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I am submitting herewith a thesis written by Constance J. Wilmes entitled "Binding of Aflatoxin B_1 to Dietary Fiber Components in vitro." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Technology and Science.

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BINDING OF AFLATOXIN B_1 TO DIETARY FIBER COMPONENTS IN VITRO

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

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ABSTRACT

Experiments were undertaken to define the binding of a chemical carcinogen, aflatoxin B_1, to dietary fiber preparations (neutral detergent fiber, acid detergent fiber, acetone fiber and lignin). Five product sources were used: wheat bran, corn bran, peanut hull, alfalfa meal and pear.

The results of this study indicated dietary fiber from different plant sources has the ability to bind aflatoxin B_1 in a stable complex. Based on these findings the following conclusions were made.

With regard to the main effects of product and fiber types, peanut hull was found to have the greatest aflatoxin B_1 binding ability of the products. Lignin possessed the greatest aflatoxin B_1 binding ability among the four fiber types.

With regard to interaction of product and fiber type, peanut hull lignin had the greatest ability to bind aflatoxin B_1 and wheat bran NDF showed the least aflatoxin B_1 binding ability. Duncan's Multiple Range Test ($\alpha = 0.05$) partitioned the treatment combinations into four groups with overlapping, while the Student-Newman-Keuls Test ($\alpha = 0.05$) produced a more conservative division of three groups with overlapping.

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CHAPTER I

INTRODUCTION

Numerous epidemiological studies have been interpreted to suggest Western Man consumes a diet deficient in dietary fiber (9, 10, 11, 16). Researchers have postulated a close relationship between low residue diets and such conditions as atherosclerosis, appendicitis, hemorrhoids, diverticulosis, ulcerative colitis and cancer of the colon and rectum (2, 6, 8, 9, 11).

Hypotheses have been suggested to explain mechanisms whereby dietary fiber may exert a protective action (15, 23, 24, 45, 46). One such mechanism is as an antitoxic agent (17, 23). Scientists have theorized the toxic effects of certain stressor agents may be lessened if the agent is bound to dietary fiber. Dietary fiber has been found to bind water, bile salts and acids and to act as a weak monofunctional cation exchanger (14, 15, 16, 26, 38, 41). This suggests its ability to bind the major classifications (polar, nonpolar and ionic) of matter. Binding studies of other stressor agents, such as carcinogens, would seem appropriate to further evaluate possible protective actions of dietary fiber.

This experiemental study was undertaken to define the binding of a carcinogenic metabolite of Aspergillus flavus,

aflatoxin B_1, to preparations of neutral detergent fiber, acid detergent fiber, acetone fiber and lignin. Five fiber sources were used: wheat bran, corn bran, peanut hull, alfalfa meal and pear mesoderm and skin.

The goals of the investigation included:

1. the extent of aflatoxin B_1 binding to the major classes of dietary fiber (i.e., lignin, cellulose, hemicellulose and their complexes—ADF and NDF); and,

2. the confounding effects on aflatoxin B_1 binding to the major classes of dietary fiber of the plant from which dietary fibers were extracted (i.e., wheat bran, corn bran, alfalfa meal, peanut hull and pear).

CHAPTER II

REVIEW OF LITERATURE

I. DEFINITION AND HISTORICAL SIGNIFICANCE

Dietary fiber has been defined as the skeletal remains of plant cells that are resistant to digestion by gastrointestinal enzymes of man (46, 48). This is in contrast to crude fiber which is the portion of carbohydrate that resists extraction from foods by boiling first with sulfuric acid and subsequently with sodium hydroxide. Crude fiber is often stated to be merely cellulose and hemicellulose, but lignin, extremely resistant to sulfuric acid, is also present (46). Dietary fiber is composed not only of those materials measured by crude fiber determinations (cellulose, hemicellulose and lignin), but such substance as hemicellulose, pectins, gums and mucilages (24).

The detergent extraction system, described in Table I, yields three unique fractions of dietary fiber: neutral detergent fiber (NDF), acid detergent (ADF) and lignin. This system not only allows the estimation of the amount of cell wall but provides relatively rapid procedures for the estimation of lignin, cellulose and hemicellulose contents (50).

TABLE I

BASIC SCHEME FOR THE ANALYSIS[a] OF FIBER USING DETERGENTS

Fraction	Reagent	Treatment	Yield
Neutral Detergent Fiber (NDF)	Na lauryl sulfate EDTA pH 7.0	Boil 1 hour	Plant cell wall—pectins
Acid Detergent Fiber (ADF)	Cetyl trimethyl ammonium bromide	Boil 1 hour	Lignocellulose and insoluble minerals
Lignin	640 ml H_2SO_4/liter	3 hours at 20°C	Crude Lignin
Cellulose		Calculate as ADF—lignin	
Hemicellulose		Calculate as NDF—ADF	

[a]Van Soest and McQueen (50).

Extensive literature has suggested an inadequate intake of plant fibers may constitute one of the major deficiencies in the diet of Western man (9, 10, 16, 24, 42). Development of technology for refining carbohydrates and milling flour to eliminate bran has often been deemed responsible for decreases in fiber consumption (17, 24).

Study of the unabsorbable fiber portion of our food has almost been ignored, possibly because of:

1. it has been thought to be "indigestible" and of negligible nutrient value (10); and,
2. limited knowledge of its chemistry and problems of definition (38).

Advances in analytical techniques now provide means of exploring physical and chemical characteristics of dietary fiber that can be subsequently evaluated in epidemiological and nutritional studies (15, 36, 37, 44, 49, 51).

II. FIBER-DEPLETED DIETS AND DISEASE

There is increasing interest in the role played by dietary fiber in the etiology of a number of common diseases. Diverticular disease of the colon, cardiovascular diseases and both benign and malignant tumors of the colon and rectum have been suggested to be associated with a decrease in dietary fiber consumption (2, 6, 8, 9, 11).

All of the studies relating dietary fiber and certain diseases testify to the complexity of the therapeutic effects and interactions involved. Mechanisms have been postulated to explain the physiology of dietary fiber in gastrointestinal behavior including: (1) as a hypocholesterolemic and antiatherosclerotic agent, (2) as a promoter of normal bowel function; and, (3) as an antitoxic agent (15, 23, 45, 46).

Fiber and Atherosclerosis

Atherosclerosis and coronary heart disease have been suggested to be linked with a lack of fiber in the diet (9, 14). Atherosclerosis, the cause of most cardiovascular diseases, has been characterized by material deposited on and within the arterial walls called "plaque," which consisted of fat, protein and cholesterol (42, 45, 46).

Epidemiological studies relating fiber intake and coronary heart disease have suggested serum triglyceride and cholesterol levels might be lowered by the addition of fiber to the diet (34, 42). Such data have led several researchers to postulate that dietary fiber may have a hypocholesterolemic function and protect against atherosclerosis (45, 47, 52).

The mechanism by which fiber is hypothesized to exert influence as a hypocholesterolemic agent involves its interaction with bile acids. Investigations have shown

dietary fiber affects the absorption and reabsorption of cholesterol and bile acids, respectively (42). People on a high fiber diet have been suggested to excrete more bile acids, sterols and fat. This implies fiber sequesters bile acids and sterols, thereby preventing bile acid reabsorption (17, 28, 29, 45). Since bile acid excretion is the main elimination pathway of internally produced cholesterol, the increase in excretion reduces the cholesterol pool. This reduction is thought to be followed by a lowering of blood cholesterol (42, 43).

Fiber and Diverticulosis

Diverticular disease is the most common intestinal disease of the western world. It is characterized by small "blow-out" type protrusion lesions of the large intestine which become inflamed and often burst producing infection (39, 42).

It has been suggested the muscular abnormality underlying diverticular disease is the result of the colon having for many years to propel small hard feces which result from eating a refined, fiber-depleted diet (30). Extensive literature has indicated dietary fiber, more specifically cereal fiber, is very effective in promoting normal bowel function and reducing intracolonic pressure associated with diverticulosis (10, 32, 39, 42).

Investigators have suggested dietary fiber creates normal bowel function by promoting regularity, softer stools, more frequent elimination and decreased transit times (6, 10, 20, 21). Fibrous foods are therefore suggested to have a physiological effect on the gastrointestinal system which is beneficial in diverticular disease (30, 42).

Fiber and Cancer

Intestinal cancer causes the second highest death rate among cancer diseases in adults (2, 8, 42). Epidemiologists continue to search for relationships which might exist between dietary fiber and cancer of the alimentary tract (2). They have suggested mechanisms to explain how adequate fiber intake may reduce the incidence of colon cancer (3). These include:

1. influencing the intestinal flora,
2. decreasing intestinal transit time,
3. exerting a solvent-like effect in that it dilutes potential carcinogens.

Intestinal flora. It has been postulated that intestinal bacteria might be able to produce carcinogens from dietary fats or from bile steroids and that the variations in the incidence of colon cancer might depend partly on differences in the composition of the intestinal bacteria brought about by differences in diet (17, 31). Degradation by intestinal

flora has been found to be an important factor with regard to in vivo production of carcinogens but not with regard to the potency of consumed carcinogens.

In general, it has been found that anaerobic bacteria cause degradation of bile acids more actively than aerobes (31). The bile acid mainly implicated is deoxycholic acid which can be converted to the carcinogen 20-methylcholanthrene (31, 40). Hill et al. (31) showed feces from Western communities, where incidence of colon cancer is high, contained a higher ratio of anaerobic bacteria and lower counts of aerobes than feces from people of India, Africa or Japan, where incidence of the disease is low.

The degradation of bile salts by bacteria could explain the reduced quantity of those salts in people prone to bowel cancer on reduced-fiber diets compared to that found in areas where bowel cancer is low (31). Figure I represents a hypothesized mechanism for a possible relationship involving (1) diet, (2) bacterial intestinal content, (3) bile salts, and (4) tumorgenesis.

Intestinal transit time. Researchers have found a correlation between intestinal transit time, stool bulk and the fiber content of food (2, 8, 53). Evidence in humans has suggested that a diet high in fiber results in shorter transit time of digesta, greater frequency of defecation

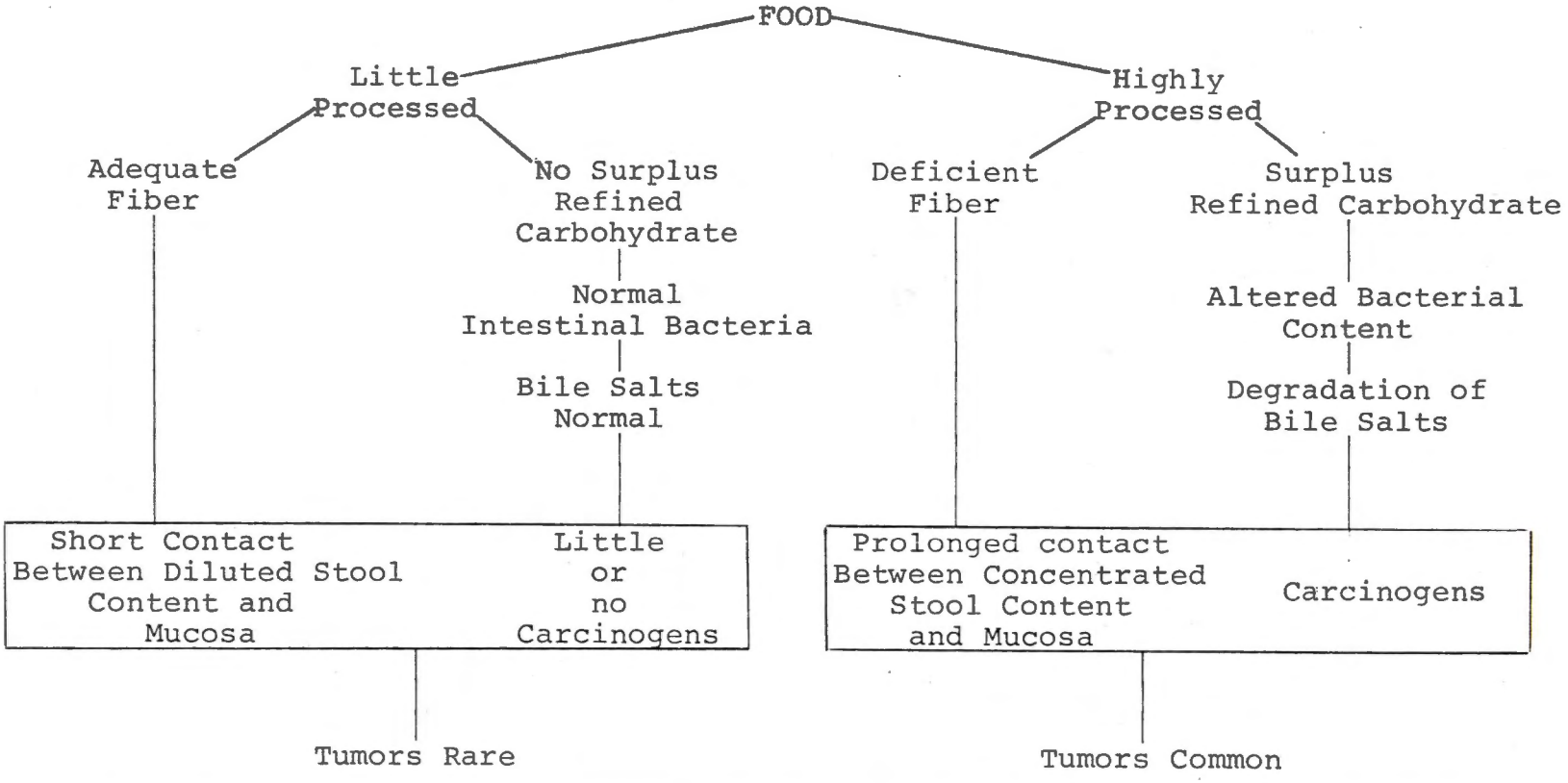


Figure I. Diagrammatic representation of a possible relationship between diet and cancer of the bowel.

Burkitt (8).

and the voiding of larger stools, which are softer and more unformed (10, 38, 54).

If cancer is caused by consumption of a foreign material which has access through the diet, it will be in the gut for a longer period of time if the diet is low in fiber. In short, if chemical A causes cancer and it spends a longer time in the intestine on a low fiber diet, then it is more likely to interact with the body and cause cellular transformation (13, 42).

Dilution. In addition to influencing bacteria of the intestine and transit time, researchers have found dietary fiber also exerts a solvent-like effect in fecal material. High residue foods act as stool expanders, increasing the amount of feces and absorbing and retaining water (19, 40). In this way potential carcinogens are suggested to be diluted by the larger fecal mass of the high fiber diet (9, 15).

Although decreased transit times and dilution are suggested to reduce the exposure time and concentration of carcinogens in the gut, it is difficult to accept a hypothesis suggesting the intestinal mucosa may not still undergo contact and possible interaction with carcinogens. The reason for this is that even the fastest transit times reported after ingestion are still greater than fourteen

hours and maximum dilution is only four-fold for high versus low residue diets (5, 8, 10, 11).

If transit times were reduced to the order of seconds or dilution was several orders of magnitude, this would appear to be a more logical hypothesis. However, this is not the case.

Thus another mode of fiber action may be feasible, whereby a protective action against carcinogens is exerted. Namely, the components of dietary fiber (i.e., cellulose, hemicellulose, lignin and pectin) may directly bind chemical carcinogens. In this way the carcinogen would be eliminated in the fecal mass without allowing the possibility of interaction with the gut. This hypothesis is based on researchers' data concerning the ability of dietary fiber to bind various molecules and ions.

III. HYPOTHESIS OF BINDING

It has been suggested that the colon filled with dietary fiber is acting as a chromatographic column with adsorptive, ion-exchange, and gel filtration properties (38). In this respect researchers have studied the ability of dietary fiber to interact with a variety of materials.

Water-Holding

One important property of fiber is its ability to swell when exposed to water (25).

Water-holding is defined as the weight of water that is retained by dry fruit, vegetable or cereal grain under specific conditions of soaking and centrifugation (26, 38).

Studies have found a close relationship between ADF and water-holding capacity but not between lignin and water-holding capacity. This suggests it is the polysaccharide content of the plant that determines its ability to hold water (38). McConnel et al. (37) found that dried fruits, vegetables and cereal grains had variable capacities to adsorb water. Values for the water-holding capacity of various fiber sources are listed in Table II. Water-holding capacity appeared to be greatest in vascular tissues such as roots, stems and leaves and less marked in storage organs.

Ions

Dietary fiber has been found to serve as an ion exchanger (16, 38). Ion exchange can be defined as a reversible process in which ions are exchanged between a liquid and an insoluble solid. This solid is the ion exchanger and undergoes no substantial changes (41).

A study by McConnell et al. (37) has shown the potential of dietary fiber to act as a cation exchanger. They found most of the types of dietary fiber acted as a monofunctional weak cation exchange resin (Table II). This suggested the electrolyte content of the stool could be

TABLE II

WATER-HOLDING CAPACITY AND CATION EXCHANGE CAPACITY OF VARIOUS FIBER SOURCES[a]

Source	Water-Holding g water/g fiber	Cation Exchange meq/g fiber
Oatmeal	1.82	—
Potato	2.00	0.3
Bran	3.00	—
Broad bean	4.10	0.9
Pea	4.60	0.8
Cauliflower	5.90	1.0
Pear	7.40	0.6
Green beans	8.10	1.4
Turnip	9.00	2.3
Winter cabbage	9.70	1.5
Tomato	10.80	1.0
Spring cabbage	11.30	2.4
Brussels sprouts	11.40	1.1
Apple	12.10	1.9
Orange	12.40	2.4
Onion	13.90	1.3
Rhubarb	14.50	1.7
Aubergine	17.30	1.8
Celery	19.20	1.5
Cucumber	20.90	1.1
Carrot	23.40	2.4
Lettuce	23.70	3.1

[a]McConnell, Eastwood, and Mitchell (37).

influenced by diet and especially by constituent plants in the diet (37).

Other studies have related cation exchange capacity to specific fiber components. Intestinal absorption of strontium is inhibited by orally administered sodium alginate while calcium absorption is not significantly reduced. This inhibition results from the presence of guluronic acid moieties in the alginate. Presumably other cations are also sequestered to a variable degree and their loss in the stool is accordingly affected by such binding characteristics (15). Therefore these researchers suggested the cation exchange property could result by ion binding to acidic polysaccharide groupings in dietary fiber.

Bile Salt Binding

Researchers have postulated that adsorption of bile salts and acids is a function of the lignin component of fiber (14, 18). They have suggested the adsorption is pH dependent, being most strong at an acid pH and weakest at an alkaline pH (14, 16). The polarity of the bile salt was also suggested to be important. The more polar the bile salt, the less strong the affinity (18). The order or adsorption being monohydroxy > dihydroxy > trihydroxy (14). Since absorption was abolished in the presence of 6 M urea the binding was postulated to be hydrophobic (18).

The binding of sodium tauro- or glycocholate by different foodstuffs has been investigated. Alfalfa (natural nonnutritive fiber) was found to bind about 20% as much bile salt as cholestyramine and about 30% as much as colestipol. Cholestyramine and cholestipol are anion exchange resins, pharmaceutically prepared specifically to bind bile acids and bile salts (33, 35).

More recently the sodium taurocholate binding capacity of various fiber sources was studied. In Table III, alfalfa meal was found to bind the highest (11.05 µg/200 mg fiber). Based on the coefficient of correlation ($r = 0.18$) between acid-detergent lignin and sodium taurocholate binding, it was suggested that this value does not reflect the general suggestion that bile salt binding is a function of the lignin (1). However, a further study is needed to determine the actual component(s) of dietary fiber responsible for bile salt binding.

IV. AFLATOXIN B_1

The aflatoxins are a group of acutely toxic and highly carcinogenic mold metabolites produced by Aspergillus flavus. The aflatoxins have been suggested to be the most potent hepatoma-inducing agents known (27). The acute toxicity and carcinogenicity of the aflatoxins has been studied extensively in rats, guinea pigs, ducklings, dogs and monkeys. Work has also been done with hamsters, mice and ferrets.

TABLE III

BINDING IN VITRO OF SODIUM TAUROCHOLATE[1, 2, 3] TO DIETARY FIBER

Fiber Source	μ Moles Bound/200 mg Fiber
Wheat bran	4.56 ± 1.08[b]
Corn bran	4.22 ± 2.12[b]
Peanut hull	2.28 ± 0.87[b]
Oatmeal	6.89 ± 1.35[a, b]
Alfalfa	11.05 ± 0.85[a]
Alpha-cell (cellulose)	2.21 ± 1.21[b]

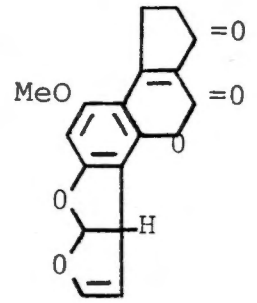
[1]Abajian (1).

[2]Mean of 12 observations with ± standard deviation.

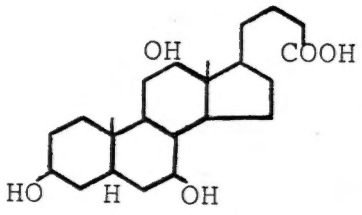
[3]Means within the column followed by the same letter are not significantly different at $\alpha = 0.05$.

Aflatoxins have closely similar structures and form a group of highly oxygenated, naturally occurring heterocyclic compounds. The major toxin, aflatoxin B_1, exhibits blue fluorescence, has a molecular weight of 312 and the composition $C_{17}H_{12}O_6$ (Figure II). Aflatoxin B_1 represents a highly unsaturated molecule (27).

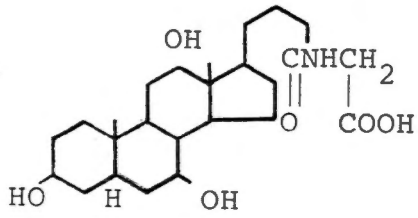
The aflatoxins are similar in structure to bile acids, both being polycyclic molecules (Figure II). Because bile acids have been found to be bound by dietary fiber, the structural similarity of aflatoxins and bile acids would suggest that aflatoxins might be a plausible candidate for binding studies with dietary fiber.



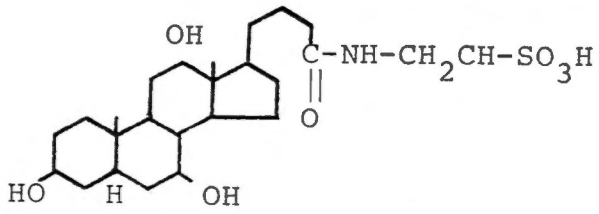
Aflatoxin B_1



Cholic Acid



Glycodeoxycholoic Acid



Taurolithocholic Acid

Figure II. Diagram showing the similarity in structure between aflatoxin B_1 and the principle bile acids.

CHAPTER III

MATERIALS AND METHODS

I. PREPARATION OF SAMPLES

Five different fiber sources were investigated in this experiment including: wheat bran, corn bran, peanut hull, alfalfa meal and pear.

Wheat bran and corn bran were received from White Lily Food of Knoxville, TN. Alfalfa meal was purchased from a local feed market in Knoxville. Raw peanuts and Bartlett pears were purchased from a local supermarket. Peanuts were shelled by hand and the nuts and skins were removed from the hulls. Pears were washed and the mesocarp and peel were cut from the core. The mesocarp and peel of each pear were homogenized with 40 ml of water. The homogenate was poured into glass dishes (0.6 cm deep) and dried overnight in an oven at 60°C. The dried pear was scraped from each dish and dry blended.

The particle size of all samples was reduced in a Wiley Mill until the particles would pass a sieve with 0.25 mm holes (U.S. Std. Sieve No. 60), but none of the particles would pass through a finer sieve of 0.17 mm (U.S. Std. Sieve No. 80). The ground samples were held in a desiccator at room temperature throughout the experiment.

II. METHODOLOGY OF FIBER PREPARATIONS

Acetone Fiber

The method of McConnel et al. (37) was used to prepare acetone fiber for each sample. This procedure diagrammed in Figure III, produces a whole fiber preparation washed free of some soluble materials and protein with warm water and dried with acetone.

Ten g dry sample were homogenized in 40 ml of warm (40°C) water. The homogenate was filtered with applied suction through a Buchner funnel and Whatman's No. 4 filter paper. The filter cake was scraped from the filter paper and resuspended in 20 ml of warm (40°) water and 40 ml redistilled acetone. The mixture was filtered in the same manner. The procedure was repeated four times. The samples were freeze-dried for 24 hours to achieve constant weight of the acetone fiber.

Acid Detergent Fiber

The Van Soest (49) method (Figure IV) was used for ADF preparation. One g of dry sample was weighed and placed in a 600 ml refluxing beaker. One hundred ml of acid-detergent solution (20 g cetyl trimethylammonium bromide—technical grade—to 1 liter 1.00 N H_2SO_4, previously standardized) were added to the beaker and the mixture was refluxed for 60 minutes from the onset of boiling. The solution was

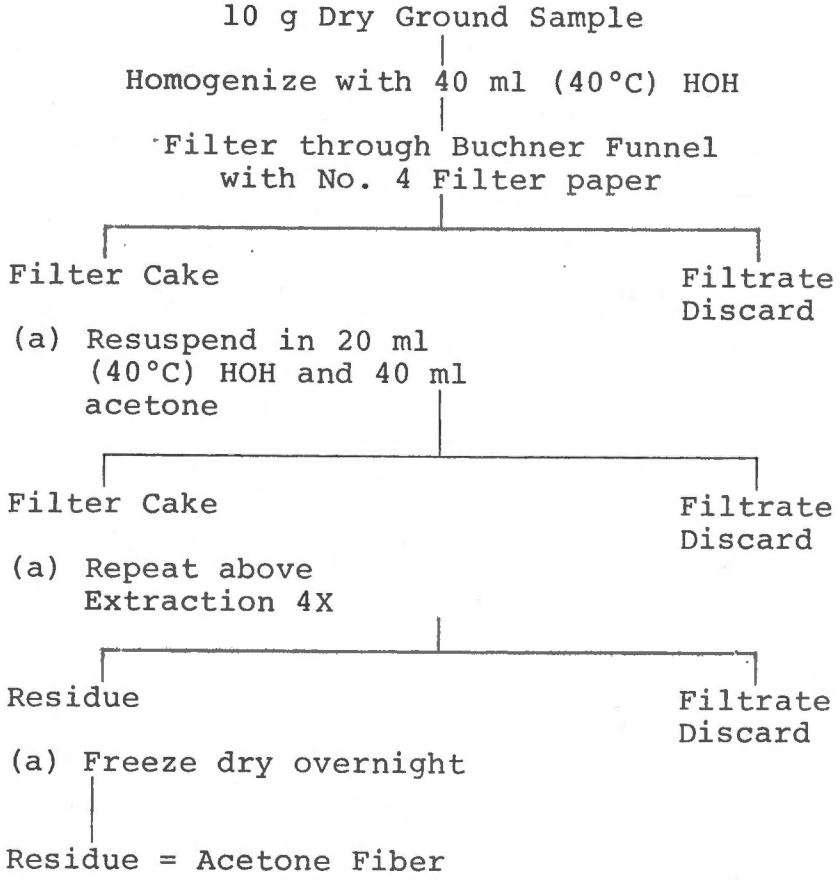


Figure III. Flow diagram for the preparation of acetone fiber.

McConnell (37).

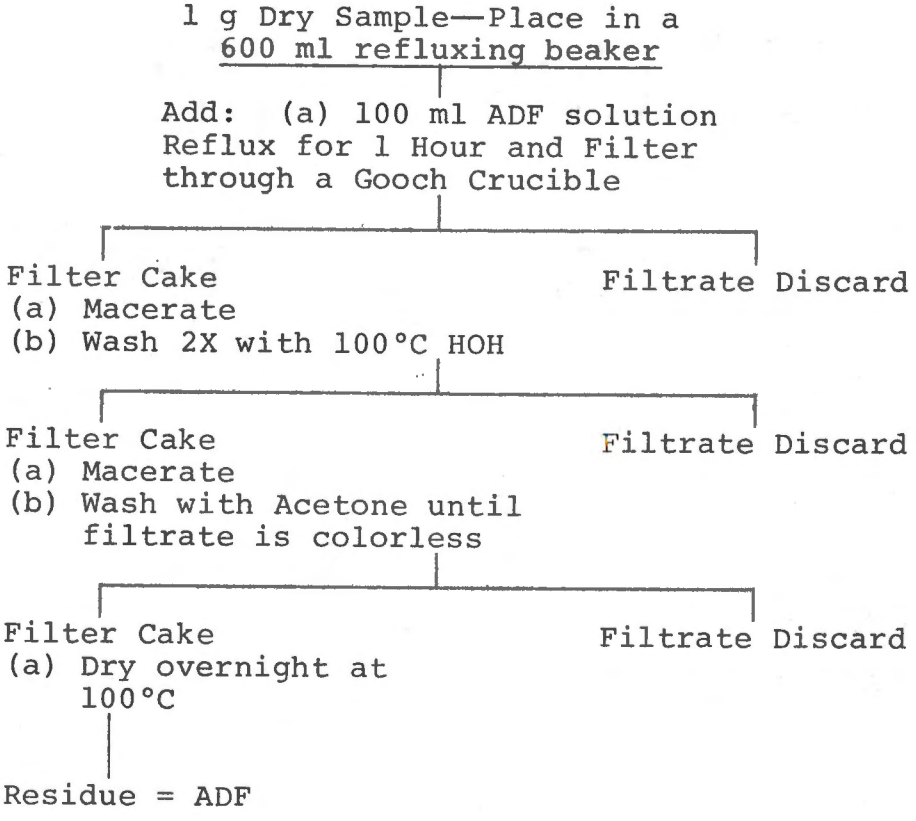


Figure IV. Flow diagram for the preparation of acid detergent fiber.

Van Soest (49).

filtered with suction through a fritted glass crucible. The filter cake was broken apart with a glass rod and washed twice with hot (90-100°C) water. Washing was repeated with redistilled acetone until the filtrate was colorless. The filter cake was broken apart with each washing so that solvent came into contact with all particles of the sample. The ADF was dried in the crucibles overnight at 100°C.

Neutral Detergent Fiber

The method of Van Soest and Wine (51), as outlined in Figure V, was used to prepare NDF. This procedure for cell wall constituents provides a rapid method for total fiber (lignocellulose, hemicellulose and pectins) in plant sources.

One g of dry sample was weighed into a 600 ml refluxing beaker. One hundred ml of room temperature neutral detergent solution (30 g sodium lauryl sulfate, USP; 19.61 g disodium ethylenediaminetetraacetate, technical grade; 6.81 g sodium borate decahydrate, reagent grade; 4.56 g disodium hydrogen phosphate, anhydrous, reagent grade; and 10 ml 2-ethoxy ethanol, purified grade—added to 1 liter distilled water, adjusted to pH 6.9-7.1), 2 ml decahydronaphthalene and 0.5 g anhydrous sodium sulfite were added to the beaker. The mixture was refluxed for 60 minutes from the onset of boiling. The solution was filtered through a fritted glass crucible. The filter cake was broken apart with a glass rod

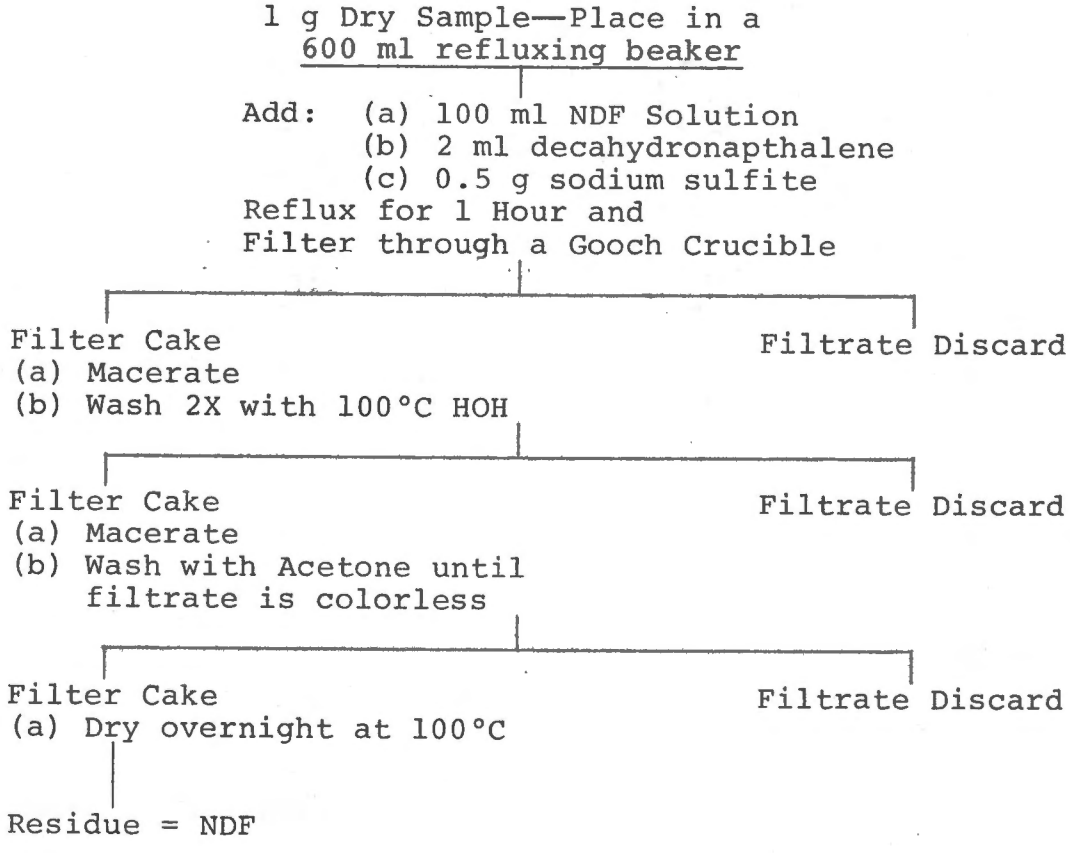


Figure V. Flow diagram for the preparation of neutral detergent fiber.

Van Soest and Wine (51).

while still in the crucible and washed twice with hot (100°C) water. The filter cake was macerated before each washing so that the solvent came into contact with all portions of the sample. The NDF was dried at 100°C overnight.

Lignin

The method of Southgate (44) was used to prepare lignin. Figure VI presents a diagram of the procedure. Ten g of dry sample were weighed into a 250 ml erlenmeyer, 50 ml of 85% boiling methanol were added and the mixture shaken on a wrist action shaker for 30 minutes. This was repeated three times, being filtered through a fritted (Gooch) crucible after each extraction. The residue was resuspended in 50 ml of diethyl ether and filtered through a Gooch crucible, applying suction with a water aspirator. Extraction with diethyl ether was repeated twice and the filter cake allowed to air dry. The residue was resuspended in 50 ml of hot (80°C) water and centrifuged at 1000 × g for 20 minutes. The extraction was repeated twice.

The residue was resuspended in 50 ml of hot (80°C) water, and heated for 10 minutes in a boiling water bath. After cooling, 40 ml of 2 M acetate buffer (2 M acetic acid diluted to 1 liter and titrated with 3 M NaOH to pH 4.5) and 100 ml of 1% amylase (0.5% bacterial amylase and 0.5% α-fungal amylase, purchased from Novo Industries-Copenhagen, Denmark)

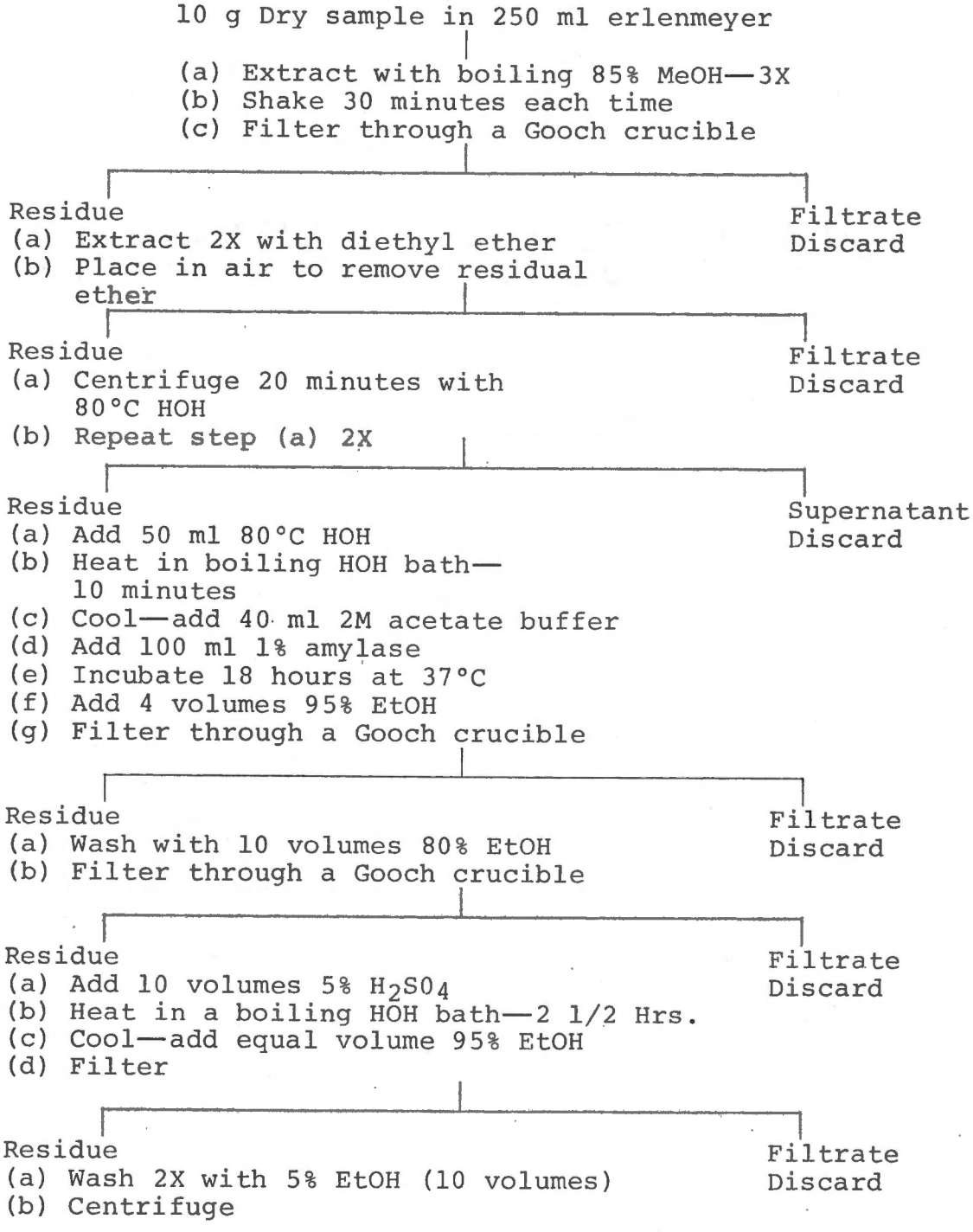


Figure VI. Flow diagram for the preparation of lignin.

Southgate (44).

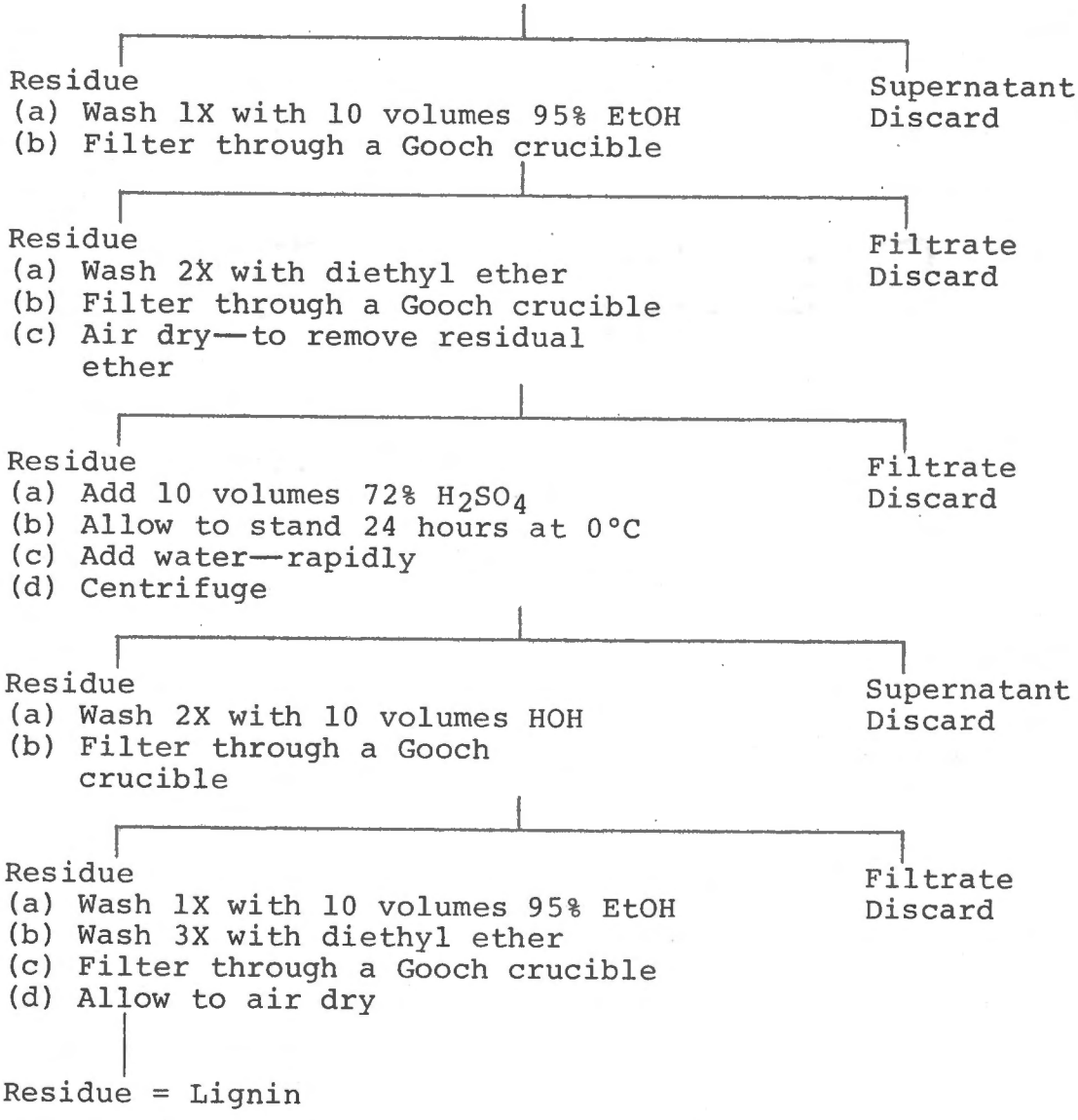


Figure VI. (continued)

were added. The mixture was incubated at 37°C for 18 hours. After incubation, 4 volumes of 95% ethanol were added and the mixture filtered through a Gooch crucible. The residue was resuspended in 10 volumes of 50% ethanol and centrifuged for 20 minutes at 1000 × g. This was repeated twice. The residue was resuspended in 10 volumes of 95% ethanol and filtered through a Gooch crucible. The filter cake was extracted with diethyl ether twice, filtered and allowed to air dry.

Ten volumes of 72% H_2SO_4 at 4°C were added to the dry residue and allowed to stand at 4°C for 24 hours. The acid mixture was diluted rapidly with 2 volumes of water and centrifuged to remove the supernatant. The remaining residue was washed twice with 10 volumes of water and centrifuged. Washing with 10 volumes of 95% ethanol followed and the mixture was filtered through a Gooch crucible. The filter cake was macerated with a glass rod and washed three times in the crucible with diethyl ether which was removed by applying suction with a water aspirator. The remaining residue, representing the lignin portion of the sample, was air dried.

In the lignin preparation, 5% H_2SO_4 hydrolyzes hexoses, pentoses and uronic acids. The amylase solution hydrolyzes starch and treatment with 72% H_2SO_4 dissolves cellulose (44).

Storage of Samples

All fiber preparations were packaged in sterilized plastic bags and stored at room temperature in a desiccator throughout the experiment.

III. DETERMINATION OF AFLATOXIN B_1 BINDING CAPACITY

The binding of aflatoxin B_1 to ADF, NDF, acetone fiber and lignin of corn bran, wheat bran, peanut hull, alfalfa meal and pear was investigated.

Aflatoxin B_1 crystals were purchased from Calbiochem, Inc. A standard solution of 0.4 µg aflatoxin B_1/µl was prepared in redistilled acetone. The standard solution was stored at 0°C through the experiment.

Experimental Design

The experimental design included 4 classes of dietary fiber prepared from 5 fiber sources, yielding 20 treatment combinations (Table IV).

Five replicates of each treatment combination were arranged in a completely randomized design (Table V). The design contained 16 sets of 6 experimental treatment combinations and one set of 4 treatment combinations. Each set was run individually, with 3 sets being run per day.

TABLE IV

EXPERIMENTAL DESIGN FOR TYPES OF DIETARY FIBER FROM FIVE PRODUCT SOURCES

Product	Fiber Types			
	NDF	ADF	Lignin	Acetone
Peanut hull	"	"	"	"
Wheat bran	"	"	"	"
Corn bran	"	"	"	"
Alfalfa meal	"	"	"	"
Pear	"	"	"	"

TABLE V

TREATMENT COMBINATIONS FOR COMPLETELY RANDOMIZED EXPERIMENTAL DESIGN

Day	Treatment Combinations					
	I	II	III	IV	V	VI
1	Peanut lignin	Peanut lignin	Wheat ADF	Corn NDF	Corn acetone	Alfalfa lignin
1	Peanut acetone	Pear ADF	Peanut ADF	Alfalfa NDF	Alfalfa acetone	Wheat ADF
1	Wheat acetone	Wheat acetone	Pear NDF	Corn lignin	Peanut acetone	Alfalfa ADF
2	Corn acetone	Wheat ADF	Pear lignin	Pear acetone	Peanut NDF	Corn NDF
2	Corn NDF	Peanut NDF	Wheat lignin	Corn lignin	Alfalfa acetone	Alfalfa ADF
2	Corn ADF	Pear acetone	Corn ADF	Wheat lignin	Corn ADF	Wheat acetone
3	Pear ADF	Pear NDF	Peanut acetone	Pear lignin	Corn lignin	Pear NDF
3	Wheat NDF	Alfalfa acetone	Pear ADF	Wheat NDF	Peanut ADF	Alfalfa NDF
3	Wheat lignin	Alfalfa NDF	Pear acetone	Wheat ADF	Alfalfa ADF	Alfalfa lignin
4	Alfalfa lignin	Peanut NDF	Corn acetone	Peanut NDF	Pear lignin	Peanut lignin
4	Corn lignin	Peanut lignin	Peanut ADF	Wheat lignin	Alfalfa lignin	Pear acetone
4	Wheat ADF	Peanut ADF	Corn acetone	Corn NDF	Pear ADF	Alfalfa ADF
5	Wheat acetone	Wheat lignin	Pear acetone	Peanut NDF	Wheat NDF	Wheat ADF
5	Wheat NDF	Pear NDF	Alfalfa acetone	Peanut lignin	Pear lignin	Alfalfa acetone
5	Corn ADF	Pear lignin	Peanut NDF	Peanut acetone	Alfalfa acetone	Corn lignin
6	Corn acetone	Wheat acetone	Alfalfa lignin	Peanut acetone	Corn NDF	Alfalfa NDF
6	Corn ADF	Pear ADF	Alfalfa NDF	Pear NDF		

Binding Assay

Dry fiber preparation (0.0100 ± 0.0001 g) was weighed into a 15 ml glass centrifuge tube. A 5 ml aliquot of warm (60°C) 0.15 M NaCl and 375 µl of aflatoxin B_1 standard (150 µg aflatoxin B_1/5 ml 0.15 M saline) were added to the tube. Aflatoxin B_1 is soluble in aqueous solutions to the extent of 30 µg/µl (7). Therefore, this was an aflatoxin B_1/saline solution at the saturation limit. Since a solution at the saturation limit was being used and the liquid/fiber ratio was 500/1, it is suggested that the fiber sample was being exposed to as much or more aflatoxin B_1 than it could be exposed to in the gut.

The tube was sealed by insertion of an aluminum foil covered rubber stopper and parafilm was wrapped around the tube and stopper to secure the stopper. The tubes were shaken for 30 minutes at 37.5°C. The tubes were centrifuged for 30 minutes at 1000 × g and the supernatant decanted from the fiber pellet.

Aflatoxin B_1 Extraction

The pellet was washed with a 5 ml aliquot of 0.15 M saline and vortexed 30 seconds to remove superfluous, nonbound aflatoxin B_1 from the fiber. The tube was centrifuged for 15 minutes at 1000 × g and the saline decanted from the fiber. The fiber was resuspended in a 2.5 ml

aliquot of reagent grade $CHCl_3$, mixed on a Vortex mixer for 30 seconds, and allowed to stand 1 minute to extract aflatoxin B_1 from the fiber pellet. The extract and pellet were filtered through 5 g of anhydrous sodium sulfate and Whatman's No. 4 filter paper. The filter cake was washed with an additional 2.5 ml aliquot of $CHCl_3$ and the $CHCl_3$ filtrate collected in a 13 × 100 mm test tube. The filtrate was brought to a volume of 5 ml in the tube.

Quantification of Aflatoxin B_1

Brinkman precoated (0.25 mm silica gel with gypsum) thin layer plates were heat activated four hours at 100°C before use.

Five µl of the chloroform filtrate were spotted on the plate using an Eppendorf pipette. As a reference, 5 µl of a solution containing 0.04 µg of aflatoxin B_1 were also spotted on the same plate.

The thin layer plate was placed in an aluminum foil covered chamber and developed in a solution of chloroform: acetone (90:10). The plate was removed when the solvent front reached a height of 12.5 cm.

Following chromatography, aflatoxin B_1 was quantified with a Schoffel Spectrodensitometer (SD 3000) (5, 22). The spectrodensitometer was operated as a single beam fluorescence instrument (4).

The equation used for calculation of data was:

$$\frac{\text{Ug aflatoxin } B_1 \text{ bound}}{0.01 \text{ g fiber sample}} = \frac{\dfrac{\text{Area sample peak}}{\text{Area standard peak}} \quad (0.04\ \mu g)}{5.0\ \mu l} \times 5000\ \mu l$$

IV. VALIDITY OF ANALYTICAL METHOD

Standard Curves

Spectrodensitometer standard curve. Silica gel plates were spotted with 0.04, 0.08, 0.12, 0.16, 0.20, and 0.24 µg aflatoxin B_1. The mean peak areas of two replicates were used to obtain a standard curve and a regression analysis of the standard curve was performed to define the regression equation.

Figure VII is the standard curve obtained with a correlation coefficient of 0.9347 and a regression equation (Table VI) of:

$$Y = 175.5\ (X) + 21.71$$

where X is µg of aflatoxin B_1 and Y is area under the curve.

Spectrofluorometer standard curve. A Perkin-Elmer Fluorescence Spectrometer (Model 203) was employed to quantitatively determine the range of aflatoxin B_1 concentrations which could be measured linearly (12). The

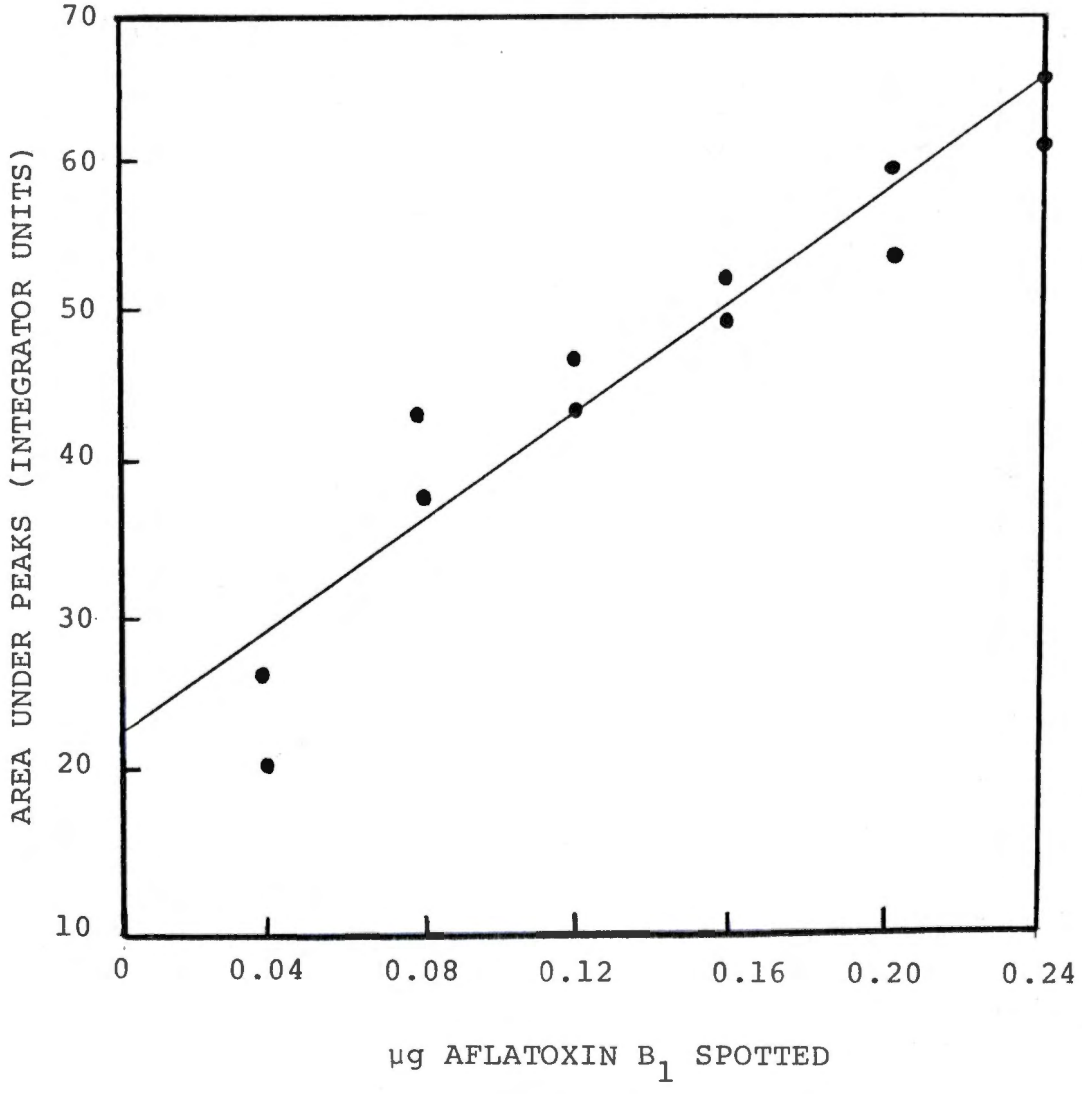


Figure VII. Spectrodensitometer standard curve obtained from the mean peak areas of two replicates containing 0.04, 0.08, 0.12, 0.16, 0.20, and 0.24 µg of aflatoxin B_1.

TABLE VI

REGRESSION ANALYSIS OF SPECTRODENSITOMETER STANDARD CURVE OBTAINED FROM THE MEAN PEAK AREAS OF TWO REPLICATES CONTAINING 0.04, 0.08, 0.12, 0.16, 0.20, AND 0.24 µg OF AFLATOXIN B_1

Concentration (µg)	Peak Area Plate I	Peak Area Plate II	Mean Peak Area
0.04	25.5	20.0	22.75
0.08	37.5	43.5	40.50
0.12	49.0	44.5	46.75
0.16	52.5	49.0	50.85
0.20	57.5	50.5	54.00
0.24	65.0	61.0	63.00

r = 0.9347.

m = 175.5357

b = 21.7166.

following instrument settings were used: (1) excitation wavelength 365 nm, (2) emission wavelength 435 nm, (3) sensitivity 9, (4) selector knob × 10.

The percent fluorescence of 4.0, 8.0, 12.0, 16.0, 20.0 and 24.0 µg of aflatoxin B_1 in 5 ml of 0.15 M saline was measured. The mean percent fluorescence of two replicates was used to obtain a standard curve and a regression analysis of the standard curve was performed to define the regression equation.

Figure VIII is the standard curve obtained with a correlation coefficient of 0.9875 and a regression equation (Table VII) of:

$$Y = 4.227\ (X) + 5.910$$

where X is µg of aflatoxin B_1 and Y is the percent fluorescence. The results indicated that the linear range for quantitative measurement of aflatoxin B_1 was < 4.0 to 24.0 µg.

Percent Recovery

The percent aflatoxin B_1 recovered was determined by adding 16.0 and 20.0 µg of aflatoxin B_1 to 0.0100 ± 0.0001 g peanut hull samples. The samples were extracted as before for bound aflatoxin B_1, except the saline supernatant was

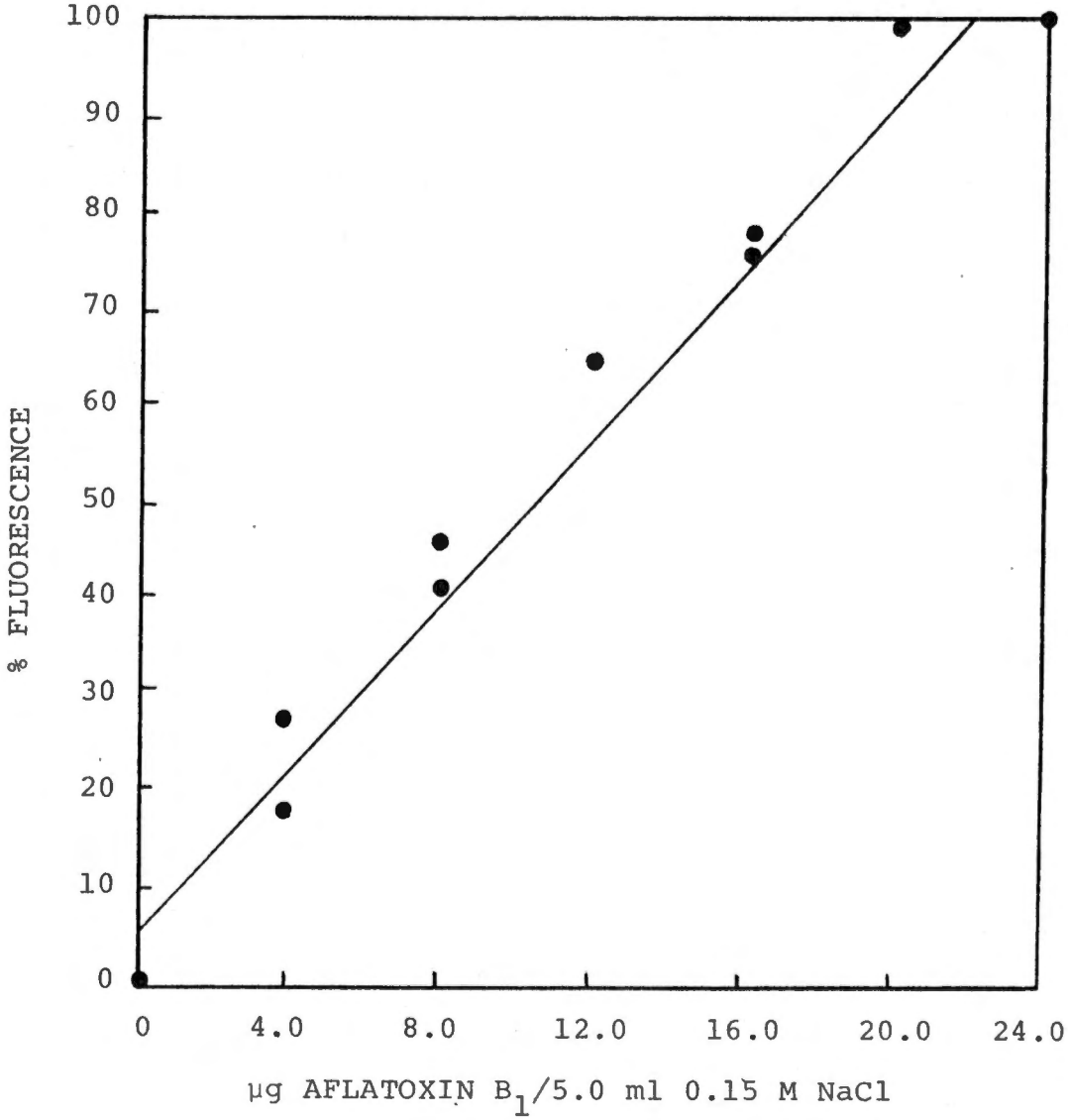


Figure VIII. Spectrofluorometer standard curve obtained from the mean percent fluorescence of two replicates containing 4.0, 8.0, 12.0, 16.0, 20.0 and 24.0 µg of aflatoxin B_1.

TABLE VII

REGRESSION ANALYSIS OF SPECTROFLUOROMETER STANDARD CURVE OBTAINED FROM THE MEAN PERCENT FLUORESCENCE OF TWO REPLICATES CONTAINING 4.0, 8.0, 12.0, 16.0, 20.0, AND 24.0 µg OF AFLATOXIN B_1

Concentration (µg)	% Fluorescence Set I	% Fluorescence Set II	Mean % F
0.0	0.0	0.0	0.0
4.0	18.5	26.5	22.5
8.0	44.5	40.5	42.5
12.0	63.5	63.5	63.5
16.0	74.5	75.5	75.0
20.0	87.5	98.5	93.0
24.0	100.0	100.0	100.0

r = 0.9875.

m = 4.2276.

b = 5.9107.

saved in order to quantify µg of aflatoxin B_1 remaining free in solution.

The amount (µg) of aflatoxin B_1 bound to the fiber pellet was measured by spectrodensitometry (5, 22) using the spectrodensitometer standard curve. The spectrofluorometer standard curve was used to measure µg of aflatoxin B_1 remaining free in solution. Percent aflatoxin B_1 recovered was calculated as:

$$\% \text{ recovered} = \frac{\mu g \text{ aflatoxin } B_1 \text{ bound} + \mu g \text{ aflatoxin } B_1 \text{ free}}{\mu g \text{ aflatoxin } B_1 \text{ added}} \times 100$$

For fiber samples containing 16 µg aflatoxin B_1 the mean percent recovery was 99.88% (Table VIII) and 98.25% for samples containing 20 µg aflatoxin B_1.

Further studies were performed on random samples from the experimental design. These data are presented in Table IX. When the added amount of aflatoxin B_1 was increased to 150 µg, percent recoveries were 98.19% for wheat bran NDF, 97.62% for pear ADF and 93.97% for peanut hull ADF.

These data indicated aflatoxin B_1 was quantitatively extracted from dietary fiber preparations with the chloroform extraction method previously detailed.

TABLE VIII

PERCENT RECOVERY FOR EXTRACTION OF BOUND AFLATOXIN B_1 FROM PEANUT HULL LIGNIN[1]

	μg Aflatoxin B_1 / 10 ml saline[1]	Rep I	Rep II	Rep III	Mean
		------μg aflatoxin B_1------			
Measured μg Aflatoxin B_1 Bound	16	6.67	2.04	4.11	4.30 ± 1.36
	20	6.64	7.51	4.00	6.05 ± 0.94
Measured μg Aflatoxin B_1 Free	16	11.89	11.88	11.29	11.68 ± 0.19
	20	13.60	12.89	14.31	13.60 ± 0.41
		% aflatoxin B_1 recovered			
% Recovery = $\frac{\text{Measured Bound + Free}}{\mu\text{g Aflatoxin } B_1 \text{ Added}} \times 100$	16	116.00%	87.00%	96.26%	99.88 ± 8.55%
	20	101.00%	102.00%	91.55%	98.25 ± 3.32%

[1]Mean values represent the μg of aflatoxin B_1 bound/0.0100 g peanut hull lignin ± standard error of the mean.

TABLE IX

PERCENT RECOVERY FOR EXTRACTION OF BOUND AFLATOXIN B_1 FROM DIETARY FIBER PREPARATIONS

Source	$\frac{\mu g \text{ Aflatoxin } B_1}{5 \text{ ml saline}}$	Measured µg Aflatoxin B_1 Bound	Measured µg Aflatoxin B_1 Free	% Aflatoxin B_1 Recovered
Wheat NDF	150.00	1.52	145.77	98.19
Pear ADF	150.00	2.36	144.07	97.62
Peanut ADF	150.00	3.68	137.28	93.97

V. STATISTICAL ANALYSIS

Data on the binding of aflatoxin B_1 to dietary fiber preparations were analyzed by a Two Way Analysis of Variance with multiple replications per cell. Duncan's Multiple Range Test and the Student-Newman-Keul Test were utilized to partition the means.

CHAPTER IV

RESULTS AND DISCUSSION

I. BINDING OF AFLATOXIN B_1 TO DIETARY FIBER

In the experimental study of aflatoxin B_1 binding to dietary fiber preparations, an analysis of variance (Table X) indicated significant differences ($\alpha = 0.05$) for both products (i.e., peanut hull, wheat bran, corn bran, alfalfa meal and pear) and fiber types (i.e., ADF, NDF, lignin and acetone fiber). At $\alpha = 0.10$, interaction of product source and fiber type was also found significant.

The extent of aflatoxin B_1 binding (Table XI) varied from 825.00 µg/g for peanut hull lignin to 204.80 µg/g for wheat bran NDF.

The marginal means (Table XI) for product type were distributed in a twofold difference range. Duncan's Multiple Range Test partitioned the means into several significantly different groups ($\alpha = 0.05$). Peanut hull and wheat bran did not indicate any significant differences ($\alpha = 0.05$) in their binding capacity of aflatoxin B_1. Corn bran and pear, while not significantly different from one another ($\alpha = 0.05$) varied significantly from peanut hull and wheat bran. Alfalfa meal varied only from peanut hull at $\alpha = 0.05$.

TABLE X

SUMMARY OF TWO WAY ANALYSIS OF VARIANCE FOR PRODUCT TYPES VERSUS FIBER TYPES IN RELATION TO BINDING OF AFLATOXIN B_1 TO DIETARY FIBER COMPONENTS

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F ratio
Product types	4	975,940.66	243,985.16	5.675[1]
Fiber types	3	1,273,924.84	424,641.62	9.877[1]
Interaction	12	963,217.26	80,268.11	1.867[2]
Error	80	3,439,453.20	42,003.16	

[1] F ratios are significant at $\alpha = 0.05$.

[2] F ratio is significant at $\alpha = 0.10$.

TABLE XI

SUMMARY OF MEANS AND DUNCAN"S MULTIPLE RANGE TEST FOR IN VITRO BINDING OF AFLATOXIN B_1 TO DIETARY FIBER PREPARATIONS[1,2,3]

Product	NDF	ADF	Lignin	Acetone Fiber	Marginal Means for Product Type
Peanut hull	456.00 ± 55.29	655.20 ± 178.54	825.00 ± 190.34	303.40 ± 21.93	559.90 ± 152.38[a]
Wheat bran	204.80 ± 35.07	564.00 ± 89.74	792.40 ± 195.46	262.20 ± 35.61	455.90 ± 148.58[a,b]
Corn bran	329.40 ± 75.51	239.20 ± 46.40	358.60 ± 72.19	329.00 ± 95.32	314.05 ± 71.15[c]
Alfalfa meal	346.80 ± 47.44	394.00 ± 108.45	431.20 ± 84.18	376.40 ± 44.51	387.10 ± 70.92[b,c]
Pear	212.40 ± 18.70	231.20 ± 29.72	489.40 ± 75.58	218.80 ± 16.74	287.95 ± 66.11[c]
Marginal mean for fiber type	309.88 ± 139.83[b]	416.72 ± 274.91[b]	579.32 ± 339.21[a]	298.00 ± 117.96[b]	

[1]Mean values represent the µg of aflatoxin B_1 bound/1.00 g fiber ± standard error of the mean.

[2]Marginal product means within the column followed by the same letter are not significantly different at $\alpha = 0.05$.

[3]Marginal fiber type means within the row followed by the same letter are not significantly different at $\alpha = 0.05$.

The marginal means for fiber types (Table XI) also varied twofold. Evaluation of the means by Duncan's Multiple Range Test indicated two significantly different groups. NDF, ADF and acetone fiber were significantly lower than lignin. Data indicated the lignin component of the products was the most efficient binding agent of aflatoxin B_1, as compared to the other three fiber types (NDF, ADF and acetone fiber). This is in agreement with researchers who have suggested adsorption of bile salts and acids is a function of the lignin component of fiber (14, 18). The lower binding capacities of ADF, NDF and acetone fiber may be due to interfering polysaccharides such as hemicelluloses, which have been theorectically linked to lignin in the plant cell wall (48) and perhaps have blocked potential aflatoxin B_1 binding sites. However, to test this hypothesis it would be necessary to evaluate the binding capacity of lignin after each specific plant polysaccharide was added individually to the lignin in the same manner as occurs in the plant cell. There is no means to accomplish this.

Because there was significant interaction of product and fiber type ($\alpha = 0.10$), all of the treatment means (Table XII) were evaluated by both Duncan's Multiple Range Test and the Student-Newman-Keuls Test. Duncan's Multiple Range Test ($\alpha = 0.05$) partitioned the means into four groups with some overlapping occurring between the groups. The

TABLE XII

MEANS OF FIVE REPLICATES FOR BINDING IN VITRO OF AFLATOXIN B_1 TO DIETARY FIBER PREPARATIONS[1,2]

Fiber Type	µg Bound 1.00 g Fiber	Duncan's Multiple Range Test	Student-Newman-Keuls Test
Peanut hull lignin	825.00 ± 190.34	a	a
Wheat bran lignin	792.40 ± 195.46	a	ab
Peanut hull ADF	655.20 ± 178.54	ab	abc
Wheat bran ADF	564.00 ± 89.74	abc	abc
Pear lignin	489.40 ± 75.58	bcd	abc
Peanut hull NDF	456.00 ± 55.29	bcd	abc
Alfalfa meal lignin	431.20 ± 84.18	bcd	bc
Alfalfa meal ADF	394.00 ± 108.45	bcd	c
Alfalfa meal acetone	376.40 ± 44.51	bcd	c
Corn bran lignin	358.60 ± 72.19	cd	c
Alfalfa meal NDF	346.80 ± 47.44	cd	c
Corn bran NDF	329.40 ± 75.51	cd	c
Corn bran acetone	329.00 ± 95.32	cd	c
Peanut hull acetone	303.40 ± 21.93	cd	c
Wheat bran acetone	262.20 ± 35.61	cd	c
Corn bran ADF	239.20 ± 46.40	d	c
Pear ADF	231.20 ± 29.72	d	c
Pear acetone	218.80 ± 16.74	d	c
Pear NDF	212.40 ± 18.70	d	c
Wheat bran NDF	204.80 ± 35.07	d	c

[1]Mean of five replicates with ± the standard error of the mean.

[2]Means followed by the same letter are not significantly different at $\alpha = 0.05$.

Student-Newman-Keuls Test partitioned the means into three groups also with overlapping.

In general, the marginal means (Table XI, page 47) and the ranked means (Table XII) indicated peanut hull was the product of greatest aflatoxin B_1 binding ability. Three out of four treatment combinations which included peanut hull ranked in the upper six product/fiber means (Table XII). Likewise three out of four treatment combinations containing lignin ranked in the upper six. This would indicate lignin is the fiber component with the greatest aflatoxin B_1 binding ability.

Variance in noted with wheat bran binding large (792.40 µg/g) and small (204.80 µg/g) quantities of aflatoxin B_1 and NDF binding 456.00 µg/g and as little as 204.80 µg aflatoxin B_1/g. Such inconsistencies (Table XII) would indicate the complexity of both product source and fiber type and interaction of their treatment combinations.

In addition, the small number of partitioned mean groups and their wide ranges reflects the variation in the data as is shown by the standard error of the means in Table XII. This variation may have been due to:

1. Nonuniformity of the fiber types although care was taken to produce a homogeneous sample of uniform particle size (< 60 mesh), well mixed for randomnization. In addition, experiments were performed with larger amounts of fiber

sample (up to 0.10 g). There was no decrease in the amount of variation. If the variation was due to nonuniformity, a tenfold increase in sample size would be expected to lower the variation in aflatoxin B_1 binding. However, it did not.

2. Variation inherent in the analytical technique in the range of fluorodensitometric measurement. A standard spot of 0.04 µg of aflatoxin B_1 (Figure VII, page 36) gave peak readings of 25.5 and 20.0 integrator units, for two replicates. This difference may have contributed to data variation for aflatoxin B_1 binding, but would not logically be responsible for standard errors of up to 25% of the mean for 5 replicates.

Therefore the two logical sources of data variation-sample homogeneity and analytical precision have been evaluated. The cause of variation therefore, must lie beyond the scope of this scrutiny.

II. SIGNIFICANCE AND LIMITATIONS

The results of this study show that dietary fiber from different plant sources has the ability to bind aflatoxin B_1 in a stable complex. However, the data cannot be interpreted without taking into account factors which could not be evaluated in the experiment such as: (1) the type of bonding between dietary fiber and aflatoxin B_1; and, (2) effects of bacterial activity in the cecum which may

alter physiological function of dietary fiber. Because aflatoxin B_1 was bound to dietary fiber *in vitro*, the possibility that this might occur *in vivo* and perhaps be a mechanism for protection against dietary carcinogens requires further study.

III. FUTURE INVESTIGATION

The following studies should be undertaken.

Definition of the Type of Bonding

Definition of the type of bond between dietary fiber preparations and aflatoxin B_1 is important for determining the stability of the fiber/aflatoxin B_1 complex.

The fiber/aflatoxin B_1 pellets as prepared in this study could be slurried with solutions of the following compounds: (1) NaCl (breaks ionic bonds), (2) urea (breaks hydrogen bonds), (3) sodium lauryl sulfate (breaks hydrophobic bonds); and (4) B-mercapto ethanol (reduces a number of bonds). After incubation and centrifugation the slurry could be quantified for released aflatoxin B_1 by fluorescence spectrophotometry (12). The amount of aflatoxin B_1 released into the supernatant would give an indication of the type of bonding between aflatoxin B_1 and dietary fiber. However, it could not prove the nature of the complex.

In Vivo Studies

To evaluate the biological significance of *in vitro* aflatoxin B_1 binding to dietary fiber, effects of fiber on the toxicity of aflatoxin B_1 in a physiological system should be evaluated. A suggested investigation would involve experimental studies with rats. These studies would be aimed at:

1. determining whether or not aflatoxin B_1 will bind to dietary fiber and be excreted in the fecal matter and,

2. establishing if a diet high in dietary fiber will lower aflatoxin B_1 absorption from the gut and lower the incidence of death from aflatoxicosis.

CHAPTER V

SUMMARY

The binding of the chemical carcinogen, aflatoxin B_1, to dietary fiber preparations (NDF, ADF, acetone fiber and lignin) was studied. Five fiber sources were used: wheat bran, corn bran, peanut hull, alfalfa meal and pear.

The results of this study indicated dietary fiber from different plant sources has the ability to bind aflatoxin B_1 in a stable complex. Based on these findings the following conclusions were made.

1. There were significant differences ($\alpha = 0.05$) in the main effects of product and fiber type of aflatoxin B_1 binding.

2. Of the five product sources, peanut hull had the greatest aflatoxin B_1 binding ability and pear the least.

3. Among fiber types, lignin possessed the greatest aflatoxin B_1 binding ability and acetone fiber the least.

4. Interaction of product and fiber type was found to be significant ($\alpha = 0.10$).

5. Peanut hull lignin possessed the greatest aflatoxin B_1 binding ability and wheat bran NDF the least, among treatment combinations. The means were partitioned into four groups with overlapping according to Duncan's Multiple Range Test ($\alpha = 0.05$) and three groups with

overlapping according to the Student-Newman-Keuls Test ($\alpha = 0.05$).

In view of these results, dietary fiber may bind the chemical carcinogen, aflatoxin B_1, in the gut and reduce its absorption by the body.

It would appear further research is needed to ascertain:

1. type of bonding between dietary fiber and aflatoxin B_1,
2. whether or not dietary fiber may actually modify the dose response relationship for aflatoxin B_1 in vivo.

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BIBLIOGRAPHY

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