In Vitro Hepatic Bioactivation of Aflatoxin B_1 in the Pregnant Rat

by

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ABSTRACT

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The specific aim of this study was to determine the capacity of the maternal rat liver to bioactivate aflatoxin B_1 on day 6 of gestation. Lowry protein determinations on rat liver microsome preparations revealed an increase in the total amount of microsomal protein per gram of liver tissue in pregnant Sprague-Dawley rats on day 6 of gestation as compared to nonpregnant control animals. *In vitro*, microsomes from day 6 pregnant animals showed an enhanced capability to bioactivate aflatoxin B_1 resulting in a two-fold increase in the level of binding of aflatoxin B_1 to DNA when compared to nonpregnant controls. It was thus concluded that there is gestation-specific activation of aflatoxin B_1 in pregnant Sprague-Dawley rats with greater hepatic aflatoxin B_1 activation present on day 6 of gestation as compared to nonpregnant control rats. This increased hepatic bioactivation indicates the enhanced production of microsomal enzymes capable of activating aflatoxin B_1.

ACKNOWLEDGEMENTS

I wish to express my gratitude to Dr. Rick Irvin for his guidance, support, and patience. Also, I would like to thank Elizabeth Maull for always being there to help, and a special note of thanks to Rudy Zarate who was always there to help me through the rough times.

DEDICATION

This paper is dedicated to my parents, Mr. and Mrs. William C. Hobbes. Without their support, encouragement, love, and understanding, I could have never made it this far.

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INTRODUCTION

Biochemical teratology is a relatively new scientific discipline which involves the study of birth defects induced by exogenous substances. Figures from the March of Dimes Foundation indicate that, in the United States, 200,000 birth defects are recorded per year and 560,000 infant deaths, spontaneous abortions, stillbirths, and miscarriages are recorded due to defective fetal development (43). Of these deaths and birth defects, 25% are attributed to underlying genetic causes while the other 75% result from an interplay of the four known classes of teratogens and prenatal toxins: radiation, viruses, drugs, and environmental chemicals. Of this 75% only 10-20% have been ascribed to the first three causative factors (22). These figures clearly implicate toxic environmental and food-borne chemicals as principal agents in human teratogenicity and prenatal mortality.

The main sources of human contact with prenatal toxins and teratogens have been identified as occupational exposure, exposure via food intake, and exposure to chemical toxins emitted as environmental pollutants. The human population is exposed to a dangerous situation as 2000 new chemicals are synthesized per year (31). The principal questions that remain unanswered are:

* This paper follows the format of Cancer Research.

1) Of the environmental chemicals to which humans are exposed, which are the principal agents causing prenatal deaths and birth defects, and what is their mechanism of action?

2) Do naturally-occuring mixtures of chemicals act synergistically to potentiate or alter the prenatal toxicity of the individual chemical components?

In an attempt to answer these questions, epidemiologists must examine disease in populations with a high exposure to a given environmental agent. Unfortunately, the insensitivity of the tools of epidemiology make it difficult to accurately identify serious human prenatal toxins and access the risk of chronic low level exposure to these chemicals. Laboratory animal studies are therefore used to determine potential human health hazards upon exposure to a chemical. Many times however animal studies are too lengthy and costly to provide proper indications of prenatal toxic potential. Whole animal models also do not provide accurate assessments of potential synergistic properties of environmental chemical mixtures as prenatal toxins. What are needed are short term in vitro assay systems which can determine the prenatal toxicity, teratogenicity, and mechanism of action of specific environmental toxins and mixtures of toxins. These assays would indicate which chemicals require further whole animal testing as a function of dose and gestation in trial studies.

This study utilizes a model in vitro assay system to characterize the capacity of maternal tissues to transform an environmental toxin into chemically-reactive cytotoxic

intermediates which are responsible for the observed toxic effect. A description of maternal metabolism during gestation may provide the answers necessary to explain a mechanism through which chemicals exert their teratogenic effects.

LITERATURE REVIEW

Human and Animal Exposure to Prenatal Toxins and Teratogens

Of all the known causes of human birth defects, chemicals represent the most preventable class of agents. Unfortunately, through poor safety procedures, careless disposal of materials, and neglect, humans are in constant contact with these agents. As mentioned previously, the major sources of human exposure to prenatal toxins and teratogens are occupational exposure, food contamination, and environmental pollutants. The disasters that have manifested from these exposures clearly implicate the need for extensive testing of all suspected prenatal toxins and teratogens.

Chemicals in the workplace having prenatal toxicity and teratogenic properties have been documented. Female anesthesiologists show a higher risk of spontaneous abortions, infertility, and birth defects. Smithells (1976) reported that the rate of spontaneous abortions is elevated two-fold, and the American Society of Anesthesiologists found a 60% increase in malformations in exposed individuals (56). These detrimental effects are believed to be caused by the anesthetic gas, nitrous oxide, which was found to be lethal to chick embryos, and fetotoxic and teratogenic to Sprague-Dawley rats (17). Tetrachloroethylene, a solvent in dry cleaning fluid, is fetotoxic causing cholestatic jaundice in a breast fed infant. The mother was exposed to chronic low levels of the vapors, and the chemical accumulated in her milk (5). Occupational exposure

to lead among lead factory workers resulted in a 60% stillbirth rate, and a 70% newborn mortality rate by age three. Its primary effects on the fetus include low birth weights, brain damage with convulsions, and other central nervous system effects (2).

Chemicals can enter the food supply in several ways, including the processing procedures, residues on plant products or in animals tissues, and as food additives. For the most part, the teratogenic properties of these chemicals in humans is not well characterized. There are incidences however which implicate chemical food contaminants as prenatal toxins and teratogens. In 1968 rice oil in Japan was contaminated with polychlorinated biphenyls (PCBs), a family of compounds used as heat exchangers. Leakage of the heat exchange fluid through a small hole in the heating coil during processing contaminated the rice oil to levels of 2000 to 3000 ppm. In those infants born to exposed women, stillbirths, growth retardation, dark pigmentation of the skin, gingiva, and conjunctivitis, and neonatal jaundice were recorded (30).

Diethylstilbesterol (DES) was used to fatten beef and poultry exposing millions of people to trace amounts of the substance. The effects of this food-borne exposure have not been determined, but as a prescription drug used by women from 1940-1970, DES has shown harmful effects. Herbst (1977) reported that infants exposed to DES during fetal development suffered carcinogenic and teratogenic effects. Females developed uterine abnormalities and cervical and vaginal adenosis. Males showed a higher incidence of sperm abnormalities, undescended testes,

hypospadias, and epididymal cysts (24). The question that arises from this incident is, will DES show similar deleterious effects in those people exposed to low levels in contaminated beef and poultry products?

Caffeine is a food additive in soft drinks, analgesics, and chocolate, and occurs naturally in tea and coffee. In 1980 the Food and Drug Administration issued a warning for pregnant women to limit their use of caffeine because studies showed it to be teratogenic in rats. Offspring of rats receiving an equivalent of 12-24 cups of coffee per day had delayed skeletal development, missing digits, and growth retardation (18). More severe defects, such as limb reduction and cleft palate were observed at higher doses in two strains of rats and three strains of mice (42). Lechat (1980) found an increase in the frequency of birth defects in women who were heavy coffee drinkers (over 7 cups a day) (34).

Careless disposal of chemicals into the environment has resulted in contamination of air, land, water, and food sources. Humans are exposed to carbon monoxide from industrial combustion, automobile exhaust, and cigarette smoke. Carbon monoxide has a higher affinity for hemoglobin than oxygen, and readily diffuses across the placenta. Fetal carboxyhemoglobin levels can exceed maternal levels by 10-15% yeilding a reduction in fetal arteriole oxygen tension. A survey of human carbon monoxide poisonings, revealed an increased rate of stillbirths when the carboxyhemoglobin concentration was 20-40%. Surviving infants showed signs of mental retardation, seizures, and spasticity (35).

Mercury in its inorganic form was used as a catalyst in a Japanese plastic factory in 1953. It was discarded into Minimata Bay where anaerobic bacteria converted the inorganic mercury to its organic alkyl form. Through bioconcentration, large amounts of mercury accumulated in fish flesh. Subsequent poisonings of pregnant women resulted in mental retardation, spasticity, chronic seizures, and blindness of their offspring (36). As mentioned before PCBs are used in heat exchange fluids and careless disposal of this group of compounds has resulted in contamination of aquatic environments. Due to their high lipid solubility, PCBs pass through the marine food chain and accumulate in high concentrations in fish flesh. In man, PCBs also tend to accumulate in the fatty tissues. High concentrations of PCBs have been found in breast milk of women who were heavy fish eaters. Infants who received PCB contaminated breast milk revealed some neurological and developmental impairments (51).

Humans are not only susceptible to chemical prenatal toxins and teratogens, but food producing animals as well. Exposure of livestock to prenatal toxins results in economic losses due to suppressed growth rates, poor development, and decreased production. Accidental poisonings of chickens with PCB contaminated fish meal for example resulted in decreased hatchability, central nervous system disorders, and ocular defects (11). Contamination of cattle feed with polybrominated biphenyls (PBBs), a commonly used fire retardant, led to an increased incidence of stillbirths and a 40% drop in production rate (36). In addition, chemicals create potential public health

problems due to chemical residues in animal products, such as the DES incident mentioned earlier.

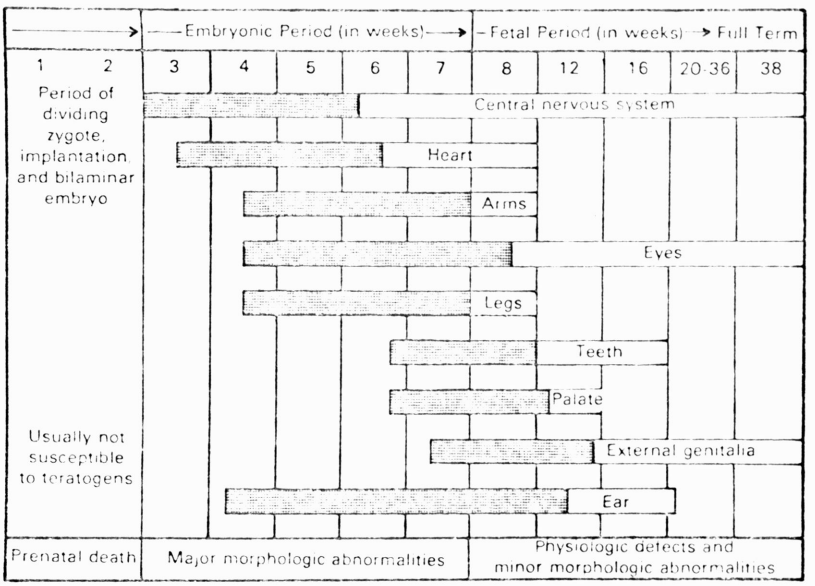
Mechanism of Action for Chemical Prenatal Toxins and Teratogens

An important finding central to the development of model *in vitro* systems to explain the mechanisms underlying chemically-induced human prenatal toxicity and teratogenicity is that maternal and fetal/embryonic tissues are capable of metabolically activating environmental and food-borne xenobiotic compounds to chemically-reactive intermediates. Studies of the interactions of chemicals with biological systems have shown that many times the parent compound undergoes biotransformation and is converted to a metabolite or metabolites which are responsible for the observed toxic effect (27).

Damage to a developing organism by a chemical occurs upon the injury or death of certain cells. The normal sequence of cell, tissue, and organ development is damaged and results in morphological change. The degree of damage done by a chemical will be dependent upon time of administration, the dosage, the number of doses, maternal health, and species variation (31). There are three important periods of development for a newborn: fertilization and implantation, the embryonic period, and the fetal period (39). (Figure 1).

In humans, day one to day seventeen is the period of fertilization and implantation. Injury at this stage results in embryonic cell death. If recovery follows, there is no structural deformity to the embryo. If the damage is

FIGURE 1



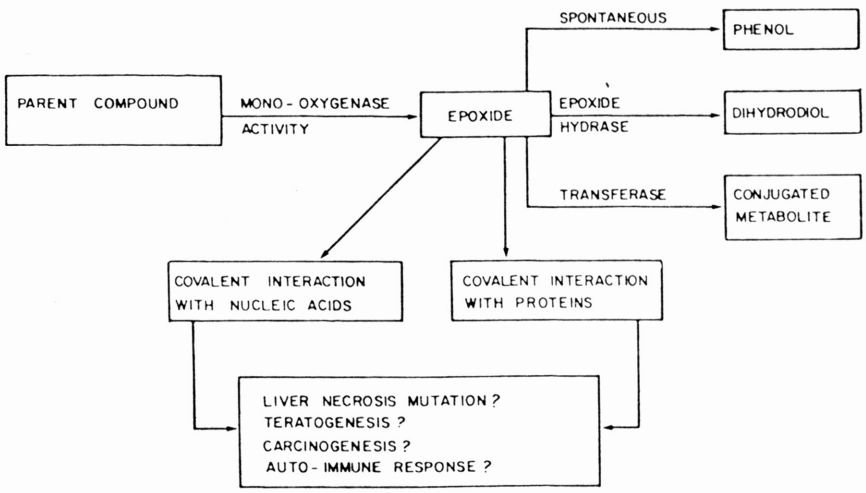
Schematic representation of human development and sensitive periods for production of maldevelopment. Cross-hatching represents highly sensitive periods; clear area represents stages that are less sensitive to teratogens.

irreversible, resorption will normally occur. The human embryonic period follows from day 18 to day 55. During this period, accurate and complete development of each organ system occurs. The fetal period follows from day 56 to term. Exposure to agents at this stage can result in reduced cell size and number, growth retardation, and central nervous system impairments.

The mechanisms through which chemicals induce their teratogenic actions remain poorly understood. The recent finding that maternal and fetal/embryonic tissues are capable of metabolically activating foreign compounds to chemically-reactive intermediates plays a principal role in determining the teratogenic properties of chemical toxins. (Figure 2) Liver enzymes, such as monooxygenases, dehydrogenases, and nitroreductases, catalyze the oxidation, reduction, and hydrolytic reactions which produce the reactive metabolites. Factors which can influence this bioactivation include substrate specificity, metabolic products formed, and metabolite stability (27).

Studies have shown that some chemical carcinogens and mutagens must undergo bioactivation in order to display their oncogenic or mutagenic properties (3,23). Bioactivation is the conversion of an inactive or less active parent compound to an active or more active metabolite. The reactive metabolites formed during this process covalently bind to critical macromolecules, such as DNA, RNA, and protein, and are believed to be responsible for the observed toxic effects (6,41).

FIGURE 2



Simplified scheme illustrating the possible metabolic pathways for reactive epoxide (or arene oxide) intermediates subsequent to oxygenation of the parent compound by the cytochrome P-450 monooxygenase system

Cellular tissue necrosis (13,44), allergic reactions, methemoglobinemia, and bone marrow aplasia (20) are other pathological effects which have resulted from reactive metabolites generated through bioactivation. From the evidence supporting bioactivation of chemical carcinogens and mutagens, it is highly probable that bioactivation also could play a major role in the mechanisms of chemically-induced prenatal toxicity and teratogenicity (27).

Previous studies of bioactivation reactions have focused almost exclusively on the liver as the principal organ mediating the conversion of inactive or less active parent compounds to active, more active, or differentially active metabolites. However, with respect to the teratogenic effects of environmental chemicals, attention has also been focused on extrahepatic tissues as operative in chemical-mediated prenatal toxicity; the tissues of greatest interest in this case being the placenta and fetal liver. Known bioactivating reactions are catalyzed by enzymes or enzyme systems present in these maternal extrahepatic tissues which produce cytotoxic and mutagenic chemical species (27). Some intermediates are sufficiently stable to be carried in the circulation, whereas others appear to act principally or exclusively at the sites generated.

Rationale for a Model System Design-aflatoxin B[1]

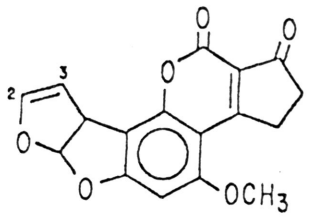
Aflatoxins are toxic metabolites produced by strains of Aspergillus flavus and Aspergillus parasiticus which are common food spoilage fungi (62). The aflatoxins first came to the

attention of the scientific community when they were associated with mass outbreaks of poultry poisoning (Turkey X Disease) in 1960. They occur under any conditions which will support mold growth on any natural substrate. Contamination commonly occurs in agricultural commodities, such as corn, peanuts, cottonseed, soybeans, tree nuts, milk, milk products, and meat products. Contamination can be direct through the growth of fungi on agricultural products at anytime from field exposure to harvesting, transport, storage, and processing or indirectly through residues in meat or milk derived from aflatoxin metabolism in exposed animals (57,60). Aflatoxin contamination is a major food safety problem in human and animal health and has stimulated public interest in its adverse toxic effects and means of control.

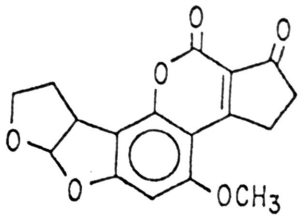
Aflatoxins occur in two series: aflatoxin B_1 and its derivatives, and aflatoxin G_1 and its derivatives. (Figure 3) These compounds are closely related structurally but differ significantly in their potency. Thirteen naturally occurring derivatives of aflatoxin B_1 have been structurally identified. Aflatoxin B_1 is the compound of primary interest because it is the major component of the toxin mixtures and is the most potent toxin and carcinogen in the series.

Aflatoxin B_1 has been proven to be a potent hepatotoxin in several animal species (7,8,45). Acute toxicity results in liver cirrhosis, and long term chronic exposure leads to central lobular necrosis and bile duct proliferation (63). Dietary aflatoxin B_1 levels of 10-100 ppm will produce poisonings in

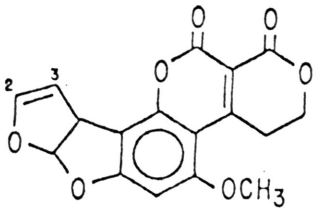
FIGURE 3



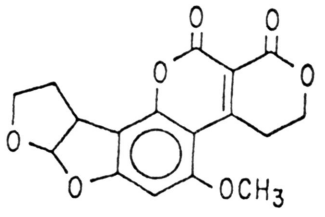
AFB_1



AFB_2



AFG_1



AFG_2

Structures of the four principal aflatoxins produced by molds

domestic animals. Cattle, swine, chickens, ducks, and turkeys are all sensitive to the acute toxic effects of aflatoxin B_1; lethal dosages for this toxin range from 0.3 ppm in the ducklings to 2.2 ppm in calves (62). Accepted LD_{50} values for aflatoxin B_1 are in the range of 0.5-10 mg/kg for most experimental animal species (60). Species sensitivity is however highly variable; for the rainbow trout (the most sensitive species), the LD_{50} value it less than 0.5 mg/kg, and for the mouse (the least sensitive species), the LD_{50} value is 60 mg/kg. Biochemical changes associated with acute aflatoxin B_1 toxicity in rodents include impairment of protein synthesis and pronounced inhibition of DNA and RNA polymerases through the reduction of template activity. Similar effects are also seen in human and animal cell cultures (63).

Aflatoxin B_1 has, in addition, carcinogenic activity in many laboratory animal species including rodents, birds, and fish (9,10,46). Once again, the liver is the principal organ affected in which the toxin induces hepatocellular carcinoma and cholangiocarcinoma (63). Lancaster et. al. (1961) showed the earliest evidence of the carcinogenic properties of aflatoxin B_1. In this study, a higher incidence of liver tumors was reported in rats fed for 30 weeks on diets containing a high level of aflatoxin contamination (33). Subsequent studies in rats revealed that an aflatoxin B_1 level of 0.015 ppm of the diet resulted in 100% tumor incidence in rats within 68-80 weeks (61). In ducks fed an aflatoxin B_1 level of 30 ppb of the diet, eight of eleven survivors developed liver tumors (10). At a dose range

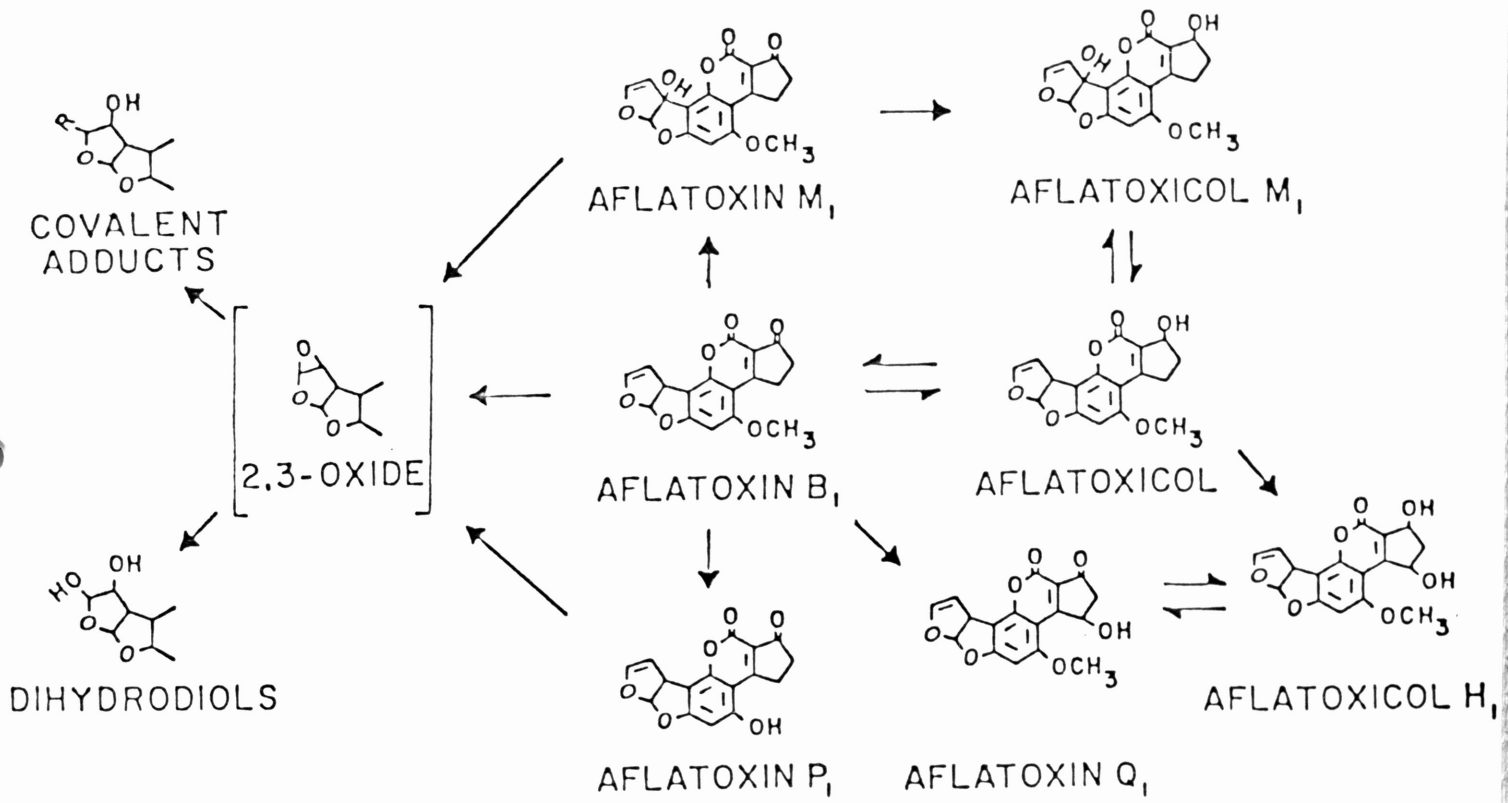
of 4-20 ppb, a logarithmic dose-relationship between aflatoxin B_1 level in the diet and liver tumor incidence was noted in rainbow trout (54).

Aflatoxin B_1 is metabolized in the liver by the mixed function oxidases in the smooth endoplasmic reticulum producing a variety of derivatives. Figure 4 diagramatically summarizes all of the known metabolic transformations of aflatoxin B_1. *In vitro* studies in several animal species show that the species sensitivity and target organ susceptibility to the toxic and carcinogenic effects of aflatoxin B_1 correlate with the capacity of target tissues to metabolically activate aflatoxin B_1 to aflatoxin B_1-2,3-oxide. This highly reactive electrophilic intermediate is lethal and mutagenic to bacteria, and is believed to mediate the toxic effects of aflatoxin B_1 by binding to critial macromolecules such as DNA and RNA (64). Lesions at the DNA site are hypothesized to interfer with transcription (12,21) leading to carcinogenesis and mutagenesis (3,48). Epoxidation appears to be the key step in aflatoxin B_1 bioactivation since toxicity is dependant upon the rate of biotransformation. Although the epoxide has not been isolated due to its great reactivity, a more stable electrophilic analogue has been synthesized, aflatoxin B_1-2,3-dichloride, and is available for use in chemical assays and biological systems (63).

Studies have also shown aflatoxin B_1 to be mutagenic. It induced mutations of 4-23 fold above the spontaneous level in transforming DNA of *B. subtilis* (38). In *Drosophila melanogaster*, aflatoxin B_1 induced recessive lethal mutations

FIGURE .4

THE DIHYDRODIOL (A CHARACTERISTIC HYDROLYSIS PRODUCT OF EPOXIDES)
HAS BEEN FOUND AS A PRODUCT OF MICROSOMAL OXIDATION



(32), and at a dose of 0.01-0.5 µg/ml aflatoxin B_1 damaged chromosomes of lymphocytes prepared from the blood of healthy human donors; a high rate of structural chromosome aberrations, such as breaks and interchanges were observed (15). Studies indicate a correlation between bacterial mutagenicity and *in vivo* carcinogenicity (65). In the *Salmonella*/microsome system, aflatoxin B_1 is potently mutagenic, and *in vivo* experiments with the different aflatoxins have shown aflatoxin B_1 to the most potent member of the aflatoxin family. Furthermore, it has been shown that microsomal activation is necessary for aflatoxin B_1 to exert its mutagenic effects (40).

Aflatoxin B_1 is a potent teratogen which causes fetal death and abnormalities at a parts per million exposure level. At dose levels of 5 µg/g and 10 µg/g decreased egg production and decreased hatchability of fertile eggs were observed in hens (25). Upon necropsy, enlarged fatty livers and enlarge spleens were observed in the hens. Pregnant female hamsters injected with 4 mg/kg of aflatoxin B_1 on day 8 of gestation produced offspring with gross abnormalities of anencephaly, disorganization of the cranial end of the neural tube, ectopia cordis, formation of soft tumors on the head, harelips, and umbilical hernias (14). Toxic lesions of subcutaneous hemorrhage, edema, ascites, and myocardial necrosis were also seen in the fetuses. Growth retardation upwards of 20-30% was observed on day 11 of gestation. Hepatic and renal necrosis were seen in the treated pregnant females (52). Encephaly, open eye lids, and protrusion of the intestine were seen in fetuses of pregnant mice dosed with

4 mg/kg of aflatoxin B_1 on day 8 and 9 of gestation (4). A sublethal dose of 3 mg/kg on day 6 of gestation resulted in 100% mortality in pregnant Sprague-Dawley rats (26).

The mechanism of action of aflatoxin B_1 during pregnancy is not known. However, since the carcinogenic and mutagenic effects of aflatoxin B_1 center around bioactivation to transfrom aflatoxin B_1 to reactive intermediates which covalently adduct and damage critical cellular macromolecules, it is reasonable to hypothesize that bioactivation by fetal and maternal tissues plays a major role in aflatoxin B_1 induced teratogenicity and prenatal toxicity.

While aflatoxin contamination of the human food supply presents a major problem to human health, livestock animals are at a much greater risk for exposure to aflatoxins due to the feeding of old, low quality, and moldy feed. Short term insestion of low levels of aflatoxins result in suppressed growth rates, lower carcass quality, and reduced milk and egg production in domestic animals (29,47,49,55). This low level exposure results in large economic losses to producers, and introduces aflatoxin residues into animal products (milk, eggs, and meat) which creates an important human food safety problem.

Humans are seldom exposed to large amounts of aflatoxin. Their exposure is primarily long-term and low level especially in areas where aflatoxin contamination of foodstuffs is frequent. In underdeveloped countries, aflatoxin contamination is more common due to the lack of knowledge in food cultivation, harvesting techniques, and proper food storage procedures.

Studies show a positive relationship between aflatoxin ingestion and liver cancer incidence (63). Aflatoxin analysis of peanuts collected in various localities in Swaziland revealed a higher liver cancer disease risk in those areas with the highest frequency of aflatoxin contamination (28). In Uganda, samples of major diet ingredients from home granaries were collected and analyzed for aflatoxin contamination. When the frequency of aflatoxin contamination was compared regionally with tribal distribution of liver cancer incidence, the highest incidence of the disease was recorded in regions where aflatoxin contamination was also high (1). Although, there is no direct evidence identifying aflatoxin as a major cause of human liver carcinoma, there is strong circumstantial evidence linking the two together, such as:

1) the proven carcinogenicity of aflatoxin B1 in laboratory animals.

2) widespread detection of aflatoxins in the human food supply.

3) epidemiological evidence associating aflatoxin exposure with increased incidence of primary liver cancer in humans.

The risk assessment of human suceptibility to the carcinogenic, mutagenic, and teratogenic effects of aflatoxin is very difficult since data must be extrapolated from animal studies. The lack of information necessary to establish regulation guidelines and tolerance levels for humans makes control of aflatoxin contamination a diffucult and complex task. The best way to control aflatoxin contamination is by preventing mold growth. This requires the proper application of food

science technology and the development of good quality control procedures to remove contaminated products from the market before these products reach the consumer.

SPECIFIC AIMS

The specific aim of this thesis project is to characterize the capacity of maternal tissues to metabolize environmental toxins using aflatoxin B_1 as the model toxin and the rat as the model animal species. Aflatoxin B_1 has been chosen as the model environmental chemical in the study because:

1) it is a known potent prenatal toxin/teratogen in laboratory animals.

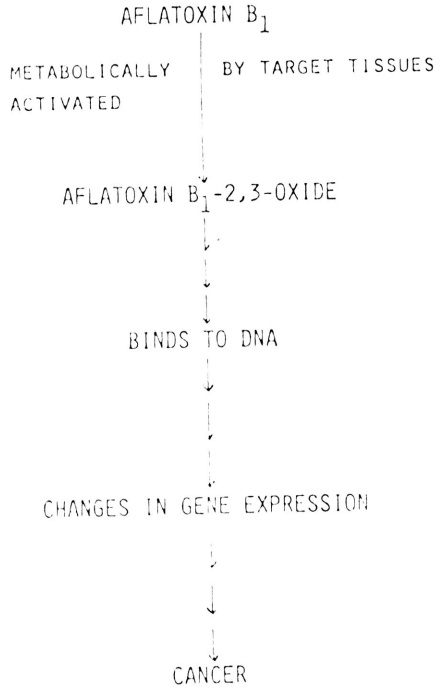
2) the metabolic routes of aflatoxin B1 as a carcinogen are well characterized.

3) the aflatoxins have been causally related to increased incidences of prenatal mortality in human populations known to exhibit high rates of liver cancer due to elevated levels of aflatoxin B_1 in the diet.

The pregnant rat was chosen as the model animal species because it has been shown to be sensitive to the toxic effects of aflatoxin B_1 on day 6 of gestation.

By using a model *in vitro* metabolic activation system and a DNA binding assay, the capacity of the maternal liver to metabolize an environmental toxin at a specific point in gestation can be measured. A comparison between pregnant rats on day 6 of gestation and nonpregnant control rats will determine if the liver is more active in metabolism during pregnancy, and thus renders the mother more susceptible to the toxic effects of aflatoxin B_1. (Figure 5)

AFLATOXIN B_1 AS A CARCINOGEN:



AFLATOXIN B_1 AS A TERATOGEN:

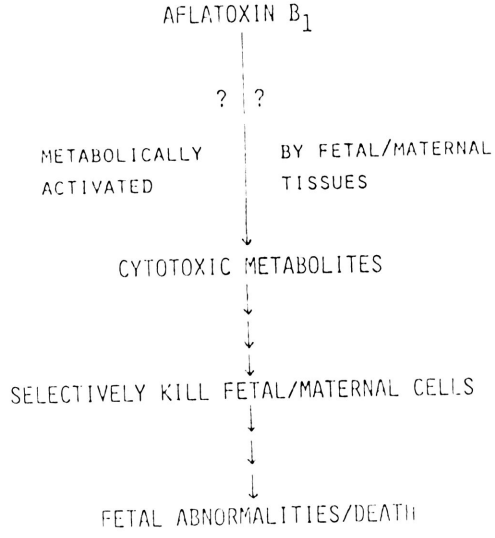


FIGURE 5

MATERIALS AND METHODS

Special Chemicals

Aflatoxin B_1 was purchased from the Sigma Chemical Company, St. Louis Mo. and [^{3}H] aflatoxin B_1 was obtained from Moravek Biochemicals, Brea, Ca.

Animals

Female Sprague-Dawley rats were obtained from Timco Breeding Labs at age 10 weeks. The animals were divided into three groups. One group was composed of pregnant females on day 6 of gestation. This group was then subdivided into three sections composed of two animals each. These six female rats were arbitrarily chosen for breeding, and each one was mated with a male. A successful mating was determined by the presence of a vaginal plug during morning examinations. The control group was composed of five nonpregnant females. The last group was a positive control group of five nonpregnant females who were given 0.1% phenobarbital water ad libum for eight days. (Phenobarbital was used as a liver enzyme inducer.)

Isolation of Liver Microsomes

Liver microsomes were prepared from each group of animals according to the procedures of Roebuck et. al. (50). For each group, the livers were perfused with microsomal homogenizing

media (H Buffer) and weighed. The liver tissue was then minced with scissors and homogenized in a dounce homogenizer with H Buffer at a volume of 5 ml H Buffer/gm of liver tissue. The homogenate was centrifuged in a preparatory centrifuge for 15 minutes, 4° C, at 8800 rpm to principally remove nuclei. The nonlipid supernatant was removed and centrifuged in an ultracentrifuge for 1 hour, 4° C, and 37,500 rpm in a Ti 60 Rotor to form the microsomal enzyme pellet. The microsomal pellets were subsequently removed and homogenized in 2 ml H Buffer/gm liver tissue. The final homogenate was held on ice for 30 minutes to allow air to escape, and stored at -70° C. The procedure for the liver microsome preparation is detailed in Appendix I.

Lowry Protein Analysis

In order to determine the mg of protein present in each group of microsomes, a Lowry protein determination was done according to the procedure of Lowry et. al. (37). A standard curve was first constructed using increasing volumes of a standard bovine albumin at a concentration of 1 mg/ml, and decreasing volumes of water to form a 1.0 ml solution. In a span of fifteen minutes, the solutions necessary to form the colormetric reaction were added, and absorbance was read at 600 nm on a spectrophotometer; the standard curve was subsequently plotted as absorbance versus mg of protein. From each group of microsomes, a 0.01 ml sample was added to 0.99 ml of water to form a 1.0 ml mixture. The solutions were added in the same

manner as for the standard curve, and absorbance was read at 600 nm. The mg of protein for each group of microsomes was obtained by extrapolation from the standard curve. Appendix II gives a detailed description of the Lowry Protein Analysis procedure.

DNA Binding Assay

In order to determine the level of binding of [^{3}H] aflatoxin B$_{1}$ to DNA, a DNA binding assay as described by Essigmann et. al. (16) was done for each group. The assay consists of a 25.0 ml incubation mix, composed of 0.05 M Tris-HCl Buffer (pH 7.5), 0.1M MgCl$_{2}$, 120 mM Glucose-6-Phosphate, 5 mM NADP, Glucose-6-Phosphate Dehydrogenase, calf thymus DNA, microsomal enzyme fraction, and [^{3}H] aflatoxin B$_{1}$ dissolved in dimethyl sulfoxide. This incubation mix was incubated for 30 minutes in a shaking water bath at 37^{o} C, and then centrifuged for 30 minutes, 4^{o} C, and 39,000 rpm in a Ti 60 rotor to remove the microsomes. The supernatant was removed and incubated with 5 M NaCl, 5% Na dodecyl sulfate, and phenol-CHCl$_{3}$-isoamyl alcohol for 30 minutes in a shaking water bath at 37^{o} C. The resulting emulsion was centrifuged for 15 minutes, 4^{o} C, and 15,000 rpm; the aqueous phase was removed, and DNA was precipitated from this phase with freezer cold ethanol. Precipitant DNA was spooled onto a glass rod, and dried at -70^{o} C. The procedure for the DNA binding assay is described completely in Appendix III.

Isolation of Pure DNA Faction

Once the DNA has been collected from the DNA binding assay, it must be purified. A DNA-cesium salt gradient composed of DNA, Sarkosyl Buffer, 3 M Cs_2SO_4 and 6 M CsCl was prepared for each group. The salt mixture was placed into quick seal polyallomer tubes, capped with mineral oil, and centrifuged for 10-12 hours, 20° C, at 45,000 rpm in a VTi 50 Rotor. The DNA can then be collected using a density gradient fractionator designed to monitor the gradient contents for absorbance at 254 nm. This procedure, however, did not work due to some unknown technical problem.

As an alternative, the DNA was purified by digesting away the protein contaminants and RNA with protease and RNAse. A diphenylamine assay as described by Giles and Myers (19) was then performed on the purified DNA sample to quantitate the amount of DNA present. A small amount of the DNA was dissolved in 2.0 ml of 1 N perchloric acid. Two ml of 4% diphenylamine in glacial acetic acid and 0.1 ml of aqueous acetaldehyde (1.6 mg/ml) were added to the DNA solution. After standing at room temperature overnight, the optical density difference at 595-700 nm was read against a 1 N perchloric acid blank. From these results, the amount of DNA in the sample was determined.

Quantitative Determinations

In order to measure the level of binding of [^{3}H] aflatoxin B_1 to DNA, the specific activity of the aflatoxin dose and the amount of radioactivity present in the DNA sample was determined

using a liquid scintillation counter. Appendix IV contains the procedures and equations necessary to make the quantitative determinations.

RESULTS

Table 1 shows the mg protein per gram of liver tissue obtained for each group from the Lowry protein determinations. In the microsome preparations of the pregnant animals on day 6 of gestation, the average obtained from the three groups was 8.7 mg protein/gram of liver tissue. The control group microsomes had 5.4 mg protein/gram of liver, and the positive control group (phenobarbital induced) microsomes had 7.6 mg protein/gram of liver tissue. The positive control group showed a 40% increase in the mg protein/gram of liver as compared to the control animals, and the pregnant animals showed an even greater increase in microsomal protein when compared to the control group.

The level of binding of [^{3}H] aflatoxin B$_1$ to DNA as determined by liquid scintillation counting is shown in Table 2. It was reported in picomoles aflatoxin B$_1$ per mg of DNA, and represents the average computed from multiple trials. The level of binding in the pregnant animals at day 6 of gestation was 6750 pmoles aflatoxin B$_1$/mg DNA. The control group showed a level of binding of 3100 pmoles aflatoxin B$_1$/mg DNA, and the level of binding for the positive control group (phenobarbital induced) was 6000 pmoles aflatoxin B$_1$/mg DNA. There was a two-fold increase in the level of binding in the positive control group compared to the control group. In the pregnant animals, a 2.2 fold increase in the level of binding was reported when compared

to the control group. The positive control group and pregnant animals showed a significant increase in the level of binding of aflatoxin B_1 to DNA in comparison to the control group.

TABLE 1

Amount of Microsomal Protein in the Microsome Preparations

GROUP	MG PROTEIN/GM LIVER
Pregnant Animals	8.7 ±0.87
Control	5.4
Positive Control	7.6

TABLE 2

LEVEL OF BINDING OF [^{3}H] AFLATOXIN B$_{1}$ TO DNA

GROUP	pMOLES AFB$_{1}$ /mg DNA
Pregnant Animals	6750 ±550
Control	3100 ±440
Positive Control	6000 ±620

DISCUSSION

From the Lowry protein determinations, it is evident that there is an increase in the mg microsomal protein/gram of liver tissue in the positive control group (phenobarbital induced) compared to the control group. This increase in microsomal protein is characteristic of phenobarbital metabolism. When phenobarbital is taken into the body, it is metabolized within the liver and is specific for the increased production of the cytochrome P-450 enzymes. It stimulates the liver cell to accelerate the rate of transcription and translation which results in increased protein production. It appears that a similar type of mechanism exists in the pregnant animals. Through some type of unknown stimulation, the liver cells produce more microsomal protein/gram of liver tissue in the pregnant animal.

The two-fold increase in the level of binding of aflatoxin B_1 to DNA seen in the positive control group (phenobarbital induced) again correlates with phenobarbital metabolism. Since there are more microsomal enzymes in the phenobarbital treated animals, more aflatoxin B_1 is activated and there is more binding of the reactive metabolites to DNA. This same type of mechanism exists in the pregnant rats on day 6 of gestation. More bioactivation and increased levels of binding of aflatoxin B_1 to DNA result due to larger amounts of microsomal enzymes available to metabolize the toxin.

CONCLUSIONS

From the increase in microsomal protein levels and increased levels of binding of aflatoxin B_1 to DNA, it was concluded that the gestation dependent toxicity of aflatoxin B_1 in the pregnant rat correlates with the increased production of microsomal protein per gram of liver tissue which results in more bioactivation and increased levels of binding to DNA. Thus, it is evident that bioactivation plays a major role in the gestation dependent toxicity of aflatoxin B_1 in the pregnant rat. From here, further investigations into fetal metabolism and maternal metabolism at other specific times in gestation will aid in understanding the role that bioactivation plays in the teratogenic properties of aflatoxin B_1 and other chemical toxins.

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APPENDIX I

Liver Microsome Preparation: The following procedure describes the preparation of rat liver microsomes in order to isolate the liver enzymes involved in metabolism.

Solutions: Microsomal Homogenizing Media (H Buffer)

0.25 M Sucrose and 50 mM Tris Buffer at pH 7.5

1. Sacrifice the animal.
   Open abdominal cavity.
   Perfuse liver with 10 ml of cold H Buffer.
   Weigh liver tissue.
2. Remove liver to cold H Buffer at a concentration of 5 ml H Buffer/gm liver. All operations that follow are performed at 4^{o} C.
3. Mince liver with scissors into small fine pieces. Homogenize with 10 strokes in a loose dounce 40 ml homogenizer.
4. Centrifuge homogenate in a Beckman Preparative Centrifuge (15 minutes; 8800 rpm; 4^{o} C). Remove the supernatant with a pipet being careful not to take any of the solid pellet on bottom or lipid layer on the top. Recentrifuge the supernatant in a Beckman Ultracentrifuge (1 hour; 37,500 rpm; 4^{o} C; Ti 60 Rotor).
5. Discard the supernatant. Wipe off excess fluid on the inside of the tube with a kimwipe. Scrape the microsomal pellet into a 15 ml dounce homogenizer. Add H Buffer at 1 ml/2 gm of total liver tissue. Homogenize with 10 strokes.

6. Let second homogenate sit on ice for 30 minutes to let air escape. Dispense into vials prerinsed with H Buffer. Freeze in 1 ml aliquots at -70°C until further use.

APPENDIX II

Lowry Method for Protein Analysis: The following procedure describes the determination of the amount of protein in the microsomes.

Solutions: Solution A 2% Na_2CO_3 in 0.1 M NaOH

Solution B 0.5% $CuSO_4 \times 5 H_2O$ in 1% Sodium Citrate

Solution C 49 ml Solution A to 1 ml Solution B
Make fresh for each analysis just prior to use.

Solution D 1 Part Folin Ciocalteau reagent to 1 part water
Make fresh for each analysis just prior to use.

Standard 10 mg bovine albumin to 1 ml water, then dilute 1:10 for a 1 mg/ml solution.

Procedure for Standard Curve:

1. Place increasing concentration of the Standard in test tubes. 0.05ml,0.10ml,0.15ml,0.20ml,0.25ml,0.30ml,0.35ml, and 0.40ml.
2. Bring each tube to 1.0 ml volume with water. 0.95ml,0.90ml,0.85ml,0.80ml,0.75ml,0.70ml,0.65ml, and 0.60ml.
3. Time 0: Add 5.0 ml of Solution C to each tube. Mix and wait 10 minutes from Time 0 for Cu to complex.
4. Add 0.5 ml of Solution D to each tube, and mix immediately.
5. Wait 15 minutes from Time 0 and read absorbance at 600 nm.
6. Plot Absorbance vs. Mg protein on a graph. (Should have a linear relationship)

Procedure for Microsomes:

1. Place 0.01 ml of microsome in each test tube.
2. Bring each tube to 1.0 ml volume with water.
3. Follow steps 3-5 on the procedure for the Standard Curve.
4. Extrapolate mg protein for the microsome from the Standard Curve.

Quantitative Determination:

$$\frac{\text{mg protein}}{0.01\ \text{ml}} \times \frac{\text{ml of H Buffer}}{\text{total gm liver}} = \frac{\text{mg protein}}{\text{gm liver}}$$

APPENDIX III

DNA Binding Assay: The following procedure describes the in vitro system used to isolate calf thymus DNA which was used to determine the level of binding of [^{3}H] aflatoxin B$_{1}$ to DNA.

Solutions:	Volumes:
0.05 M Tric-HCl pH 7.5	Variable
0.1 M MgCl$_{2}$	0.75 ml
120 mM Glucose-6-PO$_{4}$	1.05 ml
5 mM NADP	4.00 ml
Glucose-6-PO Dehydrogenase	0.20 ml
Calf Thymus DNA (Type I)	Variable
Microsome Preparation (approx. 15 mg protein/ml)	Variable
[^{3}H] aflatoxin B$_{1}$ in DMSO	0.75 ml
Total Volume	25.00 ml

Procedure:

1. Measure Tris-HCl Buffer, MgCl$_{2}$, and Glucose-6-PO$_{4}$ into a 150 ml ice-cold Erlenmeyer flask previously rinsed with buffer.
2. Just prior to incubation, add the Glucose-6-PO$_{4}$ Dehydrogenase and NADP.
3. Preincubate the flask at 37^{o} C for 5 minutes in a Dubnoff shaker (Approx. 100 oscillations/min.) to generated NADPH.
4. Add the DNA and microsome preparations after 4 minutes of incubation. Add the [^{3}H] aflatoxin B$_{1}$ in DMSO 1 minute later.

5. Terminate the assay after 30 minute incubation by placing the flask in ice. Immediately transfer the material into centrifuge tubes, and cetrifuge for 30 minutes, 39,000 rpm, 4^{o} C, Ti 60 Rotor.

6. Transfer the supernatant into the original cleaned flask and add 1.6 ml 5 M NaCl and 5.0 ml 5% Na dodecyl sulfate (SDS).

7. After a 1 minute incubation at 37^{o} C in the Dubnoff shaker add 32.0 ml of phenol-$CHCl_3$-isoamyl alcohol. Stopper and shake flask.

8. Reincubate the flask for 30 minutes at 37^{o} C in the Dubnoff shaker.

9. Transfer the emulsion to centrifuge tubes and centrifuge for 15 minutes, 15,000 rpm, 4^{o} C.

10. Remove the aqueous epiphase with a pipet and place in an ice-cold beaker. Precipitate and spool the DNA onto a glass rod after adding 100 ml of freezer-cold absolute ethanol to the solution.

11. Store the DNA overnight in ethanol at -70^{o} C.

APPENDIX IV

These procedures and equations exrlain how the level of binding of $[^{3}H]$ aflatoxin B_1 to DNA was determined.

1. Amount of Radioactivity in a DNA Sample:

Procedure:

a) Place a 0.10 ml sample of DNA into a scintillation vial.

b) Add 1.0 ml of water and 12 ml of scintillation fluid.

c) Mix thoroughly and allow to stand overnight.

d) Place in a liquid scintillation counter.

Equations:

a) $$\frac{\text{average count in cpm-background}}{\text{efficiency of machine (0.38)}} = \text{counts in dpm}$$

b) $$\text{count in dpm} \times \frac{\mu Ci}{2.2 \times 10^{2}\ dpm} = \frac{\mu Ci}{0.10\ ml} = \frac{\mu Ci}{ml\ DNA}$$

2. Specific Activity of $[^{3}H]$ aflatoxin B_1 Dose:

Procedure:

a) Place a 0.01 ml sample of $[^{3}H]$ aflatoxin B_1 into a 10ml volumetric flask diluted with methanol.

b) Monitor absorbance of this sample at 362 nm.

c) Take a 0.10 ml sample from step a and dilute with methanol in a 10 ml volumetric flask.

d) Place a 0.10 ml aliquot of this sample in a scintillation vial with 12 ml of scintillation fluid.

e) Allow to stand overnight and then place in a liquid scintillation counter.

Equations:

Absorbance dilution factor = 10

Scintillation dilution factor = 10,000

a) $$\frac{\text{Average count in cpm-background}}{\text{Efficiency of machine (0.425)}} = \text{count in dpm}$$

b) $$\frac{(\text{Count in dpm})(10{,}000)(21{,}100)(4.5\times10^{-10})}{(\text{Average Absorbance at 362 nm})(10)} = \frac{\mu\text{Ci}}{\mu\text{mole AFB}_1}$$

3. Determination of Level of Binding of [^{3}H] aflatoxin B_1 to DNA:

Equations:

a) $$\frac{\mu\text{Ci}}{\text{ml DNA}} \times \frac{\text{ml}}{\text{mg DNA}} = \frac{\mu\text{Ci}}{\text{mg DNA}}$$

b) $$\frac{\mu\text{mole AFB1}}{\mu\text{Ci}} \times \frac{\mu\text{Ci}}{\text{mg DNA}} = \frac{\mu\text{mole AFB1}}{\text{mg DNA}}$$