Characterize the *in vitro* and *in vivo* sorption efficacy of NSP for AFB_1 and potential interaction with VA. Determine sorption affinity and capacity of NSP for AFB_1 and VA and characterize the thermodynamics of the AFB_1-NSP interaction *in vitro* in order to predict *in vivo* activities. Investigate

the sorption of AFB_1 by NSP in Sprague-Dawley (S-D) rats, as measured by the AFM_1 metabolite in urine. Determine the minimal effective dietary inclusion level of NSP that can protect broiler chicks from the effects of AFs and hepatic VA depletion.

2) Evaluate potential adverse effects of chronic (6.5 months) ingestion of NSP in the diets of male and female S-D rats based on measurements of feed intake, change in body weights, terminal organ weights, complete blood counts and leukocyte differentials, typical serum biochemical parameters, selected trace metals in tissues, and qualitative organ histology.

3) Establish representative baseline data on human exposure to AFs by collecting and analyzing AFM_1 in urine samples from volunteers in Ghana. Determine localities with high dietary aflatoxin exposure, which will eventually be the populations of choice for the clay-based intervention studies in the country.

CHAPTER II

IN VITRO AND *IN VIVO* CHARACTERIZATION OF NOVASIL CLAY: AFB_1 AND VITAMIN A INTERACTIONS

2.1 Introduction

Aflatoxins (AFs), naturally occurring metabolites of *Aspergillus* spp. that are produced primarily by *Aspergillus flavus* and *Aspergillus parasiticus*, have been shown to be acutely toxic, carcinogenic, mutagenic, teratogenic, and immunosuppressive to mammalian species (Busby and Wogan, 1984; Miller and Wilson, 1994; Pestka and Bondy, 1994; Turner et al., 2003; 2004; Glahn et al., 1991). Specifically, B_1, B_2, G_1, and G_2, are considered human carcinogens (group 1) by IARC (WHO-IARC, 1993). Among these, AFB_1 is the most predominant metabolite and the most potent hepatocarcinogen (Busby and Wogan, 1984; Flaherty and Payne, 1997; Lopez et al., 2002).

AF contamination of livestock feed often results in poor growth and feed conversion efficiency, increased disease susceptibility, and increased mortality rates (Smith and Hamilton, 1970; Jones et al., 1982; Huff et al., 1986). The impact of AFs on micronutrient status is also a major concern, specifically the association between dietary AF exposure, vitamin A (VA) deficiency, and disease susceptibility. In a recent study, dietary exposure to AFB_1 in growing barrows resulted in a reduction of serum VA levels, suggesting that AF exposure may exacerbate VA deficiencies (Harvey et al., 1994). In addition, VA

deficiencies have been linked to compromised immune responses and increased disease susceptibility in poultry (Aye et al., 2000 a,b; Dalloul et al., 2002).

The inclusion of non-nutritive enterosorbents to sequester AFs in contaminated feeds is both practical and economically feasible for large-scale detoxification of contaminated feedstuffs. Calcium montmorillonite clays such as parent NovaSil (NS) and currently available NovaSil Plus (NSP) are commonly added to animal feeds as anticaking agents. NS has been shown to diminish the adverse effects of AFs in multiple animal species including rats (Abdel-Wahhab et al., 2002; Mayura et al., 1998), chickens (Phillips et al., 1988; Kubena et al., 1990b), turkeys (Kubena et al., 1991), pigs (Lindermann et al., 1993), lambs (Harvey et al., 1991a), dairy goats (Smith et al., 1994a), and dairy cows (Harvey et al., 1991b). In addition, NS has been shown to effectively reduce AFB_1 exposure in the gastrointestinal tract as evidenced by a reduction of AFM_1 (a dose-dependent biomarker of dietary AFB_1 exposure) levels in the urine of rats dosed with AFB_1 (Sarr et al., 1995). Previous research has shown that the inclusion of 0.5% of NS in AF-contaminated diets of animals effectively reduced the symptoms of aflatoxicosis (Phillips et al., 1995) and did not interfere with nutritional utilization in chicks (Chung et al., 1990). However, the efficacy and minimal effective dose of NSP that is sufficient to protect chicks from aflatoxicosis, as well as the potential for NSP interaction with important vitamins, such as vitamin A (VA), have not been reported.

In this research, the major objectives were to: 1) determine the sorption affinity and capacity of NSP for AFB_1 and VA and characterize the thermodynamics of the AFB_1-NSP interaction *in vitro*; 2) investigate the potential of NSP to reduce exposure of AFB_1 in Sprague-Dawley rats, as measured by the AFM_1 metabolite in urine; and 3) determine the minimal effective dietary inclusion level of NSP that will protect broiler chicks from the effects of aflatoxicosis and hepatic VA depletion.

2.2 Materials and Methods

2.2.1 Chemicals and Reagents

For all experiments, purified water (ddH_2O) was prepared by processing deionized water through a Milli-Q^{UF+} water purification system. All solvents were HPLC grade. VA (retinol, 95% purity), cetylpyridinium (CP) chloride, AFB_1, and AFM_1 were purchased from Sigma Chemical Co. (St. Louis, MO). Activated carbon (AC) was purchased from Aldrich Chemical Co. (Milwaukee, WI). NSP and low-pH montmorillonite (LPHM) were obtained from Engelhard Chemical Corp. (Iselin, NJ).

2.2.2 Sorbent Preparation

Both NSP and LPHM were sieved to ≤45 μm prior to use. Heat-collapsed NSP (col-NSP) was prepared by first heating the clay at 200 °C for 30 min and then increasing the temperature to 800 °C for 1 hr. CP-exchanged organoclays

were prepared according to methods previously reported (Lemke et al., 1998). Briefly, LPHM was washed in H_2O (100 ml/g of clay) for 120 hr while shaking. Afterwards, the suspension was centrifuged and the supernatant was discarded. The washed clay was resuspended in H_2O (100 ml/g clay), and CP was added based on the estimated cation exchange capacity (CEC) for LPHM (1 CEC, 90 $cmol_{charge}$/kg of LPHM, obtained from Engelhard Corp.). The clay was exchanged for 24 hr, centrifuged, and the supernatant was again discarded. Unexchanged organic cations were removed by washing in H_2O for an additional 24 hr. The washed clay was centrifuged and the supernatant discarded before the clay was dried, ground, and sieved to a particle size of <45 μm.

2.2.3 AFB_1 Sorption Studies with NSP Clay

The efficiency of AFB_1 sorption by NSP was evaluated over various equilibration time periods and pH ranges. A 100 μg sample of the sorbent was added to a 5 μg/ml solution of AFB_1 was agitated at 1000 rpm at 25 °C for 5, 10, 15, and 30 min, and 1, 2, 4, and 24 hr. After shaking, samples were centrifuged at 800 x *g* for 10 min, and residual AFB_1 in the supernatant was measured by UV-visible spectrophotometry (362 nm; ε= 21,865 $M^{-1}cm^{-1}$) with Beckman DU-65 scanning UV-visible spectrophotometer. Based on these results, a minimum of at least 2 hr was selected as an appropriate equilibration time period and was used to evaluate the effect of sorption at pH 2, 7, and 10. All solutions were adjusted to the corresponding pH value with either HCl or NaOH prior to

shaking. The samples and controls were agitated at 1,000 rpm for 2 hr at 25 °C. After shaking, the samples were centrifuged at 800 x *g* for 10 min, and the supernatants were analyzed for AFB_1 content as stated above.

2.2.4 Isothermal Analysis of Vitamin A and AFB_1 with NSP

Isothermal analyses were conducted based on previously described methodology (Grant, 1998; Grant and Phillips, 1998; Lemke et al., 1998; Huebner et al., 1999; Pimpukdee, 2000). Briefly, stock solutions of VA (10 μg/ml) and AFB_1 (8 μg/ml) were prepared in purified water (pH 6.5 ± 0.2) and verified by UV-visible spectrophotometry (absorbance at 292 nm ε= 6634 M^{-1} cm^{-1} and 362 nm ε= 21,865 M^{-1} cm^{-1}, respectively). Test samples using 5 ml aliquots were prepared in triplicate containing VA ranging from 0.5 to 10 μg/ml or AFB_1 ranging from 0.4 to 8 μg/ml made by diluting the stock solutions with ddH_2O. Respective sorbent suspensions (2 mg/ml) were prepared then vortexed for 1 min and 50 µl portions were added to the dilutions. Controls consisted of ddH_2O alone, ddH_2O + 10.0 µg VA/ml or 8 μg AFB_1/ml, and ddH_2O + 50 µl sorbent suspension. The tubes were capped, agitated at 1,000 rpm for 2 hr (VA) or 24 hr (AFB_1) at 25 °C in the absence of light, then centrifuged at 800 x *g* for 30 min at 25 °C. Residual VA or AFB_1 in supernatant was measured by UV-visible spectrophotometry. CP-LPHM and AC were used as positive controls for the VA isotherms since they have been shown to be effective for organic compounds such as VA (Paune et al., 1998; Srinivasan and Fogler, 1990a,b). In

the case of AFB_1, the procedure was repeated with 15 and 37 °C in order to evaluate the thermodynamic parameters of AFB_1-sorbent interactions, specifically, to estimate the enthalpy of sorption (ΔH_{ads}) of AFB_1 on the surface of the test sorbent. Isothermal data was transferred to Table Curve 2D v3 and fit to appropriate models based on shape, r^2 and standard deviation. Estimates of the capacity values (Q_{max}) and the distribution constants (K_d) for each sorbent were calculated using various equations (Table 5). Enthalpy of sorption (ΔH_{ads}) was determined using the van't Hoff equation (eq 1, Table 5) by substituting individual K_d values at different temperatures into the equation. The Gibbs free energy change of adsorption (ΔG^o_{ads}) of NSP-AFB_1 reaction was calculated using eq 2 (Table 5) with the K_d value from the isotherm conducted at 25 ^oC.

2.2.5 Biomarker Determination of AFB_1 Exposure in Rats

Four-month old male Sprague–Dawley rats (Harlan, Houston, TX) were randomly assigned to 1 of 3 treatment groups (3 rats/group). Treatment groups consisted of: 1) basal feed in the absence of clay; 2) basal feed containing 0.0625% NSP; 3) basal feed containing 0.25% NSP. Water and feed (Teklad rodent diet 8604; Harlan Feeds, Madison, WI) were provided *ad libitum*. On day 6 of the study, each rat was weighed and dosed with 1 mg/kg (bw) AFB_1 (dissolved in corn oil) by gavage as previously described (Sarr et al., 1995). Additionally, rats in treatment groups 2 and 3 were dosed with an NSP equivalent of either 0.0625% or 0.25% (based on the estimated maximum daily

TABLE 5

Equations used for plots and calculations in NSP-AFB$_1$ adsorption

Isotherm Model	Equation[a]
Langmuir Model (LM)	$q = Q_{max}\ [(K_dC_w)/(1 + K_dC_w)]$
Multi-Langmuir Model (MLM)	$q = Q_{max1}[(K_dC_w)/(1 + K_dC_w)] + Q_{max2}[(K_dC_w)/(1 + K_dC_w)] + \ldots.$
Toth Model (TM)	$q = Q_{max}\ [(K_dC_w)/\{1 + (K_dC_w)^n\}^{1/n}]$
Freundlich Model (FM)	$q = K(C_w)^n$
Generalized-Freundlich Model (GFM)	$q = Q_{max}\ ((K_dC_w)/(1 + K_dC_w))^n$
Langmuir-Freundlich Model (LFM)	$q = Q_{max}\ [(K_dC_w)^n/(1 + (K_dC_w)^n)]$
Equations used for Thermodynamic Calculations	
van't Hoff equation	$\Delta H_{ads} = -R\ ln\ (K_{d2}/K_{d1})/\{(1/T_2) - (1/T_1)\}$ …… (1)
Gibbs free energy change of Adsorption at Equilibrium	$\Delta G^o_{ads} = -RT\ ln\ K_d$ …… (2)
Rearrangement of Langmuir Model	$K_d = q/(Q_{max} - q)C_w$ …… (3)

Note. q = concentration of AFB$_1$ adsorbed (mol/kg), Q_{max} = maximum capacity of clay (mol/kg), K_d = distribution constant, C_w = equilibrium concentration of AFB$_1$ (mol/L). n = exponent, K = distribution constant. T = temperature (Kelvin) and R = gas constant.

intake of feed (30 g)) concomitantly with the toxin as previously described (Mayura et al., 1998). Each rat was placed in a metabolism cage and urine was collected over the entire 48 hr time period following dosing. Urine volumes were recorded and samples were frozen at –80 °C in cryogenic vials prior to AFM_1 quantification.

2.2.6 Urinary AFM_1 Analysis

AFM_1 in urine was quantified by HPLC following immunoaffinity cleanup based on procedures previously described by Groopman et al. (1992b) with modifications by Wang et al.(1999) and Sarr et al. (1995). Briefly, a 1 ml aliquot of urine was eluted through a Aflatest P® immunoaffinity column (Vicam, Watertown, MA) at a flow rate of approximately 0.3 ml/min. After elution, the column was rinsed with 5 ml PBS and AFs were then eluted with 1 ml dimethylsulfoxide (DMSO):PBS (1:1).

Quantitative measurements of AFM_1 were performed by HPLC with fluorescence detection utilizing a 250 mm x 4.6 mm LiCrospher RP-18 endcapped column with pore size 100 Å and particle size 5 µm (Alltech Associates, Deerfield, IL). The mobile phase contained 22% ethanol buffered with 20 mM ammonium formate (pH 3.0). Chromatographic separation of AFs was achieved by isocratic elution of the mobile phase for a total run time of 35 min (flow rate 1.0 ml/min) and injection volume of 50 µl. AFM_1 quantification was based on peak area and retention time as compared to external standard.

2.2.7 Preparation of Poultry Diets

AFs were produced through the fermentation of rice by *Aspergillus parasiticus* NRRL 2999 as described by Kubena et al. (1990a). Briefly, fermentation was carried out in 2.5 L fernbach flasks containing 50 g of white rice (free of preservatives). Water (25 ml) was added to each flask and rice was autoclaved then shaken to break up clumps. Each flask was inoculated with 1 ml of an *Aspergillus* spore suspension then shaken on a platform shaker at 220 to 240 rpm at 15 °C for 24 hr. Ten ml of water was added at 48 hr. The initial temperature of the rice fermentation was increased from 15 °C to 21 °C after 24 hr of incubation and then to 28 °C after 48 hr. Incubation was continued for a total of 5 d and during the fermentation process, grains were kept separated to prevent excessive hyphae formation. After incubation, the rice was autoclaved, dried, then ground into a powder. The total aflatoxin content of the rice powder was initially measured by spectrophotometry (Nabney and Nesbitt, 1965) as modified by Wiseman et al. (1967) and consisted of approximately 79% AFB_1, 16% AFG_1, 4% AFB_2, and 1% AFG_2. The rice powder was incorporated into a corn-soybean meal basal diet (free of detectable mycotoxin contamination) to provide 5 mg AFB_1/kg diet as confirmed by HPLC methods (Hutchins and Hagler, 1983). The basal diet contained no antibiotics, coccidiostats, growth promoters, or inorganic sorbents and met or exceeded the levels of critical nutrients recommended by the National Research Council (1994).

2.2.8 Experimental Design for the Broiler Chick Study

One-day-old male broiler chicks (Peterson x Hubbard) were obtained from a commercial hatchery. Chicks were reared in wire-floored brooding cages, with continuous fluorescent illumination and forced ventilation at the USDA/ARS, Southern Plains Agricultural Research Center (College Station, TX). Chicks were individually weighed, wing-banded, and randomly distributed to the different treatment groups consisting of four replicates of six chicks per dietary treatment group (pen). Chicks were grouped by the following dietary treatments: 1) basal feed (BF) free of toxins (control); 2) BF containing 0.5% NSP; 3) BF containing 5 mg AFB_1/kg; 4) BF containing 0.125% NSP + 5 mg AFB_1/kg; 5) BF containing 0.25% NSP + 5 mg AFB_1/kg; and 6) BF containing 0.5% NSP + 5 mg AFB_1/kg. Broilers were provided with water and feed *ad libitum* for the duration of the study (3 weeks). Body weights of chicks and feed consumption were measured weekly and chick mortalities were recorded as they occurred. After termination of the study, liver and kidney weights were recorded and were evaluated as relative organ weight changes (g/100 g bw). Random liver samples (3/pen) were also collected and stored at –75 °C in preparation of hepatic VA level determination. Control birds were evaluated via necropsy by scientists and an attending veterinarian for underlying diseases. No evidence of preexisting problems was observed.

2.2.9 Hepatic Vitamin A (Retinol) Determination

A 1 g portion of liver was mixed with 100 mg butylated hydroxytoluene and 25 g silica gel. The mixture was ground in a mortar, then extracted with 30 ml hexane:acetone (1:1) for 30 min with agitation. Afterwards, 10 ml ddH_2O was added to the mixture, which was then vortexed for 30 sec and centrifuged at 1,000 rpm for 15 min at 4 °C. Ten ml of the hexane fraction was removed from the mixture and dried under nitrogen. The oily residue was resuspended in 3 ml of saturated KOH, incubated for 30 min at 65 °C, then re-extracted with hexane:water (5:1) and centrifuged at 1,000 rpm for 15 min at 4 °C. The resulting hexane fraction was again removed and dried under nitrogen, and resuspended in methanol:ammonium acetate (9:1; pH 5.6) prior to analysis.

Quantitative measurements of VA were performed by HPLC with photodiode array detection methods (325 nm) utilizing an ODS column (4.5 x 150 mm) maintained at 30°C. The mobile phase consisted of a gradient elution of 40 mM ammonium acetate buffer (pH 5.6):methanol (40:60) for 12 min followed by 100% methanol elution for 4 min, then ammonium acetate buffer:methanol (40:60) for 9 min (flow rate of 1 ml/min). Injection volume was 20 μl with a total run time of 25 min. VA was quantified based on retention time and peak area as compared with an external retinol standard.

2.2.10 Statistical Analysis

Data were subjected to analysis of variance using the General Linear Models procedure to establish differences between means. Means showing significant differences in ANOVA were compared using Fisher's protected least significant difference procedure (Snedecor and Cochran, 1967). All statements of differences were based on a significance of $p \leq 0.05$.

2.3 Results

2.3.1 Sorption of AFB_1 with NSP Clay *In Vitro*

Initial sorption studies indicated that removal of AFB_1 from aqueous solution occurred rapidly (within 5 min) and an equilibrium state was achieved within 2 hr (data not shown). Using this 2 hr equilibration time period, isothermal analysis of AFB_1 sorption by NSP was further shown to be pH-dependent. At pH 7 and 10, NSP exhibited greater sorption than at pH 2 (Figure 9). Experimental data for removal of AFB_1 from aqueous solution by NSP at pH 7 and 25 °C were fit to various isotherm equations to select the most comprehensive model (Table 6). Considering the Q_{max}, K_d, n, and the residuals, the Langmuir Model (LM) and Toth Model (TM) were determined to be most appropriate for modeling AFB_1 sorption on NSP. However, the LM had a much higher F-value making it a better model than TM (Table 6). Based on this set of data, the LM was selected for quantitative and qualitative analysis of the binding affinity (K_d) and capacity (Q_{max}) of NSP for AFB_1. The average Q_{max} values (± SEM) for AFB_1 sorption

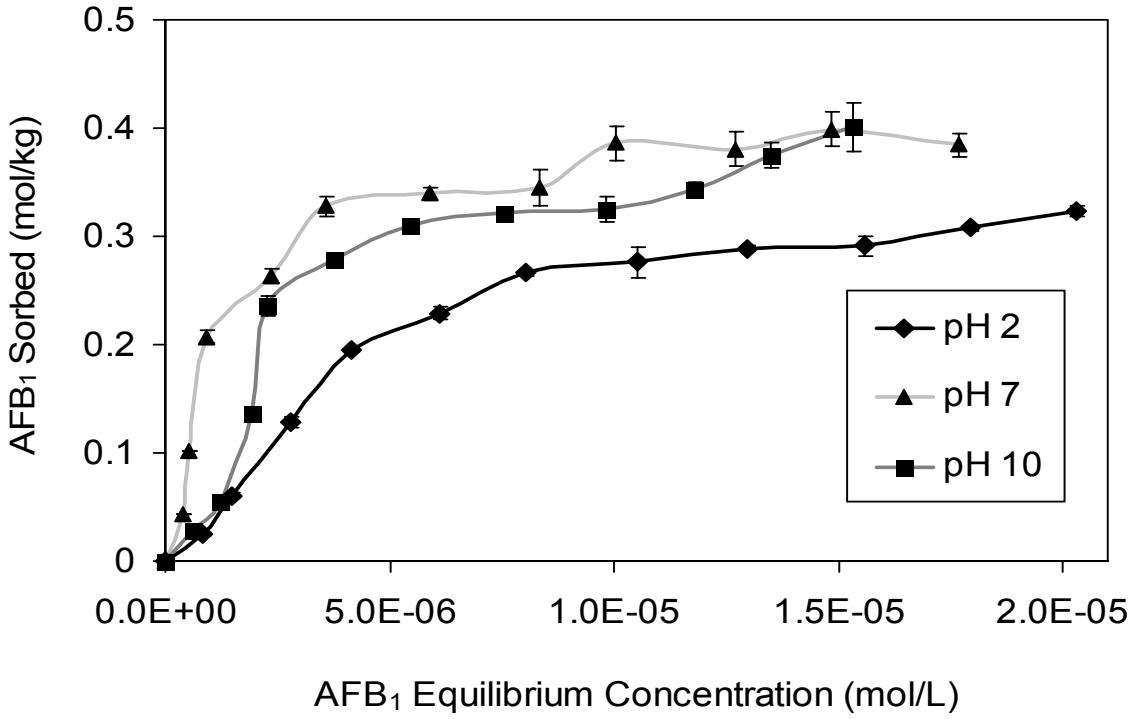

Figure 9. Isotherm analysis of AFB_1 sorption by NSP at pH 2, 7, and 10 at 25 ^{o}C. Samples and controls were agitated at 1,000 rpm (25 °C) for 2 hr, centrifuged, and supernatants were analyzed for residual AFB_1 utilizing UV-visible spectrophotometry. Data represent means (± SD) from three independent experiments.

TABLE 6

Summary of isotherm fit parameters of AFB_1 adsorption by NSP

AFB_1 Sorption at 25 ^oC (pH 7)						
Isotherm Model	(Q_{max}) ± SE (mol/kg NSP)	K_d ± SE (x10^5)	Exponent (n)	r^2	F-value	Residual (Runs)
LM	0.456 ± 0.007	7.479 ± 0.498	NA	0.996	2814.27	3
TM	0.488 ± 0.021	8.957 ± 1.152	0.811	0.997	1830.19	5
FM	N/A	N/A	0.311	0.960	239.42	2
LFM	0.119 ± 0.093	6.646 ± 0.705	0.896	0.997	1587.78	5
GFM	0.462 ± 0.009	5.806 ± 1.535	0.867	0.997	1379.30	3
MLM[1]	0.341 ± 1.797	0.118 ± 0.825	NA	0.998	1718.92	5
MLM[1]	0.401 ± 0.044	9.512 ± 1.521	NA	0.998	1718.92	5

Note. [1]Two sets of data were obtained for MLM. NA = not applicable. LM = Langmuir Model, TM = Toth Model, FM = Freundlich Model, LFM = Langmuir-Freundlich Model, GFM = Generalized- Freundlich Model, MLM = Multi-Langmuir Model.

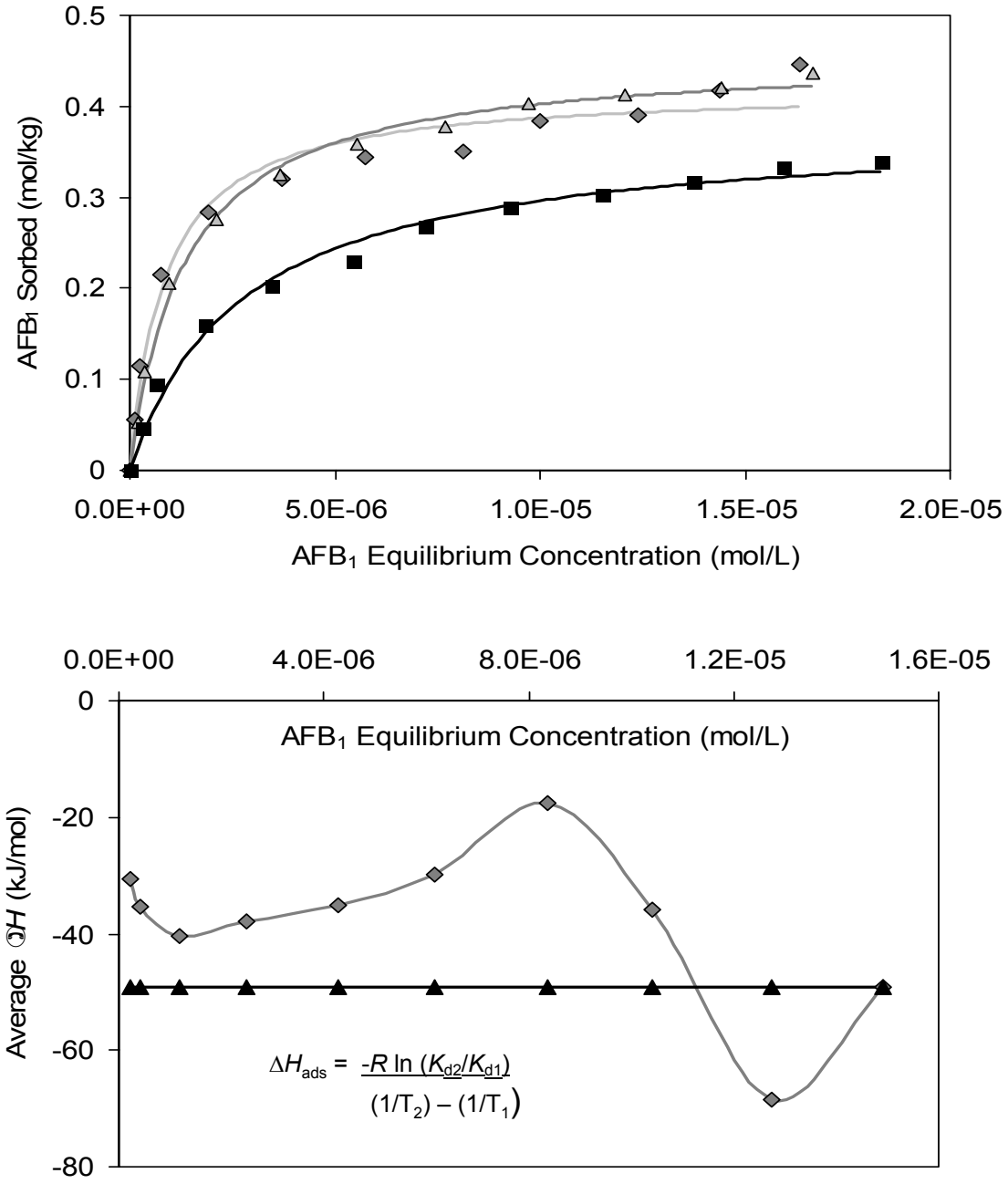

Figure 10. Isothermal analysis and enthalpy of sorption (ΔH_{ads}) plots for AFB_1 by NSP at 15 °C, 25 °C, and 37 °C. **A.** Isotherm plots for NSP-AFB_1 sorption. Data were fit with Langmuir model (LM). Curve fits are displayed as solid lines, and experimental data as average points. **B.** Enthalpy of sorption plots based on isothermal analysis. ΔH_{ads} was calculated using the van't Hoff equation (see inset). Data show average enthalpy values derived from observed experimental data and estimated values based on LM data.

onto NSP at 15 °C, 25 °C, and 37 °C were 0.420 ± 0.013 mol/kg, 0.456 ± 0.007 mol/kg, and 0.379 ± 0.001 mol/kg, respectively (Figure 10A). Similarly, the average K_d constants were determined to be 1.18 x 10^6, 7.48 x 10^5, and 3.61 x 10^5, respectively. Sorption was shown to be exothermic and spontaneous with an average enthalpy (ΔH_{ads}) equal to -49.2 kJ/mol (Figure 10B) and Gibbs free energy change of adsorption (ΔG^o_{ads}) equal to -33.5 kJ/mol. From the LM, the maximum observed concentration of AFB_1 sorbed onto NSP was calculated to be 0.437 mol/kg, 96% of the Q_{max} of 0.456 mol/kg. In contrast, isothermal analysis of col-NSP indicated that the Q_{max} and K_d were 0.0267 mol/kg (94% less than unmodified NSP) and 2.06 x 10^2 (99% less than unmodified NSP) (Figure 11).

2.3.2 Interaction of NSP with Vitamin A *In Vitro*

In isothermal analyses experiments, NSP exhibited negligible interactions with VA, whereas CP-LPHM and AC (positive controls) both showed high affinity binding for the ligand (Figure 12). However, only AC appeared to have a saturable binding site for VA. Data for VA sorption were best fit to the Freundlich Model (FM) with K_d (± SEM) equal to 1.59 x 10^2 ± 1.50 x 10^2 for NSP-VA interactions and 6.44 x 10^4 ± 1.24 x 10^4 for CP-LPHM-VA interactions. The shapes of the data plots suggest sorption occurs primarily by partition processes. In contrast, AC-VA isotherm data were fit best to the LM showing a Q_{max} of 0.552 ± 0.027 and a K_d value of 4.15 x 10^5 ± 0.89 x 10^5.

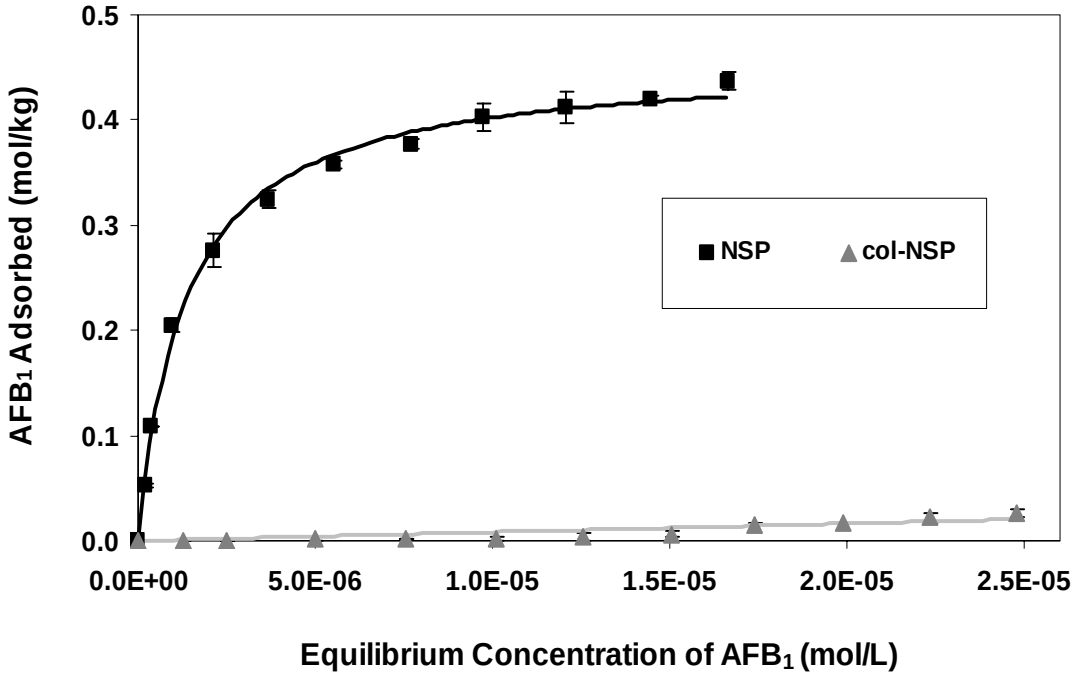

Figure 11. Comparison of isotherm sorption plots for AFB_1 on NovaSil PLUS (NSP) vs. collapsed NSP (col-NSP) surfaces at 25 ^{o}C (pH 7). Isotherm data were fitted to the Langmuir Model. The solid lines show the curve fits while the experimental data are represented by the average data points ± SEM.

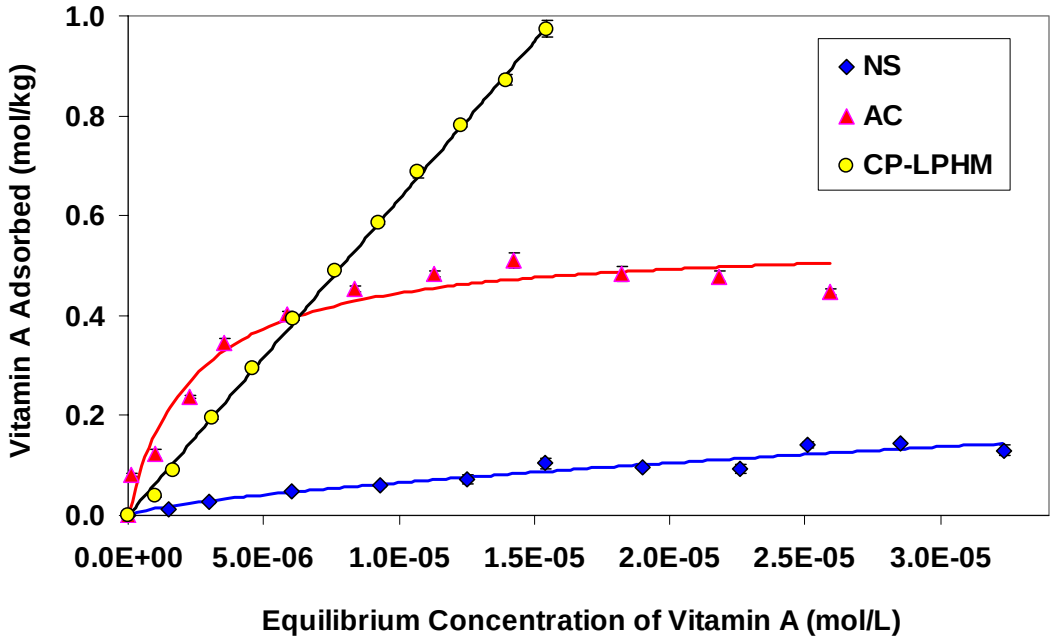

Figure 12. Comparison of isotherm plots for vitamin A sorption onto NSP, CP-LPHM, and AC surfaces at 25 ^{o}C (pH 7). AC Isotherm data were fit to Langmuir Model while CP-LPHM and AC data were fit to Freundlich Model. The solid lines show the curve fits while the experimental data are represented by the average data points ± SEM.

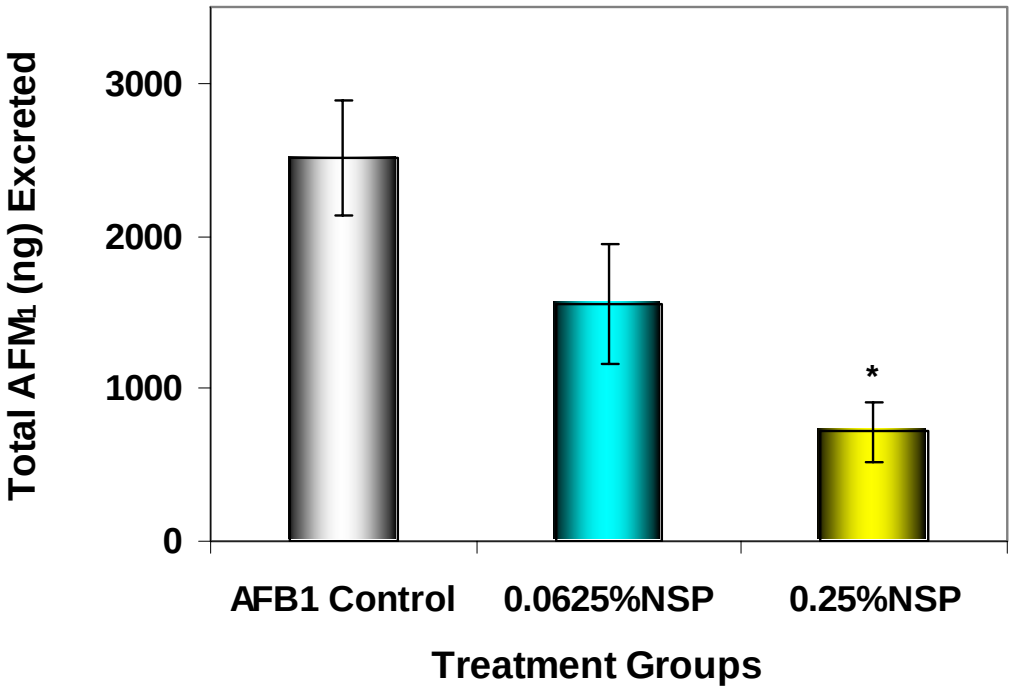

Figure 13. Total AFM_1 in urine over 48 hr of rats dosed 1.0 mg/Kg (bw) AFB_1. Values represent data for 3 rats ± SEM. *****Indicates significant reduction of AFM_1 levels in rats dosed with the toxin and treated with NSP versus untreated controls.

2.3.3 Analysis of Urinary Biomarker AFM_1 in Rats

AFM_1 was used as a biomarker indicative of AFB_1 exposure. In control animals dosed with 1.0 mg/kg (bw) AFB_1 in the absence of clay, the average concentration (± SEM) of AFM_1 was 2511.2 ± 380.0 ng/ml of urine. Those animals exposed to AFB_1 and NSP showed lower AFM_1 levels. Co-exposure to 0.0625% NSP decreased AFM_1 in urine to 1555.3 ± 397.6 ng (38%) and 0.25% NSP decreased the biomarker to 719.2 ± 196.5 ng (71%) (Figure 13).

2.3.4 Feeding Studies using Broiler Chicks

In the feeding study, consumption of AFB_1-contaminated diet in the absence of NSP resulted in significantly decreased feed consumption (FC) in broiler chicks (Table 7). Treatment with NSP ranging from 0.125 to 0.5% NSP showed protection from these effects, although not to the extent of untreated controls. Compared to the controls, AFB_1 reduced the FC by 30% while reductions in the presence of the clay with the toxin were 9.8% (0.25% NSP), 13.0% (0.50%NSP), and 15.7% (0.125% NSP (Table 7). Also, dietary exposure to NSP alone and at the greatest amount used in the study (0.5%) did not significantly affect feed consumption.

In addition to feed consumption, dietary exposure to 5 mg/kg AFB_1 significantly affected body weight gain (Table 8). In chicks fed diets containing 5 mg/kg AFB_1, body-weight gains were reduced by 19% after 21 d, although significant reductions were apparent after only 7 d. Exposure to 0.5% NSP in

TABLE 7

Effects of dietary NSP inclusion either alone or in combination with AFs on average feed consumption and feed:gain ratios in broiler chicks

Treatment Group		Feed Consumption[1] (g)	% Reduction of FC from control	Feed:Gain Ratio[1]
AFB_1 (mg/kg)	NSP (%)			
0	0	1369.0 ± 45.7^{a}	0.0	1.628 ± 0.058^{a}
0	0.500	1275.8 ± 38.5^{a}	6.8	1.535 ± 0.029^{a}
5	0	957.8 ± 183.1^{b}	30.0	1.480 ± 0.117^{a}
5	0.125	1153.8 ± 103.4^{ab}	15.7	1.483 ± 0.084^{a}
5	0.250	1235.0 ± 54.2^{ab}	9.8	1.460 ± 0.050^{a}
5	0.500	1191.5 ± 22.9^{ab}	13.0	1.538 ± 0.073^{a}

Note. [1]Data are reported as the mean ± SEM of four replicate pens of six broiler chicks each per treatment group. [a,b] Mean values with no common superscript differ significantly ($p \leq 0.05$). FC = feed consumption.

TABLE 8

Effects of dietary NSP inclusion either alone or in combination with AFs on temporal body weight gains in broiler chicks

Treatment Group		Body Weight Gain[1] (g)				
AFB_1 (mg/kg feed)	NSP (%)	---------- Days ----------				% Reduction from Control (21 days)
		1-7	8-14	15-21	1-21	
0	0	145 ± 2.9^{a}	265 ± 13.0^{ab}	446 ± 13.2^{a}	866 ± 12.7^{a}	0
0	0.500	132 ± 5.9^{a}	287 ± 11.0^{a}	418 ± 26.1^{a}	839 ± 42.6^{a}	3
5	0	126 ± 9.6^{a}	229 ± 18.5^{b}	338 ± 22.7^{b}	699 ± 38.5^{b}	19
5	0.125	138 ± 4.7^{a}	264 ± 8.9^{ab}	390 ± 31.2^{ab}	791 ± 43.5^{ab}	9
5	0.250	144 ± 6.1^{a}	284 ± 6.9^{a}	427 ± 12.0^{a}	854 ± 22.7^{a}	1
5	0.500	136 ± 3.4^{a}	290 ± 8.9^{a}	385 ± 30.5^{ab}	816 ± 37.6^{a}	6

[1]Data are reported as the mean $\pm$ SEM of four replicate pens of six broiler chicks each per treatment group.
[a,b]Mean values with no common superscript differ significantly ($p \leq 0.05$).

the absence of AFB_1 did not significantly decrease body weight gain as compared to those animals consuming diets without clay. Throughout the study, chicks consuming diets containing AFs in the absence of NSP had significantly lower gains in body weight and the inclusion of NSP prevented growth inhibition with varying levels of effectiveness. After 21 d of continuous exposure to AFs, body weights of chicks fed diets containing either 0.25 or 0.50% NSP did not significantly differ from controls. However, this effect was not apparent in those chicks consuming only 0.125% NSP. No significant differences were observed for feed:gain ratios among treatment groups and controls (Table 7).

A clear dose-response relationship was observed for protection from the effects of AFB_1 exposure on liver and kidney weights with various dietary levels of NSP (Table 9). Dietary inclusion of AFB_1 caused significant increases in both relative liver and kidney weights in broiler chicks (Table 9). In chicks exposed to the greatest amount of NSP (0.5%), neither relative organ weight was significantly different from those animals consuming the control diet. The relative liver weights of chicks maintained on diets containing either 0.5% NSP in addition to 5.0 mg/kg AFB_1 were comparable to untreated controls, whereas levels of only 0.25 or 0.125% NSP failed to show significant protection in comparison to AFB_1 controls. A similar trend was observed for relative kidney weights.

Hepatic VA in broiler chicks was significantly reduced in those animals consuming diets containing 5.0 mg/kg AFB_1 (Figure 14). Further, co-exposure

TABLE 9

Effects of dietary NSP inclusion either alone or in combination with AFs on relative organ weights in broiler chicks

Treatment Group	Relative Liver Weight[1] (g/100g bw.)	Relative Kidney Weight[1] (g/100g BW)
Control Feed	2.99 ± 0.15^{b}	0.441 ± 0.034^{bc}
0.5% NSP	2.82 ± 0.14^{b}	0.371 ± 0.023^{c}
AFB_1 (5.0 mg/kg feed)	3.87 ± 0.49^{a}	0.589 ± 0.055^{a}
0.125% NSP + AFB_1	3.54 ± 0.32^{ab}	0.547 ± 0.046^{ab}
0.25% NSP + AFB_1	3.12 ± 0.03^{ab}	0.503 ± 0.024^{ab}
0.5% NSP + AFB_1	2.81 ± 0.16^{b}	0.449 ± 0.024^{bc}

[1]Data are reported as the mean ± SEM of four replicate pens of three broiler chicks each per treatment group. [a,b,c] Mean values with no common superscript differ significantly ($p \leq 0.05$).

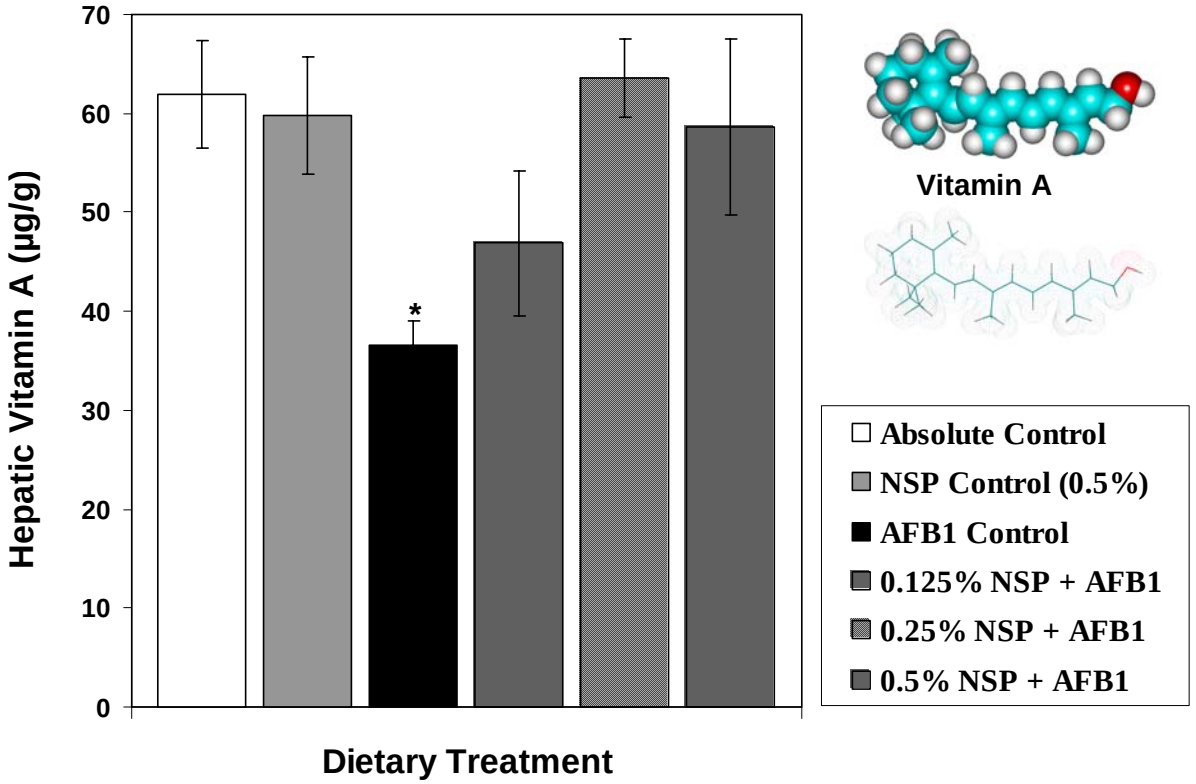

Figure 14. Dose response effects of dietary NSP addition on hepatic vitamin A in broiler chicks. Data are shown as the means ± SEM of four replicate pens of three broiler chicks each per treatment group. *Indicates that average hepatic VA content of chicks exposed to AFB_1 control was significantly reduced compared to all the other treatments.

to NSP ranging from 0.125 to 0.5% in the diet showed complete protection, particularly at 0.25% level of NSP, from the effects of AFB_1 on hepatic VA.

2.4 Discussion

The present study characterized the sorption of AFB_1 and evaluated the potential interaction with VA by NSP, a calcium montmorillonite clay that is commonly added to animal feeds as an anti-caking agent. Overall, the *in vitro* data suggests that NSP selectively provides the capacity and affinity for AFB_1 equivalent to or greater than that previously reported for NS, another calcium montmorillonite anti-caking agent (Grant and Phillips, 1998). In both cases, calculation of thermodynamic parameters suggests strong sorption of AFB_1 (Figure 10B). However, the estimated enthalpy of sorption for NSP (-49.2 kJ/mol) was higher than values previously reported for NS (-40 kJ/mol), suggesting a tighter binding to the clay (Grant and Phillips, 1998). Based on ΔH_{ads}, the adsorption of AFB_1 to the surface of either sorbent is characterized as chemisorption, a chemical reaction and/or sharing of electrons between the chemical and the sorbent surface that is indicated by enthalpy values > -20 kJ/mol (Gatta, 1985). This suggests a stronger association between the toxin and ligand, predicting a greater efficiency as an enterosorbent. The variability of ΔH_{ads} in these experiments may reflect multiple binding mechanisms and/or sites as well as the heterogeneity of the clay mineral (Figure 2B). For the col-NSP, isothermal analysis indicated that the capacity (Q_{max}) and affinity (K_d) were 94%

and 99% less than unmodified NSP, respectively. This evidence is similar to Grant's report on NS and suggests that a large portion of AFB_1 binding to NSP occurs within the interlayer region (and Ca^{2+} ions) of the clay (Grant, 1998).

NSP interaction with VA was negligible *in vitro*, predicting a lack of interaction with the nutrient *in vivo* (Figure 12). Sorbents used as positive controls were CP-LPHM, a hydrophobic organoclay, and AC because they have been shown to be effective for organic compounds such as VA (Paune et al., 1998; Srinivasan and Fogler, 1990a,b). Both showed high affinity for VA under these conditions. The FM was used to fit data for NSP- and CP-LPHM-VA interactions and C-type isotherm curves were observed, indicating partition processes - involving weak associations such as van der Waals, dipole-dipole, induced dipole, and hydrogen bonding (Gu et al., 1994). However, this model was not appropriate for fitting the AC-VA data. The LM showed an H-type curve, suggesting a saturable site for VA sorption that is indicated by the presence of a plateau. This is important because the data show that not all sorbents are appropriate for intervention of the AF problem in animals and/or humans.

Based on the prevalence in urine and the dose-dependent linearity observed with dietary AFB_1 ingestion, AFM_1, an oxidative metabolite of AFB_1, was selected as a biomarker of exposure (Groopman et al., 1992a; Hsieh and Wong, 1994). Previous research indicated that 0.5% NS in the diet was sufficient to reduce AFM_1 in the urine of Fisher 344 rats by 58% following a single 1 mg/kg (bw) AFB_1 gavage dose (Sarr et al., 1995). At an identical

dietary inclusion level, NS reduced the urinary metabolite in Sprague-Dawley rats exposed to a single 2 mg/kg (bw) AFB_1 gavage dose by 76% (Mayura et al. 1998). In this study, inclusion of 0.25% NSP reduced AFM_1 in the urine of Sprague-Dawley rats following a 1 mg/kg (bw) AFB_1 dose by 71% (Figure 13), suggesting that NSP is perhaps more efficient than NS in reducing the bioavailability of AFB_1 in the gastrointestinal tract.

In broiler chicks, the health manifestations of AFB_1 exposure were expressed as reduced weight gains, increased liver and kidney weights, and decreased feed consumption. These effects are in agreement with those previously reported (Huff et al., 1986; Kubena et al., 1990a, 1993b). The feed consumption of broiler chicks exposed to AFB_1 in the presence of NSP was greater than for those consuming diets that did not contain the sorbent, although not as great as in the untreated controls. Of the 3 treatment levels tested in this study, dietary inclusion of 0.25% NSP was the most effective in reducing the negative impact of AFB_1 on body weight gains (95%). At inclusion levels of 0.5 and 0.125%, NSP lessened the AFB_1-dependent decrease in body weight gain by 68 and 53%, respectively. Based on both feed consumption and body weight gain, 0.25% NSP appeared to be the most effective in broiler chicks exposed to 5 mg/kg in the diet.

The liver is considered to be a target organ for AFs and the effects are typically expressed as an increase in weight (Huff et al., 1986). Renal toxicity has also been linked to AF in some animals including swine (Sisk and Carlton,

1972) and rabbits (Clark et al, 1982). In this study, exposure to 5 mg/kg AFB_1 in the absence of sorbent significantly increased the relative liver weights in broiler chicks. The relative liver weights of birds treated with NSP (0.5% and 0.25%) plus 5 mg/kg AFB_1 were not only comparable to controls, but were also significantly lower when compared to birds treated with AFB_1 alone. These data indicate that NSP effectively protected chicks from AFB_1 hepatotoxicity at levels as low as 0.25%. Also, relative kidney weights were reduced to levels comparable to the controls in the presence of 0.5% NSP in diet of chicks exposed to 5 mg/kg AFB_1. A similar protective effect by NS against AFB_1 was observed on relative kidney weights and was consistent with previously published reports (Kubena et al., 1990a, 1993b).

VA is vital for vision, controlling the differentiation program of epithelial cells in the digestive tract and respiratory system, skin, bone, the nervous system, the immune system, and for hematopoiesis (Gursu et al., 2002). VA also induces lymphoproliferation which results in a stimulated immune system. A deficiency can decrease specific antibody production, the number of circulating lymphocytes, and lymphocyte proliferation (West et al., 1991). In a previous report, camels with aflatoxicosis had about half of retinol (VA) levels found in the plasma of healthy camels (Abbas and Ali, 2001). However, it was not clear if VA deficiency predisposed the camels to aflatoxicosis or the AFs induced the VA deficiency. Also, potential for interaction between nutrients (such as VA) and ingested clay minerals had been unclear until this study was conducted.

Importantly, this study indicated that NSP does not significantly alter hepatic VA content at the levels tested (0.125, 0.25, and 0.5%) against a dietary inclusion level of 5 mg AFB_1/kg. These results are in agreement with isothermal measurements, suggesting negligible interactions between NSP and VA in the presence or absence of the AFB_1. Thus, in the presence of NSP, the overall health and immune defenses of AF-challenged broilers would not be compromised by VA deficiency since the sorbent does not interact with VA.

Overall, *in vitro* and *in vivo* results suggest that NSP strongly binds AFB_1 from the gastrointestinal tract, but does not interfere with VA levels, indicative of strong specificity. Also, in a study by Wiles et al (2004), concentrations as high as 2% of dietary NSP neither produced maternal or fetal toxicity in pregnant rats, nor significantly affected the bioavailability of selected trace metals in a variety of tissues.

This clay-based application, when used in combination with other good management practices, may be a valuable tool for the development of an integrated approach to the preventive management of mycotoxin-contaminated commodities. Due to the observed strong specificity for AFB_1 sorption, NSP may also be a viable candidate for studies designed to prevent AF exposure in humans. However, long-term animal studies are required to comprehensively evaluate any hidden potentials for toxicity prior to the initiation of human feasibility studies. Future research is needed to identify other sorbents to effectively sorb other mycotoxins found in food and feed. Also, studies are

needed to evaluate NSP and other sorbents for potential interactions with other nutrients such as Fe, Zn, Ca, K, vitamins E, D, K, etc.

CHAPTER III

DIETARY INCLUSION OF NOVASIL: *IN VITRO* ANALYSIS, SUBCHRONIC AND CHRONIC TOXICITY EVALUATION IN SPRAGUE-DAWLEY RATS

3.1 Introduction

Aflatoxins (AFs) are common foodborne mycotoxins that have been identified as a result of *Aspergillus* growth in grains, such as peanuts, corn, cottonseed, and tree nuts (CAST, 2003). The compounds are carcinogenic (Wild et al., 1992; WHO-IARC, 1993a,b), genotoxic (Wang et al., 1999; Smela et al., 2001), and immunotoxic (Hinton et al., 2003; Turner et al., 2003). In addition, the presence of AFs has been found to significantly reduce hepatic vitamin A in animals, including broiler chicks (Pimpukdee et al., 2004). Importantly, vitamin deficiency is a common cause of blindness, especially in developing countries (Fieldler et al., 2000).

The naturally occurring AFs (e.g., B_1, B_2, G_1, and G_2) are characterized as ubiquitous and can contaminate food/feed sources either separately or concurrently (CAST, 1989). Among the naturally occurring AFs, aflatoxin B_1 (AFB_1) is the most predominant, and the most potent hepatocarcinogen known in humans (Flaherty and Payne, 1997; Lopez et al., 2002). It is estimated that ingestion of 2-6 mg AF/day for one month can lead to acute hepatitis and death in humans (Patten, 1981). In the U.S., a 20 ppb action level for AFs in foods

intended for human consumption has been set, however, a recent outbreak of AF poisoning in Kenya was linked to consumption of foods containing levels as high as 8,000 ppb (CDC, 2004). Because these toxins can affect both animal and human health, effective (yet economically feasible) intervention strategies are imperative.

Several intervention strategies have been previously reviewed and include diverse physical, chemical, and biological methods (Phillips, 1999; Phillips et al., 1994, 2002). One practical approach of current interest is the inclusion of non-nutritive clay minerals in contaminated food and feed to bind AFs in the gastrointestinal tract, thus reducing toxin bioavailability and distribution to the blood and target organs (Phillips, 1995, 1999).

The dietary consumption of clay (known as geophagy) is not a new phenomenon. It has been observed among both animals and humans on all continents for centuries (Carretero et al., 2002). The potential health benefits of geophagy (although ill-defined in the scientific literature) may include the enterosorption and decreased bioavailability of toxic chemicals and hazardous microbes from contaminated food and water.

For example, a naturally-occurring calcium montmorillonite clays (e.g. NovaSil and NovaSil Plus) that is routinely added to feeds as an anti-caking agent, has been used as a novel dietary intervention to decrease external exposure to aflatoxins. NovaSil (NS) and NovaSil Plus (NSP) in the diet has resulted in significant protection from the adverse effects of AFs in multiple

animal species, including pregnant rodents (Mayura et al., 1998), chickens (Phillips et al., 1988; Kubena et al., 1990a; Pimpukdee et al., 2004), turkeys (Kubena et al., 1991), swine (Lindemann et al., 1993), lambs (Harvey et al., 1991a), goats (Smith et al., 1994), and dairy cattle (Harvey et al., 1991b).

In addition, no observable adverse effects have been reported in numerous short-term animal studies utilizing NSP clay for the prevention of aflatoxicosis. In particular, a study in which Sprague-Dawley (S-D) rats ingested the clay at dietary concentrations as high as 2% throughout pregnancy showed neither maternal nor fetal toxicity, and did not show significant trace metal bioavailability in a variety of tissues (Wiles et al., 2004).

Importantly, the potential effects of NSP clay following chronic dietary ingestion have not been established. These include weight gains and feed conversions, common hematological and serum biochemical parameters, histology, and nutrient levels (e.g., vitamins A and E, Fe and Zn). Thus, the objective of this study was to evaluate the potential adverse effects of sub-chronic and chronic dietary inclusion of NSP in S-D rats to predict the safety of this product for future use in human populations burdened by AF contamination.

3.2 Materials and Methods

3.2.1 Chemical Reagents

For all experiments, purified water (ddH_2O) was prepared by processing deionized water through a Milli-Q^{UF+} purification system (Millipore, Benford, MA).

Novasil clay was kindly provided by the Engelhard Chemical Corporation (Iselin, NJ). All-trans retinol (>95% purity) and α–tocopherol (>95% purity), normal human serum, tetrahydrofuran (THF), ascorbic acid, PBS, and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich (St. Louis, MO). Individual stock standard solutions of all-trans-retinol (4.34 μg/ml) and α-tocopherol (32.8 μg/ml) were prepared in absolute ethanol and stored at -20 °C. Concentrations were calculated based on absorbance values in ethanol at 325 nm (all-trans-retinol) and 292 nm (α-tocopherol) wavelengths. Dilutions were performed immediately prior to use. HPLC grade acetone, acetonitrile (ACN), and methanol (MeOH) were obtained from Burdick and Jackson (Muskegon, MI). Ethanol was obtained from Anachemia (Rouses Point, NY). HPLC grade ammonium acetate (AA) was purchased from Fisher (Fair Lawn, NJ).

3.2.2 Quantification of PCDD/PCDF in NSP Clay

Polychlorinated dibenzo-*p*-dioxin/furan analysis was performed by Columbia Analytical Services, Inc. (Houston, TX). Procedures followed the United States Environmental Protection Agency (USEPA) methods for sample preparation, cleanup, and analysis with high resolution capillary column gas chromatography/high resolution mass spectrometry (USEPA Method 1613b). The 17 USEPA priority compounds analyzed included: 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin; 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin; 1,2,3,4,7,8-hexachloro-dibenzo-*p*-dioxin; 1,2,3,6,7,8-hexachlorodibenzo-*p*-dioxin; 1,2,3,7,8,9-hexa-chlorodibenzo-*p*-dioxin; 1,2,3,4,6,7,8-heptachlorodibenzo-*p*-dioxin; octachloro-

dibenzo-*p*-dioxin (OCDD); 2,3,7,8-tetrachlorodibenzo-*p*-furan; 1,2,3,7,8-pentachlorodibenzo-*p*-furan; 2,3,4,7,8-pentachlorodibenzo-*p*-furan; 1,2,3,4,7,8-hexachlorodibenzo-*p*-furan; 1,2,3,6,7,8-hexachlorodibenzo-*p*-furan; 1,2,3,7,8,9-hexachlorodibenzo-*p*-furan; 2,3,4,6,7,8-hexachlorodibenzo-*p*-furan; 1,2,3,4,6,7,8-heptachlorodibenzo-*p*-furan; 1,2,3,4,7,8,9-heptachlorodibenzo-*p*-furan; and octachlorodibenzo-*p*-furan.

3.2.3 Feed Preparation and Experimental Design

Five to six week old male (102 to 163 g) and female (79 to 135 g) Sprague-Dawley (S-D) rats were purchased from Harlan (Houston, TX) and maintained on feed (Teklad rodent diet 8604, Harlan, Madison, WI) and water *ad libitum.* All animals were housed in a climate-controlled environment (temperature 22 to 25 °C) that was artificially illuminated (12 hr dark/12 hr light) and free from chemical contamination. After a short acclimation period, the animals were randomly divided into treatment groups receiving 1 of 5 dietary formulations containing basal rodent feed with various concentrations of NS ranging from 0 to 2% (w/w). Diets consisted of: 1) absolute control (Abs), 2) 0.25% NSP, 3) 0.5% NSP 4) 1.0% NSP, and 5) 2.0% NSP. Dietary clay concentrations were based on the highest level previously allowed (2.5% w/w) by the Association of American Feed Control Officials (AAFCO) for use in pelleting processes and as an anti-caking agent in non-medicated livestock feeds (Ferrario and Byrne, 2000). Each treatment group consisted of 13 male

and 13 female rats, with the exception of the 0.5% NS group which contained 12 males and 14 females. Animals were inspected daily for general appearance, behavioral changes and signs of morbidity and mortality. Feed consumption was recorded daily for the first month and every fourth day thereafter. Body weights were measured initially then once per week throughout the course of the study. Necropsy and histopathological evaluations were conducted on 30 animals (3 rats/sex/group) at 90 d and at 28 weeks for the remaining 100 animals (10 rats/sex/group). After 28 weeks, final body weights were recorded and blood was drawn via cardiac puncture under isoflurane anesthesia. Following euthanasia at both time points, tissues of interest (liver, kidneys, lungs, heart, brain, spleen, tibia, uterus and ovaries) were removed and assessed for anatomic abnormalities. Wet weights were recorded at both termination time points and portions of all tissues were fixed in 10% formalin for subsequent histological analysis. A portion of skin (including hair) and the gastrointestinal tract were also collected and fixed in the same manner.

3.2.4 Hematological and Serum Biochemical Parameters

Hematological and serum biochemical parameters were analyzed by the Clinical Pathology Lab, Texas Veterinary Medical Diagnostics Lab (TVMDL) (College Station, TX). Hematological analysis of collected whole blood samples was conducted using a Cell-Dyn 3500 counter (Abbott Laboratories, Abbott Park, IL) and included red blood cell (RBC) count, hemoglobin (Hb)

concentration, percent corpuscular volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and white blood cell (WBC) count. Leukocyte differential counting was performed manually from microscopic examination of blood smears and included nuetrophils, lyphocytes, monocytes, and eosinophils. Serum biochemical parameters were assessed using a Hitachi 911 (Roche Laboratories, Indianapolis, IN) and included total protein (TP), albumin (ALB), calcium (Ca), phosphorous (P), glucose (GLUC), blood urea nitrogen (BUN), creatinine (CRT), total bilirubin (T-BIL), alkaline phosphatase (ALP), creatine kinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), globulin (GLOB), A/G ratio, gamma glutamyl-transferase (GGT), amylase (AMYL), and cholesterol (CHOL). In addition, serum micronutrients iron (Fe) was also quantified using a Hitachi 911. Also, serum zinc (Zn) levels were analyzed using a Perkin Elmer Analyst 100 (PerkinElmer, Shelton, CT).

3.2.5 Serum Vitamins A and E Analysis

Serum vitamins A and E were extracted under yellow light following previously described methodology (Ruperez et al, 2004). Briefly, rat serum (50 μl) was mixed with 150 μl ethanol:chloroform (3:1, v/v) in a 1.5 ml microcentrifuge tube, vortexed for 1 min, allowed to stand for 5 min, and vortexed for an additional 1 min. Samples were then centrifuged at 13,200 x *g* for 8 min at 4 °C and the supernatant was analyzed by HPLC.

3.2.6 Hepatic Vitamin A & E Analysis

Liver vitamins A & E were extracted following a modification of procedures described by Hosotani and Kitagawa (2003). Briefly, 0.2 g liver was mixed with 10 ml 1X PBS containing 1% ascorbic acid. The mixture was homogenized for 5 min on ice under reduced light. Following homogenization, 100 µl of ddH_2O was added to 50 µl of homogenate, and the mixture was vortexed for 20 s. For protein precipitation, 300 µl of ethanol:chloroform (3:1, v/v) containing 0.01% BHT was added and the mixture was vortexed for 1 min. Hexane (600 µl) was added and the solution was vortexed for 1 min and then centrifuged at 13,200 x *g* for 10 min at 4 °C. Following centrifugation, the hexane layer was removed and the sample was dried using a CentriVap Cold Trap (Labconco, Kansas City, MO). The residue was reconstituted in 200 µL of mobile phase A - ACN: THF: MeOH: AA (85:5:5:5, v/v/v/v), 50 µl of which was used for the HPLC analysis.

3.2.7 HPLC Analysis Procedures

HPLC analysis of VA and VE was performed using previously reported methodology (Burri et al., 2003) with minor modifications using a Thermo Finnigan Liquid chromatograph with a P4000 pump, an AS3000 autosampler with a 100 µl loop, and a UV6000 LP photodiode array detector (Thermo Separation Products, Riviera Beach, FL). For serum VA and VE, chromatographic separation was achieved with a Microsorb 100-5 C_{18} column

with 150 mm × 4.6 mm ID and 5 μm particle size (Varian, Palo Alto, CA) using mobile phase A (ACN:THF:MeOH:AA at 85:5:5:5, v/v/v/v); and mobile phase B (ACN:THF:MeOH:AA at 55:35:5:5, v/v/v/v) under a flow rate of 1 ml/min with an injection volume of 50 μl. The elution profile consisted of 95% A and 5% B for the first 5 min, followed by a gradient to 5% A and 95% B over 13 min. Afterwards, conditions were maintained for 2 min and then the column was washed with 95% A and 5% B for 8 min. The total run time was 28 min. Analysis of hepatic VA and VE was conducted using a Zorbax Eclipse XDB-C_{18} column with 250 mm × 4.6 mm ID and 5 μm particle size (Agilent, Palo Alto, CA). The elution profile consisted of 95% A and 5% B for the first 5 min, followed by a gradient to 5% A and 95% B over 13 min. Afterwards, conditions were maintained for 10 min and then the column was washed with 95% A and 5% B for 8 min. The total run time was 36 min. Quantification of both vitamins was based on comparison of peak areas and retention times to reference standards. In the case of liver analysis, final vitamin concentrations were adjusted based on individual tissue weights.

3.2.8 Histopathology

Formalin-fixed tissues (liver, kidney, heart, lungs, brain, stomach, intestine, skin, uterus and ovaries) were processed and embedded in paraffin. The tissues were sectioned at 5 μm and stained with hematoxylin and eosin (H&E) for microscopic examination. Severity of microscopic changes was

graded as: 0, normal; 1, minimal change; 2, mild change; 3, moderate change; or 4, severe change.

3.2.9 Statistics

All experimental data was subjected to ANOVA utilizing the General Linear Models procedures to establish differences among treatment groups with variable means. Means were compared using Tukey's multiple comparison procedure and considered significant at $p \leq 0.05$.

3.3 Results

3.3.1 Quantification of PCDD/PCDF in NSP Clay

Of the 17 USEPA priority PCDDs/PCDFs, only OCDD (2.34 parts per trillion, ppt) was present above the limits of detection (LOD = 1.02 ppt) in NSP. Based on this value, the toxic equivalent (TEQ) of this contaminant was calculated to be 0.000234 in the clay.

3.3.2 Feeding Study

Throughout the study, all animals with the exception of 2 males remained healthy and active with no noticeable behavioral changes. One male rat (0.25% NSP) exhibited dark discharge from the left eye on day 92 that disappeared within 3 days and an ulceration was observed on the right hind limb of a second male rat (absolute control) on day 132 that healed within 1 week.

TABLE 10

Total feed consumption (TFC), total body weight gain (TBWG), and feed conversion efficiency (FCE) in S-D rats following the dietary ingestion of 0 - 2.0% NSP for 28 weeks

Treatment Group (% NSP)	Total Animals	TFC[a] (g)	TBWG[a] (g)	FCE[a]
		Males		
Abs. control	10	4305.5 ± 55.3	339.2 ± 8.8	12.74 ± 0.24
0.25%	10	4286.4 ± 66.6	341.9 ± 5.5	12.55 ± 0.21
0.5%	9	4581.9 ± 149.5	350.7 ± 11.4	13.11 ± 0.35
1.0%	10	4688.5 ± 199.9	349.9 ± 12.7	13.41 ± 0.34
2.0%	10	4622.2 ± 177.2	348.0 ± 7.2	13.28 ± 0.40
		Females		
Abs. control	10	3102.4 ± 82.7	156.4 ± 7.9	20.07 ± 0.64
0.25%	10	3204.1 ± 84.6	164.5 ± 7.0	19.70 ± 0.70
0.5%	11	3185.5 ± 68.4	160.4 ± 4.4	19.95 ± 0.53
1.0%	10	3195.8 ± 68.1	173.9 ± 7.1	18.59 ± 0.67
2.0%	10	3212.0 ± 50.5	172.4 ± 6.9	18.89 ± 0.79

[a]Data represent mean values (± SEM) of 10 animals/sex/group with the exception of the 0.5% NSP treatment which contained 9 males and 11 females.

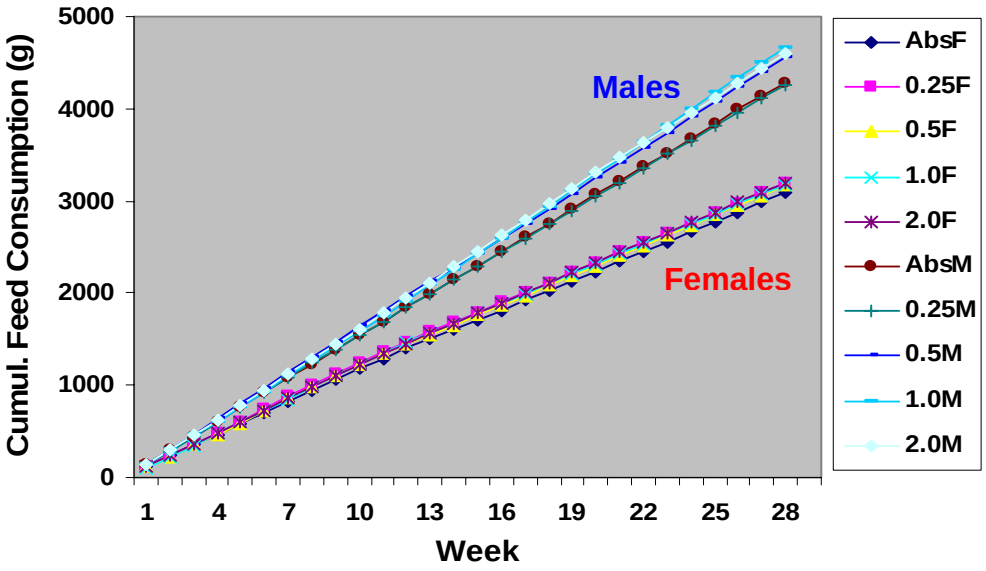

Figure 15. Mean cumulative weekly feed consumption of male (M) and female (F) S-D rats fed diets containing 0 - 2% NSP for 28 weeks.

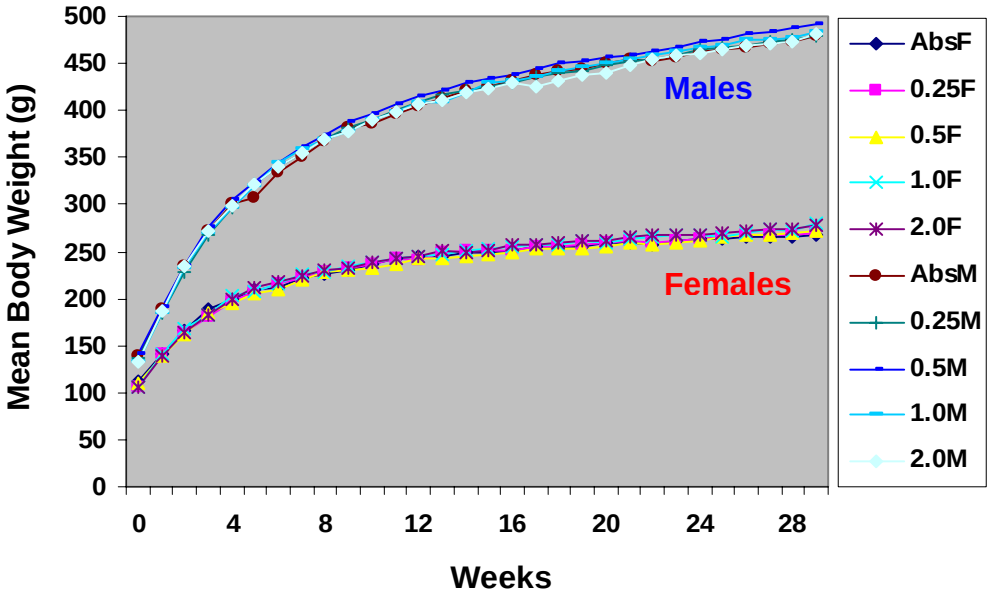

Figure 16. Mean body weight (g) of male (M) and female (F) S-D Rats fed diets containing 0% - 2% NSP for 28 weeks.

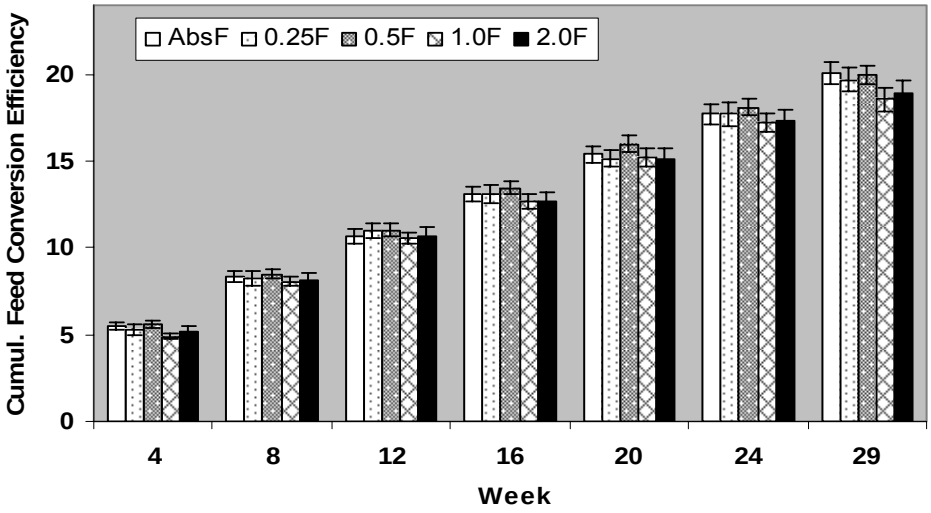

Figure 17. Mean cumulative feed conversion efficiency (± SEM) calculated as the ratio of cumulative feed consumption to the cumulative body weight gain of female S-D rats with dietary inclusion levels of 0-2% NSP over 28 weeks.

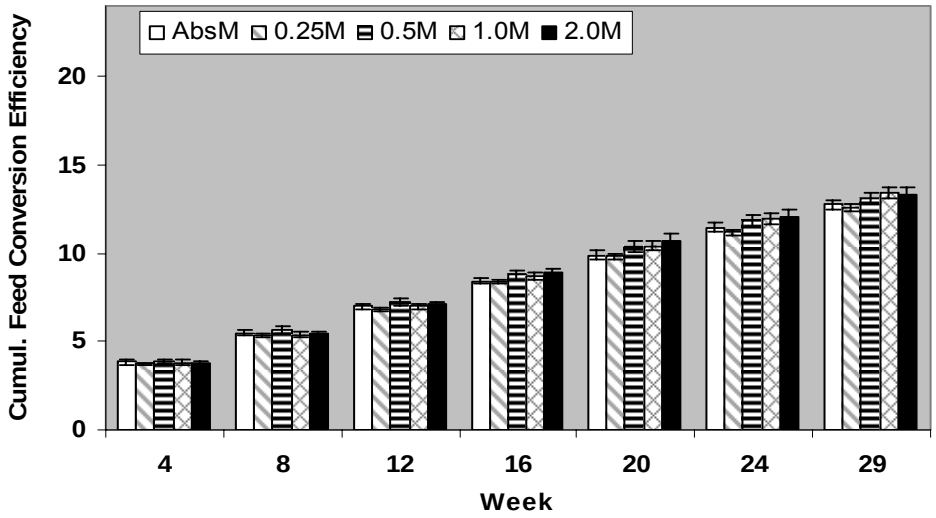

Figure 18. Mean cumulative feed conversion efficiency (± SEM) calculated as the ratio of cumulative feed consumption to the cumulative body weight gain of male S-D rats with dietary inclusion levels of 0-2% NSP over 28 weeks.

There were no statistically significant differences between rats (within each sex) consuming treated versus untreated diets with regard to: total feed consumption (TFC), cumulative feed consumption (CFC) - calculated as the sum of feed consumption from initiation to each additional week, body weight (BW), total body weight gain (TBWG), feed conversion efficiency (FCE) - measured as TFC/TBWG, and cumulative feed conversion efficiency (CFCE) - calculated as the sum of FCE from initiation to each additional 4 week period throughout the study (Table 10 and Figures 15, 16, 17, and 18). However, FCE increased over the course of the study in both sexes in treated and untreated animals, and this increase was independent of the amount of NSP added to the diet (Figures 17 and 18).

At necropsy on day 90 and day 196, there were no obvious gross abnormalities in the major organs of interest (liver, kidneys, lungs, heart, brain, spleen, tibia, uteri and ovaries) in NSP-treated rats. In addition, the relative organ weight (ROW) for these organs (data on day 90 not shown), measured as the ratio of wet organ weight (WOW) to final body weight (FBW) was not significantly different in rats consuming treated versus untreated diets (Table 11). Histopathological evaluation of H&E stained sections of the major organs showed no substantial differences between animals consuming NSP-containing diets and untreated controls at 90 d (Tables 12 and 13) or 28 weeks (Table 14 and 15). Any lesions present were interpreted as background lesions and were of similar incidence and severity in NSP-treated and control animals.

TABLE 11

Relative organ weights of S-D rats following the dietary addition of 0 - 2.0% NSP for 28 weeks

Organ	Treatment Group				
	Abs. control	0.25%	0.5%	1.0%	2.0%
			Males		
Brain ($x10^3$)	3.75 ± 0.07	3.85 ± 0.06	3.72 ± 0.08	3.78 ± 0.11	3.78 ± 0.10
Heart ($x10^3$)	2.95 ± 0.05	2.88 ± 0.06	2.98 ± 0.07	2.79 ± 0.06	2.90 ± 0.05
Kidneys ($x10^3$)	5.95 ± 0.16	5.83 ± 0.13	5.91 ± 0.12	5.77 ± 0.12	5.75 ± 0.16
Liver ($x10^2$)	3.31 ± 0.10	3.28 ± 0.09	3.27 ± 0.10	3.22 ± 0.07	3.25 ± 0.09
Lungs ($x10^3$)	3.36 ± 0.08	3.60 ± 0.09	3.68 ± 0.12	3.44 ± 0.09	3.28 ± 0.14
Spleen ($x10^3$)	1.67 ± 0.06	1.78 ± 0.07	1.74 ± 0.04	1.76 ± 0.05	1.75 ± 0.06
Tibia ($x10^3$)	1.67 ± 0.02	1.65 ± 0.02	1.63 ± 0.02	1.70 ± 0.04	1.71 ± 0.03
			Females		
Brain ($x10^3$)	6.17 ± 0.13	6.07 ± 0.13	6.19 ± 0.10	6.02 ± 0.14	5.92 ± 0.09
Heart ($x10^3$)	3.20 ± 0.05	3.37 ± 0.06	3.26 ± 0.07	3.28 ± 0.11	3.24 ± 0.07
Kidneys ($x10^3$)	6.00 ± 0.11	5.91 ± 0.10	5.84 ± 0.12	5.63 ± 0.10	5.84 ± 0.07
Liver ($x10^2$)	2.94 ± 0.05	2.83 ± 0.04	2.85 ± 0.04	2.90 ± 0.05	2.83 ± 0.06
Lungs ($x10^3$)	4.72 ± 0.15	4.89 ± 0.14	4.64 ± 0.12	4.58 ± 0.09	4.65 ± 0.11
Spleen ($x10^3$)	2.06 ± 0.08	1.98 ± 0.09	2.02 ± 0.06	1.99 ± 0.04	1.91 ± 0.09
Tibia ($x10^3$)	2.05 ± 0.12	1.93 ± 0.06	1.98 ± 0.07	2.03 ± 0.05	2.09 ± 0.05
Uterus/ovaries ($x10^3$)	2.47 ± 0.18	2.35 ± 0.10	2.76 ± 0.19	2.70 ± 0.24	2.64 ± 0.17

Note. Data are reported as mean values (± SEM) for 10 animals/sex/group with the exception of the 0.5% NSP treatment which contained 9 males and 11 females.

TABLE 12

Histological findings for male S-D rats following the dietary ingestion of 0 - 2.0% NSP for 90 days

Treatment group	Control	0.25%	0.5%	1.0%	2.0%
	Incidence (group mean severity)				
Liver					
Focal inflammation	0	0	2 (1.0)	0	0
Kidney					
Focal interstitial inflammation/fibrosis	2 (1.5)	1 (1.0)	2 (1.0)	1 (1.0)	2 (1.5)
Focal cortical tubular epithelial hyperplasia	3 (1.7)	3 (1.3)	3 (1.7)	2 (1.5)	3 (1.7)
Focal tubular dilatation with protein casts	3 (1.7)	3 (1.3)	3 (2.0)	2 (1.5)	2 (2.5)
Heart					
Focal non-suppurative interstitial inflammation	2 (1.0)	0	1 (1.0)	0	0
Lungs					
Focal peribronchiolar alveolar vacuolated macrophages	0	1 (1.0)	0	0	1 (1.0)
Focal peribronchiolar arteriolar mineralization	0	0	1 (1.0)	0	0
Brain	0	0	0	0	0
Stomach	0	0	0	0	0
Intestine (duodenum, jejunum, ileum, and colon)	0	0	0	0	0
Skin	0	0	0	0	0

Note. Group mean severity was calculated by adding severity scores 1-4 (see Methods) for animals with lesions and dividing by the number of animals with lesions. Tissue samples examined were obtained from 3 male rats per treatment.

TABLE 13

Histological findings for female S-D rats following the dietary ingestion of 0 - 2.0% NSP for 90 days

Treatment group	Control	0.25%	0.5%	1.0%	2.0%
	Incidence (group mean severity*)				
Liver					
Focal inflammation	0	0	0	1 (1.0)	0
Kidney					
Focal interstitial inflammation/fibrosis	1 (1.0)	1 (1.0)	1 (2.0)	1 (2.0)	1 (1.0)
Focal cortical tubular epithelial hyperplasia	2 (1.0)	1(1.0)	1 (1.0)	1 (2.0)	1 (2.0)
Focal tubular dilatation with protein casts	0	0	0	1 (1.0)	1 (1.0)
Heart					
Focal non-suppurative interstitial inflammation	0	1 (1.0)	0	0	0
Lungs					
Focal peribronchiolar alveolar vacuolated macrophages	0	1 (1.0)	0	0	0
Focal peribronchiolar arteriolar mineralization	0	0	0	0	0
Brain	0	0	0	0	0
Stomach					
Intestine (duodenum, jejunum, ileum, and colon)	0	0	0	0	0
Skin	0	0	0	0	0

Note. Group mean severity was calculated by adding severity scores 1-4 (see Methods) for animals with lesions and dividing by the number of animals with lesions. Tissue samples examined were obtained from 3 female rats per treatment.

TABLE 14

Histological findings for male S-D rats following the dietary ingestion of 0 - 2.0% NSP for 28 weeks

Treatment group	Control	0.25%	0.5%	1.0%	2.0%
	Incidence (group mean severity)				
Liver[a]					
Focal inflammation	4 (1.0)	3 (1.0)	3 (1.0)	5 (1.0)	3 (1.0)
Basophilic focus of hepatocellular alteration	0	0	1 (1.0)	0	0
Kidney[a]					
Focal interstitial inflammation/fibrosis	10 (1.2)	10 (1.1)	10(1.1)	10(1.2)	10 (1.1)
Focal cortical tubular epithelial hyperplasia	10 (2.0)	10 (2.2)	10 (2.1)	10 (2.0)	10 (1.8)
Focal tubular dilatation with protein casts	10 (1.6)	10 (1.8)	10 (2.1)	10 (1.7)	9 (1.9)
Mineralization	3 (1.0)	3 (1.0)	2 (1.0)	3 (1.0)	4 (1.0)
Heart (focal non-suppurative interstitial inflammation)[b]	0	1 (1.0)	0	0	0
Lungs[b]					
Focal peribronchiolar/perivascular/subpleural vacuolated macrophages	2 (1.5)	2 (2.0)	2 (1.5)	2 (1.0)	2 (2.0)
Focal peribronchiolar arteriolar mineralization	0	0	0	1 (1.0)	1 (1.0)
Focal peribronchiolar/perivascular eosinophil accumulation	0	0	1 (1.0)	0	1 (1.0)
Focal osteosis	0	0	0	0	1 (1.0)
Brain[b]	0	0	0	0	0
Stomach[b]					
Focal gland dilation and epithelial attenuation)	1 (1.0)	1 (2.0)	0	0	0
Intestine (duodenum, jejunum, ileum, and colon)[b]	0	0	0	0	0
Skin[b]	0	0	0	0	0

Note. Group mean severity was calculated by adding severity scores 1-4 (see Methods) for animals with lesions and dividing by the number of animals with lesions. [a]Tissue samples examined were obtained from 10 rats per treatment except the 0.5% group that contained 9 animals. [b]Tissue samples examined were obtained from the first three animals of each group.

TABLE 15

Histological findings for female S-D rats following the dietary ingestion of 0 - 2.0% NSP for 28 weeks

Treatment group	Control	0.25%	0.5%	1.0%	2.0%
	Incidence (group mean severity)				
Liver[a]					
Focal inflammation	5 (1.0)	6 (1.0)	6 (1.0)	2 (1.0)	7 (1.0)
Basophilic focus of hepatocellular alteration	0	0	0	0	0
Kidney[a]					
Focal interstitial inflammation/fibrosis	5 (1.0)	8 (1.1)	7 (1.0)	5 (1.0)	7 (1.0)
Focal cortical tubular epithelial hyperplasia	6 (1.0)	7 (1.4)	8 (1.3)	7 (1.1)	8 (1.3)
Focal tubular dilatation with protein casts	6 (1.0)	9 (1.3)	8 (1.1)	9 (1.1)	9 (1.2)
Mineralization	1 (1.0)	1 (1.0)	0	2 (1.0)	2 (1.0)
Heart (focal non-suppurative interstitial inflammation)[b]	0	0	0	1 (1.0)	0
Lungs[b]					
Focal peribronchiolar/perivascular/subpleural vacuolated macrophages	0	2 (1.5)	0	1 (1.0)	2 (1.0)
Focal peribronchiolar arteriolar mineralization	0	0	0	0	0
Focal peribronchiolar/perivascular eosinophil accumulation	0	0	0	0	0
Focal osteosis	0	0	1 (1.0)	0	0
Brain[b]	0	0	0	0	0
Stomach[b]					
Focal gland dilation and epithelial attenuation)	1 (1.0)	1 (1.0)	0	0	0
Intestine (duodenum, jejunum, ileum, and colon)[b]	0	0	0	0	0
Skin[b]	0	0	0	0	0
Ovary (paraovarian cyst)[b]	0	0	0	0	0
Uterus[b]	0	0	0	0	0

Note. Group mean severity was calculated by adding severity scores 1-4 (see Methods) for animals with lesions and dividing by the number of animals with lesions. [a]Tissue samples examined were obtained from 10 rats per treatment except the 0.5% group that contained 11 animals. [b]Tissue samples examined were obtained from the first three animals of each group.

Analysis of whole blood samples showed no significant differences in hematological parameters between rats fed diets containing NSP compared to untreated controls in either males or females with the exception of males consuming 0.25% NSP which showed a significantly higher mean MCH (18.6 ± 0.2 pg) (Table 16). There was no evidence of hemoparasites. Erythrocytosis or anemia was not observed in the treated compared to untreated animals as evidenced by the RBC count and hemoglobin levels. RBC morphology was normal for most rats, although some exhibited polychromasia (Table 17). Poikilocytosis was observed in 1 female consuming 0.5% NSP and 1 male consuming 0.25% NSP in the diet, while anisocytosis was present in only 1 absolute control male. No evidence of leukocytosis or leukopenia was detected between the treated and the control groups in both males and females. Microscopic examination of the blood samples revealed that the WBCs in all rats were normal and morphologically similar. All other hematological parameters assessed in this study were normal.

Serum biochemical analysis indicated that Ca was slightly increased in the 0.5% (10.79 ± 0.13 mg/dl) and 2.0% (10.83 ± 0.12 mg/dl) NSP-treated females compared to absolute controls (10.31 ± 0.09 mg/dl) (Table 18). All other parameters were unchanged between NSP-treated and untreated groups within both sexes.

Serum and hepatic vitamin A (VA) and vitamin E (VE) and serum Fe and Zn were also analyzed. Serum and hepatic VA and VE levels were unchanged

TABLE 16

Hematology of S-D rats following the dietary ingestion of 0 - 2.0% NSP for 28 weeks

Hematological Parameter	Treatment Group				
	Abs. control	0.25%	0.5%	1.0%	2.0%
			Males		
WBC/ul	5840 ± 713	6180 ± 637	5667 ± 464	8120 ± 369	5365 ± 704
Neutrophils (%)	14.1 ± 3.0	14.1 ± 3.3	14.1 ± 2.7	10.3 ± 1.6	13.9 ± 2.3
Lymphocytes (%)	83.2 ± 2.9	81.9 ± 3.5	83.6 ± 3.1	87.2 ± 1.8	83.7 ± 2.6
Monocytes (%)	1.8 ± 0.2	1.8 ± 0.5	1.4 ± 0.2	1.0 ± 0.0	1.3 ± 0.3
Eosinophils (%)	2.3 ± 0.5	3.2 ± 0.7	2.3 ± 0.7	2.4 ± 0.4	2.5 ± 0.8
RBCs/ul ($x10^6$)	7.5 ± 0.1	7.4 ± 0.1	7.5 ± 0.1	7.5 ± 0.1	7.5 ± 0.1
Hb (g/dl)	13.4 ± 0.2	13.6 ± 0.1	13.6 ± 0.2	13.6 ± 0.1	13.5 ± 0.2
PCV (%)	39.5 ± 0.4	39.9 ± 0.3	40.4 ± 0.5	40.0 ± 0.4	39.7 ± 0.5
MCV (fl)	52.6 ± 0.5	54.3 ± 0.6	53.7 ± 0.3	53.6 ± 0.6	53.0 ± 0.4
MCH (pg)	17.8 ± 0.2	18.6 ±0.2*	18.1 ± 0.1	18.2 ± 0.2	18.0 ± 0.1
MCHC (g/dl)	33.9 ± 0.3	34.2 ± 0.2	33.7 ± 0.2	33.9 ± 0.3	34.0 ± 0.2
			Females		
WBC/ul	3930 ± 431	4250 ± 487	3936 ± 356	3950 ± 452	3411 ± 486
Neutrophils (%)	17.7 ± 2.6	13.5 ± 1.7	13.0 ± 2.7	13.9 ± 1.1	14.0 ± 2.7
Lymphocytes (%)	79.7 ± 2.9	83.4 ± 2.1	85.0 ± 2.8	78.4 ± 6.6	82.8 ± 3.1
Monocytes (%)	1.2 ± 0.2	2.3 ± 0.5	1.3 ± 0.3	1.0 ± 0.0	1.8 ± 0.6
Eosinophils (%)	2.2 ± 0.6	3.1 ± 0.7	1.9 ± 0.2	1.8 ± 0.3	2.2 ± 0.4
RBCs/ul ($x10^6$)	6.5 ± 0.1	6.4 ± 0.1	6.5 ± 0.1	6.2 ± 0.1	6.5 ± 0.1
Hb (g/dl)	12.4 ± 0.2	12.4 ± 0.2	12.6 ± 0.1	12.3 ± 0.2	12.5 ± 0.2
PCV (%)	36.5 ± 0.6	36.1 ± 0.5	37.2 ± 0.5	35.7 ± 0.5	36.5 ± 0.7
MCV (fl)	56.4 ± 0.6	56.8 ± 0.5	57.1 ± 0.5	57.2 ± 0.3	56.3 ± 0.5
MCH (pg)	19.1 ± 0.2	19.5 ± 0.2	19.4 ± 0.2	19.7 ± 0.1	19.3 ± 0.1
MCHC (g/dl)	33.9 ± 0.2	34.3 ± 0.1	33.9 ± 0.1	34.4 ± 0.1	34.3 ± 0.1

Note. Data are reported as mean values (± SEM).
*Indicates statistical significance compared to untreated controls ($p \leq 0.05$).

TABLE 17

Morphological evaluation of red blood cells of S-D rats following dietary ingestion of 0 - 2.0% NSP for 28 weeks

Treatment Group	Total Samples	Cell Morphology			
		Normal	Polychromasia[a]	Poikilocytosis	Anisocytosis
			Males		
Abs. control	10	3	6	-	1 (rare)
0.25%	10	4	5	1(few)	-
0.5%	9	3	6	-	-
1.0%	10	6	4	-	-
2.0%	10	6	4	-	-
			Females		
Abs. control	10	6	4	-	-
0.25%	10	6	4	-	-
0.5%	11	6	4	1 (rare)	-
1.0%	10	5	5	-	-
2.0%	9	4	5	-	-

Note. Data represent the number out of total samples in which the selected morphological characteristic was observed. [a]Polychromatophilic cells were “rare or few” in all the samples in which they were identified.

TABLE 18

Terminal serum biochemical parameters in S-D rats following the dietary addition of 0 - 2.0% NSP for 28 weeks

Test	Treatment Group				
	Abs. control	0.25%	0.5%	1.0%	2.0%
			Males		
TP (g/dl)	5.91 ± 0.09	5.92 ± 0.04	5.99 ± 0.03	5.90 ± 0.06	5.91 ± 0.07
ALB (g/dl)	3.40 ± 0.09	3.53 ± 0.09	3.48 ± 0.08	3.51 ± 0.07	3.49 ± 0.09
Ca (mg/dl)	10.25 ± 0.15	10.51 ± 0.08	10.67 ± 0.12	10.50 ± 0.09	10.56 ± 0.07
P (mg/dl)	5.83 ± 0.18	5.70 ± 0.22	5.71 ± 0.27	5.45 ± 0.17	5.24 ± 0.15
GLUC	258.6 ± 6.3	283.1 ± 15.2	287.9 ± 9.9	264.1 ± 14.6	271.3
BUN (mg/dl)	22.65 ± 0.86	22.68 ± 0.64	21.57 ± 0.50	22.27 ± 0.42	22.53 ± 0.52
CRT (mg/dl)	0.26 ± 0.02	0.30 ± 0.00	0.29 ± 0.01	0.28 ± 0.01	0.29 ± 0.01
T-BIL (mg/dl)	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00
ALP (U/l)	104.6 ± 2.7	106.2 ± 4.5	111.7 ± 8.5	110.6 ± 4.2	119.6 ± 3.9
CK (U/l)	1455 ± 1002	376 ± 57	3358 ± 2903	994 ± 617	318 ± 35
AST (U/l)	127.0 ± 17.4	87.3 ± 7.9	142.8 ± 49.2	134.6 ± 26.7	94.2 ± 6.3
ALT (U/l)	64.3 ± 3.9	58.9 ± 2.3	71.4 ± 9.2	70.2 ± 6.8	59.8 ± 2.9
GLOB (g/dl)	2.51 ± 0.08	2.39 ± 0.11	2.51 ± 0.08	2.39 ± 0.12	2.42 ± 0.09
A/G Ratio	1.37 ± 0.06	1.51 ± 0.09	1.40 ± 0.07	1.52 ± 0.11	1.47 ± 0.08
GGT (U/l)	<3.0	<3.0	<3.0	<3.0	<3.0
AMYL (U/l)	2671 ± 89	2633 ± 87	2759 ± 120	2457 ± 74	2532 ± 113
CHOL	120.0 ± 10.1	112.6 ± 11.7	109.8 ± 6.6	102.7 ± 9.7	107.7 ± 12.4
			Females		
TP (g/dl)	5.96 ± 0.09	5.96 ± 0.09	6.01 ± 0.10	6.06 ± 0.10	6.24 ± 0.10
ALB (g/dl)	4.05 ± 0.08	4.05 ± 0.07	4.10 ± 0.06	4.06 ± 0.06	4.17 ± 0.09
Ca (mg/dl)	10.31 ± 0.09	10.51 ± 0.05	10.79 ± 0.13*	10.62 ± 0.11	10.83 ±
P (mg/dl)	4.81 ± 0.29	5.21 ± 0.20	4.87 ± 0.27	4.99 ± 0.13	4.91 ± 0.28
GLUC	223.7 ± 7.3	218.5 ± 2.9	218.5 ± 9.5	211.0 ± 5.0	212.4 ± 4.3
BUN (mg/dl)	20.96 ± 0.78	21.14 ± 0.73	20.69 ± 0.78	20.38 ± 1.05	20.27 ± 0.52
CRT (mg/dl)	0.32 ± 0.01	0.33 ± 0.02	0.34 ± 0.02	0.35 ± 0.03	0.33 ± 0.02
T-BIL (mg/dl)	0.11 ± 0.01	0.11 ± 0.01	0.17 ± 0.06	0.10 ± 0.00	0.10 ± 0.00
ALP (U/l)	96.9 ± 7.7	93.8 ± 5.7	94.5 ± 6.9	103.5 ± 9.5	100.5 ± 8.3
CK (U/l)	1019 ± 781	5455 ± 2362	3525 ± 1760	3676 ± 1939	1333 ± 595
AST (U/l)	147.0 ± 37.9	268.2 ± 72.6	314.0 ± 104.8	179.5 ± 43.1	130.1 ± 20.6
ALT (U/l)	73.0 ± 6.4	77.1 ± 8.5	243.2 ± 158.5	66.3 ± 8.7	67.8 ± 7.2
GLOB (g/dl)	1.91 ± 0.03	1.91 ± 0.05	1.91 ± 0.06	2.00 ± 0.08	2.07 ± 0.05
A/G Ratio	2.13 ± 0.06	2.13 ± 0.07	2.16 ± 0.06	2.05 ± 0.08	2.02 ± 0.06
GGT (U/l)	<3.0	<3.0	<3.0	<3.0	<3.0
AMYL (U/l)	1703 ± 52	1690 ± 35	8665 ± 6937	1639 ± 64	1713 ± 85
CHOL	100.3 ± 6.7	91.3 ± 4.9	88.9 ± 3.3	95.6 ± 6.1	94.6 ± 3.4

Note. Data are reported as mean values (± SEM) of 10 animals/sex/group with the exception of the 0.5% NSP treatment which contained 9 males and 11 females.
*Indicates statistical significance compared to untreated controls ($p \leq 0.05$).

TABLE 19

Analysis of vitamin A (VA), vitamin E (VE), Fe, and Zn in liver and serum of S-D rats following the dietary ingestion of 0 - 2.0% NSP for 28 weeks

Selected Parameter	Treatment Group				
	Abs. control	0.25%	0.5%	1.0%	2.0%
			Male		
[a]Serum VA (µg/L)	567.4 ± 24.8	571.2 ± 17.4	532.3 ± 18.4	542.3 ± 21.8	561.7 ± 20.9
[b]Hepatic VA (µg/g)	28.7 ± 3.2	28.4 ± 2.6	27.4 ± 0.9	27.3 ± 1.3	28.9 ± 1.7
[a]Serum VE (µg/L)	13391 ± 1220	13217 ± 1309	11561 ± 478	11822 ± 1221	12042 ± 1315
[b]Hepatic VE (µg/g)	395.3 ± 2.8	393.4 ± 3.7	384.6 ± 5.0	407.1 ± 3.4	393.2 ± 2.4
[a]Serum Fe (µg/dl)	136.7 ± 8.9	153.3 ± 8.7	131.1 ± 12.2	179.6 ± 12.4*	150.8 ± 4.9
[a]Serum Zn (mg/L)	1.29 ± 0.04	1.37 ± 0.04	1.35 ± 0.05	1.38 ± 0.06	1.22 ± 0.05
			Female		
[a]Serum VA (µg/L)	254.1 ± 6.0	229.4 ± 8.3	247.5 ± 9.9	302.1 ± 10.7*	287.9 ± 13.2
[b]Hepatic VA (µg/g)	60.4 ± 3.9	61.0 ± 4.6	61.5 ± 4.7	51.3 ± 5.1	53.5 ± 5.7
[a]Serum VE (µg/L)	14535 ± 1168	13574 ± 568	13804 ± 628	14675 ± 1018	15731 ± 715
[b]Hepatic VE (µg/g)	482.1 ± 14.2	471.2 ± 1.7	468.9 ± 13.3	469.4 ± 16.1	480.9 ± 41.7
[a]Serum Fe (µg/dl)	321.0 ± 13.7	309.0 ± 18.1	344.1 ± 24.0	337.0 ± 22.5	344.1 ± 27.4
[a]Serum Zn (mg/L)	1.05 ± 0.03	1.11 ± 0.03	1.16 ± 0.04	1.11 ± 0.04	1.11 ± 0.06

[a]Data are reported as mean values (± SEM) of 10 animals/sex/group with the exception of the 0.5% NSP treatment which contained 9 males and 11 females. [b]Data represent mean values (± SEM) of 3 randomly selected liver samples.
*Indicates statistical difference compared to untreated controls ($p \leq 0.05$).

with dietary inclusion of NSP at all treatment levels with the exception of a slight increase in VA in the 1.0% NSP-treated females (302.1 ± 10.7 µg/L) compared to untreated controls (254.1 ± 6.0 µg/L). Serum Fe and Zn levels were also unaffected by the dietary inclusion of up to 2.0% NSP except in 1% NSP-treated males which showed increased Fe levels (179.6 ± 12.4 µg/dl) in comparison to untreated control animals (136.7 ± 8.9 µg/dl) (Table 19). There was no trend toward dose-dependency observed in either case.

3.4 Discussion

In this study, the potential for adverse effects resulting from chronic dietary ingestion of up to 2% (w/w) of NSP clay was evaluated in S-D rats. Previously, evaluation of up to 96 compounds in various animal species found no significant added benefit to repeat-dose animal tests of greater than 6 months duration; hence, 28 weeks was chosen as sufficient for this study (Lumley and Walker, 1985a, 1986; Lumley et al, 1992). Based on standards set by the International Conference on Harmonization (ICH) of Technical Requirements for Registry of Pharmaceuticals for Human Use this constitutes a chronic study (D'Arcy and Harron, 1992) whereas the 90 d termination was a subchronic pilot study.

At termination, mean TFC, CFC, BW, TBWG, FCE, and CFCE of all the NS-treated groups were not statistically different from the controls in either sex. However, FCE, measured as the ratio of TFC to TBWG, increased as the study

progressed, suggesting impairment of feed utilization with age (Figure 17 and 18). However, comparison between NSP-treated rats and untreated controls suggested that this was not NSP-dependent. In addition, no NSP-dependent gross or histopathological changes or differences in relative organ weights were observed, further suggesting that NSP is not overtly toxic. There was a single basophilic focus of hepatocellular alteration in one or two sections of liver from one treated rat (in the 0.5% group). Such foci may occur spontaneously as a background lesion in rats. They may represent progenitor lesions from which hepatocellular neoplasia may arise. However, the incidence of spontaneously occurring basophilic foci is generally much lower than in the liver of rodents treated with carcinogens (Cattley and Popp, 2002). In this case, the single basophilic focus is most likely a spontaneous occurring, background lesion as it is present in only 1 of 2 sections of the liver from 1/80 treated rats and it is not in the higher dose groups (1% and 2%).

Analysis of whole blood samples showed no significant differences in hematological parameters between rats fed diets containing NSP compared to untreated controls in either males or females with the exception of males consuming 0.25% NSP which showed a significantly higher mean MCH over their untreated counterparts (Table 16). However, this difference was not dose-dependent and therefore, was not attributed to the dietary inclusion of the NSP. In addition, the RBC counts were not affected and no NSP-related morphological differences were detected in RBCs, suggesting that NSP ingestion is unlikely to

produce hemolytic, hemorrhagic, or other forms of anemia, or to affect RBC physiology or metabolism (George-Gay and Parker, 2003). Further, WBCs and leukocyte differentials were found to be quantitatively and morphologically similar between NSP-treated and untreated animals and were considered normal in all treatment groups. This suggests that NSP is unlikely to impair immunity or inflammatory processes, lead to increased incidence of infections, or cause alterations to bone marrow (Goodnough et al., 1999; George-Gay and Parker, 2003).

In terms of serum biochemical analysis, Ca was the only parameter found to be altered by NSP ingestion, with increases in females upon dietary inclusion levels of either 0.5 or 2.0% (Table 18). Because this was apparent only in females and in a manner that was independent of NSP dose, it is unlikely that NSP was the source of these changes. Overall, analysis of the serum biochemical parameters used in this study suggests that NSP ingestion (at dietary inclusion levels up to 2.0% w/w) does not adversely affect biochemical or physiological processes.

VA is vital for visual acuity, controlling the differentiation program of epithelial cells in the digestive tract and respiratory system, skin, bone, the nervous system, the immune system, and for hematopoiesis (Gursu et al., 2002). VA deficiency is the leading cause of childhood blindness in developing countries, and is a major contributor to childhood morbidity and mortality resulting from common infections (Fieldler et al., 2000). Dietary AFs (5 ppm)

have been shown to reduce hepatic VA concentrations in broiler chicks (Pimpukdee et al., 2004). In the same study, inclusion of 0.25% NSP to the AF-contaminated diet not only protected the animals against aflatoxicosis, but also preserved the hepatic VA. Dietary NSP alone (0.5%) did not affect VA levels. VE is an essential antioxidant for various biochemical functions including normal reproduction, muscle development, and resistance of RBCs to hemolysis (Lehninger et al., 1997b) and has been associated with prevention of cancer and heart disease (Packer, 1993). Previous studies have suggested that this antioxidant may reduce AF toxicity via reduction of both oxidative damage and bioavailability of the toxins (Odin, 1997). In this study, serum and hepatic VA and VE levels remained unchanged with dietary inclusion of NSP at treatment levels ranging from 0 to 2.0% with the exception of a slight increase in VA in 1.0% NSP-treated females (Table 19). The fact that the same effect was not observed in the 2.0% group or across both sexes suggests that this elevation was not NS-dependent.

Fe and Zn are also essential in the body as cofactors in several enzymatic and metabolic functions (Lehninger et al., 1997a). Fe is required for the synthesis of heme proteins including hemoglobin and myoglobin, metalloenzymes, and cytochromes (Nicholls, 1996; George-Gay and Parker, 2003). Zn deficiency can lead to reduced growth rate, skin lesions, impaired immune response, low sperm count, fetal abnormalities, and cognitive impairment (Chesters, 1996; Prasad et al., 2001). In addition to VA and VE

levels, serum Fe and Zn levels were unaffected by the dietary inclusion of up to 2.0% NSP except in 1.0% NSP-treated males which showed increased Fe levels. Like the increase observed in VA, it is unlikely that this increase is NSP-dependent because Fe levels in animals ingesting 2.0% NSP were unaffected.

PCDD/PCDF congeners are ubiquitous environmental contaminants. Upon exposure, they bioaccumulate in fatty animal tissues and subsequently can be highly toxic to humans (Startin *et al*., 1990; Schecter *et al*., 1997; Jensen, 2001). In 1997, use of ball clay as an anti-caking and pelleting agent in animal feeds was discontinued because it was found to contain high levels of these contaminants (Headrick et al, 1999). Studies attributed the source of contamination in chicken products to the use of this ball clay in chicken feed (Ferrario and Byrne, 2000; Headrick et al, 1999). Ball clay consists primarily of poorly defined crystalline kaolinite, while the clay used in this study (NSP) is a smectite (Ferrario and Byrne, 2000). For purposes of comparison, OCDD was detected at 205,663 ppt in the ball clay source from which the animals were contaminated. In contrast, analysis of 17 USEPA priority dioxins/furans confirmed that the NSP clay used in this study was not contaminated with high levels of any of these pollutants. In fact, OCDD (2.34 ppt) was the only contaminant present above the limits of detection (LOD = 1.02 ppt), approximately 0.001% of the amount detected in ball clay. According to the World Health Organization (WHO), OCDD exhibits the least toxicity among the priority PCDDs with a Toxic Equivalency Factor (TEF) of 0.0001 (developed by

WHO, reported by Van den Berg et al., 1998). Based on the measurement value, the toxic equivalent (TEQ) of this contaminant in NSP was calculated to be 0.000234, in comparison to the TEQ of 21 that was calculated for the same contaminant in ball clay (Ferrario and Byrne, 2000).

Nonselective sorbents may bind enzymes and critical nutrients, and may release sorbed contaminants, making prolonged use inadvisable. In previous research, concentrations as high as 2% NSP in the diet throughout pregnancy produced neither maternal nor fetal toxicity in S-D rats, and did not affect metal bioavailability in a variety of tissues (Wiles et al., 2004). In this study, prolonged (28 weeks) dietary inclusion of 0 to 2.0% NSP in S-D rats did not appear to affect TFC, CFC, BW, TBWG, FCE, CFCE, generally accepted hematological and serum biochemical parameters, or selected micronutrients (VA, VE, Fe and Zn). Although statistically significant changes in some parameters were noted, these differences did not appear to be NSP-related or dose-dependent, suggesting that NSP at a dietary inclusion level as high as 2.0% (w/w) does not produce overt toxicity. Importantly, this information increases the feasibility for use of NSP clay in the diet of humans to reduce foodborne exposure to aflatoxins and improve liver cancer management in high risk populations.

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CHAPTER IV

ASSESSMENT OF ACUTE AFLATOXIN EXPOSURE IN A GHANAIAN POPULATION PRIOR TO INTERVENTION WITH NOVASIL CLAY

4.1 Introduction

Aflatoxins (AFs) are a group of structurally similar, highly toxic fungal metabolites produced primarily by *Aspergillus flavus* and *A. parasiticus*, frequently identified in corn, groundnuts, cottonseed, and tree nuts (Bhatnagar *et al.,* 2002; Lopez *et al.,* 2002; CAST, 2003). The fungi have been found to be most abundant between latitudes 26° to 35° north or south of the equator, primarily in tropical areas including Ghana and other parts of Africa. Conditions that favor AF production and subsequent contamination of grain in these areas include a temperature of 30.5 °C, relative humidity of greater than 85%, and a grain moisture content of 14-32% (Cole *et al*., 1995; Payne, 1998; Shephard, 2003).

Unfortunately, the basic dietary staples that are produced and consumed by rural populations in Ghana and other West African countries are poorly handled and stored and therefore are frequently contaminated by toxigenic fungi and AF (Jolly et al, 2004). For example, analysis of randomly selected groundnut samples obtained from twelve major markets across Ghana revealed

AF contamination ranging from 5.7 to 22,168 ppb (Awuah and Kpodo, 1996). In a separate study, samples of kenkey (a corn-based product) exhibited AF contamination that ranged from 6.15 to 196.1 ppb with a mean concentration of 50.55 ppb (Jesperson et al., 1994).

AFs, particularly AFB_1, have been found to be acutely toxic, carcinogenic, mutagenic and immunosuppressive. They also cause death and disease in mammalian species (WHO-IARC, 1993; Miller and Wilson, 1994; Pestka and Bondy, 1994; Turner, 2003). In addition, dairy animals consuming AFB_1-contaminated rations can excrete aflatoxin M_1 (AFM_1), a hydroxylated metabolite of AFB_1, in milk and subsequently taint other dairy-based products such as cheese and ice cream (Phillips et al., 1994; Neal, 1998; Chopra et al., 1999). In humans, AFM_1 secreted in mother's milk may enhance the morbidity of children with kwashiorkor or malnutrition. Kwashiorkor is characterized by severe protein deficiency and a variety of symptoms including hypoalbuminemia, retarded growth, changes in skin and hair pigmentation, diarrhea, loss of appetite, nervous irritability, edema, anemia, fatty liver, and immunosuppression. It is often accompanied by infection and vitamin deficiencies (Hendrickse et al., 1983; Hendrickse, 1991; Adhikari, 1994). In developing countries, where foodborne exposure to AF is significant, studies have reported a dose-dependent association between AFM_1 in the urine and an increased relative risk of developing hepatocellular carcinoma (HCC) (Qian et al., 1994; Yu et al.,

1997). Thus, there is a critical need for practical interventions or therapies to ensure the health of humans burdened by AF contamination of food.

Trials are ongoing in the U.S. to assess the feasibility of NS clay for use as a potential enterosorbent of AF in humans. Future work is planned to investigate the effectiveness of this approach for reducing aflatoxin exposure in high risk populations in Ghana, West Africa using AFM_1 in the urine as a molecular biomarker (Groopman et al 1992a; Hsieh and Wong, 1994). The objective of this study was to quantitate this biomarker in humans from four communities in the Ejura district of Ghana in order to establish baseline AF exposure levels prior to NS clay intervention studies.

4.2 Materials and Methods

4.2.1 Chemicals and Reagents

Aflatoxin M_1 (AFM_1) and creatinine measurement kits were purchased from Sigma Chemical Co. (St. Louis, MO) and Sigma Diagnostics, Inc. (St. Louis, MO), respectively. Preparative Aflatest P monoclonal antibody columns were purchased from Vicam (Watertown, MA). All solvents were HPLC grade. Purified water (ddH_2O) was prepared by processing deionized water through a Milli-Q^{UF+} purification system (Millipore Corp., Bedford, MA).

4.2.2 Urine Collection

Approximately 120 ml of the first urine in the morning was collected from 91 volunteers in four communities in the Ejura district of Ghana at designated community health centers. This district was selected because the communities are predominantly agricultural and the main crops include maize, groundnuts, and other cereal-based products. The study subjects consisted of males and females with ages ranging from 19 to 86 years. The investigative team contacted and recruited the volunteers through the district health director and the chief of each community. Each participant signed an informed consent prior to recruitment. All samples were kept cool during transport to the Kwame Nkrumah University of Science and Technology Medical Center where three 4.5 ml aliquots of urine from each volunteer were pipetted into 5 ml cryogenic vials (Corning Glass Works, Corning, NY). All samples were stored at -80 °C prior to shipment to Texas A&M University (TAMU). Upon arrival, all samples were stored at -80 °C before AFM_1 analysis.

4.2.3 Extraction of AFM_1 from Urine

AFM_1 in urine was quantified by high performance liquid chromatography (HPLC) following immunoaffinity cleanup of samples. Affinity chromatography cleanup procedures and HPLC analysis were based on methodologies described by Groopman et al. (1992b), with modifications by Sarr et al. (1995) and Wang et al. (1999). Urine samples were centrifuged at 500 x *g* for 5 min to

remove particulate matter. Five milliliters of supernatant was adjusted to an acidic pH with 0.5 ml of 1.0 M ammonium formate (pH 4.5), and increased to a final volume of 10 ml with ddH_2O. Samples were vortexed and loaded onto a 1-ml preparative Aflatest P monoclonal antibody column (Vicam, Watertown, MA) at a flow rate of approximately 0.3 ml/min (Wang et al 1999). The column was washed twice with 5 ml of phosphate-buffered saline (pH 7.4) and once with 10 ml of ddH_2O to remove nonspecifically bound analytes. AFs were eluted with 2 ml of 80% methanol and evaporated to dryness under nitrogen. The eluates were re-suspended in a solution consisting of 100 µl of 80% methanol and 100 µl of 20 mM of ammonium formate (pH 3.0). Samples were analyzed by HPLC.

4.2.4 AFM_1 Quantification by HPLC Methods

HPLC analysis was conducted using a Waters HPLC system (Waters Corporation, Milford MA) with fluorescence detection capabilities. A 250 mm x 4.6 mm LiCrospher RP-18 endcapped column with pore size 100 Å and particle size 5 µm (Alltech Associates, Deerfield, IL) was employed for chromatographic separation. The mobile phase consisted of 22% ethanol, buffered with 20 mM ammonium formate (pH 3.0). Chromatographic separation of aflatoxins was achieved by isocratic elution of the mobile phase. Samples were injected (100 µl) on the column at a flow rate of 1.0 ml/min. AFM_1 quantification was based on peak area and retention time compared to the standard peak (LOD = 0.01 ng/ml). Urinary concentrations of AFM_1 metabolites were expressed two

different ways: ng/ml urine to view the actual toxin levels in each urine without considering the dilution factor, and pg/mg creatinine to correct for individual sample dilution variations.

4.2.5 Quantitative Analysis of Urinary Creatinine

Measurement of creatinine (CRT) was conducted using a creatinine measurement kit (Sigma Diagnostics, St. Louis, MO) based on the manufacturers enclosed instructions (Sigma Diagnostics, 1997). Positive and negative controls consisted of 0.3 ml CRT standard (3 mg/dl creatinine) and 0.3 ml ddH_2O, respectively. Alkaline picrate solution (APS) was prepared by mixing CRT color reagent (0.6% picric acid, sodium borate and surfactant) with sodium hydroxide solution (5:1, v/v), both of which were included in the kit. Urine samples were diluted 10 to 15 fold in water depending on the concentration of the urine and 3.0 ml of APS was added to each 0.3 ml aliquot. The resulting solutions were vortexed and allowed to stand at 25 °C for 8 to 12 min. Initial absorbance (A_I) values were taken for standard (ST) and sample (SA) solutions in comparison to controls using a UV/visible spectrophotometer at 500 nm. Following initial absorbance measurements, 0.1 ml acid reagent (a mixture of sulfuric and acetic acid) was added to each test tube, the mixture was vortexed, and allowed to stand 5 min at 25 °C. Absorbance (A_F) measurements were again taken against control solutions and recorded as the final absorbance (A_F).

Urinary CRT concentration (mg/dl) was calculated using the following equation:

Urine CRT (mg/dl) = [(A_I of SA – A_F of SA) / (A_I of ST – A_F of ST)]*3.

4.3 Results

4.3.1 Urinary Concentrations of AFM_1

Urinary levels of AFM_1 metabolite were quantified for 91 study volunteers in the Ejura district of Ghana. AFM_1 was found to be present above the limits of detection (LOD = 0.01 ng/ml) in 83 (91.2%) of the 91 samples. Values ranged from 0.01 to 17.24 ng/ml urine with a mean value (± SEM) of 1.47 ± 0.31 ng/ml urine. In 8 (8.8%) samples, AFM_1 was not found above the limits of detection. Of the 83 samples that contained detectable levels of AFM_1, 22 (26.5%) were found to contain low to moderate levels of AFM_1 (0.01 to 0.17 ng/ml), 59 (71.1%) contained moderate to very high levels (0.18 to 5.26 ng/ml), and 2 (2.4%) were considered to be extremely high (16.77 to 17.24 ng/ml). Figure 19 illustrates representative chromatograms of urinary AFM_1 detected by the HPLC methods.

4.3.2 AFM_1 Standardized to Urinary Creatinine Levels

To correct for individual variations in hydration status AFM_1 was standardized to urinary CRT levels. CRT concentrations in the 91 study participants ranged from 11.35 to 182.83 mg/dl and a mean (± SEM) of 68.21 ± 3.96 mg/dl. Based on this correction factor, the levels of AFM_1 in the urine

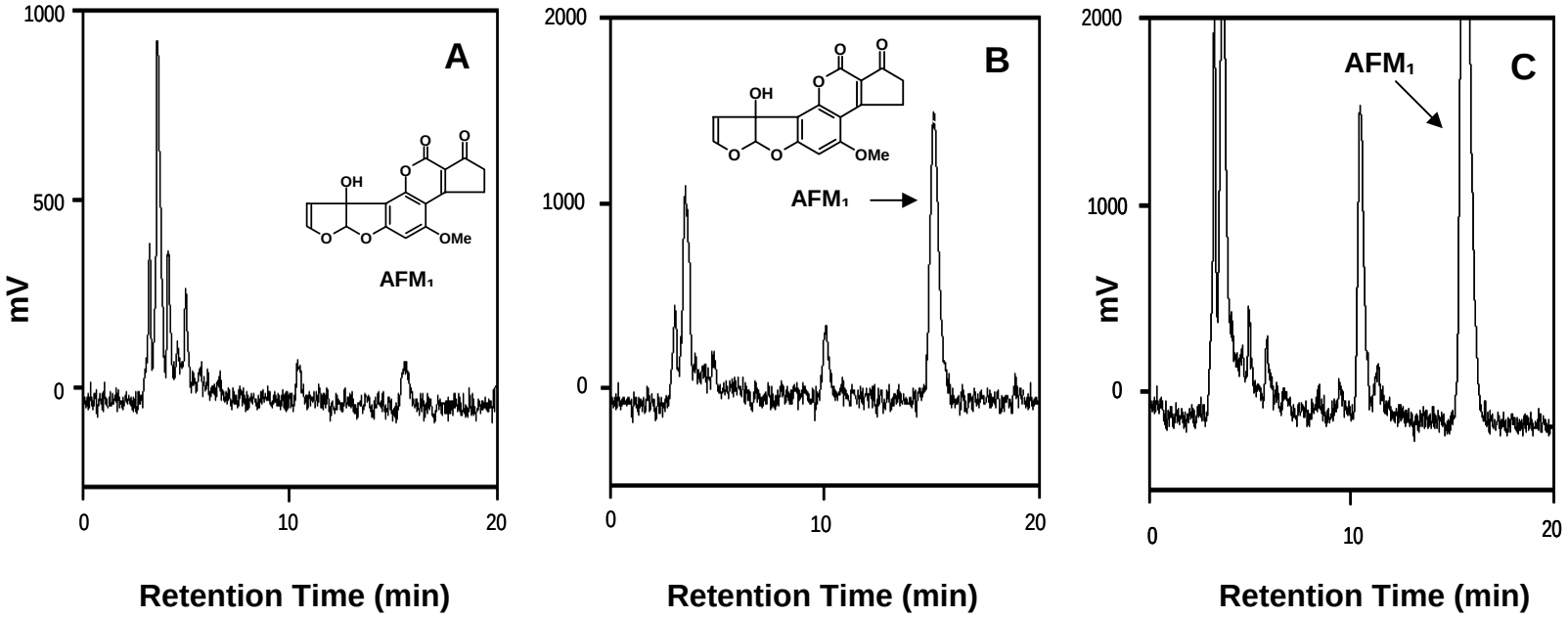

Figure 19. Representative HPLC fluorescence chromatographs of aflatoxin M_1 (AFM_1) detected in urine samples collected from 91 study participants in the Ejura district of Ghana. Of the 91 urine samples, 83 (91.2%) tested positive for AFM_1. **A.** Represents samples with low to moderate levels of M_1. **B.** Represents samples with moderate to very high levels of M_1. **C.** Represents samples that had extremely high levels of M_1.

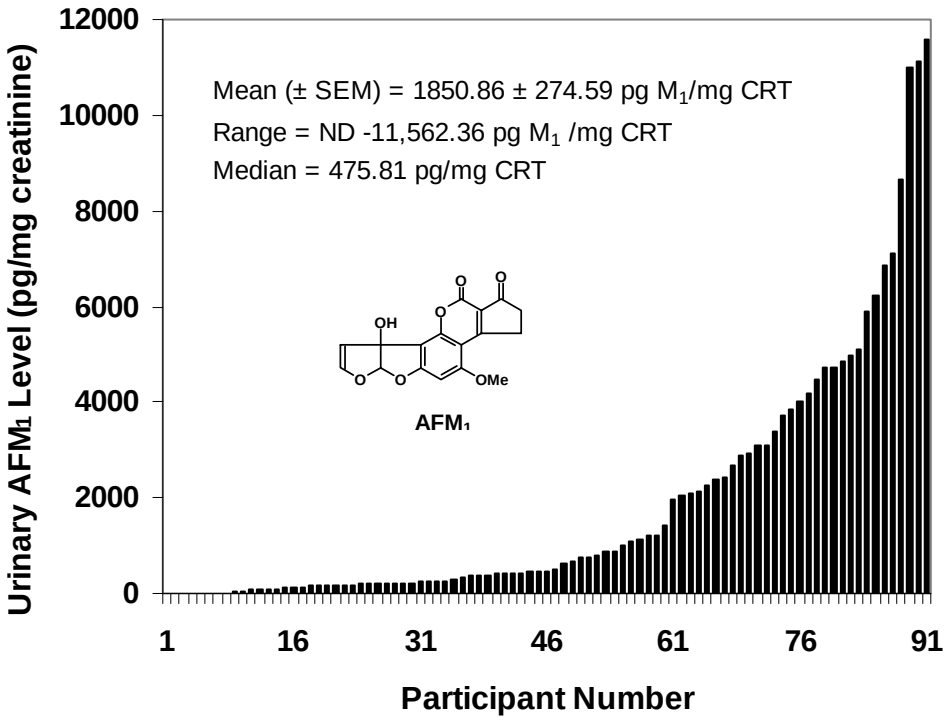

Figure 20. Diagramatic representation of aflatoxin M_1 (AFM_1) concentrations in urine samples (standardized to creatinine (CRT) levels) obtained from study participants in four communities in the Ejura district of Ghana.

samples ranged from less than 25.59 to 11,562.36 pg/mg CRT with a mean (± SEM) of 1,850.86 ± 274.59 pg/mg CRT (median = 475.81 pg/mg) (Figure 20).

4.4 Discussion

The objective of this study was to establish baseline data for human exposure to AFs in four communities in the Ejura district of Ghana. Urinary AFM_1 has been shown to be a dose-dependent biomarker for dietary AFB_1 exposure (Groopman et al., 1992; Wang et al., 1999). In this study, 91.2% of samples tested positive for AFM_1. This is comparable to previous research showing the presence of AFM_1 in 88.9% of urine samples analyzed in a Chinese population consuming high levels of AF in the diet and at high risk for liver cancer (Wang et al., 2001). Based on urine volumes, AFM_1 ranged from less than 0.01 to 17.2 ng/ml with a mean of 1.5 ng/ml. These results are comparable to those reported by Qian et al. (1994) in China (0.17 to 5.2 ng/ml) and with an average of 4.2 ng/ml found in Zimbabwe (Nyathi et al 1987). In the present study, the data were grouped into three concentration categories based on the previous report by Qian et al. (1994). These authors reported that AFM_1 levels ranging from 0.17 to 5.2 ng/ml urine were associated with a 4-fold increased relative risk of incidence of HCC. Levels of AFM_1 were categorized as low to moderate if samples contained 0.01 to 0.17 ng/ml urine, moderate to very high for samples that contained 0.18 to 5.26 ng/ml urine, and extremely high for samples with AFM_1 concentrations > 5.26 ng/ml urine.

However, because urine production is influenced largely by individual hydration status (Masi et al., 2004), AFM_1 levels in this study were further standardized based on creatinine production in individual study participants. Although this is a common method of standardization for a variety of urinary toxins and hormones (Greenberg, 1989; Wang et al., 1999), creatinine production is influenced by muscle mass and varies between ethnic groups (James et al., 1988; Goldwasser et al., 1997; Masi, 2004). Controlling for muscle mass in creatinine measurements was unrealistic under these study conditions and hence homogeneity was assumed. However, all participants in this study were black Ghanaians, eliminating ethnicity/race as a potential confounder.

Upon creatinine standardization, urinary AFM_1 ranged from less than 26 to 11,562 pg/mg creatinine with a mean value of 1,851 ± 275 pg/mg creatinine. Approximately one-third of study participants had AFM_1 levels greater than the mean value and considerably larger than those reported in China where AFM_1 levels ranged from less than nondetectable to 144.8 pg/mg creatinine (Wang et al. 1999).

Although urinary AFM_1 is dependent on several factors (e.g., sample size, diet, extent of aflatoxin exposure, methods of urine collection and aflatoxin analyses, urine concentration, genetic susceptibility to aflatoxins, health, and individual nutritional status) (Jolly et al., 2004), the levels of AFM_1 in this study were found to be substantially high, indicating that this particular study

population is highly exposed to AFs. Regrettably, medical records in this district provide little to no information on diseases and deaths associated with liver problems and in these regions of Ghana, AF-related health problems are not considered a high priority for therapy. Thus, the identification of this high risk population will facilitate human trials to assess the ability of NS clay to reduce biomarkers of AF exposure and promote the management of human aflatoxicosis in Ghana.

CHAPTER V

SUMMARY AND CONCLUSIONS

The aflatoxins (AFs) represent the major class of mycotoxins produced by *Aspergillus* species of fungi. They are a group of structurally similar, highly toxic fungal metabolites that frequently occur in foods (e.g. corn and groundnuts) and are strongly implicated in the etiology of disease and death in humans and animals (CAST, 2003). Among the AFs, aflatoxin B_1 (AFB_1) is usually the most predominant in foods/feeds, most toxic, and the most potent hepatocarcinogen in humans (Busby and Wogan, 1984; Bhatnagar *et al.,* 2002; Lopez *et al*., 2002). The AF occurrence in food/feed has been described as an unavoidable problem and difficult to control even with good manufacturing practices. This is because the AFs are invisible to the naked eye, heat stable, and not neutralized by common food processing techniques, (CAST, 1989; Phillips *et al.,* 2002). Food contamination with AF remains a serious burden in many parts of the world and is a serious impediment to improving food safety, especially in areas where food supplies are scarce (McAlpin *et al.,* 2002; Shephard, 2003).

Previous studies have demonstrated that calcium montmorillonite clays (e.g. NovaSil or NS and NovaSil Plus or NSP) in the diet can significantly protect multiple animal species against the insults associated with AFs (Phillips et al., 1988; 1994; 1999). Unfortunately, comparable studies in humans have not yet been published. Therefore, the overall goal of this research was to investigate

the suitability of NSP for human use through *in vitro* characterization followed by *in vivo* evaluation of AFB_1 sorption and parent clay safety. To address this concern, the main objectives for this research were to:

1) Characterize the *in vitro* and *in vivo* sorption efficacy of NSP for AFB_1 and evaluate potential interactions with vitamin A (VA).
2) Evaluate the potential adverse effects of chronic ingestion of NSP in the diets of male and female Sprague-Dawley (S-D) rats.
3) Establish representative baseline data for human AF exposure in four communities in the Ejura district of Ghana.

The first objective of this research was to characterize the *in vitro* and *in vivo* sorption efficiency of NSP for AFB_1 and to evaluate potential interactions with VA. Specifically, these studies were designed to 1) determine the sorption affinity and capacity of NSP for AFB_1, 2) characterize the thermodynamics of the AFB_1-NSP interaction *in vitro*; 3) investigate the sorption of AFB_1 by NSP in S-D rats through quantification of urinary AFM_1, and 4) determine the minimal effective dietary inclusion level of NSP that can protect broiler chicks from the effects of aflatoxicosis and hepatic VA depletion.

Isothermal analysis was used to show that NSP had high affinity, capacity, and specificity for AFB_1 and did not appear to interact with VA in aqueous solution. The shape of the NSP-VA isotherm curve was the C-type with negligible affinity value, which represents a constant partition process of binding with no saturable site for the nutrient VA. On the other hand, the shape

of the isothermal curve for NSP was the L-type, indicating saturation of a real binding site in the clay (Giles et al., 1960).

Further, NSP may have an equivalent or greater capacity and affinity for AFB_1 than that previously reported for NS, an earlier and closely related product (Grant and Phillips, 1998). Although the binding capacity (Q_{max}) value (± SEM) of NSP (0.456 ± 0.007 mol AFB_1/kg sorbent) and that previously reported for NS (0.461 ± 0.015 mol AFB_1/kg sorbent) for AFB_1 sorption were comparable, the affinity (K_d) parameter of NSP (7.48×10^5) was more than 6-fold higher than that reported by Grant and Phillips (1998) for NS (1.21 ± 10^5). This evidence suggests that both clays may have the same binding capacity but NSP exhibits tighter binding compared to NS. In both cases, calculation of thermodynamic parameters suggested strong sorption of AFB_1. However, the estimated enthalpy of sorption for NSP (-49.2 kJ/mol) was higher than values previously reported for NS (-40 kJ/mol), suggesting a tighter toxin binding onto NSP clay (Grant and Phillips, 1998). Based on ΔH_{ads} values, the adsorption of AFB_1 to the surface of either sorbent is characterized as chemisorption, a chemical reaction and/or sharing of electrons between the ligand and the sorbent surface that is indicated by enthalpy values > -20 kJ/mol (Gatta, 1985). Therefore, since NS has shown protection against AFs in the gastrointestinal (GI) tract of multiple animals, the prediction is that NSP may also bind AFB_1 in the GI tract of animals with high efficacy, thus providing protection from aflatoxicosis.

Additionally, this *in vitro* evidence indicates that NSP can selectively sorb the AF without interfering with the physiological levels of VA *in vivo*. Also, the use of isothermal analysis for VA serves as a useful model to investigate potential NSP interactions with other important nutrients such as vitamin E, iron, and zinc. Future *in vivo* studies will include measurements of nutrients such as VA, VE, Fe, and Zn in AF-sensitive animals (i.e., young chickens, turkeys, swine, lambs, and rats) ingesting NSP.

On the basis of its prevalence in urine and the dose-dependent linearity observed with dietary AFB_1 ingestion, AFM_1, an oxidative metabolite of AFB_1, was selected as a biomarker of exposure (Groopman et al., 1992a; Hsieh and Wong, 1994). Previous research has indicated that 0.5% NS in the diet of Fisher 344 rats was sufficient to reduce urinary AFM_1 levels by 58% following a single gavage dose of 1 mg/kg (bw) AFB_1 (Sarr et al., 1995). However, in the current study, S-D rats fed only half the amount of NSP (0.25%) showed a greater reduction (71%) of the metabolite following the same AFB_1 dose. Although the two studies used different animal strains, the results suggest that NSP is at least equivalent to, if not more efficient, than NS in reducing the bioavailability of AFs in the GI tract. Therefore, this 48 hr study serves as a useful model to quickly evaluate the *in vivo* sorption of diverse sorbents for their ability to sorb AFs or other mycotoxins in future studies in animals. Notice that the *in vitro* and *in vivo* characteristics of NSP suggest that NSP, which is the one

currently available in animal feeds, is a highly efficient binder for AFs and hence could also be applied to reduce biological exposure to AFs in humans.

In addition to reducing urinary AFM_1 in rats, dietary inclusion of 0.25% NSP was also shown to effectively reduce (95%) the negative health manifestations associated with AFB_1 consumption in broiler chicks (e.g., reduced weight gains, increased liver and kidney weights, and decreased feed consumption). Importantly, at dietary inclusion levels as great as 0.5%, NSP protected from AFB_1-dependent hepatotoxicity without affecting hepatic VA levels. This lack of interaction is important because VA deficiency is the leading cause of childhood blindness in developing countries, and is a major contributor to childhood morbidity and mortality resulting from common infections (Fieldler et al., 2000). In addition, VA has been reported to prevent or delay carcinogenesis induced in animals by chemicals such as AFB_1 (Sporn and Newton, 1981; Frayssinet and La-farge-Frayssinet, 1989), and therefore, VA has been considered a potential inhibitor of human cancer (Kummet and Meyskens, 1983). Specifically, it is reported that VA appears to have an exceptional ability in rat liver microsomes to suppress AFB_1 metabolic activation (Firozi et al., 1987) and to modulate DNA-AFB_1 adduct formation (Bhattacharya et al., 1984). Also, VA deficiency has been shown to enhance liver microsomal activity for AFB_1 activation (Bhattacharya et al., 1989). With all these effects of VA, the clay-based intervention strategy would be counter productive if NSP affects the

physiological levels of this nutrient. Therefore, VA was used as a model for future investigations regarding nutritional analysis in AF prevention studies.

Nonselective sorbents may bind enzymes and critical nutrients, making prolonged use inadvisable. Therefore, any clay intended for human consumption must show high specificity for the toxin of interest, without interfering with normal physiological functions or nutrient utilization. For example, in a previous study, inclusion of a common zeolite known as clinoptilolite (0.5% w/w) in an AFB_1-contaminated diet enhanced the occurrence of preneoplastic lesions in the liver of pregnant rats. These lesions were not observed in the presence of AFB_1 or clinoptilolite alone (Mayura *et al.,* 1998). Unlike NS clay, zeolites are nonselective in their action like carbon. In this same study, NS clay protected adults and developing fetuses from the effects of AFs and did not produce changes in the liver like clinoptilolite. Taken together, the results obtained for studies outlined in the first objective (*in vitro* and *in vivo*) indicate that NSP strongly and specifically sorbs AFB_1 without interfering with hepatic VA. Thus, in future studies, NSP added to the diet at up to 0.5% would not be expected to compromise health nor would the clay be expected to non-specifically sorb VA.

Previous research in pregnant rats indicated that concentrations as great as 2.0% NSP in the diet throughout gestation did not result in either maternal or fetal toxicity, and did not significantly affect the bioavailability of a variety of trace metals in selected tissues (Wiles et al., 2004). However, the effects of chronic

ingestion of NSP remain unpublished. Therefore, the second objective of our research was to evaluate the potential adverse effects of chronic ingestion of NSP in the diets of male and female S-D rats.

Studies addressing the second objective showed that feed consumption, body weight gain, feed conversion efficiency, and relative organ weights in male or female S-D rats were not affected by dietary NSP concentrations as great as 2% over 6.5 months, suggesting that the clay did not produce overt toxicity upon chronic ingestion. In addition, no observable NSP-dependent gross anatomical or histopathological changes to major organs were observed, and there were no NSP-dependent changes to common diagnostic hematological or serum biochemical parameters, further confirming a lack of overt toxicity. Specifically, red blood cell (RBC) count was not affected and there were no NSP-related morphological differences detected in RBCs in NSP-treated versus untreated groups, suggesting that NSP ingestion is unlikely to produce hemolytic, hemorrhagic, or other forms of anemia, or to affect RBC physiology or metabolism (George-Gay and Parker, 2003). Further, white blood cell and leukocyte differentials were found to be quantitatively and morphologically similar between NSP-treated and untreated animals, suggesting that the clay is unlikely to impair immunity or inflammatory processes, increase incidence of infections, or cause alterations to bone marrow (Goodnough et al., 1999; George-Gay and Parker, 2003). Finally, serum and hepatic VA and vitamin E (VE) and micronutrients Fe and Zn did not appear to be adversely impacted by

NSP ingestion. Overall, this study indicated that chronic ingestion of NSP at levels as great as 2.0% (w/w) in the diet does not produce overt toxicity.

The information gained in the second objective increases the feasibility for dietary inclusion of NSP in humans in order to reduce foodborne AF exposure and improve quality of life in high risk populations. Future studies will test this product in healthy human volunteers in order to reduce potential confounding variables (i.e., malnourishment, hepatitis B and C viral infection, liver and kidney problems, HIV/AIDS, and immune suppression) before translating dietary NSP intervention to susceptible populations.

Although future research may be designed to characterize the efficacy of NSP to protect humans from AF contamination, biomarker exposure data are first needed to identify high risk populations. Thus, the third main objective of this research was to establish representative baseline data for human AF exposure in four communities in the Ejura district of Ghana. Individuals in this area of the world are frequently exposed to AFs and have the potential to greatly benefit from clay-based dietary intervention strategies/therapies.

Research for this objective was accomplished by analyzing urinary AFM_1 as a biomarker for AF exposure in volunteers within these communities. Measurement of urinary AFM_1 is a non-invasive method of AFB_1 exposure characterization, as it is not only prevalent but also exhibits dose-dependent excretion with dietary AFB_1 ingestion (Groopman et al 1992a; Hsieh and Wong, 1994).

The results of a survey of 91 subjects in Ghana, revealed high urinary AFM_1 levels (71.1% contained moderate to very high levels), indicating substantial AF exposure in the selected study population. With the identification of this high risk population, future intervention trials in Ghana will be designed to characterize the efficacy of NSP to decrease AF exposure from contaminated food. However, since urinary AFM_1 levels reflect short-term exposure status (over the previous 24 hr period) (Groopman, 1993; Wild, 1998) all volunteers will also be screened for aflatoxin-albumin adducts, which reflect chronic exposure to the toxin.

In order to make this technology more accessible to people who frequently ingest contaminated foods, efforts should be made to educate Ghanaians in the use of NSP clay for AF prevention. This would involve the participation and collaborations with the ruling government, community leaders (e.g. the chief and his entourages), and district health professionals. It would also require the development and implementation of a comprehensive, layperson-oriented, and culturally acceptable educational curriculum for effective translation of this clay-based approach for the prevention of aflatoxicosis. Perhaps, the most efficient and effective methods to facilitate the AF prevention educational process could be to train some community leaders and the district health professionals who will in turn convey the message to their counterparts. The media will also play a vital role in reaching the majority of the people in the country.

Additionally, future research should investigate local clay minerals for efficacy and specificity for AFs. For instance, clays that are commonly sold in Ghanaian marketplaces and considered “edible”, as well as those from clay mining sites can be tested for their ability or lack thereof, to selectively sorb AFs *in vitro* and *in vivo*. It will be useful to collaborate with mineralogists in any country or area of interest to explore the possibilities of identifying clay minerals with properties comparable to those of NSP with $Q_{max} \geq$ 0.3 mol AFB_1/kg sorbent and $K_d \geq 1.0 \times 10^5$. Also, the enthalpy of sorption should be > -30 KJ/mol to predict tight sorbent-ligand chemisorption. Although imported clay minerals such as NSP appear to be cost effective, success will be contingent upon affordability. Based on this and previously described methodology (Phillips et al., 1988; Grant and Phillips, 1998; Lemke, 2000), efficacy and specificity of each clay may be investigated in a relatively short period of time. It must be noted that, although NSP has been shown to significantly protect against aflatoxicosis, the effects of other clay minerals are ill-defined in the scientific literature which spells out the essentiality of thorough investigation before application to humans.

Future studies may also focus on the identification of populations that exhibit genetic sensitivity to AFs. Genes that code for synthesis of drug metabolizing enzymes, such as epoxide hydrolases (EH) and glutathione *s*-transferase (GST), which are involved in detoxification of AFs and other xenobiotics, may be defective or mutated. Gene mutations may lead to

enhanced susceptibility due to alterations in the levels and/or activities of these AF-metabolizing enzymes. Therefore, the proposed clay-based intervention strategy for the reduction of AF bioavailability may be highly beneficial to individuals possessing a genetically defective allele in an enzyme involved in its metabolism.

Of particular significance is the fact that this technology is relatively cost effective, culturally acceptable, environmentally friendly, and has a high potential for sustainability. However, delivery methods that will not destroy or alter the structural integrity of the clay and/or affect the taste of foods need to be further explored. Thus far, dietary inclusion of NSP for animals has been shown to be effective against AFB_1 and the chronic toxicity study showed that the amounts of feed consumption in rats that ingested dietary NSP were similar to those of the controls. In the case of humans, the following are pertinent questions that need to be addressed regarding NSP usage. For example, will encapsulating NSP be an appropriate delivery option for humans? What about making NSP slurries that can be consumed before each meal? Does dietary inclusion of NSP affect the palatability of finished food products? What about the effects of cooking temperature and pH changes? These are some of the questions that need to be answered in future research of NS clay.

Dietary NSP studies should be conducted utilizing several local and imported foodstuffs to determine not only the delivery method but also the minimal effective concentration and the impact of the selected amount on

palatability. One possibility is the use of capsules, like vitamin supplements, to encase selected amounts of the clay mineral that may then be ingested with food. However, the efficacy of capsule delivery, mechanisms and kinetics of release, hydration and ligand sorption by NS will require verification.

It is well-established that AF contamination of foods/feeds presents both economic and public health burdens worldwide, resulting in an increase in research initiatives to address the problem. Clay-based enterosorption of the toxin within the GI tract has shown promise for the prevention of the AF burden in multiple animal species. The overall goal of this research was to investigate the suitability of NSP for human use through *in vitro* characterization followed by *in vivo* evaluation of not only AFB_1 sorption but also parent clay safety. In these studies, NSP demonstrated high affinity, capacity, and specificity for AFs without affecting anatomical or physiological parameters at dietary inclusion levels as great as 2.0% (w/w) following long-term ingestion. The overall findings of this research suggest that NSP may be feasible and appropriate for the management of aflatoxicosis in high risk populations.

REFERENCES

Abbas, T. A. and Ali, B. H. (2001). Retinol values in the plasma of the Arabian camel (*Camelus dromedaries*) and the influence of aflatoxicosis. *Vet. Res. Comm*. **25,** 517-522.

Abdel-Wahhab, M. A., Nada, S. A., Khalil, F. A. (2002). Physiological and toxicological responses in rats fed aflatoxin-contaminated diet with or without sorbent materials. *Animal Feed Science Technol.* **97,** 209-219.

Abdulrazzaq, Y. M., Osman, N., Yousif, Z. M., and Trad, O. (2004). Morbidity in neonates of mothers who have ingested aflatoxins. *Ann Trop Paediatr.* **24,** 145-51.

Abrahams, P. W. and Parsons, J. A. (1996). Geophagy in the tropics: A literature review. *Geog J.* **162,** 63-72.

Adhikari, M., Ramjee, G., and Berjak, P. (1994). Aflatoxin, Kwashiorkor and morbidity. *Natural Toxins*. **2,** 13.

AflaTest® Instruction Manual. (1999). Vicam. Watertown, MA.

Aguilar, F., Hussain, S. P., and Cerutti, P. (1993). Aflatoxin B_1 induces the transversion of G $\rightarrow$ T in codon 249 of the p53 tumor suppressor gene in human hepatocytes. *Proc. Natl. Acad. Sci. USA*. **90,** 8586-8590.

Authrup, H., Esssigman, J. M., Croy, R. G., Trump, B. F, Wogan, G. N., and Harris, C. C. (1979). Metabolism of aflatoxin B_1 and identification of the major aflatoxin B_1-DNA adducts formed in cultured human bronchus and colon. *Cancer Res.* **39,** 694-698.

Authrup, H., Schwartz, R. D., Esssigman, J. M., Smith, L., Trump, B. F, and Harris, C. C. (1980). Metabolism of aflatoxin B_1, bezo[a]pyrene, and 1,2-dimethylhydrazine by cultured rat and human colon. *Terat. Carc. Mutagen.* **1,** 3-13.

Awuah, R. T. and Kpodo, K. A. (1996). High incidence of *Aspergillus flavus* and aflatoxins in stored groundnuts in Ghana and the use of microbial assay to assess the inhibitory effects of plant extracts on aflatoxin synthesis. *Mycopathologia*, **134,** 109-114.

Aye, P. P., Morishita, T. Y., Saif, Y. M., and Jonas, M. (2000a). The effect of hypovitaminosis A on the pathogenesis of *Pasteurella multocida* in turkeys. *Avian Dis.* **44,** 8-26.

Aye, P. P., Morishita, T. Y., Saif, Y. M., Latshaw, J. D., Harr, B. S., and Cihla, F. B. (2000b). Induction of vitamin A deficiency in turkeys. *Avian Dis.* **44,** 9-17.

Baxter, C. C., Wey, H. E., and Burg, W. R. (1981). A prospective analysis of the potential risks associated with inhalation of aflatoxin-contaminated grain dusts. *Food Cosmet. Toxicol.* **19,** 765-769.

Becroft, D. M. and Webster, D. R. (1972). Aflatoxins and Reye's disease. *Br. Med. J.* **4,** 117.

Bhatnagar, D., Cleveland, T. E., and Cotty, P. J. (1994). Mycological aspects of aflatoxin formation. In *The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance* (D. L. Eaton and J. D. Groopman, Eds.), pp. 327-346. Academic Press, Inc., San Diego, CA.

Bhatnagar, D., Ehrlich, K. C., and Cleveland, T. E. (2003). Molecular genetic and regulation of aflatoxin biosynthesis. *Applied Microbiol Biotechnol.* **61,** 83-93.

Bhatnagar, D., Yu, J., and Ehrlich, K. C. (2002). Toxins of filamentous fungi. *Chem. Immunology*. **81**, 167-206.

Bingham, A. K., Phillips, T. D., and Bauer, J. E. (2003). Potential for dietary protection against the effects of aflatoxins in animals. JAVMA **222,** 591-596.

Blount, W. P. (1961). Turkey “X” disease. *J. British Turkey Fed*. **9,** 52-54.

Bodine, A. B., Fisher, S. F., and Gangjee, S. (1984). Effect of aflatoxin B_1 and major metabolites on fetohemeglutinin-stimulated lymphoblastogenesis of bovine lymphocytes. *J. Dairy Sci.* **67,** 110-114.

Bohn, H. L., McNeal, B. L., and O’Connor, G. A. (1979). *Soil Chemistry,* pp. 80-105. John Wiley & Sons, New York, NY.

Boix-Ferrero, J., Pellin, A., Blesa, R., Adrados, M., and Llombart-Bosch, A. (1999). Absence of p53 gene mutation in hepatocarcinomas from a mediterranean area of Spain. A study of 129 archival tumor samples. *Virch. Arch.* **434,** 497-501.

Borchardt, G. (1989). Smectites. In *Minerals in Soil Environments* (J. B. Dixon, J. and S. B. Weed, Eds.), pp. 675-727. Soil Science Society of America, Madison, WI.

Bressac, B., Kew, M., Wands, J., and Ozturk, M. (1991). Selective G to T mutations of p53 gene in hepatocellular carcinoma from Southern Africa. *Nature* **350,** 429-431.

Brown, R. W., Pier, A. C., Richard, J. L., and Krogstad, R. E. (1981). Effects of dietary aflatoxins on existing bacterial intramammary infections of dairy cows. *Am. J. Vet. Res.* **42,** 927-933.

Burri, B. J., Dopler-Nelson, M., and Neidlinger, T. R. (2003). Measurements of the major isoforms of vitamins A and E and carotenoids in the blood of people with Spinal-cord injuries. *J Chromatography A*, **987,** 359-366.

Busby, W. F., and Wogan, G. N. (1984). Aflatoxins. In *Chemical Carcinogens* (C. D. Seale, Ed.), pp. 945-1136. American Chemical Society, Washington, DC.

Butler, F. C. (1947). Ear, cob, and grain rots of maize. *Agri Gaz New South Wales* **58**.

Butler, W. H. and Hempsall, V. (1981). Histochemical studies of hepatocellular carcinomas in the rat induced by aflatoxin. *J. Pathol.* **134,** 157-170.

Carretero, M. I. (2002). Clay minerals and their beneficial effects upon human health. *Appl. Clay Sci.* **21,** 155–163.

Cary, J. W., Linz, J. E, Bhatnagar, D. (2000a). Aflatoxins: Biological significance and regulation of biosynthesis. In *Microbial Foodborne Diseases: Mechanisms of Pathogenesis and Toxin Synthesis* (J. W. Cary, J. E. Linz, and D. Bhatnagar, Eds.), pp 317–361. Technomic, Lancaster, PA.

CAST (Council for Agricultural Science and Technology) (1989). *Mycotoxins: Economic and Health Risks* (K. Niyo, Ed.), pp. 1-91. CAST, Task Force Report No. 116. Ames, IA.

CAST (Council for Agricultural Science and Technology). (2003). Mycotoxins: Risks in plant, animal, and human systems. Task Force Report No. 139. pp. 13-85. CAST, Ames, IA.

Centers for Disease Control and Prevention (CDC). (2004). Outbreak of aflatoxin poisoning - Eastern and Central Provinces, Kenya. *Morbidity and Mortality Weekly Rep.* **53,** 790-793.

Chopra, R. C., Chhabra, A., Prasad, K. S. N., Murphy, T. N., Prasad, T. (1999). Carryover of aflatoxin M_1 in milk of cows fed aflatoxin B_1 contaminated ration. *Indian J. Anim. Nutr.* **16,** 103-106.

Christensen, C. M., Mirocha, C. I., and Meronuck, R. A. (1977). Mold, mycotoxins, and mycotoxicoses. In *Agricultural Experiment Station, Report 142.* University of Minnesota, St. Paul, MN.

Chung, T. K., Erdman, J. W. Jr., and Baker, D. H. (1990). Hydrated sodium calcium aluminosilicate: Effects on zinc, manganese, vitamin A and riboflavin utilization. *Poultry Sci*. **69,** 1364.

Clark, J. D., Hatch, R. C., Jain, A. V., and Weiss, R. (1982). Effect of enzyme inducers and inhibitors and glutathione precursor and depleter on induced acute aflatoxicosis in rabbits. *Am. J. Vet. Res.* **43,** 1027-1033.

Clifford, J. I. and Rees, K. R. (1967). The action of AFB_1 on rat liver. *Biochem. J.* **103,** 65-75.

Cockcroft, P. D. (1995). Sudden death in dairy cattle with putative acute aflatoxin B poisoning. *Vet. Rec.* **136,** 248.

Cole, R. J. (1989). Technology of aflatoxin decontamination. In *Mycotoxins and Phycotoxins '88* (S. Natori, K. Hashimoto, and Y. Ueno, Eds.), p. 177. Elsevier Scientific, Amsterdam.

Cole, R. J., Dorner, J. W., and Holbrook, C. C. (1995). Advances in mycotoxin elimination and resistance. In *Advances in Peanut Science* (H. E. Pattee and H. T. Stalker, Eds.), pp. 456-474. American Peanut Research and Education Society, Stillwater, OK.

Cole, R. J., Sanders, T. H., Dorna, J. W., and Blankenship, P. D. (1989). Environmental conditions required to induce preharvest aflatoxin contamination of groundnuts: Summary of six years research. In *Aflatoxin Contamination of Groundnuts* (D. McDonald and V. K. Mehan, Eds.), pp. 279-287. The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India.

Cole, R. J., and Cox, R. H. (1981). *Handbook of Toxic Fungal Metabolites* (R. J. Cole and R. H. Cox, Eds.), pp 1-903. Academic Press, New York.

Conway, H. F., Anderson, R. A., and Bagley, E. B. (1978). Detoxification of aflatoxin-contaminated corn by roasting. *Cereal Chem.* **55,** 115.

Coppeck, R. W., Reynolds, R. D., Buck, W. B., Jacobsen, B. J., Ross, S. C., and Mostrom, M. S. (1989). Acute aflatoxicosis in feeder pig, resulting from improper storage of corn. *J. Am. Vet. Med. Assoc.* **195,** 1380-1381.

Coulombe, R. A. (1994). Nonhepatic Disposition and Effects of Aflatoxin B_1. In *The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance* (D. L. Eaton and J. D. Groopman, Eds.), pp. 89-101. Academic Press, Inc., San Diego, CA.

Cullen, J. M., and Newberne, P. M. (1994). Acute hepatotoxicity of aflatoxins. In *The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance* (D. L. Eaton and J. D. Groopman, Eds.), pp. 3-26. Academic Press, Inc., San Diego, CA.

Cysewski, S. J., Wood, R. L., Pier, A. C., and Baetz, A. L. (1978). Effects of aflatoxin on the development of acquired immunity to swine erysipelas. *Am J. Vet. Res.* **39,** 445-448.

Dalloul, R. A., Lillehoj, H. S., Shellem, T. A., and Doerr, J. A. (2002). Effect of vitamin A deficiency on host intestinal immune response to *Eimeria acervulina* in broiler chickens. *Poult. Sci.* **81,**1509-1515.

D'Arcy, P. F. and Harron, D. W. G. (1992). Technical requirements for registry of pharmaceuticals for human use. *Proceedings of the First International Conference on Harmonization*. The Queens University of Belfast, Ireland.

Di Paolo, J. A., Ellis, J., and Ewin, H. (1967). Teratogenic response by hamsters, rats and mice to aflatoxins. *Nature.* **215,** 638-639.

Diamond, J. M. (1999). Dirty eating for healthy living. *Nature.* **400,** 120-121.

Dickens, W. J. and Whitaker, T. B. (1975). Efficacy of electronic color sorting and hand picking to remove aflatoxin contaminated kernels from commercial lots of shelled peanuts. *Peanut Sci.* **2,** 45.

Diener, U. L., Cole, R. J., Sanders, T. H., Payne, G. A., Lee, L. S., and Klich, M. A. (1987). Epidemiology of aflatoxin formation by *Aspergillus flavus*. *Ann. Rev. Phytopath.* **25,** 249-270.

Dimri, U., Rao, V. N., Joshi, H. C. (1994). Effect of chronic aflatoxin B_1 feeding on serum-calcium, magnesium and iron profiles in chicken. *Indian Vet. J.* **71,** 907-910.

Doerr, J. A., Huff, W. E., Tung, H. T., Wyatt, R. D., and Hamilton, P. B. (1974). A survey of T-2 toxin, ochratoxin, and aflatoxin for their effects on the coagulation of blood in young broiler chickens. *Poultry Sci.* **53,** 1728-1734.

Dugyala, R. R. and Sharma, R. P. (1996). The effects of aflatoxin B_1 on cytokines mRNA and corresponding protein levels in peritoneal macrophages and splenic lymphocytes. *Intl. J. Immunophamacol.* **18,** 599-608.

Eaton, D. L., Ramsdell, H. S., and Neal, G. E. (1994). Biotransformation of aflatoxins. In *The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance* (D. L. Eaton and J. D. Groopman, Eds.), pp. 45-72. Academic Press, San Diego, CA.

Eddins, A. H. (1930). Corn diseases in Florida. Florida Agricultural Experiment Station. *Bull. 210*. Gainesville, FL.

Ehrlich, K. C., Montalbano, B. G., and Cary, J. W. (1999). Binding of the C6-zinc cluster protein, AFLR, to the promoters of aflatoxin pathway biosynthesis genes in Aspergillus parasiticus. *Gene*. **230,** 249-257.

Ellis, J. A., Harvey, R. B., Kubena, L. F., Clement, B. A., and Phillips, T. D. (1990). Reduction of aflatoxin M_1 residues in milk utilizing hydrated sodium calcium aluminosilicate. *Toxicol.* **10,** 63.

Essigmann, J. M., Croy, R. G., Nadzan, A. M., Busby, W. F. Jr., Reinhold, V. N., Buchi, G., and Wogan, G. N. (1977). Structural identification of the major DNA adduct formed by aflatoxin B_1 *in vitro*. *Proc. Natl. Acad. Sci.* **74,** 1870-1874.

Essigmann, J. M., Green, C. L., Croy, R. G., Fowler, K. W., Buchi, G. H., Wogan, G. N. (1983). Interactions of aflatoxin B_1 and alkylating agents with DNA: Structural and functional studies. *Cold Spring Harb Symp Quant Biol.* **47,** 327-37.

Ferrario, J. B. and Byrne, C. J. (2000). 2,3,7,8-Dibenzo-p-dioxins in mined clay products from the United States: Evidence for possible natural origin. *Environ. Sci. Technol.* **34,** 4524-4532.

Fiedler, J. L., Dado, D. R., Maglalang, H., Juban, N., Capistrano, M., and Magpantay, M. C. (2000). Cost analysis as a vitamin A program design and evaluation tool: A case study of the Philippines. *Social Science & Medicine* **51**, 223-242.

Fink-Gremmels, J. (1999). Mycotoxins: Their implications for human and animal health. *The Veterinary Quarterly* **21,** 115-120.

Firozi, P. F., Aboobaker, V. S., and Bhattacharya, R. K. (1987). Action of vitamin A on DNA adduct formation by aflatoxin B_1 in a microsome catalysed reaction. *Cancer Lett.* **34,** 213–220.

Fischer, R. B., and Peters, D. G. (1970). *Chemical Equilibrium,* pp. 1-61. W. B. Saudners and Co., Philadelphia, PA.

Flaherty, J. E. and Payne, G. A. (1997). Overexpression of aflR leads to upregulation of pathway gene transcription and increased aflatoxin production in *Aspergillus flavus*. *Applied and Environ. Microbiol.* 3995-4000.

Foster, P. L., Eisenstadt, E., and Miller, J. H. (1983). Base substitution mutations induced by metabolically activated aflatoxin B_1. *Proc. Natl. Acad. Sci.* **80,** 2695-2698.

Frayssinet, C., and Lafarge-Frayssinet, C. (1989). Malnutrition et cancerogenese exprimentale induite par les substances chimiques, In *Les Carences Nutritionnelles dans les Pays en Vole de Developpement* (D. Lemonnier and Y. Ingenbleek, Eds.), pp. 507-518. Col. INSERM-ACCT, Paris.

Gatta, G. D. (1985). Determination of adsorption heats. *Thermochim. Acta.* **96,** 9-363.

Geissler, P. W., Prince, R. J., Levene M., Poda, C., Beckerleg, S. E., Mutemi, W., and Shulman, C. E. (1999). Perceptions of soil-eating and anemia among pregnant women on the Kenyan coast. *Soc. Sci. Med.* **48,** 1069–1079.

Gentil, A., Cabral-Neto, J. B., Mariage-Samson, R., Margot, A., Imbach, J. L., Rayner, B., and Sarasin, A. (1992). Mutagenicity of a unique apurinic/apyrimidinic sites in mammalian cells. *J. Mol. Biol.* **227,** 981-984.

George-Gay, B., and Parker, K. (2003). Understanding the complete blood count with differential. *J. PeriAnesthesia* **18,** 96-117.

Ghosh, R. C., Chauhan, H. V. S., and Roy, S. (1990). Immunosuppression in broilers under experimental aflatoxicosis. *Br. Vet. J.* **146,** 457-462.

Gilardi, J. D., Duffey, S. S., Munn, C. A., and Tell, L. A. (1999). Biochemical functions of geophagy in parrots: Detoxification of dietary toxins and cytoprotective effects. *J. Chem. Ecol.* **25,** 897-922.

Gilber, J., and Anklam, E. (2003). Validation of analytical methods for determining mycotoxins in foodstuffs. *Trends in analytical chem.* ***2*****1,** 6-7.

Giles, C. H., D'Silva, A. P., and Easton, I. A. (1974b). A general treatment and classification of the solute adsorption isotherm, part II: Experimental interpretation. *J. Colloid Interface Sci.* **47,** 766-778.

Giles, C. H., MacEwan, T. H., Nakhwa, S. N., and Smith, D. (1960). Studies in adsorption. Part XI. A system of classification of solution adsorption isotherms, and its use in diagnosis of adsorption mechanisms and in measurement of specific areas of solids. *J. Chem. Soc.* 3973-3993.

Giles, C. H., Smith, D., and Huitson, A. (1974a). A general treatment and classification of the solute adsorption isotherm, part I: Theoretical. *J. Colloid Interface Sci.* **47,** 755-765.

Glahn, R., Beers, K., Bottje, W., Wideman, R. J., Huff, W., and Thomas, W. (1991). Aflatoxicosis alters avian renal function, calcium, and vitamin D metabolism. *J. Toxicol. Environ. Health*. **34**, 309-321.

Goerttler, K., Lohrke, H., Schweizer, H., and Hesse, B. (1980). Effects of aflatoxin B_1 on pregnant inbred Sprague-Dawley rats and their F_1 generation. A contribution to transplacental carcinogenesis. *J. Natl. Cancer Inst.* **64,** 1349-1354.

Goldwasser, P., Aboul-Magd, A., and Maru, M. (1997). Race and creatinine excretion in chronic renal insufficiency. *Am. J. Kidney Dis.* **30,** 16–22.

Gong, Y. Y., Cardwell, K., Hounsa, A., Egal, S., Turner, P. C., Hall, A. J., and Wild, C. P. (2002). Dietary aflatoxin exposure and impaired growth in young children from Benin and Togo: Cross sectional study. *Br. Med. J.* **325,** 20-21.

Goodnough, L. T., Brecher, M. E., and Katner, M. H. (1999). Transfusion medicine: Blood transfusion. *N. Eng. J. Med.* **340,** 438-447.

Gopalakrishnan, S., Harris, T. M., and Stone, M. P. (1990). Intercalation of aflatoxin B_1 in two oligodeoxynucleotide adducts: Comparative 1H NMR analysis of d(ATCAFBGAT).d(ATCGAT) and d(ATAFBGCAT)$_2$. *Biochemistry.* **29,** 10438-10448.

Gorman, D. P. and Kang, N. S. (1991). Preharvest aflatoxin contamination in maize: Resistance and genetics. *Plant Breeding* **107,** 1-10.

Grant, P. G. (1998). Investigation of the mechanism of aflatoxin B1 adsorption to clays and sorbents through the use of isothermal analysis. Ph.D. dissertation, Department of Veterinary Anatomy and Public Health, Texas A&M University, College Station.

Grant, P. G., and Phillips, T. D. (1998). Isothermal adsorption of aflatoxin B_1 on HSCAS clay. *J. Agric. Food Chem.* **46,** 599-605.

Greenberg, G. N., and Levine, R. J. (1989). Urinary creatinine excretion is not stable: A new method for assessing urinary toxic substance concentration. *J. Occup. Med.* **31,** 832–838.

Groopman, J. D. (1994). Molecular dosimetry methods for assessing human aflatoxin exposures. In *The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance* (D. L. Eaton and J. D. Groopman, Eds.), pp. 259-279. Academic Press, Inc., San Diego, CA.

Groopman, J. D., Hasler, J. A., Trudel, L. J., Pikul, A., Donahue, P. R., and Wogan, G. N. (1992b). Molecular dosimetry in rat urine of aflatoxin-N7-guanine and other aflatoxin metabolites by multiple monoclonal antibody affinity chromatography and mmunoaffinity/high performance liquid chromatography. *Cancer Res.* **52,** 267-274.

Groopman, J. D., Zhu, J., Donahue, P. R., Pikul, A., Zhang, L. S., Chen, J. S., and Wogan, G. N. (1992a). Molecular dosimetry of urinary aflatoxin DNA adducts in people living in Guangxi Autonomous Region People's Republic of China. *Cancer Res.* **52,** 45-51.

Gu, B., Schmitt, J., Chen, Z., Liang, L., and McCarthy, J. F. (1994). Adsorption and desorption of natural organic matter on iron oxide: Mechanisms and models. *Environ. Sci. Technol.* **28,** 38-46.

Guengerich, F. P. and Mason, P. S. (1979). Immunological comparison of hepatic and extrahepatic cytochromes P-450. *Mol. Pharmacol.* **15,** 154-64.

Gupta, S. K., and Venkitasubramanian, T. A. (1975). Production of aflatoxin on soybeans. *Appl. Microbiol.* **29,** 834-836.

Gursu, M. F., Sari, M., Sahin, N., and Sahin, K. (2002). Effects of vitamins E and A supplementation on lipid peroxidation and concentration of some mineral in broilers reared under heat stress (32°C). *Nutr. Res.* **22,** 723-731.

Harvey, R. B., Clark, D. E., Huff, W. E., Kubena, L. F., Corrier, D. E., and Phillips, T. D. (1988). Suppression of serum iron-binding capacity and bone marrow cellularity in pigs fed aflatoxin. *Bull. Environ. Contam. Toxicol.* **40,** 576-583.

Harvey, R. B., Kubena, L. F., Elissalde, M. H., Corrier, D. E., and Phillips, T. D. (1994). Comparison of two hydrated sodium calcium aluminosilicate compounds to experimentally protect growing barrows from aflatoxicosis. *J. Vet. Diag. Invest*. **6,** 88-92.

Harvey, R. B., Kubena, L. F., Elissalde, M. H., and Phillips, T. D. (1993). Efficacy of zeolitic ore compounds on the toxicity of aflatoxin to growing broiler chickens. *Avian Dis.* **37,** 67-73.

Harvey, R. B., Kubena, L. F., Phillips, T. D., Corrier, D. E., Elissalde, M. H., and Huff, W. E. (1991a). Diminution of aflatoxin toxicity to growing lambs by dietary supplementation with hydrated sodium calcium aluminosilicate. *Am. J. Vet. Res.* **52,** 152-156.

Harvey, R. B., Kubena, L. F., Phillips, T. D., Huff, W. E., and Corrier, D. E. (1989). Prevention of aflatoxicosis by addition of hydrated sodium calcium aluminosilicate to the diets of growing barrows. *Am. J. Vet. Res.* **50**, 416-420.

Harvey, R. B., Phillips, T. D., Ellis, J. A., Kubena, L. F., Huff, W. E., and Petersen, H. D. (1991b). Effects of aflatoxin M_1 residues in milk by addition of hydrated sodium calcium aluminosilicate to aflatoxin-contaminated diets of dairy cows. *Am. J. Vet. Res.* **52**, 1556-1559.

Hatori, Y., Sharma, R. P., and Warren, R. P. (1991). Resistance of C57B1/6 mice to immunosuppressive effects of aflatoxin B_1 and relationship with nueroendocrine mechanisms. *Immunopharmacology* **22,** 127-136.

Hayes, A. W. (1981). *Mycotoxin Teratogenicity and Mutagenicity*. CRC Press, Boca Raton, FL.

Headrick, M. L., Hollinger, K., Lovell, R. A., and Matheson, J. C. (1999). PBBs, PCBs, and dioxins in food animals, their public health implications. *Vet Clin N. Am. Food Animal Practices.* **15,** 109-131.

Heathcote, J. G. and Hibbert, H. R. (1978). Aflatoxins: Chemical and biological aspects. In *Developments in Food Science I*. pp. 1-186. Elsevier, New York.

Hendrickse, R. G. (1985a). Kwashiorkor: 50 years of myth and mystery. Do aflatoxins provide a clue? *Acta Leiden.* **53,** 11-30.

Hendrickse, R. G. (1985b). The influence of aflatoxins on child health in the tropics with particular reference to Kwashiorkor. *Trans. Royal Soc. Trop. Med. Hyg.* **78,** 427-435.

Hendrickse, R. G., Coulter, J. B., Lamplugh, S. M., MacFarlane, S. B., Williams, T. E., Omer, M. I., Suliman, G. I., and El-Zorgani, G. A. (1983). Aflatoxins and Kwashiorkor. Epidemiology and clinical studies in Sudanese children and findings in autopsy liver samples from Nigeria and South Africa. *Bull. Soc. Pathol. Exot. Filiales.* **76,** 559-566.

Henry, S. H., Bosch, F. X., and Bowers, J. C. (2002). Aflatoxins, hepatitis, and worldwide liver cancer risks. *Adv. Exp. Med. Biol.* **504,** 229-233.

Herrold, K. M. (1969). Aflatoxin induced lesions in Syrian hamsters. *Br. J. Cancer* **23,** 655-660.

Hill, R. A., Wilson, D. M., McMillian, W. W., Widstrom, N. W., Cole, R. J., Sanders, T. H., and Blankenship, P. D. (1985). Ecology of the A. flavus group and aflatoxin formation in maize and groundnut. In *Trichothecenes and Other Mycotoxins* (J. Lacey, Ed.), pp. 79-95. John Wiley and Sons, Chichester, England.

Hinton, D. M., Myers, M. J., Raybourne, R. A., Francke-Carroll, S., Sotomayor, R. E., Shaddock, J., Warbritton, A., and Chou, M. W. (2003). Immunotoxicity of aflatoxins in rats: Effects on lymphocytes and the inflammatory response in a chronic intermittent dosing study. *Tox. Sci.* **73,** 362-377.

Holeski, C. J., Eaton, D. L., Monroe, D. H., and Bellamy, G. M. (1987). Effects of phenobarbitol on the biliary excretion of aflatoxin P_1-glucuronide and aflatoxin B_1-*S*-glutathione in the rat. *Xenobiotica* **17,** 139-153.

Hosotani, K., and Kitagawa, M. (2003). Improved simultaneous determination method of beta-carotene and retinol with saponification in human serum and rat liver. *J Chromatogr B, Analyt Technol Biomed Life Sci.* **791,** 305-313.

HSDB. Hazardous Substances Data Bank. (2003). Aflatoxins. Online database produced by the National Library of Medicine.http://toxnet.nlm.nih.gov/cgi-bin/sis/search/r?dbs+hsdb. Accessed in October 2004.

Hsieh, D. P. H. (1985). An assessment of liver cancer risk posed by aflatoxin M_1 in the western world. In *Trichothecenes and Other Mycotoxins* (J. Lacey, Ed.), pp. 521-527. Wiley & Sons, Chichester, England.

Hsieh, D. P. H. and Wong, J. J. (1994). Pharmacokinetics and excretion of aflatoxins. In *The toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance* (D. L. Eaton and J. D. Groopman, Eds.), pp. 73-88. Academic Press, Inc., San Diego, CA.

Hsu, I. C., Metcalf, R. A., Sun, T., Welsh, J. A., Wang, N. J., and Harris, C. C. (1991). Mutational hotspot in the p53 gene in human hepatocellular carcinomas. *Nature* **350,** 427-428.

Huebner, H. J., Lemke, S. L., Ottinger, S. E., Mayura, K., and Phillips, T. D. (1999). Molecular characterization of high affinity, high capacity clays for the equilibrium sorption of ergotamine. *Food Addit. Contam.* **16,** 159-171.

Huebner, H. J., Mayura, K., Pallaroni, L., Ake, C. L., Lemke, S. L., Herrera, P., and Phillips, T. D. (2000). Development and characterization of a carbon-based composite material for reducing patulin in apple juice. *J. Food Protection*. **63,** 106-110.

Huff, W. E., Kubena, L. F., Harvey, R. B., Corrier, D. E., and Mollenhauer, H. H. (1986). Progression of aflatoxicosis in broiler chickens. *Poult. Sci.* **65,** 1891-1899.

Huff, W. E., Kubena, L. F., Harvey, R. B. and Phillips, T. D. (1992). Efficacy of a hydrated sodium calcium aluminosilicate to reduce the individual and combined toxicity of aflatoxin and ochratoxin A. *Poultry Sci.* **71,** 64.

Hussein, H. S. and Brasel, J. M. (2001). Toxicity, metabolism, and impact of mycotoxins on humans and animals. *Toxicology.* **167**, 101-134.

Hutchins, J. E., and Hagler, W. M. (1983). Rapid liquid chromatographic determination of aflatoxins in heavily contaminated corn. *J AOAC*. **61,** 861-868.

Huwig, A., Friedmund, S., Kappeli, O., and Dutler, H. (2001). Mycotoxin detoxification of animal feed by different adsorbents. *Toxicol. Lett.* **122,** 179-188.

IARC (International Agency for Research on Cancer). (1987). Overall evaluations of carcinogenicity*. Monographs on the Evaluation of Carcinogenic Risks to Humans, Supplement 7*, pp. 440, Lyon, France.

Jackson, P. E. and Groopman, J. D. (1999). Aflatoxin and liver cancer. *Bailliere's Clin Gastroenterol.***13,** 545-555.

Jaimez, J., Fente, C. A., Vasquez, B. I., Franco, C. M., Cepeda, A., Mahuzier, G., and Prognon, P. (2000). Review: Application of the assay of aflatoxins by liquid chromatography with fluorescence detection in food analysis. *J. Chromatography*. **882,** 1-10.

James, G. D., Sealey, J. E., Alderman, M., Ljungman, S., Mueller, F. B., Pecker, M. S., and Laragh, J. H. (1988). A longitudinal study of urinary creatinine and creatinine clearance in normal subjects: Race, sex, and age differences. *Am. J. Hypertens.* **1,** 124–131.

Jensen, E. and Bolger, P. M. (2001). Exposure assessment of dioxins/furans consumed in dairy foods and fish. *Food Add. Contam.* **18,** 395-403.

Jespersen, L., Halm, M., Kpodo, K., and Jacobsen, M. (1994). Significance of yeasts and molds occurring in maize dough fermentation for “kenkey” production. *Intl. J. Food Microbiol.* **24,** 239-248.

Johns, T. and Duquette, M. (1991). Detoxification and mineral supplementation as functions of geophagy. *Am J Clin Nutr*. **53,** 448-456.

Jolly, P., Jiang, Y., Ellis, W., Awuah, R., Nnedu, O., Phillips, T. D., Wang, J-S., Tang, L., Afriyie-Gyawu, E., Person, S., Williams, T., Jolly, C., and Adjei, O. (2004). Determinants of aflatoxin levels in Ghanaians: Sociodemographic factors, knowledge of aflatoxin and food handling and consumption practices. *Environ. Sci. Public Health: Environmental Geosciences.* (In Press).

Jones, F. T., Hagler, W. H., and Hamilton, P. B. (1982). Association of low levels of aflatoxin in feed with productivity losses in commercial broiler operations. *Poult. Sci*. **61,** 861-868.

Jones, J. M. (1995). Molds and mycotoxins. In *Food Safety,* pp. 141-149. Eagan Press, St. Paul, MN.

Kadian, S. K., Monga, D. P., and Goel, M. C. (1988). Effect of aflatoxin B_1 on the delayed type hypersensitivity and phagocytic activity of reticuloendothelial system in chickens. *Mycopathologia.* **104,** 33-36.

Kalorey, D. R., Daginawala, H. F., Ganorkar, A. G., Mangle, N. (1996). Serum zinc and iron status in experimental aflatoxicosis in chicks. *Indian J Vet Res.* **5,** 28-32.

Katiyar, S., Dash, B. C., Thakur, V., Guptan, R. C., Sarin, S. K., and Das, B. C. (2000). p53 tumor suppressor gene mutations in hepatocellular carcinoma patients in India. *Cancer* **88,** 1565-1573.

Kelly, J. D., Eaton, D. L., Guengerich, F. P., and Coulombe, R. J. (1997). Aflatoxin B_1 activation in human lung. *Toxicol. Appl. Pharmacol.* **144,** 88-95.

Kew, M. C. (2003). Synergistic interaction between aflatoxin B1 and hepatitis B virus in hepatocarcinogenesis. *Liver Intl.* **23,** 405-409.

Kinniburgh, D. G. (1986). General purpose adsorption isotherms. *Environ. Sci. Technol.* **20,** 895-904.

Kirk, G. D., Kamus-Randon, A. M., Mendy, M., Goedert, J. J., Merle, P., Trepo, C., Brechot, C., Hainaut, P., and Montesano, R. (2000). Ser-249 p53 mutations in plasma DNA of patients with hepatocellular carcinoma from The Gambia. *J. Natl. Cancer Inst.* **92,** 148-153.

Klich, M. A., Tiffany, L. H., and Knaphus, G. (1994). Ecology of the aspergilli of soils and litter. In *Aspergillus Biology and Industrial Applications* (M. A. Klich and J. W. Bennett, Eds.), pp. 329-353. Butterworth-Heineman, Boston, MA.

Kolars, J. C., Benedict, P., Schmiedlin-Ren, P. and Watkins, P. B. (1994). Aflatoxin B_1-adduct formation in rat and human small bowel enterocytes. *Gastroenterology* **106**, 433–439.

Krishnamachari, K. A., Bhat, V. R., Nagarajan, R. V., and Tilac, T. B. G. (1975b). Hepatitis due to aflatoxicosis. *Lancet.* **1,** 1061-1063.

Krishnamachari, K. A., Bhat, V. R., Nagarajan, R. V., and Tilac, T. B. G. (1975a). Investigations into an outbreak of hepatitis in West India. *Indian J. Med. Res.* **63,** 1036-1048.

Krishnamani, R. and Mahaney, W. C. (2000). Geophagy among primates: Adaptive significance and ecological consequences. *Anim. Behav.* **59,** 899-915.

Kubena, L. F., Harvey, R. B., Corrier, D. E., Phillips, T. D., and Huff, W. E. (1990a). Diminution of aflatoxicosis in growing chickens by the dietary addition of a hydrated sodium calcium aluminosilicate*. Poult. Sci.* **69,** 727-735.

Kubena, L. F., Harvey, R. B., Huff, W. E., Corrier, D. E., Phillips, T. D., and Rottinghaus, G. E. (1990b). Efficacy of hydrated sodium calcium aluminosilicate to reduce the toxicity of aflatoxin and T-2 toxin. *Poult. Sci.* **69,** 1078-1086.

Kubena, L. F., Harvey, R. B., Huff, W. E., Elissalde, M. H., Yersin, G. A., Phillips, T. D., and Rottinghaus, G. E. (1993a). Efficacy of a hydrated sodium calcium aluminosilicate to reduce the toxicity of aflatoxin and diacetoxyscirpenol. *Poult. Sci.* **72,** 51-59.

Kubena, L. F., Harvey, R. B., Phillips, T. D., and Clement, B. A. (1993b). Effects of hydrated sodium calcium aluminosilicates on aflatoxicosis in broiler chicks. *Poult. Sci*. **72,** 651-657.

Kubena, L. F., Huff, W. E., Harvey, R. B., Yersin, A. G., Elissalde, M. H., Witzel, D. A., Giroir, L. E., and Phillips, T. D. (1991). Effects of hydrated sodium calcium aluminosilicate on growing turkey poults during aflatoxicosis. *Poultry Sci.* **70,** 1823-1830.

Kummet, T. and Meyskens, F. L. (1983). Vitamin A: A potential inhibitor of human cancer. *Semin. Oncol.* **10,** 281-289.

Kurtzman, C. P., Horn, B. W., and Hesseltine, C. W. (1987). *Aspergillus nominus*: A new aflatoxin-producing species related to *Aspergillus flavus* and *Aspergillus tamari. Antonie van Leeuwenhoek.* **53,** 147.

Lancaster, M. C., Jenkins, F. P, and Philp, J. M. (1961). Toxicity associated with certain samples of groundnuts. *Nature.* **192,** 1095-1096.

Langmuir, I. (1916). The constitution and fundamental properties of solids and liquids. *J. Am. Chem. Soc.* **38,** 2221-2294.

Larsson, P. and Tjalve, H. (1995). Extrahepatic bioactivation of aflatoxin B_1 in fetal, infant and adult rats. *Chem.-Biol. Interact.* **94**, 1–19.

Lawrence, C. W., Gorden, A., Banerjee, S. K., and LeClerc, J. E. (1990). Mutation frequency and spectrum resulting from a single abasic sites in a single-stranded vector. *Nucleic Acids Res.* **18,** 2153-2157.

Leeson, S., Diaz, G. J., and Summers, J. D. (1995). *Poultry Metabolic Disorders and Mycotoxins*, pp. 190-309. University Books, Guelph, Ontario, Canada.

Lehninger, A. L., Nelson, D. L., and Cox, M. M. (1997a). Enzymes. In *Principles of Biochemistry, 2nd Edition* (V. Neal, Ed.), pp. 198-200. Worth Publishers, New York.

Lehninger, A. L., Nelson, D. L., and Cox, M. M. (1997b). Lipids: Vitamins A, D, E, and K are fat-soluble. In *Principles of Biochemistry, 2nd Edition* (V. Neal, Ed.), pp. 259-260. Worth Publishers, New York.

Lemke, S. L. (2000). Investigation of clay-based strategies for the protection of animals from the toxic effects of selected mycotoxins. Ph.D. dissertation, Department of Veterinary Anatomy and Public Health, Texas A&M University, College Station.

Lemke, S. L., Grant, P. G., and Phillips, T. D. (1998). Adsorption of zearalenone by organophilic montmorillonite Clay. *J Agric. Food Chem.* **46,** 3789-3796.

Levi, C. P. (1980). Mycotoxins in coffee. *J AOAC.* **63,** 1282.

Lindermann, M. D., Blodgett, D. J., Kornegay, E. T., Schurig, G. G. (1993). Potential ameliorators of aflatoxicosis in weanling/growing swine. *J. Anim. Sci.* **71,** 171-178.

Lindermann, M. D., Blodgett, D. J., Schurig, G. D., and Kornegay, E. T. (1989). Evaluation of potential ameliorators of aflatoxicosis in weanling/growing swine. *J. Anim. Sci.* **67,** 36.

Lisker, N., and Lillehoj, E. B. (1991). Prevention of mycotoxins contamination (principally aflatoxins and *Fusarium* toxins) at the preharvest stage. In *Mycotoxins and Animals Foods* (J.E. Smith and R.E. Henderson, Eds.), pp. 689-719. CRC Press, Boca Raton, FL.

Liu, B. H., Yu, F. Y., Chan, M. H., and Yang, Y. L. The effects of mycotoxins, fumonisin B_1 and aflatoxin B_1, on primary swine alveolar macrophages. *Toxicol. Appl. Pharmacol.* **180,** 197-204.

Lopez, C., Ramos, L., Bulacio, L., Ramadan, S., and Rodriguez, F. (2002). Aflatoxin B_1 content in patients with hepatic diseases. *Medicina.* **62,** 313-316.

Lubulwa, A. S. G., and Davis, J. S. (1994). Estimating the social costs of the impacts of fungi and aflatoxins. In *Stored Products Protection, Proceedings of the 6th International Working Conference in Stored-product Protection, National Convention Centre Canberra, Australian Capital Territory* (Highley et al., Eds), pp. 17-23. CAB, International, Wallingford, Oxford, England.

Lumley, C. E., Parkinson, C., and Walker, S. R. (1992). An international comparison of the maximum duration of chronic animal toxicity studies. *Hum. Exp. Toxicol.* **11,** 155-162.

Lumley, C. E. and Walker, S. R. (1985). The value of chronic animal toxicology studies of pharmaceutical compounds: A retrospective analysis. *Fundam. Appl. Toxicol.* **5,** 1007-1024.

Lumley, C. E. and Walker, S. R. (1986). A critical appraisal of the duration of chronic toxicity studies. *Reg. Toxicol. Pharmacol.* **6,** 66-72.

Lunn, R. N., Zhang, Y-J., Wang, L-Y., Chen, C. J., Lee, P. H., Lee, C. S., Tsai, W. Y., and Santella, R. M. (1997). p53 mutations, chronic hepatitis B virus infection, and aflatoxin exposure in hepatocellular carcinoma in Taiwan. *Cancer Res.* **57,** 3471-3477.

Luter, L., Wyslouzil, W., and Kashyap, S. C. (1982). The destruction of aflatoxins in peanuts by microwave roasting. *Can Inst. Food Sci. Technol. J.* **15,** 236.

Mahaney, W. C. and Krishnamani, R. (2003). Understanding geophagy in animals: Standard procedures for sampling soils. *J. Chem. Ecol.* **29,** 1503-1523.

Manns, M. P., and Kubicka, S. (1997). Hepatocellular carcinoma. *Digestion.* **58,** 69-75.

Marasas, W. F. O., and Nelson, P. E. (1987). Mycotoxicology. In *Introduction to the Mycology, Plant Pathology, Chemistry, Toxicology, and Pathology of Naturally Occurring Mycotoxicoses in Animals and Man,* pp. 1- 99. The Pennsylvania State University Press, University Park, PA.

Masi, C. M., Rickett, E. M., Hawkley, L. C., and Cacioppo, J. T. (2004). Gender and ethnic differences in urinary stress hormones: The population-based Chicago Health, Aging, and Social Relations Study. *J. Appl. Physiol.* **97,** 941–947.

Mayura, K., Abdel-Wahhab, M. A., McKenzie, K. S., Sarr, A. B., Edwards, J. F., Naguib, K., and Phillips, T. D. (1998). Prevention of maternal and developmental toxicity in rats via dietary inclusion of common aflatoxin sorbents: Potential hidden risks. *Toxicol. Sci*. **41,** 175-182.

McAlpin, C. E., Wicklow D. T., and Horn, B. W. (2002). DNA fingerprinting analysis of vegetative compatibility groups in *Aspergillus flavus* from a peanut field in Georgia. *Plant Dis*. **86,** 254-258.

McKenzie, K. S., Kubena, L. F., Denvir, A. J., Rogers, T. D., Hitchens, G. D., Bailey, R. H., Harvey, R. B., Buckley, S. A., and Phillips, T. D. (1998). Aflatoxicosis in turkey poults is prevented by treatment of naturally contaminated corn with ozone generated by electrolysis. *Poult. Sci.* **77,**1094-1102.

McKenzie, K. S., Sarr, A. B., Mayura, K., Bailey, R. H., Miller, D. R., Rogers, T. D., Norred, W. P., Voss, K. A., Plattner, R. D., Kubena, L. F., and Phillips, T. D. (1997). Oxidative degradation and detoxification of mycotoxins using a novel source of ozone. *Food Chem. Toxicol.* **35,** 807-820.

McMahon, G., Davis, E., and Wogan, G. N. (1987). Characterization of c-Ki-ras oncogene alleles by directing sequencing of enzymatically amplified DNA from carcinogen-induced tumors. *Proc. Natl. Acad. Sci. USA.* **84,** 4974-4978.

McMahon, G., Hanson, L., Lee, J. J., and Wogan, G. N. (1986). Identification of an activated c-Ki-ras oncogene in rat liver tumors induced by aflatoxin B_1. *Proc. Natl. Acad. Sci. USA.* **83,** 9418-9422.

Mehan, V. K., McDonald, D., and Ramakrishna, N. (1986). Varietal resistance of peanuts to aflatoxin production. *Peanut Sci.* **13,** 7-10.

Mendez, M. A., and Adair, L. S. (1999). Severity and timing of stunting in the first two years of life affect performance on cognitive tests in late childhood*. J. Nutr.* **129,** 1555-1562.

Mendez-Albores, A., De Jesus-Flores, F., Castaneda-Roldan, E., Arambula-Villa, G., and Moreno-Martinez, E. (2004). The effect of toasting and boiling on the fate of B-aflatoxins during pinole preparation. *Journal of Food Engineering.* **65,** 585–589.

Miller, D., and Marasas, W. (2002). Ecology of mycotoxins in maize and groundnuts. In *Leisa* (Suppl.), pp. 23-24.

Miller, E. C. (1978). Some current perspectives on chemical carcinogenesis in humans and experimental animals: Presidential address. *Cancer Res.* **38,** 1479-1496.

Miller, M. D., and Wilson M. D. (1994). Veterinary Diseases Related to Aflatoxins. In *The Toxicology of Aflatoxins* (L. D Eaton. and J. D. Groopman., Eds.)", pp. 347-364. Academic Press, San Diego, CA.

Millot, G. (1979). Clay. *Scientific American*. **240,** 109-118.

Minto, R. E., and Townsend, C. A. (1997). Enzymology and molecular biology of aflatoxin biosynthesis. *Chem. Rev.* **97,** 2537-2555.

Mocchegiani, E., Corradi, A., Santarelli, L., Tibaldi, A., DeAngelis, E., Borghetti, P., Bonomi, A., Fabris, N., and Cabassi, E. (1998). Zinc, thymic endocrine activity and mitogen responsiveness (PHA) in piglets exposed to maternal aflatoxicosis B1 and G1. *Vet Immunol Immunopathol.* **62,** 245-260.

Moss, E. J. and Neal, G. E. (1985). The metabolism of aflatoxin B_1 in human liver. *Biochem. Pharmacol.* **34,** 3193-3197.

Muller, R. D., Carlson, C. W., Semeniuk, G., and Harshfield,G. S. (1970).The response of chicks, ducklings, goslings, pheasants and poults to graded levels of aflatoxin. *Poult. Sci.* **49,** 1346-1350.

Nabney, J., and Nesbitt, B. F. (1965). A spectrophotometric method of determining the aflatoxins. *Analyst.* **90,** 155-160.

National Research Council. (1994). *Nutrient Requirements of Poultry*. National Academy Press, Washington, DC.

Neal, G. E., Eaton, D. L., Judah, D. J., and Verma, A. (1998). Metabolism and toxicity of aflatoxins M1 and B1 in human-derived in vitro systems. *Toxicology and Applied Pharmacology*. **151**(1), 152-158.

Nesbitt, B. F., O'Kelly, J., Sargeant, K., and Sheridan, A. (1962). Toxic metabolites of *Aspergillus flavus*. *Nature.* **195,** 1062-1063.

Newberne, P. M. (1986). Aging animal models for the study of drug-nutrient interactions. In *Nutrition of Aging* (S. Gershoff, Ed.), pp. 99-115. Academic Press, New York.

Newberne, P. M., de Camargo, J. L. V., and Clark, A. J. (1982). Choline deficiency, partial hepatectomy, and liver tumors in rats and mice. *Tox. Pathol.* **10,** 95-109.

Newberne, P. M., Russo, R., and Wogan, G. N. (1966). Acute toxicity of aflatoxin B1 in the dog. *Pathol Vet J.* **3,** 331-340.

Ngindu, A., Kenya, P. R., Ocheng, D. M., Omonde, T. N., Ngare, W., Gatei, D., Johnson, B. K., Ngira, J. A., Nandwa, H., Jansen, A. J., Kaviti, J. N., and Siongok, T. A. (1982). Outbreak of acute hepatitis caused by aflatoxin poisoning in Kenya. *Lancet.* **1,** 1346-1348.

Nyathi, C. B., Mutiro, C. F., Hasler, J. A., and Chetsanga, C. J. (1987). A survey of urinary aflatoxin in Zimbabwe. *International Journal of Epidemiology.* **6,** 516-519.

Odin, A. P. (1997). Vitamins as antimutagens: Advantages and some possible mechanisms of antimutagenic action. *Mutat. Res.* **386,** 39–67.

Ominski, K. H., Marquardt, R. R., Sinha, R. N., and Abramson, D. (1994). Ecological aspects of growth of mycotoxin production by storage fungi. In *Mycotoxins in Grain: Compounds other than aflatoxin* (J. D. Miller and L. H. Trenolm, Eds.), pp. 287-312. Eagan Press, St. Paul, Minnesota.

Ong, T. M. (1975). Aflatoxin mutagenesis. *Mutat. Res.* **32,** 35-53.

Otsuki, T., Wilson, J. S., and Sewadeh, M. (2001). Saving two in a billion: Quantifying the trade effect of European food safety standards on African exports. *Food Policy* **26,** 495-514.

Park, D. L. (2002). Effect of processing on aflatoxin. In *Mycotoxins and Food Safety, Advances in Experimental Medicine and Biology* (J. W. DeVries, M. W. Trucksess, and L. S. Jackson, Eds.), vol. 504, pp. 173-179. Kluwer Academic / Plenum Publishers, New York.

Park, D. L., Lee, L. S., Price, R. L., and Pohland, A. E. (1988). Review of decontamination of aflatoxin by ammoniation: Current status and regulation. *J AOAC*. **71,** 685.

Park, D. L. and Price, W. D. (2001). Reduction of aflatoxin hazard using ammoniation. *Rev. Environ. Contam. Toxicol*. (In Press).

Patten, R. C. (1981). Aflatoxins and disease. *Am J Trop Med Hyg*. **30,** 422-425.

Patterson, E. C. and Staszak, D. J. (1977). Effects of geophagia (kaolin ingestion) on the maternal blood and embryonic development in the pregnant rat. *J. Nutr*. **107,** 2020–2025.

Paune, F. J., Ciaxach, I., Espadaler, J. O., and Rivera, J. (1998). Assessment of the removal of organic chemicals from raw and drinking water at a Llobregat river water works plant using GAC. *Water Res.* **32,** 3313-3324.

Payne, G. A. (1998). Process of contamination by aflatoxin-producing fungi and their impact on crops. In *Mycotoxins in Agriculture and Food Safety* (K. K. S. Sinha and D. Bhatnagar, Eds.), pp. 279-306. Marcel Dekker, Inc., New York.

Payne, G. A., Hagler, W. M., Jr, and Adkins, C. R. (1988). Aflatoxin accumulation in inoculated ears of field-grown maize. *Plant Dis.* **72,** 422-424.

Peers, F. G. and Linsell, C. A. (1975). Aflatoxin contamination and its heat stability in Indian cooking oils. *Trop. Sci.* **17,** 229.

Pestka, J. J., Bondy, G. S. (1994). Mycotoxin-induced immunomodulation. In *Immunotoxicology and immunopharmacology*. (J. H. Dean, M. I. Luster, A. E. Munson, and I. Kimber, Eds.), pp. 163-182. Raven Press, New York.

Phillips, T. D. (1999). Dietary clay in the chemoprevention of aflatoxin-induced disease. *Tox. Sci*. **52,** 118-126.

Phillips, T. D., Clement, B. A., and Park, D. L. (1994). Approaches to reduction of aflatoxins in foods and feeds. In *The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance* (D. L. Eaton and J. D. Groopman, Eds.), pp. 383-406. Academic Press, San Diego, CA.

Phillips, T. D., Kubena, L. F., Harvey, R. B., Taylor, D. R., and Heidelbaugh, N. D. (1988). Hydrated sodium calcium aluminosilicate: A high affinity sorbent for aflatoxin. *Poult. Sci.* **67,** 243-247.

Phillips, T. D., Lemke, S. L., and Grant, P. G. (2002). Characterization of clay-based enterosorbents for the prevention of aflatoxicosis. In *Advances in Experimental Medicine and Biology: Mycotoxins and Food Safety*, (J. W. DeVries, M. W. Trucksess and L. S. Jackson, Eds.), **504,** pp. 157-171. Kluwer Academic/Plenum Publishers, New York.

Phillips, T. D., Sarr, A. B., Grant, P. G. (1995). Selective chemisorption and detoxification of aflatoxins by phyllosilicate clay. *Nat. Toxins*. **3,** 204-213.

Pier, A.C. (1981). Mycotoxins and animal heath. *Advances in Veterinary Science & Comparative Medicine.* **25**, 185-243.

Pier, A. C., Belden, E. L., Ellis, J. A., Nelson, E. W., and Maki, L. R. (1989). Effects of cyclopiazonic acid and aflatoxin singly and in combination on selected clinical, pathological, and immunological responses of guinea pigs. *Mycopathologia* **105,** 135-142.

Pimpukdee, K. (2000). *In vitro* and *in vivo* characterization of potential enterosorbents for aflatoxin B_1. Ph.D. dissertation, Department of Veterinary Anatomy and Public Health, Texas A&M University, College Station.

Pimpukdee, K., Kubena, L. F., Bailey, C. A., Huebner, H. J., Afriyie-Gyawu, E., and Phillips, T. D. (2004). Aflatoxin-induced toxicity and depletion of hepatic vitamin A in young broiler chicks: Protection of chicks in the presence of low levels of NovaSil PLUS in the diet. *Poult. Sci.* **83,** 737-744.

Pisani, P., Bray, F., and Parkin, D. M. (2002). Estimates of the world-wide prevalence of cancer for 25 sites in the adult population. *Int J Cancer* **97,** 72-81.

Prasad, A. S., Bao, B., Beck, F. W. J., and Sarkar, F. H. (2001). Zinc activates NF-kappaB in HUT-78 cells. *Lab Clin Med.* **138,** 250-256.

Qian, G. S., Ross, R. K., Yu, M. C., Yuan, J. M., Gao, Y. T., Henderson, B. E. (1994). A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in Shanghai, People's Republic of China. *Cancer Epidemiol. Biomarkers Prev.* **3,** 3-10.

Raisuddin, S., Zaidi, S. I., Singh, K. P., and Ray, P. K. (1991). Effects of subchronic aflatoxin exposure on growth and progression of Ehrlich's ascites tumor in mice. *Drug Chem. Toxicol.* **15,** 185-206.

Ramsdell, H. S. and Eaton, D. L. (1990). Species susceptibility to aflatoxin B_1 carcinogenesis: Comparative kinetics of microsomal biotransformation. *Cancer Res.* **50,** 615-620.

Reddy, R. V., Taylor, M. J., and Sharma, R. P. (1987). Studies of immune function of CD-1 mice exposed to aflatoxins B_1. *Toxicol.* **43,** 123-132.

Robens, J. F. and Richard, J. L. (1992). Aflatoxins in animal and human health. *Environ. Contam. Toxicol.* **127,** 69-94.

Roebuck, B. D. and Maxuitenko, Y. Y. (1994). Biochemical Mechanisms and Biological Implications of the Toxicity of Aflatoxins as Related to Aflatoxin Carcinogenesis. In *The Toxicology of Aflatoxins* (D. L. Eaton and J. D. Groopman., Eds.), pp. 27-43. Academic Press, San Diego, CA.

Rogers, A., Vaughn, P., Prentice, T., Edejer, T. T., Evans, D., and Lowe, J. (2002). Reducing risks, promoting healthy life. In *The World Health Report* (B. Campanini and A. Haden, Eds.), World Health Organization, Geneva.

Ryan, N. J., Hogan, G. R., Hayes, A. W., Unger, P. D., and Siraj, M. Y. (1979). Aflatoxin B_1 : Its role in the etiology of Reye's syndrome. *Pediatrics* **64,** 71-74.

Sabbioni, G., Skipper, P. L., Buchi, G., and Tannenbaum, S. R. (1987). Isolation and characterization of the major serum albumin adduct formed aflatoxin B_1 *in vivo* in rats. *Carcinogenesis* **8,** 819-824.

Sarr, A. B., Mayura, K., Kubena, L. F., Harvey, R. B., and Phillips, T. D. (1995). Effects of phyllosilicate clay on the metabolic profile of aflatoxin B_1 in Fisher-344 rats. *Tox. Lett.* **75,** 145-151.

SAS Institute. (1982). *SAS User's Guide: Statistics*. SAS Institute, Cary, NC.

Schecter, A. and Li, L. (1997). Dioxins, dibenzofurans, dioxin-like PCBs, and DDE in US fast food, 1995. *Chemosphere* **34,** 1449-1457.

Schroeder, H. W. (1969). Factors influencing the development of aflatoxins in some field crops. *Journal Stored Prod. Res*. **5,** 187-190.

Schulze, D. G. (1989). An Introduction to soil mineralogy. In *Minerals in soil environments; (*J. B. Dixon and S. B. Weed Eds.), pp. 1-34. Soil Science Society of America, Madison, WI.

Shank, R. C. (1977). Epidemiology of aflatoxin carcinogenesis. *Adv. Mod. Toxicol*. **3**, 291-318.

Shank, R. C., Bourgeois, C. H., Keschamras, N., and Chandavimol, P. (1971). Aflatoxins in autopsy specimens from Thai children with an acute disease of unknown aetiology. *Food Cosmet Toxicol.* **9,** 501-507.

Shephard, G. (2003). Aflatoxin and food safety: Recent African perspectives. *J. Toxicol.* **22,** 267-286.

Shimizu, Y., Zhu, J. J., Han, F., Ishikawa, T., and Oda, H. (1999). Different frequencies of p53 codon-249-hotspot mutations in hepatocellular carcinomas in Jiang-Su Province of China. *Intl. J. Cancer* **82,** 187-190.

Shupe, T. and Sell, S. (2004). Low hepatic glutathione *S*-transferase and increased hepatic DNA adduction contribute to increased tumorigenicity of aflatoxin B_1 in newborn and partially hepatectomized mice. *Toxicol. Lett.* **148,** 1-9.

Sigma Diagnostics® Creatinine. (1997). Procedure. No. 555. Sigma Diagnostics®, Inc., St. Louis, MO.

Siller, W. G., and Ostler, D. C. (1961). The histopathology of an enterohepatic syndrome of turkey poults. *Vet. Rec.* **73**, 134-138.

Sinnhuber, R. O., Hendricks, J. D., Wales, J. H., and Putman, G. B. (1977). Neoplasms in rainbow trout, a sensitive animal model for environmental carcinogenesis. *Ann NY Acad. Sci.* **298,** 389-408.

Sisk, D. B., and Carlton, W. W. (1972). Effects of dietary protein concentration on response of miniature swine to aflatoxins. *Am. J. Vet. Res.* **33,** 107-114.

Smela, M. E., Currier, S. S., Bailey, E. A., and Essigmann, J. M. (2001). The chemistry and biology of aflatoxin B_1: From mutational spectrometry to carcinogenesis. *Carcinigenesis.* **22,** 535-545.

Smith, E. E., Phillips, T. D., Ellis, J. A., Harvey, R. B., Kubena, L. F., Thompson, J. and Newton, G. M. (1994a). Dietary hydrated sodium calcium aluminosilicate reduction of aflatoxin M_1 residue in dairy goat milk and effects on milk production and components. *J. Anim. Sci.* **72,** 677-682.

Smith, J. W., and Hamilton, P. B. (1970). Aflatoxicosis in the broiler chicken. *Poult. Sci.* **49,** 207-215.

Somogyi, A., and Beck, H. (1993). Nurturing and breast-feeding: Exposure to chemicals in breast milk. *Environ. Health Perspect*. **101,** 45-52.

Sorenson, W. G., Jones, W., Simpson, J., and Davidson, J. I. (1984). Aflatoxin in respirable airborne peanut dust. *J. Toxicol. Environ. Health* **14,** 525-533.

Sorenson, W. G., Simpson, J. P., Peach, M. J., Thedell, T. D., and Olenchock, S. A. (1981). Aflatoxin in respirable corn dust particles. *J. Toxicol. Environ. Health* **7,** 669-672.

Sporn, M. B., and Newton, D. L. (1981). Retinoids and chemoprevention of cancer. In *Inhibition of Tumor Induction and Development* (M. S. Zedeck and M. Lipkin, Eds.), pp. 71-100. Plenum, New York.

Srinivasan, K. R., and Fogler, H. S. (1990a). Use of inorgano-organo-clays in the removal of priority pollutants from wastewaters: Structural aspects. *Clay Miner.* **38,** 277-286.

Srinivasan, K. R., and Fogler, H. S. (1990b). Use of inorgano-organo-clays in the removal of priority pollutants from industrial wastewaters: Adsorption of benzo(a)pyrene and chlorophenols from aqueous solutions. *Clay Miner.* **38,** 287-293.

Statin, J. R., Rose, M., Wright, C., Packer, I., and Gilbert, J. (1990). Surveillance of British foods for PCDDs and PCDFs. *Chemosphere* **20,** 793-798.

Stumm W., Sigg, L., and Sulzberger, B. (1992). *Chemistry of the Solid-Water Interface*. pp. 87-155. John Wiley and Sons, New York.

Sylvia, D., Fuhrmann, J., Hartel, P., and Zuberer, D. (1997). *Principles and Applications of Soil Microbiology* (D. Sylvia, J. Fuhrmann, P. Hartel and D. Zuberer, Eds.). Prentice Hall Publishers, Upper Saddle River, NJ.

Taubenhaus, J. J. (1920). A Study of the Black and Yellow Molds of Ear Corn. Texas Agricultural Experiment Station. *Bull. No. 270*. College Station, TX.

Trenholm, H. L., Charmley, L. L., Prelusky, D. B., and Warner, R. M. (1991). Two physical methods for decontamination of four cereals contaminated with deoxynivalenol and zearalenone. *J. Agric. Food Chem.* **39,** 356-360.

Turner, P. C., Mendy, M., Whittle, H., Fortuin, M., Hall, A. J., and Wild, C. P. (2000). Hepatitis B infection and aflatoxin biomarker levels in Gambian children. *Trop. Med. Int. Health* **5,** 837-841.

Turner, P. C., Moore, S. E., Hall, A. J., Prentice, A. M., and Wild, C. P. (2003). Modification of immune function through exposure to dietary aflatoxin in Gambian children. *Environ. Health Persp.* **111,** 217-220.

Ueng, Y.-F., Shimada, T., Yamazaki, H., and Guengerich, F. P. (1995). Oxidation of aflatoxin B_1 by bacterial recombinant human cytochrome P450 enzymes. *Chem. Res. Toxicol.* **8,** 218-225.

Ulloa, S. M., and Herrea, T. (1970). Persistencia de las aflatoxinas durante la fermantacion del pozol. *Rev. Lat-Am Microbiol.* **12,** 19.

U.S. Food and Drug Administration (FDA). (2000). Potential economic costs of mycotoxins in the United States. Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, College Park, MD.

U.S. Food and Drug Administration (FDA). (2003). Aflatoxins. Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, College Park, MD.

Van den Berg, M., Birnbaum, L., Bosveld, A. T., Brunstrom, B., Cook, P., Feeley, M., Giesy, J. P., Hanberg, A., Hasegawa, R., Kennedy, S. W., Kubiak, T., Larsen, J. C., van Leeuwen, F. X., Liem, A. K., Nolt, C., Peterson, R. E., Poellinger, L., Safe, S., Schrenk, D., Tillitt, D., Tysklind, M., Younes, M., Waern, F., and Zacharewski T. (1998). Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. *Environ Health Perspect.* **106,** 775-792.

Vautier, G., Bomford, A. B., Portmann, B. C., Metivier, E., Williams, R., and Ryder, S. D. (1999). p53 mutations in British patients with hepatocellular carcinoma: Clustering in genetic hemochromatosis. *Gastrointerology* **117,** 154-160.

Verma, R. J. and Nair, A. (2002). Effect of aflatoxins on testicular steroidogenesis and amelioration by vitamin E. *Food Chem. Toxicol.* **40,** 669–672.

Wang, J. S., Huang, T., Su, J., Liang, F., Wei, Z., Liang, Y., Luo, H., Kuang, S. Y., Qian, G. S., Sun, G., He, X., Kensler, T. W., and Groopman, J. D. (2001). Hepatocellular carcinoma and aflatoxin exposure in Zhuqing Village, Fusui, PRC (Short Communication). *Cancer Epidemiol., Biomarkers Prev.* **10,** 143-146.

Wang, J. S., Shen, X., He, X., Zhu, Y. R., Zhang, B. C., Wang, J. B., Qian, G. S., Kuang, S. Y., Zarba, A., Egner, P. A., Jacobson, L. P., Munoz, A., Helzlsouer, K. J., Groopman, J. D., and Kensler, T. W. (1999). Protective alterations of phase 1 and 2 metabolism of aflatoxin B_1 by oltipraz in residents of Qidong, PRC. *J Nat. Cancer Inst.* **91,** 347-354.

Wannop, C. C. (1960). Disease of Turkey Poults. *Vet. Res.* **72,** 671-672.

West, C. E., Rombout, J. M., Van der Ziypp, A. J., and Siytsma, S. R. (1991). Vitamin A and immune function*. Proceedings of Nutrition Soc.* **50,** 251-262.

WHO-IARC (World Health Organization-International Agency for Research on Cancer) (1993). Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines, and mycotoxins. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*. **56,** 571.

Widstrom, N. W. (1996). The aflatoxin problem with corn grain. In *Advances in Agronomy* (D. Sparks, Ed.), pp. 219-280. Academic Press, New York.

Wild, C. P., and Hall, A. J. (2000). Primary prevention of hepatocellular carcinoma in developing countries. *Mutation Research.* **462,** 381-393.

Wild, C. P., Hudson, G. J., Sabbioni, G., Chapot, B., Hall, A. J., Wogan, G. N., Whittle, H., Montesano, R., and Groopman, J. D. (1992). Dietary intake of aflatoxins and the level of albumin-bound aflatoxin in peripheral blood in The Gambia, West Africa. *Cancer Epidemiol Biomark Prev.* **1,** 229-234.

Wild, C. P., and Turner, P. C. (2002). The toxicology of aflatoxins as a basis for public health decisions. *Mutagenesis.* **17,** 471-481.

Wiles, M. C., Huebner, H. J., Afriyie-Gyawu, E., Taylor, R. J., Bratton, G. R., and Phillips, T. D. (2004). Toxicological evaluation and metal bioavailability in pregnant rats following exposure to clay minerals in the diet. *J. Toxicol. Environ. Health,* Part A. **67**, 863–874.

Williams, P. (1991). Storage of grains and seeds. In *Mycotoxins and Animals Foods* (J. E. Smith and R. E. Henderson, Eds.), pp. 721-746. CRC Press, Boca Raton, FL.

Wilson, D. M., and Payne, G. A. (1994). Factors affecting *Aspergillus flavus* group infection and aflatoxin contamination of crops. In *The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance* (D. L. Eaton and J. D. Groopman, Eds.), pp. 309-325. Academic Press, San Diego, CA.

Wilson, M. J. (2003). Clay mineralogical and related characteristics of geophagic materials. *J Chem. Ecol.* **29,** 1525-1547.

Wilson, R., Ziprin, R., Ragsdale, S., and Busbee, D. (1985). Uptake and vascular transport of ingested aflatoxin. *Toxicol. Lett.* **29,** 169-176.

Wiseman, H. G., Jacobson, W. C., and Harmeyer, W. E. (1967). Note on removal of pigments from chloroform extracts of aflatoxin cultures with copper carbonate *J AOAC*. **50,** 982-983.

Wogan, G. N. (1969). Naturally occurring carcinogens in foods. *Progress in Exp. Tumor Res.* **11,** 134-162.

Wogan, G. N. and Newberne, P. M. (1967). Dose-response characteristics of aflatoxin B_1 carcinogenesis in rats. *Cancer Res.* **27,** 2370-2376.

Wong, Z. A., and Hsieh, D. P. H. (1978). Aflatoxicol: Major aflatoxin B_1 metabolite in rat plasma. *Science* **200,** 325-327.

Wong, Z. A., and Hsieh, D. P. H. (1980). The comparative metabolism and toxicokinetics of aflatoxin B_1 in the monkey, rat, and mouse. *Toxicol Appl. Pharmacol.* **55,** 115-125.

Yang, M., Zhou, H., Kong, R. Y., Fong, W. F., Ren, L. Q., Liao, X. H., Wang, Y., Zhuang, W., and Yang, S. (1997). Mutations at codon 249 of p53 gene in human hepatocellular carcinomas from Tongan, China. *Mutat. Res.* **381,** 25-29.

Yu, M. W., Lien, J. P., Chiu, Y. H., Santella, R. M., Liaw, Y. F., and Chen, C. J. (1997). Effect of aflatoxin metabolism and DNA adduct formation on hepatocellular carcinoma among chronic hepatitis B carriers in Taiwan. *J Hepatol.* **27,** 320-330.

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Educational Background

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Professional Experience

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September 1998 - May 2000, **Graduate Research Assistant,** School of Rural Public Health, Texas A&M University Systems Health Science Center. Grant Writing and Birth Defects Prevention and other Public Health Research.

January - August 1998, **Lab Assistant,** Clinical Pharmacology lab, TAMU. Development of Bioassays by HPLC Procedures for Analysis of Enrofloxacin and Ciprofloxacin.

Related Publications

Afriyie-Gyawu, E., Mackie, J., Dash, B., Wiles, M. C., Taylor, J., Huebner, H. J., and Phillips, T. D. Chronic toxicological evaluation of NovaSil clay in the diet of Sprague-Dawley rats. In Preparation.

Afriyie-Gyawu, E., Wiles, M. C., Huebner, H. J., Richardson, M. B., Fickey, C., and Phillips, T. D. Prevention of zearalenone-induced hyperestrogenism in prepubertal mice with activated carbon. *J. Toxicol. Environ. Health,* Part A. In Press.

Professional Affiliation

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