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Title: EFFECT OF VARIOUS PARAMETERS ON *IN VIVO* AFLATOXIN B_1 BINDING TO RAINBOW TROUT (*SALMO GAIRDNERI*) LIVER DNA

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Aflatoxin B_1 (AFB_1) is a potent liver carcinogen to a number of animal species including rainbow trout (Salmo gairdneri). Microsomal activation is required for the in vitro conversion of AFB_1 to a reactive metabolite, thought to be the AFB_1-2,3-oxide, which will bind nucleic acids, produce toxicity, and cause mutagenesis. Nucleic acid-AFB_1 adduct formation is believed to be an indication of cancer initiation.

Initial time-course and dosage experiments were conducted to establish fundamental binding data in rainbow trout. Dietary casein and fish protein concentrate (FPC) fed at 40, 50, 60, or 70% in the diet, and cyclopropenoid fatty acids (CPFA), were measured for their effect on in vivo AFB_1 binding to trout liver DNA. Both were previously reported to dramatically alter AFB_1 induced hepatocar-

cinogenesis. Rainbow trout and coho salmon (Oncorhynchus kisutch), two Salmonid species varying greatly in their sensitivity to AFB_1, were compared for their relative ability to produce AFB_1-DNA adducts. Correlations between trout liver mixed function oxidase (MFO) activity and AFB_1 binding to trout liver DNA were attempted by use of *B*-napthoflavone, a powerful enzyme inducer in rainbow trout.

Binding of AFB_1 to trout liver DNA over the 48 hour(h) period studied reached a maximum value at 24 h. Increasing AFB_1 dosage produced a linear response in AFB_1-DNA adduct formation. Binding was significantly greater in the 70% casein fed fish than in the 70% FPC group. Binding in the 40, 50, and 60% casein fish was non-significantly greater than the corresponding FPC groups. AFB_1 binding to liver DNA was greatest in each source at the 60% protein level. Binding in rainbow trout was at least 20-fold greater by comparison than in coho salmon. CPFA reduced AFB_1 binding to trout liver DNA at each protein diet employed, although non-significantly. Pretreatment of fish with *B*-napthoflavone reduced the total level of AFB_1 in the liver and AFB_1-DNA adduct formation by 55 and 40%, respectively. Dietary protein and CPFA apparently alter tumor formation through promoter effects since binding was unaffected. The tumor resistance of coho salmon and *B*-napthoflavone pretreated animals is possibly due to reduced initiation of of the toxic lesion.

Effect of Various Parameters on *in vivo* Aflatoxin B_1 Binding to Rainbow Trout (*Salmo gairdneri*) Liver DNA

by

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LITERATURE REVIEW

Activation of Aflatoxin B1 (AFB_1) and Macromolecule Binding

Aflatoxin B_1 (AFB_1) is converted metabolically to a variety of metabolites. The relative quantity of the metabolite produced is species dependent (Campbell and Hayes, 1976). Some of the conversion products are aflatoxin M_1, aflatoxin B_{2a}, aflatoxin P_1, aflatoxin Q_1, aflatoxicol (AFL), and probably AFB_1 2,3-epoxide. Metabolic activation is believed to be a requirement for production of toxic, mutagenic, and carcinogenic effects. This activation leads to a highly reactive species which binds macromolecules such as DNA, RNA, and protein.

In vitro studies using hamster liver microsomes revealed that AFB_1 binding to DNA, tRNA, or polyribonucleotides would not occur if the liver fraction or a NADPH generating system was deleted (Garner, 1973a). Similar evidence using rat liver microsomes for nucleic acid binding was reported by Gurtoo and Bejba (1974) and by Gurtoo and Dave (1975). Essigmann (1977) noted the microsomal fraction must be present for activation and microsomes denatured by boiling would not catalyze binding.

Metabolic activation is also required for AFB_1 mediated toxicity in bacteria. Production of a factor lethal to *S. typhimurium* strains TA 1530 and TA 1531 incubated with AFB_1 required rat liver post-mitochondrial supernatants (PMF), a NADPH generating system and air (Garner, 1971). The liver activity was reportedly localized in the microsomal fraction. AFB_1 alone had no effect on bacterial survival, but

increasing concentrations of PMF in the incubation decreased survival exponentially. Garner and Miller (1972) reported similar findings with S. typhimurium TA 1530 and they also increased bacterial survival six-fold by addition of the nucleophiles RNA and DNA to the incubation. They stated that this indicated the close relationship between the AFB_1 metabolite toxic to bacteria and that which binds nucleic acids. Loss of viability in bacterial lethality assays was noted to require native and fully active microsomes (Garner and Wright, 1973c; Schoenhard, 1975). Schoenhard (1975) reported that rainbow trout PMF added to an AFB_1 incubation reduced the viability of the DNA repair deficient Bacillus subtilis GSY 1057 by 60%.

The same activation requirements for bacterial lethality are also required for bacterial mutagenesis. The forward mutation to 8-azaguanine resistance was reported by Stark (1979) and required rat liver PMF and the usual cofactors. Similar mutagen assays with AFB_1 have been noted using rainbow trout liver microsomes as the activating system (Ahokas, 1977; Stott and Sinnhuber, 1978).

Formation of 2,3-dihydro-2,3-dihydroxy-AFB_1 (dihydrodiol), a presumed detoxification product of AFB_1, was reported by Lin (1978) to be dependent on the presence of rat liver microsomes, a NADPH generating system, and appropriate cofactors.

Following activation of AFB_1, a reactive metabolite is formed which binds readily to macromolecules. The importance of this binding event in carcinogenesis is unknown, but nucleic acid binding occurs readily and is believed to interfere with gene replication and gene expression. Binding to protein is thought to occur non-specifically and be

unimportant. *In vitro* incubations with hamster or rat liver microsomes have been reported to produce AFB_1 binding to calf-thymus DNA 1.5 to 11-times greater than the binding to RNA (Gurtoo and Dave, 1975; Garner, 1973a). *In vivo* studies with AFB_1 administered intraperitoneally (ip) show varied results dependent on sampling time. Binding in the rat and guinea pig 2 to 6 hours (hr) after AFB_1 treatment was approximately equal for DNA and rRNA, but the nucleic acid binding exceeded that to protein 4 to 33-fold on a per milligram basis (Garner, 1975a; Swenson, 1977; Ueno, 1980). The amount of rRNA bound by AFB_1 was reported to increase from 6 to 18 hr after treatment in the rat to where the rRNA binding was 1 to 2 times than that to DNA (Swenson, 1974; Swenson, 1977). Campbell (1975) and Garner (1975a) observed rRNA binding in rats exceeded that to DNA by two-fold at 6 hr, with nucleic acid binding at all times exceeding protein binding by 5 to 10-fold.

The nature of the AFB_1-nucleic acid bond or adduct is presumably covalent and irreversible. Several workers have subjected DNA adducts to a variety of treatments including: Sephadex gel filtration; repeated solvent extraction and precipitation; lysozyme, ribonuclease, and pronase treatment; and other DNA purification steps with only minimal loss of bound radiolabeled aflatoxin (Garner, 1973a; Gurtoo and Bejba, 1974; Gurtoo and Dave, 1975; Stark, 1979).

Considerable indirect evidence has been accumulated as to the nature of the reactive AFB_1 metabolite alkylating specific nucleic acid sites. Lin (1978) and Garner (1973b) reported the major AFB_1 metabolite formed *in vitro* in the absence of added nucleophile with rat and hamster microsomes was the 2,3-dihydro-2,3-dihydroxy-AFB_1 (dihydrodiol). Formation

of the dihydrodiol by water hydrolysis of the AFB_1 2,3-oxide generated during metabolism would be expected. Identification of 2,3-dihydro-2-(N^7-guanyl)-3-hydroxyaflatoxin B_1 (AFB_1-N^7-Gua) as the major radioactive product formed after mild acid hydrolysis of DNA adducts provides further support for *in vitro* epoxide formation (Essigmann, 1977; Lin, 1977). AFB_1-N^7-Gua was also noted as the major DNA adduct formed in cultured human bronchus or colon cells (Autrup, 1979). Identification of the dihydrodiol (Swenson, 1974; Swenson, 1977) or AFB_1-N^7-Gua (Lin, 1977; Croy, 1978; Croy et al., Unpub.) as the major products formed *in vivo* has been reported using rats, hamsters, and rainbow trout. The severity of acid and alkali conditions to which the DNA preparation is exposed determines which of these compounds is identified. Most of the label from bacterial mutagenesis assays using ratioactive AFB_1 eluted at the AFB_1-N^7-Gua peak (Stark, 1979). In these reports, formation of the AFB_1-N^7-Gua would likely occur as a result of formation of the electrophilic AFB_1 2,3-oxide and subsequent attack at the nucleophilic nitrogen of guanine bases. Garner and Miller (1972) reported that aflatoxin metabolites containing the 2,3 double bond produce the greatest lethality in bacterial lethality assays. Aflatoxin B_1 2,3-dichloride, synthesized as an appropriate model of AFB_1 2,3-oxide (Swenson, 1975), was reported to produce identical toxicity and mutational spectra as AFB_1 in *Drosophila* (Fahmy, 1978).

Alteration of AFB1 Activation

Various factors alter the activation of AFB_1 and binding or alkylation of specific macromolecular sites. Understanding these factors and

their correlation with toxic or carcinogenic responses will aid in evaluating the importance of monitoring carcinogen binding to cellular macromolecules.

Microsomes from phenobarbitone (PB) pre-treated rats were reported to greatly reduce survival in bacterial lethality assays with AFB_1 (Garner and Miller, 1971). Garner and Wright (1973c) noted similar results with PB, and they also observed that 3-methyl cholanthrene (3-MC) pretreatment or the addition of various nucleophiles or cyclohexene oxide to the incubation mixture did not affect bacterial survival. Microsomes from PB treated rats were reported to increase AFB_1-DNA binding two-fold *in vitro*, and as stated above for a bacterial assay, 3-MC or cyclohexene oxide did not alter binding (Gurtoo and Bejba, 1974). Gurtoo and Dave (1975) reported identical *in vitro* findings with PB and 3-MC pretreatment, and also SKF 525-A addition to the incubation mixture inhibited binding significantly. Lin (1978) reported that PB increased dihydrodiol formation four-fold with rats and two-fold with hamsters in the absence of added nucleophile *in vitro* compared to the untreated controls. In addition, Lin observed that 3-MC produced no increase of dihydrodiol production and SKF-525A largely inhibited its formation. *In vivo* effects are in marked contrast to *in vitro* findings, probably due to the complexity of the activation and detoxification pathways in the whole animal. Garner (1975b) noted that PB pretreatment greatly reduced AFB_1-nucleic acid adduct formation of intraperitoneally (ip) administered AFB_1 to levels comparable to protein-bound carcinogen, while protein binding was unaffected by PB. Similar *in vivo* results were reported, with PB decreasing binding 9-fold, 7-fold, and 3-fold to rat liver DNA,

rRNA, and protein, respectively (Swenson, 1977). Swenson noted in a separate feeding study that rats administered PB in the drinking water showed dramatically reduced AFB_1-induced hepatocarcinogenesis.

Flavones have also been cited to affect AFB_1 activation. Swartz and Rate (1979) reported that quercetin and tangeretin, two naturally occurring flavones, inhibited the binding of ^3H-AFB_1 to cellular DNA of cultured rat liver epithelial-like cells when added in culture.

Species differences can also affect AFB_1 binding. AFB_1 treatment ip resulted in in vivo nucleic acid binding 4-5 times greater in rat liver than in hamster liver at 2-6 hr, but protein binding was similar in both (Garner and Wright, 1975a). Confusingly, in vitro incubations with hamster liver microsomes were reported to yield 5 times more dihydrodiol than rat liver microsomes in the absence of added nucleophile (Lin, 1978). Ueno (1980) reported that ^{14}C-AFB_1 treatment produced somewhat greater in vivo AFB_1-DNA binding in rat liver than in the guinea pig, but guinea pig RNA and protein binding significantly exceeded that in the rat. In addition, Ueno noted all rat and guinea pig macromolecule binding exceeded that in the mouse by approximately 10-fold. Garner and Miller (1972) reported the following order of increasing microsomal lethality on incubation of AFB_1 with S. typhimurium: guinea pig > rats > hamsters > and mice.

Diet and sex differences have also been noted to alter AFB_1 activity. Lipotrope-deficient diets, previously reported to enhance hepatocarcinogenesis (Rogers, 1969), were observed by Campbell and Hayes (1978) to reduce DNA and RNA-AFB_1 adduct formation at 6 hr after ip AFB_1 treatment while not affecting protein binding. Male rats, known to be more

sensitive than females to the carcinogenic effects of AFB_1, were noted by Gurtoo and Motycka (1976) to produce 2-3 times more *in vitro* DNA binding in four different strains of rats. *In vivo* evidence comparing the binding in male, female, and testosterone treated females was also presented in support of this *in vitro* evidence.

Dietary Protein and AFB_1

The level and source of dietary protein appear to play a definite role in aflatoxin induced toxicity and carcinogenicity. However, correlations between liver drug metabolizing activity and dietary protein are unclear.

Early work noted that protein-deficient rats given aflatoxin daily for 20 days developed liver lesions typical of aflatoxin injury while the control protein group exhibited only mild changes (Madhavan, 1965). Madhavan (1968) later reported that 50% of the high protein-fed rats given aflatoxin orally developed hepatomas or other organ cancer at one year, while none of the low protein group developed hepatomas. Lee (1977) noted similar results at 12 months in rainbow trout fed 32 or 49.5% fish protein concentrate (FPC) at various levels of AFB_1. The high protein FPC group produced more hepatomas than the 32% group at all AFB_1 levels. However, casein-fed fish at these two protein levels produced similar liver cancer incidences. Hendricks et al. (Unpub. data) reported that rainbow trout fed AFB_1 and either 40, 50, 60, or 70% protein, from FPC or casein, produced an increased liver tumor incidence at 10 months with increasing protein levels of either source. In addition, FPC produced a greater hepatoma incidence than casein at each corresponding protein

level. In contrast, rats and young pigs fed low and optimal protein diets and administered aflatoxin exhibited greater toxic and carcinogenic effects with the low protein diets (Sisk, 1972; Temcharoen, 1978).

In a review of dietary protein effects, Campbell and Hayes (1974) reported that rats fed 5% casein exhibited a two-fold decrease in liver microsomal ethylmorphine and aniline activity, along with less microsomal protein and depressed cytochrome P-450 values relative to rats fed 20% casein. Kato (1968) noted similar results in both male and female rats. Correlating liver mixed function oxidase (MFO) activities and dietary protein in rainbow trout with AFB_1-induced hepatocarcinogenesis is difficult. Stott and Sinnhuber (1978; 1979) reported that increasing dietary protein decreased activity of both AFB_1 activation and detoxification enzymes, but increased AFB_1 to AFL conversion. Sachan (1975) using microsomes from low, normal, and high protein fed rats reported no trends in MFO activities. A decrease of *in vivo* AFB_1 activation in low protein (5% casein) fed rats compared to a 20% group was noted by a 70% decrease in AFB_1 binding to chromatin, DNA, and chromatin protein (Preston, 1976).

Cyclopropenoid Fatty Acids (CPFA) and AFB_1

CPFA are naturally occurring fatty acids containing a cyclopropene ring and present as triglycerides in the seed oils of plants such as cotton and kapok (Sinnhuber, 1976). "Pink white" and other egg disorders arose from the ingestion by hens of leaves or seeds from plants containing CPFA, and is believed to occur due to increased yolk membrane permeability (Shenstone, 1965). CPFA are the only naturally occurring compounds which give a true Halphen test, require the cyclopropene ring

for biological activity, and are most abundantly found in Sterculia foetida (SF) oil. The two major CPFA are malvalic acid (C_{18}) and sterculic acid (C_{19}), with the latter predominating 10:1 in SF oil.

Ingestion in the diet of CPFA alters lipid metabolism and normal body growth. An early report by Ellis (1931) noted that ingestion of cottonseed oil by hogs resulted in firm body fat with high melting point and high saturated acid content. SF oil (50-70% CPFA of the total acids) at 2-5% in the diet was fed to rats and reported to retard growth, increase liver to body weight ratios, increase lipid saturation, alter membrane integrity, and reduce rat pup survival (Nixon, 1974a; Nixon, 1977a). Schneider (1968) noted that dietary SF levels above 5% produced death in rats. Considerable effects have been reported in rainbow trout. Methyl sterculate feeding has been observed to reversibly increase stearic acid levels of liver triglyceride and phospholipid four and three-fold, respectively, and to significantly increase the lipid saturation to unsaturation ratios (Roehm, 1968; Roehm, 1970). Struthers (1975a) reported CPFA feeding to decrease [^{32}P]-PO_4 incorporation into trout liver lipids and to alter the distribution of perfused ^{14}C-oleic acids in the various lipid fractions obtained by centrifugation. Increases in total liver fat and liver to body weight ratios were noted in rainbow trout, with decreased liver protein content and a reduction in certain liver enzyme activities (Malevski, 1974a). Cell damage to trout resulting from CPFA feeding has been evaluated histologically and been reported to include: liver necrosis, kidney tubule degeneration, reversible liver parenchymal cell injury, olive-brown droplets in many tubule epithelial cells, unusual glycogen deposition, liver cytoplasmic "fibers",

and irreversible bile duct and blood vessel hyperplasia (Nixon, 1974a; Malevski, 1974b; Struthers, 1975b).

Nixon (1977b) reported that ^{14}C-sterculic acid was readily absorbed from the gastrointestinal tract of rats, and most of the label was excreted in the urine as short chain dicarboxylic acids (Eisele, 1977). Eisele postulated that sterculic acid must be transported through both mitochondrial and microsomal systems for oxidation to these products.

Rainbow trout liver microsomes from CPFA fed fish had decreased cytochrome (cyt) P-450 and cyt b_5 content, and decreased NADPH-cyt c reductase activity compared to controls (Eisele, 1978; Loveland, 1979). These fish were also noted to have induced benzo(α)pyrene aryl hydrocarbon hydroxylase activity, lowered ability to convert AFB_1 to aflatoxicol, lower microsomal protein levels, and a ten-fold decrease in aldrin epoxidation activity.

CPFA have been implicated as powerful tumor growth promoting agents. They were reported to be cocarcinogens when incorporated with AFB_1 into rainbow trout diets as SF or Hibiscus syriacus oil because they promoted a large increase in hepatoma incidence and tumor growth compared to AFB_1 fed controls (Sinnhuber, 1966; Lee, 1967; Sinnhuber, 1968; Lee, 1968). This synergistic effect was reported to be very weak, if present at all, in rats (Nixon, 1974b). Methyl sterculate was noted to increase tumor incidence and promote tumor growth significantly more than methyl malvalate (Lee, 1971; Lee, 1972; Sinnhuber, 1974).

CPFA also exhibits a synergistic effect on carcinogenesis of other AFB_1 metabolites. Both aflatoxin Q_1, the major in vitro metabolite of AFB_1 with monkey and human microsomes, and aflatoxin M_1, the hydroxylated

metabolite of AFB_1 found in animal milk, promote more tumors when fed together with CPFA (Sinnhuber, 1974; Hendricks, 1980).

CPFA have also been reported to be carcinogens themselves when fed as sterculic acid to rainbow trout (Sinnhuber, 1976).

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INTRODUCTION

Aflatoxin B_1 (AFB_1) is a potent liver carcinogen to a number of animal species (Wogan, 1973). Mt. Shasta strain rainbow trout (Salmo gairdneri) are extremely sensitive to the hepatocarcinogenic effects of AFB_1 and provide an excellent animal model for studying this mycotoxin (Sinnhuber, 1977).

Microsomal activation is required for the *in vitro* conversion of AFB_1 to a metabolite that will bind nucleic acids (Garner, 1973a; Gurtoo, 1974), reduce bacterial viability (Garner, 1971; Garner, 1972; Schoenhard, 1975), cause mutagenesis (Stark, 1979; Stott, 1978), and undergo detoxification to the 2,3-dihydro-2,3-dihydroxy-AFB_1 (Lin, 1978). *In vivo* macromolecule binding of AFB_1 occurs to a much greater extent with nucleic acids than with protein (Garner, 1975a; Campbell, 1975; Swenson, 1977; Ueno, 1980) and has been suggested to be a better indicator of cancer susceptibility than binding to protein (Swenson, 1977). Further evidence for the importance of nucleic acid binding is that phenobarbitol treatment of rats decreases susceptibility to AFB_1 induced hepatocarcinogenesis and decreases *in vivo* AFB_1 binding to DNA, while protein binding is unaffected (Garner, 1975b; Swenson, 1977). Considerable indirect evidence has implicated AFB_1 2,3-oxide as the reactive species binding DNA, and the nucleophilic N^7 position of guanine as the major site of attack (Essigmann, 1977; Autrup, 1979; Croy, 1978; Stark, 1979; Fahmy, 1978).

Many factors can significantly alter AFB_1 induced toxic and carcinogenic effects. Both the level and source of dietary protein affect hepatoma development in rats (Madhavan, 1965; Madhavan, 1968) and in

rainbow trout (Lee, 1977; Hendricks, Unpub.). Low protein-fed rats decrease the *in vivo* binding of AFB_1 to chromatin and DNA compared to controls fed adequate protein (Preston, 1976). Cyclopropenoid fatty acids (CPFA), naturally occurring fatty acids present in the seed oils of plants such as cotton and kapok, also markedly alter AFB_1 induced hepatocarcinogenesis (Sinnhuber, 1976). CPFA are powerful synergists increasing tumor incidence and growth when incorporated into rainbow trout diets with AFB_1 (Sinnhuber, 1966; Lee, 1967; Sinnhuber, 1968; Lee, 1968), aflatoxin M_1 (Sinnhuber, 1974), aflatoxin Q_1 (Hendricks, 1980), and are carcinogenic when fed alone to trout (Sinnhuber, 1976). Species differences drastically affect the hepatoma incidence resulting from AFB_1 treatment. The magnitude of AFB_1-nucleic acid binding in the rat, hamster, and mouse correlates well with the relative susceptibility of each species to AFB_1 induced hepatocarcinogenesis (Garner and Wright, 1975a; Ueno, 1980).

The purpose of this investigation was to measure *in vivo* binding of AFB_1 to trout liver DNA to: (a) determine the influence of source and level of dietary protein on AFB_1 binding, (b) evaluate the impact of CPFA on AFB_1 binding, (c) compare it with AFB_1 binding in coho salmon (*Oncorhynchus kisutch*), a species with different susceptibility to AFB_1-induced carcinogenesis than rainbow trout, and (d) observe the effect of β-napthoflavone, a dramatic inducer of liver mixed function oxidase (MFO) activity in rainbow trout, on binding (Haight, Unpub.; Statham, 1978).

MATERIALS AND METHODS

Aflatoxin Solutions

Aflatoxin B_1-H^3(AFB_1-H^3) was administered by intraperitoneal (ip) injection at 50 µg AFB_1/kg body weight (bw) and 0.35-0.45 µCi/µg AFB_1 in 0.05-0.25 ml of 95% ethanol. This dosage was followed except for experiments 1 and 2. Injection solutions were made by mixing unlabeled AFB_1 (grade A-Calbiochem, San Diego, CA) with AFB_1-H^3 (15Ci/mmol-Moravek Biochemicals, City of Industry, CA), evaporating off the solvent, and diluting with 95% ethanol. AFB_1-H^3 was 98% radiochemically pure as determined by radiochromatography. Chemical purity of each AFB_1 shipment was confirmed by ultraviolet (UV) scanning and by thin layer chromatography (TLC).

Animals and Diets

Mt. Shasta strain rainbow trout (Salmo gairdneri) reared and grown from brood stock at our Fish Nutrition and Toxicology Lab (Corvallis, OR) or coho salmon (Oncorhynchus kisutch) eggs obtained locally were used. Rainbow trout in all experiments were fed Oregon Test Diet (Table 1) until the dietary changes indicated below were initiated or until ip injection. Coho salmon in experiment 5 were fed Oregon Moist Pellet (Table 2) until treatment with AFB_1.

Experiment 1 was conducted to follow the time-course binding in rainbow trout. Ten month old trout weighing approximately 45 grams (g) were treated with AFB_1 (84 µg/kg bw), fasted for either 4, 12, 24, or 48 hours (hr) and then sacrificed. In experiment 2, the effect of AFB_1

dosage on total DNA binding was determined. Trout at 11 months of age and weighing approximately 53 g were administered AFB_1 at either 5, 25, 100, or 300 µg/kg bw, starved for 24 hr, and then sacrificed. Experiment 3 was carried out to determine the influence of both source and level of dietary protein on AFB_1 binding. Trout at 6 months of age were divided into separate tanks and fed 40, 50, 60, or 70% semipurified protein diets from two different protein sources, fish protein concentrate (FPC) or casein (Table 3). After 5 months on these diets, trout weighing 58-71 g were treated with AFB_1, starved 24 hr, and then sacrificed. In experiment 4, the variability in binding between individual fish was estimated. Trout 12 months of age, weighing 95-121 g, and remaining on Diet 56 from experiment 3 were used. These previously untreated fish were individually weighed and administered AFB_1, starved for 24 hr, and then sacrificed. In experiment 5, trout and coho salmon 13 months of age and weighing approximately 74 and 61 g respectively were studied to compare the DNA binding in different Salmonid species. Both groups of fish were individually weighed and treated with AFB_1, starved for 24 hr, and then sacrificed. CPFA influence on AFB_1-DNA adduct formation was determined in experiment 6. Trout 14 months of age remaining from experiment 3, and weighing 95-145 g were used. These fish were fed either Diet 54, 57, 64, or 67 for 7 months and were equally divided into two tanks from each diet. CPFA were incorporated into the diet at 100 ppm with the salmon oil in one group from each protein diet, and fed from 23-32 days prior to AFB_1 treatment. The CPFA-untreated group represented the control. Each CPFA-fed group and its respective control were simultaneously administered AFB_1, starved for

24 hr, and then sacrificed. The influence of the synthetic β-napthoflavone on AFB_1 binding was studied in experiment 7. Two groups of trout 14 months in age and weighing 80-125 g were used. They were individually weighed and injected ip with either β-napthoflavone (Aldrich Chem. Co.) at 100 mg/kg bw in 0.27-0.43 ml of corn oil or with 0.3 ml of corn oil. Both groups were starved exactly 4 days and treated with AFB_1. They were starved an additional 24 hr and then sacrificed.

Preparation of Tissue

Except for experiment 1, all fish were killed by a cranial blow 24 hr after injection with AFB_1. Livers were excised and immediately perfused with 0.9% NaCl and then nuclei isolated according to the method of Hymer and Kuff (1964). Whole livers were homogenized in 9 ml of 0.25M sucrose, 3 mM MgCl per gram of liver by use of a Potter-Elvehjem homogenizer fitted with a teflon pestle. Homogenates were then filtered through two layers of coarse cheese cloth and centrifuged at 900 x g for 15 min. (Sorvall, SS-34 rotor). The supernatant was discarded and the sediment resuspended in a solution of 0.5% Triton-X-100 (w/v), 0.25 M sucrose, and 1 mM MgCl using a transfer pipette. The suspension was spun at 900 x g for 10 min. and the crude nuclear pellet washed two additional times with the Triton solution. Time of exposure of the nuclei to the detergent should be minimized. The nuclear sediment was gently dispersed in 0.25 M sucrose, 1 mM MgCl solution for the final nuclear preparation.

Extraction and Purification of DNA

DNA was extracted from the final nuclear preparation and purified as described by Marmur (1961) and modified by Croy (1978). Briefly, nuclei were resuspended in 0.05 M Tris-HCl, pH 6.5, and appropriate volumes of 4M NaCl (in 0.05M Tris, pH 6.5) and 5% sodium dodecyl sulfate (in Tris buffer) were added to achieve final concentrations of 1M NaCl and 1% sodium dodecyl sulfate (SDS). After addition of 4M NaCl, clumps were broken by agitation and the solution was warmed to room temperature. SDS was slowly added with swirling and the solution was gently mixed for 5-10 min. To this solution of DNA (0.2-0.3 mg/ml), one volume of $CHCl_3$: isoamyl alcohol (24:1 v/v) was added and the resulting biphasic solution extracted vigorously by shaking 10-15 min. at room temperature. The resulting emulsion was centrifuged at 7000g for 10 min. to separate the $CHCl_3$:alcohol, and the aqueous phase was removed and extracted again. The final aqueous phase was carefully removed and cooled on ice. Precipitation of nucleic acids was achieved by addition of 3 volumes of ice cold ethanol. The fibrous precipitate was spooled onto glass rods and dissolved in 0.05M Tris-HCl, pH 6.5. This DNA solution was adjusted to 0.1M NaCl with 4M NaCl, ribonuclease (Worthington code R) was added to a final concentration of 20-30 µg/ml, and the mixture was incubated at 37 degrees for 30 min. Sufficient pronase (B grade, Calbiochem-Behring) was then added to the ribonuclease solution for a final pronase concentration of 220 µg/ml and then incubated at 37^{o} an additional 2 hr. The solution was adjusted to 0.9M NaCl with 4M NaCl and extracted twice with $CHCl_3$:isoamyl alcohol. DNA was precipitated from the aqueous phase, washed in ethanol, and dried *in vacuo* over P_2O_5. The purified DNA was

hydrolyzed in 0.5N perchloric acid at 80° for 15 min. (Garner and Wright, 1975).

Quantitative Determinations

Liver AFB_1-H^3 levels were determined by solubilization of 0.2 ml aliquots from whole liver homogenates in Soluene-350 (Packard) and counting in Dimilume-30 (Packard). Liver AFB_1 levels were calculated by dividing the liver radioactivity (dpm/g liver) by the specific activity (SA) of the injection solution (dpm/nmoles AFB_1). Radioactivity of DNA hydrolysates was obtained by counting aliquots in Aquasol (New England Nuclear). A measure of covalent binding, AFB_1-DNA adduct formation, was calculated by first converting radioactivity in the DNA hydrolysate to nmoles AFB_1 by dividing the amount of radioactivity by the SA of the injection solution. Binding was expressed as dmoles (10^{-10}moles) AFB_1 per mg DNA. The fraction of aflatoxin present in the liver bound to DNA was expressed by: total liver DNA dpm divided by the total liver dpm. Variability of the DNA isolation and purification technique was analyzed by measuring the binding of three separate aliquots from the same whole liver homogenate. Radioactivity was measured in a Beckman LS 7500 scintillation counter.

DNA hydrolysates were quantitated by the diphenylamine reaction (Burton, 1956), RNA contamination by the orcinol reaction (Ceriotti, 1955), and protein contamination by the Lowry-Folin procedure (1951). AFB_1 solutions were quantitated in 95% ethanol using a molar extinction coefficient of E(362 nm) = 21,800 O.D./Mcm. All colorimetric reactions

and UV absorbance readings were determined in a Beckman Acta CIII spectrophotometer.

RESULTS

Effect of time on binding of AFB_1 to trout liver DNA. To assess relationships between the covalent binding of reactive metabolites to macromolecules and toxicities, the covalent binding should be determined when it is maximal (Gillette, 1974). Studies were conducted to evaluate the time-course of covalent binding of AFB_1 to trout liver DNA by measuring binding at 4, 12, 24, and 48 hr after AFB_1 treatment. Covalent binding was maximal at 24 hr after AFB_1 administration, but there was no significant difference between any of the sampling times (Table 4).

Effect of AFB_1 dose on covalent binding. To evaluate dose-binding relationships, studies were undertaken using four different AFB_1 dose levels. The dose levels employed were 5, 25, 100, and 300 μg AFB_1/kg bw. Minimum doses for realistic metabolic challenges, and significant binding for optimum experimental analysis were criteria used in selection of the dose range. Almost a linear increase in binding was determined with increasing dose levels (Graph 1) and binding was significantly different at all doses (Table 5).

Dietary protein effects on AFB_1 binding. Dietary protein had recently been reported in this laboratory (Hendricks, Unpub.) to dramatically affect the incidence of liver tumors in rainbow trout. Binding studies were conducted with trout fed the identical diets employed by Hendricks. The diets consisted of casein or FPC as the protein source, and fed at a level of 40, 50, 60, or 70% protein in the diet. Binding of AFB_1 to trout liver DNA was significantly greater in fish fed 70% casein than with fish fed 70% FPC as a protein source (Table 6). Binding

in the 40, 50, and 60% casein fish was greater than each corresponding FPC group, but they were not significantly different. The 60% protein level from each source produced the greatest binding, but there were no significant differences between casein levels. Sixty percent FPC produced significantly greater binding than either the 50 or 70% FPC groups. However, no trends were observed due to the high value obtained for the 40% FPC group.

Binding variability among individual fish treated with AFB_1. The studies noted above were conducted with samples of pooled livers. To evaluate the possible genetic heterogeneity among the rainbow trout population studied, as has been noted for mice (Levitt, 1979), individual fish were treated and analyzed for adduct formation. The coefficient of variation was 36%, with a range of 1.62 to 4.65 dmoles AFB_1/mg DNA. This variation was computed by dividing the standard deviation by the mean.

Comparison of AFB_1-DNA adduct formation in rainbow trout and coho salmon livers. Sockeye salmon and coho salmon embryos have been reported to be more resistant to the hepatocarcinogenic effects of AFB_1 than rainbow trout (Hendricks, Per. comm.; Wales, 1972). To further investigate this, AFB_1 binding to DNA in rainbow trout and coho salmon was determined. Binding in trout was approximately 20-fold greater than in salmon (Table 7).

CPFA and the effect on AFB_1 binding to rainbow trout DNA. The influence of CPFA on AFB_1 binding was studied because of the cocarcinogenic reports noted in rainbow trout above. Also incorporated with the CPFA variable were two levels and sources of dietary protein. Rainbow trout fed CPFA were reported to produce two-fold more liver tumors with casein

rather than FPC as a protein source, and increased protein content was also noted to increase tumor incidence (Nixon, Unpub.). AFB_1 present in the liver at 24 hr was found to be greater for the CPFA treated fish at 40 and 70% FPC and at 70% casein compared to controls (Table 8). However, this difference was non-significant. The liver AFB_1 levels for the 40% casein control was non-significantly greater than the CPFA treated group. Liver AFB_1 levels were non-significantly greater for each casein treatment compared to the corresponding FPC treatment, except for the 40% CPFA comparison. AFB_1 binding to liver DNA was somewhat less for each CPFA treated group than in the untreated controls. However, these differences were non-significant in each case. Binding was greater for each casein treatment compared to the corresponding FPC treatment, except for the 40% CPFA group. Again, these differences were non-significant. Calculations were also performed from this study on the amount of AFB_1 present in liver tissue at 24 hr from 24 samples relative to the administered dose. Of the total administered ^3H-AFB_1 dose, 15.4% (range: 10.7-21.5) was present in the liver and 3.1% (range: 2.3-5.0) was covalently bound to DNA. Approximately 20.4% (range: 15.1-25.3) of the total ^3H-AFB_1 in the liver was bound to DNA.

β-napthoflavone pretreatment and its effect on in vivo AFB_1 binding to rainbow trout liver DNA. Beta-napthoflavone pretreatment of rainbow trout dramatically increases MFO activity (Statham, 1978; Haight, Unpub.). AFB_1 activation and detoxification is believed intimately related to such activity. This study was conducted to look for decreased AFB_1 binding to DNA, because previous *in vivo* studies have reported that increased MFO activity protects against challenge by chemical carcinogens

(Wattenberg, 1975). AFB_1 present in the liver was reduced two fold by β-napthoflavone (Table 9). Pretreatment with β-napthoflavone also significantly reduced binding to DNA compared to the untreated controls. Variability of binding as a result of isolation procedures was determined in this experiment and calculated to be 1.2%.

DISCUSSION

As stated by Gillette (1974), covalent binding to macromolecules in combination with toxicity studies provides a screening tool for substances that evoke toxicity through the formation of reactive metabolites. In addition, binding studies will aid in understanding if various factors increase the carcinogenic potential of a chemical carcinogen such as AFB_1 through initiation or promotion of the toxic lesion.

The measured binding of AFB_1 to rainbow trout liver DNA over the 48 hr period monitored was approximately constant. This is in marked contrast to the maximum binding observed from 2-6 hr in the rat which was followed by a rapid removal of bound AFB_1 between 6 and 24 hr (Garner, 1975a). Two possibilities become immediately evident: (1) Most of the binding occurs earlier than 4 hr after AFB_1 treatment and removal of adducts is extremely slow, (2) Excision repair or removal of bound AFB_1 occurs at a rate approximately equal with covalent binding to DNA. If total DNA binding is an indicator of cancer initiation, the longer exposure time of rainbow trout would appear to place them at greater risk to the hepatocarcinogenic activity of AFB_1 than rats. Rainbow trout in fact are the most sensitive animals known to the hepatocarcinogenic effects of AFB_1. From this experiment, 24 hr sampling time was used in subsequent work to compare the effect of various parameters on binding. The almost linear dose-binding curve (Graph 1) indicates that binding sites for AFB_1 were not saturated in the dose range employed. The slightly upward bend of this curve could possibly indicate an inductive effect caused by increasing dosage, with greater activation and subsequent binding.

Dietary protein of varied source and level produced no dramatic trends on AFB_1 binding. Dietary protein had recently been observed to alter AFB_1 induced hepatocarcinogenesis in rainbow trout (Lee, 1977; Hendricks, Unpub.). Interestingly, dietary casein at each protein level produced greater DNA binding than the corresponding FPC group. This was in marked contrast to the AFB_1 feeding studies above, where FPC at each protein level produced more tumors. No trends of increased adduct formation with increasing dietary protein were evident. Increasing dietary protein had previously been reported to increase both tumor incidence and DNA binding (Madhavan, 1968; Lee, 1977; Hendricks, Unpub.; Preston, 1976). A possible explanation for the protein effects is that promotion rather than initiation is affected. In this function, activation and binding would be unaffected, but factors after the carcinogenic event (e.g., alkylation of specific sites on DNA) would be altered by an unknown mechanism to increase or decrease tumor growth. Further evidence for the promotional influence of protein on carcinogenesis was reported in rainbow trout embryo exposure experiments (Hendricks, Unpub.). In these studies, trout were exposed to AFB_1 only as embryos, but after swimup increasing dietary protein was observed to increase tumor incidence. Also, the age of exposure of the trout to AFB_1 (11 months) did not correspond with the age class employed (5 months) in the AFB_1 feeding study by Hendricks. Toxification pathways that modify activation and binding may vary radically with age.

CPFA reduced binding of AFB_1 to DNA with each protein variable studied. The importance of this is enhanced by the observation that CPFA treatment increased liver AFB_1 levels in three out of four

comparisons. Reduction in DNA binding by CPFA treatment was non-significant, but this was probably due to their short term (3-4 weeks) incorporation into the trout rations. A reduction in AFB_1-DNA adduct formation by CPFA has been previously noted at this laboratory (Nixon, Personal comm.). As was stated above for protein effects on tumor development, CPFA may possibly produce their synergistic action with AFB_1 by acting as promoters. This does not explain the carcinogenic ability of CPFA when fed alone. The older age class fish employed (14 months) compared to previous feeding studies (2-6 months) may have some influence. CPFA alteration of membrane structure may be responsible for reduced microsomal cyt P-450 and protein content, and reduced MFO activity reported with CPFA feeding (Eisele, 1978; Loveland, 1979). Therefore, reduced microsomal activation presents an explanation for the decreased *in vivo* AFB_1-DNA binding noted in this study.

The 20-fold higher binding in rainbow trout compared with coho salmon suggests that reduced alkylation of DNA is an explanation for the resistance of coho to AFB_1 hepatocarcinogenesis. However, sampling conditions used were those previously established for rainbow trout. Data on time-course binding, and on liver uptake and removal of AFB_1 would greatly aid the further understanding and significance of this difference.

The large decrease in AFB_1 binding with β-napthoflavone pretreatment correlated well with the tremendous inductive effect on MFO activity noted in previous studies with rainbow trout liver. The simultaneous 2-fold reduction in liver AFB_1 levels with such treatment suggests that increased detoxification activity has enhanced conversion of AFB_1 to a

readily excretible metabolite. However, several fish pretreated with the water insoluble β-napthoflavone exhibited a clear gelatinous material covering some internal viscera at sacrifice time. The possibility exists that reduction in liver AFB_1 levels and binding resulted from such a material posing a mechanical barrier to uptake. The voluminous blood supply to the intraperitoneal cavity and the good membrane permeability of AFB_1 makes the latter alternative a remote possibility.

The results of this study indicate that dietary protein and CPFA produce promotional effects to increase AFB_1 induced hepatocarcinogenesis. However, binding studies done here were conducted at one small point in time and this factor puts limitations on such a hypothesis. Further study with coho salmon, a Salmonid species whose reduced AFB_1 activation and DNA binding is possibly responsible for the resistance of this animal to AFB_1 induced hepatocarcinogenesis, and with β-napthoflavone, a synthetic drug which provides tumor protection in several animal species (Wattenberg, 1975) apparently through altered activation and detoxification pathways, could provide important information in understanding mechanisms of chemical carcinogenesis.

TABLE 1-THE OREGON TEST DIET

FORMULATION	PERCENT
CASEIN (VITAMIN-FREE)	49.5
GELATIN	8.7
DEXTRIN	15.6
MINERAL MIX	4.0
CARBOXY METHYL CELLULOSE	1.0
ALPHA-CELLULOSE	8.2
CHOLINE CHLORIDE(70%)	1.0
VITAMIN MIX	2.0
SALMON OR HERRING OIL	10.0
	100.0

TABLE 2-OREGON MOIST PELLET (OMP)

FORMULATION	PERCENT
HERRING OIL/anti	6.0
CHOLINE CHLORIDE (70%)	0.5
SHRIMP (WHOLE)	35.0
HERRING MEAL	40.0
OREGON VITAMIN PREMIX	1.5
COOKED OAT GROATS	10.0
PREGELATINIZED TAPIOCA STARCH	7.0
	100.0

TABLE 3. PROTEIN DIETS
PROTEIN SOURCE - CASEIN

Ingredient	40% Protein (Diet 54)	50% Protein (Diet 55)	60% Protein (Diet 56)	70% Protein (Diet 57)
Casein	30.8	42.0	52.0	62.0
Gelatin	7.7	8.0	8.0	8.0
Dura-gel	6.0	6.0	6.0	6.0
Dextrin	15.3	15.9	6.5	0.0
α-Cell	10.7	4.1	6.5	6.0
Min.Mix	4.0	4.0	4.0	4.0
CMC	1.0	1.0	1.0	1.0
Choline Cl_2	1.0	1.0	1.0	1.0
Arg	0.6	---	---	---
L-Cys	0.2	---	---	---
Met	0.4	---	---	---
D.L.-Try	0.3	---	---	---
Vitamin Mix	2.0	2.0	2.0	2.0
Salmon Oil	20.0	15.0	12.0	9.0
	100.0%	100.0%	100.0%	100.0%

PROTEIN SOURCE - FPC

Ingredient	40% Protein (Diet 64)	50% Protein (Diet 65)	60% Protein (Diet 66)	70% Protein (Diet 67)
FPC	32.0	42.0	52.0	62.0
Gelatin	8.0	8.0	8.0	8.0
Dura-Gel	0.0	0.0	0.0	0.0
Dextrin	21.3	21.9	12.5	6.0
α-Cell	12.2	6.6	9.0	8.5
Min. Mix	2.5	2.5	2.5	2.5
CMC	1.0	1.0	1.0	1.0
Choline Cl_2	1.0	1.0	1.0	1.0
Vitamin mix	2.0	2.0	2.0	2.0
Salmon Oil	20.0	15.0	12.0	9.0
	100.0%	100.0%	100.0%	100.0%

TABLE 4-Effect of time after [H^3]-AFB_1 treatment on AFB_1 binding to trout liver DNA *in vivo*

Time after Treatment (h)	Binding[a] dMoles AFB_1 ($\times 10^{-10}$ Moles)/mgDNA
4	2.01 ± .462[b]
12	2.18 ± .234
24	2.63 ± .555
48	2.51 ± .213

[a]Seven pooled livers were used for each sample , n=3

[b]Mean ± Standard Deviation

TABLE 5-Effect of [H^3]-AFB_1 dose on AFB_1 binding to trout liver DNA *in vivo*

Dose($\mu gAFB_1$/Kg body weight)	Binding[a] dMoles AFB_1 ($x10^{-10}$Moles)/mgDNA
5	0.0923 ± .00417[b,c]
25	0.556 ± .0495[c]
100	3.15 ± .192[c]
300	12.4 ± .270[c]

[a]At least 6 pooled livers were used for each sample, n=3

[b]Mean ± Standard Deviation

[c]Significantly different, $p < .05$(students t-test)

TABLE 6-Effect of dietary protein on AFB_1 binding to trout liver DNA *in vivo*

Protein Source	Protein Level % of Diet	Binding[a] dMoles ($\times 10^{-10}$ Moles)/mgDNA
Casein	40	2.58 ± .432[b]
Casein	50	2.53 ± .347
Casein	60	3.29 ± .366
Casein	70	2.99 ± .0996[1]
FPC	40	2.28 ± .445
FPC	50	1.90 ± .387[2]
FPC	60	3.09 ± .563[2,3]
FPC	70	1.89 ± .421[1,3]

[a]At least 5 pooled livers were used for each sample, n=3
[b]Mean ± Standard Deviation

The same superscript represents significant difference, $p < .05$ (students t-test)

TABLE 7- Comparison of *in vivo* AFB_1 DNA adduct formation in rainbow trout and coho salmon livers

Fish	Binding[a] dMoles AFB_1 ($\times 10^{-10}$ Moles)/mgDNA
Trout	2.43 ± .859[b,c]
Coho Salmon	0.106 ± .0100[c]

[a]At least 5 pooled livers were used for each sample, n=3

[b]Mean ± Standard Deviation

[c]Significantly different, $p < .05$ (students t-test)

TABLE 8- Effect of CPFA on $[H^3]$-AFB_1 binding to trout liver DNA *in vivo*

Protein Source	Protein Level(%)	Control or CPFA	Liver Levels[a] dMoles AFB_1 ($x10^{-10}$moles)/gLiver	Binding[a] dMoles AFB_1 ($x10^{-10}$moles)/mgDNA
Casein	40	Control	27.2 ± 1.24[b]	2.75 ± .136
Casein	40	CPFA	24.7 ± 5.50	2.19 ± .699
Casein	70	Control	25.7 ± 3.00	2.83 ± .623
Casein	70	CPFA	27.8 ± 2.17	2.46 ± .386
FPC	40	Control	23.0 ± 1.76	2.70 ± .336
FPC	40	CPFA	26.0 ± .617	2.60 ± .0312
FPC	70	Control	18.3 ± 3.86	2.10 ± .467
FPC	70	CPFA	19.9 ± 2.73	1.89 ± .286

[a]At least 5 pooled livers were used for each sample, n=3

[b]Mean ± Standard Deviation

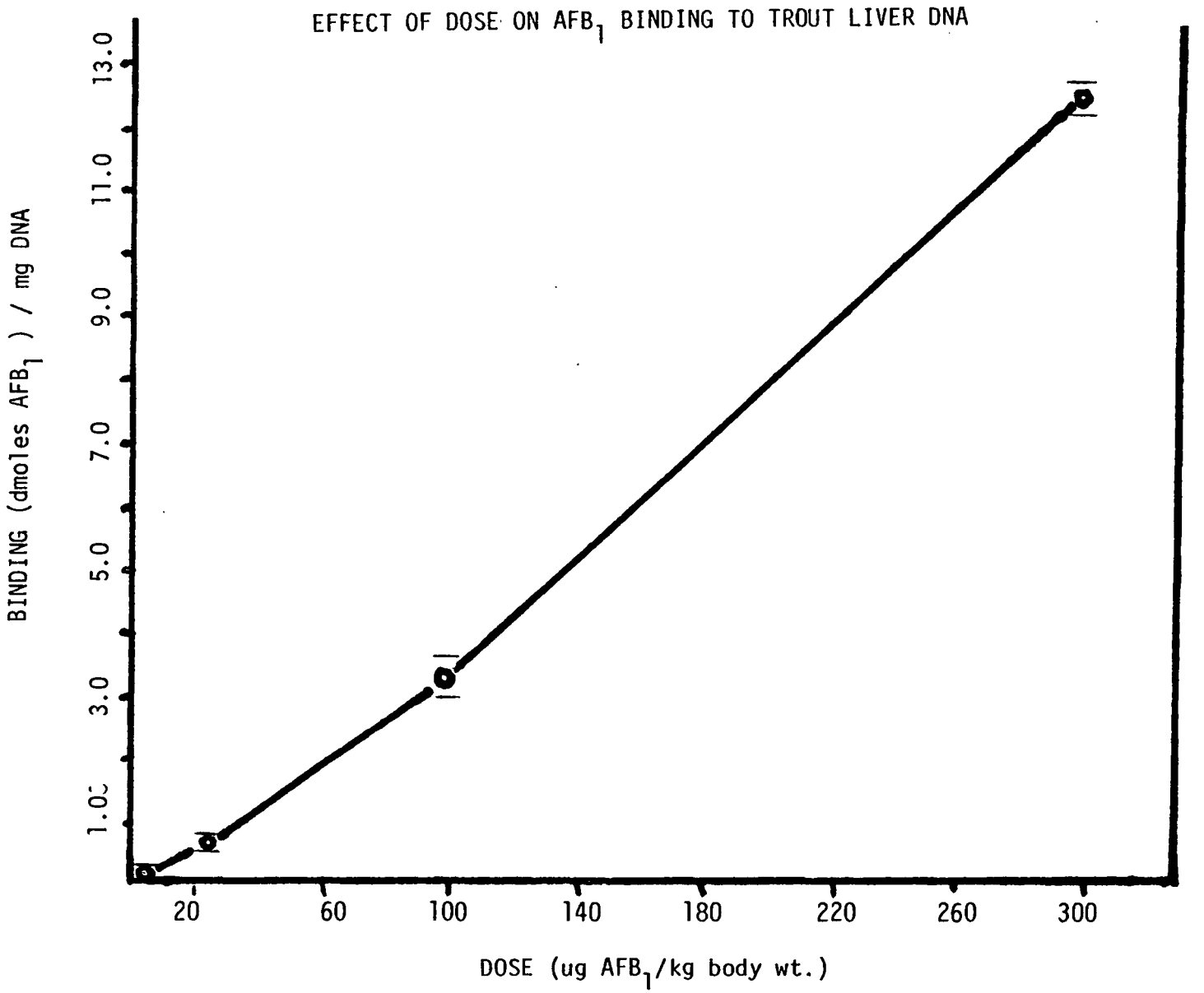
TABLE 9 - Effect of β-napthoflavone pretreatment on in vivo AFB_1 binding to trout liver DNA

Treatment	Liver Levels[a] dMoles AFB_1 ($\times 10^{-10}$moles)/gLiver	Binding[a] dMoles AFB_1 ($\times 10^{-10}$moles)/mgDNA
Control	20.9 ± .762[1]	1.62 ± .182[b,2]
β-napthoflavone	9.41 ± 1.48[1]	0.966 ± .221[2]

[a]At least 5 pooled livers were used for each sample, n=3

[b]Mean ± standard deviation

Same superscript indicates significant difference, $p<.05$ (student t-test)


EFFECT OF DOSE ON AFB1 BINDING TO TROUT LIVER DNA
BINDING (dmoles AFB1) / mg DNA
1.0
3.0
5.0
7.0
9.0
11.0
13.0
20
60
100
140
180
220
260
300
DOSE (ug AFB1/kg body wt.)

BIBLIOGRAPHY

Ahokas, J., and Paakkonen, R. 1977. In: Microsome- and drug oxidations, ed. by V. Ullrich. Oxford, Pergamon Press, pp. 435-441.

Autrup, H., and Essigmann, J.M. 1979. Metabolism of aflatoxin B_1 and identification of the major aflatoxin B_1-DNA adducts formed in cultured human bronchus and colon. Cancer Res. 39:694.

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

Campbell, T.C., and Hayes, J.R. 1974. Role of nutrition in the drug metabolizing enzyme system. Pharm. Rev. 26:171.

Campbell, T.C., and Hayes, J.R. 1976. The effect of quantity and quality of dietary protein on drug metabolism. Fed. Proc. 35:2470.

Campbell, T.C., and Hayes, J.R. 1978. Dietary lipotropes, hepatic microsomal mixed-function oxidase activities, and in vivo covalent binding of aflatoxin B_1 in rats. Cancer Res. 38:4569.

Ceriotli, G. 1955. Determination of nucleic acids in animal tissues. J. Biol. Chem. 214:59.

Croy, R.G., Essigmann, J.M., Reinhold, V.N., and Wogan, G.N. 1978. Identification of the principal aflatoxin B_1-DNA adduct formed in vivo in rat liver. Proc. Natl. Acad. Sci. USA. 75:1745.

Croy, R.G., and Nixon, J.E. 1980. Unpublished data. Dept. Food Science, Oregon State University, Corvallis.

Eisele, T.A., Yoss, J.K., Nixon, J.E., Pawlowski, N.E., and Sinnhuber, R.O. 1977. Rat urinary metabolites of [9,10-methylene-^{14}C]sterculic acid. Biochim. Bioph. Acta. 488:76.

Eisele, T.A., Nixon, J.E., Pawlowski, N.E., and Sinnhuber, R.O. 1978. Effects of dietary cyclopropene fatty acids on the mixed function oxidase system of the rainbow trout. J. Env. Path. and Tox. 1:773.

Ellis, N.R., Rothwell, C.S., and Pool, W.O. 1931. The effect of ingested cottonseed oil on the composition of body fat. J. Biol. Chem. 92:385.

Essigmann, J.M., Croy, R.G., and Wogan, G.N. 1977. Structural identification of the major DNA adduct formed by aflatoxin B_1 in vitro. Proc. Natl. Acad. Sci. U.S. 74:1870.

Fahmy, M.J., Fahmy, O.G., and Swenson, D.H. 1978. Aflatoxin B_1-2,3-dichloride as a model of the active metabolite of aflatoxin B_1 in mutagenesis and carcinogenesis. Cancer Res. 38:2608.

Garner, R.C., Miller, E.C., Miller, J.A., Garner, J.V., and Hanson, R.S. 1971. Formation of a factor lethal for S. typhimurium TA1530 and TA1531 on incubation of aflatoxin B_1 with rat liver microsomes. Biochem. Biophys. Res. Comm. 45:774.

Garner, R.C., Miller, E.C., and Miller, J.A. 1972. Liver microsomal metabolism of aflatoxin B_1 to a reactive derivative toxic to Salmonella typhimurium TA1530. Cancer Res. 32:2058.

Garner, R.C. 1973a. Microsome-dependent binding of aflatoxin B_1 to DNA, RNA, polyribonucleotides and protein in vitro. Chem.-biol. Int. 6:125.

Garner, R.C. 1973b. Chemical evidence for the formation of a reactive aflatoxin B_1 metabolite, by hamster liver microsomes. FEBS Lett. 36:261.

Garner, R.C., and Wright, C.M. 1973c. Induction of mutations in DNA-repair deficient bacteria by a liver microsomal metabolite of aflatoxin B_1. Br. J. Cancer 28:544.

Garner, R.C., and Wright, C.M. 1975a. Binding of [^{14}C]aflatoxin B_1 to cellular macromolecules in the rat and hamster. Chem.-biol. Int. 11:123.

Garner, R.C. 1975b. Reduction in binding of [^{14}C]aflatoxin B_1 to rat liver macromolecules by phenobarbitone pretreatment. Biochem. Pharm. 24:1553.

Gillette, J.R. 1974. A perspective on the role of chemically reactive metabolites of foreign compounds in toxicity - I. Correlation of changes in covalent binding of reactivity metabolites with changes in the incidence and severity of toxicity. Biochem. Pharm. 23:2785.

Gurtoo, H.L., and Bejba, N. 1974. Hepatic microsomal mixed function oxygenase: Enzyme multiplicity for the metabolism of carcinogens to DNA-binding metabolites. Biochem. Biophy. Res. Comm. 61:685.

Gurtoo, H.L., and Dave, C.V. 1975. In vitro metabolic conversion of aflatoxins and benzo(a)pyrene to nucleic acid-binding metabolites. Cancer Res. 35:382.

Gurtoo, H.L., and Motycka, L. 1976. Effect of sex difference on the *in vitro* and *in vivo* metabolism of aflatoxin B_1 by the rat. Cancer Res. 36:4663.

Haight, L. 1980. Unpublished data. Dept. Food Science, Oregon State University, Corvallis.

Hendricks, J.D., Sinnhuber, R.O., Nixon, J.E., Wales, J.H., Masri, M.S., and Hsieh, D.P.H. 1980. Carcinogenic response of rainbow trout (*Salmo gairdneri*) to aflatoxin Q_1 and synergistic effect of cyclopropenoid fatty acids. J. Natl. Cancer Inst. 64:523.

Hendricks, J.D. 1980. Unpublished data. Dept. Food Science, Oregon State University, Corvallis.

Hendricks, J.D. 1980. Personel communication. Dept. Food Science, Oregon State University, Corvallis.

Kato, R., Oshima, T., and Tomizawa, S. 1968. Toxicity and metabolism of drugs in relation to dietary protein. Jap. J. Pharm. 18:356.

Lee, D.J., Wales, J.H., and Sinnhuber, R.O. 1967. A comparison of cyclopropenes and other possible promoting agents for aflatoxin-induced hepatoma in rainbow trout. Fed. Proc. 26:99.

Lee, D.J., Wales, J.H., and Sinnhuber, R.O. 1968. Synergism between cyclopropenoid fatty acids and chemical carcinogens in rainbow trout (*Salmo gairdneri*). Cancer Res. 28:2312.

Lee, D.J., Wales, J.H., and Sinnhuber, R.O. 1971. Promotion of aflatoxin-induced hepatoma growth in trout by methyl malvalate and sterculate. Cancer Res. 31:960.

Lee, D.J., Pawlowski, N.E., Wales, J.H., and Sinnhuber, R.O. 1972. A comparison of the cocarcinogenic activity of cyclopropenoid compounds in rainbow trout. Fed. Proc. 31:733.

Lee, D.J., Sinnhuber, R.O., Wales, J.H., and Putnam, G.B. 1977. Effect of dietary protein on the response of rainbow trout (*Salmo gairdneri*) to aflatoxin B_1. J. Natl. Cancer Inst. 60:317.

Lin, J.K., Miller, J.A., and Miller, E.C. 1977. 2,3-Dihydro-2-(guan-7-yl)-3-hydroxy-aflatoxin B_1, a major acid hydrolysis product of aflatoxin B_1-DNA or -ribosomal RNA adducts formed in hepatic microsome-mediated reactions and in rat liver *in vivo*. Cancer Res. 37: 4430.

Lin, J.K., Kennan, K.A., Miller, E.C., and Miller, J.A. 1978. Reduced nicotinamide adenine dinucleotide phosphate-dependent formation of 2,3-dihydro-2,3-dihydroxy aflatoxin B_1 from aflatoxin B_1 by hepatic microsomes. Cancer Res. 38:2424.

Loveland, P.M., Nixon, J.E., and Sinnhuber, R.O. 1979. Aflatoxin B_1 and aflatoxicol metabolism in rainbow trout (Salmo gairdneri) and the effects of dietary cyclopropene. J. Env. Path. Tox. 2:707.

Lowry, O.H. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265.

Madhavan, T.V., and Gopalan, C. 1965. Effect of dietary protein on aflatoxin liver injury in weanling rats. Arch. Path. 80:123.

Madhavan, T.V., and Gopalan, C. 1968. The effect of dietary protein on carcinogenesis of aflatoxin. Arch. Path. 85:133.

Malevski, Y., Wales, J.H., and Montgomery, M.W. 1974a. Liver damage in rainbow trout (Salmo gairdneri) fed cyclopropenoid fatty acids. J. Fish. Res. Board Can. 31:1397.

Malevski, Y., Montgomery, M.W., and Sinnhuber, R.O. 1974b. Liver fat and protein metabolism in rainbow trout (Salmo gairdneri) fed cyclopropenoid fatty acids. J. Fish. Res. Board Can. 31:1093.

Nixon, J.E., Eisele, T.A., and Sinnhuber, R.O. 1974a. Effect of subacute toxic levels of dietary cyclopropenoid fatty acids upon membrane function and fatty acid composition in the rat. Lipids 9:314.

Nixon, J.E., Sinnhuber, R.O., and Lee, D.J. 1974b. Effect of cyclopropenoid compounds on the carcinogenic activity of diethylnitrosamine and aflatoxin B_1 in rats. J. Natl. Cancer Inst. 53:453.

Nixon, J.E., Eisele, T.A., Hendricks, J.D., and Sinnhuber, R.O. 1977a. Reproduction and lipid composition of rats fed cyclopropene fatty acids. J. Nutr. 107:574.

Nixon, J.E., Yoss, J.K., and Sinnhuber, R.O. 1977b. Metabolism and tissue distribution of label from [9,10-methylene-^{14}C]sterculic acid in the rat. Lipids 12:629.

Nixon, J.E. 1980. Personel communication. Dept. Food Science, Oregon State University, Corvallis.

Preston, S.R., Hayes, J.R., and Campbell, T.C. 1976. The effect of protein deficiency on the in vivo binding of aflatoxin B_1 to rat liver macromolecules. Life Sci. 19:1191.

Roehm, J.N., Lee, D.J., and Sinnhuber, R.O. 1968. Alteration of the fatty acid composition of rainbow trout fed sterculic acid. Fed. Proc. 27:105.

Roehm, J.N., Lee, D.J., and Sinnhuber, R.O. 1970. The effect of dietary sterculic acid on the hepatic lipids of rainbow trout. Lipids 5:80.

Rogers, A.E., and Newberne, P.M. 1969. Aflatoxin B_1 carcinogenesis in lipotrope-deficient rats. Cancer Res. 29:1965.

Sachan, D.S. 1975. Effects of low and high protein diets on the induction of microsomal drug-metabolizing enzymes in rat liver. J. Nutr. 105:1631.

Schneider, D.L., Sheehan, E.T., and Kemmerer, A.R. 1968. Effect of Sterculia foetida oil on weanling rat growth and survival. J. Agr. Food Chem. 16:1022.

Schoenhard, G.L., Bishop, P.E., Lee, D.J., and Sinnhuber, R.O. 1975. Bacillus subtilis GSY 1057 assay for aflatoxin B_1 activation by rainbow trout (Salmo gairdneri). J. Assoc. Off. Anal. Chem. 58:1074.

Shenstone, F.S., Vickery, J.R., and Johnson, A.R. 1965. Studies on the chemistry and biological effects of cyclopropenoid compounds. J. Agr. Food Chem. 13:410.

Sinnhuber, R.O., Wales, J.H., and Lee, D.J. 1966. Cyclopropenoids, cocarcinogens for aflatoxin-induced hepatoma in trout. Fed. Proc. 25:184.

Sinnhuber, R.O., Lee, D.J., and Wales, J.H. 1968. Dietary factors and hepatoma in rainbow trout (Salmo gairdneri). II. Cocarcinogenesis by cyclopropenoid fatty acids and the effect of gossypol and altered lipids on aflatoxin induced liver cancer. J. Natl. Cancer Inst. 41:1293.

Sinnhuber, R.O., Lee, D.J., and Wales, J.H. 1974. Hepatic carcinogenesis of aflatoxin M_1 in rainbow trout (Salmo gairdneri) and its enhancement by cyclopropene fatty acids. J. Natl. Cancer Inst. 53:1285.

Sinnhuber, R.O., Hendricks, J.D., and Putnam, G.B. 1976. Sterculic acid, a naturally occurring cyclopropene fatty acid, a liver carcinogen to rainbow trout (Salmo gairdneri). Fed. Proc. 35:505.

Sinnhuber, R.O., Wales, J.H., Hendricks, J.D., Putnam, G.B., Nixon, J.E., and Pawlowski, N.E. 1977. Trout bioassay of mycotoxins. In: Mycotoxins in Human and Animal Health, ed. by J.V. Rodricks, Pathotox Publishers, Park Forest South, pp. 731-744.

Sisk, D.B., and Carlton, W.W. 1972. Effect of dietary protein concentration on response of miniature swine to aflatoxins. Am. J. Vet. Res. 33:107.

Stark, A.A., Essigmann, J.M., and Wogan, G.N. 1979. Aflatoxin B_1 mutagenesis, DNA binding, and adduct formation in Salmonella typhimurium. Proc. Natl. Acad. Sci. USA. 76:1343.

Statham, C.N., Elcombe, C.R., and Szyjka, S.P. 1978. Effect of polycyclic aromatic hydrocarbons on hepatic microsomal enzymes and disposition of methyl-napthalene in rainbow trout in vivo. Xenobiotica 8:65.

Stott, W.T., and Sinnhuber, R.O. 1978. Dietary protein levels and aflatoxin B_1 metabolism in rainbow trout (Salmo gairdneri). J. Env. Path. Tox. 2:379.

Stott, W.T., and Sinnhuber, R.O. 1979. Dietary casein levels and aflatoxin B_1 metabolism in rainbow trout (Salmo gairdneri). ACS Sym. Ser. No. 99, paper 23, pp. 389-400.

Struthers, B.J., Lee, D.J., and Sinnhuber, R.O. 1975a. Altered lipid metabolism in livers of rainbow trout fed cyclopropenoid fatty acids. Exper. Mol. Path. 23:181.

Struthers, B.J., Wales, J.H., and Lee, D.J. 1975b. Liver composition and histology of rainbow trout fed cyclopropenoid fatty acids. Exper. Mol. Path. 23:164.

Swartz, A.G., and Rate, W.R. 1979. Inhibition of aflatoxin B_1-induced cytotoxicity and binding to DNA in cultured rat liver cells by naturally occurring flavones. J. Env. Path. Tox. 2:1021.

Swenson, D.H., Miller, E.C., and Miller, J.A. 1974. Aflatoxin B_1-2,3-oxide: Evidence for its formation in rat liver in vivo and by human liver microsomes in vitro. Biochem. Biophys. Res. Com. 60:1036.

Swenson, D.H., Miller, J.A., and Miller, E.C. 1975. The reactivity and carcinogenicity of aflatoxin B_1-2,3-dichloride, a model for the putative 2,3-oxide metabolite of aflatoxin B_1. Cancer Res. 35:3811.

Swenson, D.H., Lin, J.K., Miller, E.C., and Miller, J.A. 1977. Aflatoxin B_1-2,3-oxide as a probable intermediate in the covalent binding of aflatoxins B_1 and B_2 to rat liver DNA and ribosomal RNA in vivo. Cancer Res. 37:172.

Temcharoen, P., et al. 1978. Influence of dietary protein and vitamin B_{12} on the toxicity and carcinogenicity of aflatoxins in rat liver. Cancer Res. 38:2185.

Ueno, I., Friedman, L., and Stone, C.L. 1980. Species difference in the binding of aflatoxin B_1 to hepatic macromolecules. Tox. Appl. Pharm. 52:177.

Wattenberg, L.W. 1975. Effects of dietary constituents on the metabolism of chemical carcinogens. Cancer Res. 35:3326.

Wogan, G.N. 1973. In: Methods in Cancer Research, ed. by H. Busch, Academic Press, New York, 7:309-344.

APPENDICES

APPENDIX I

A. NUCLEI ISOLATION

EXCISE LIVER

-perfuse with 0.9% NaCl

HOMOGENIZE (Potter-Elvehjem O.K., loose pestle)

-use 9 vol. 0.25 M Sucrose/3mM $MgCl_2$ per gram liver

HOMOGENATE

-filter through 2 or 3 layers of coarse cheese cloth

FILTERED HOMOGENATE

-900 g/15 min. (2900 rpm,SS-34 Sorvall)
-use Oakridge Type Polycarbonate tubes

SEDIMENT

-resuspend gently in 0.5% Triton-X-100 (w/v)/0.25 M Sucrose/ 1 mM $MgCl_2$, using transfer pipette
-centrifuge less than 2500 rpm/10 min.
-wash 2 additional times with Triton solution

NUCLEAR SEDIMENT

-disperse in 0.25 M Sucrose/1mM $MgCl_2$

FINAL NUCLEAR PREPARATION

*Nuclei breakage makes dispersion difficult

B. EXTRACTION OF DNA

NUCLEI SUSPENSION OR CHROMATIN CLUMPS

-resuspend quickly in 0.05 M Tris-HCl, pH 6.5
-transfer to extraction flask
-nuclei will break and clump, O.K.

NUCLEI IN TRIS

-add 4 M NaCl (in 0.05 M Tris, pH 6.5) to achieve final concen. of 1 M NaCl, warm viscous solution to room temp., all clumps must be broken by shaking before proceeding
-add 5% SDS (in Tris) slowly with swirling to achieve final concentration of 1% SDS

APPENDIX I (continued)

-gently swirl for 5-10 min.

DNA SOLUTION (0.2-0.3 mg/ml)

-extract vigorously with 1 vol. $CHCl_3$:Isoamyl alcohol (24:1) for 10 min.

EMULSION

-centrifuge using Tefzel tubes at 10000 rpm/10 min(SS-34 rotor)

REMOVE AQUEOUS

-repeat extraction and centrifugation

AQUEOUS

-ppt DNA using 3 vol. i.c. ethanol
-spool out on glass rod

NUCLEIC ACIDS (dissolve at 0.2-0.3 mg/ml)

-dissolve in 0.05 M Tris-HCl, pH 6.5 using 50 ml culture tubes
-adjust to 0.1 M NaCl using 4 M NaCl (in Tris)
-RNAse (in 0.1 M NaCl) added to 30-40 µg/ml, 37 /30 min.
-adjust to 1.7% sarcosil with 22% sarcosil
-Pronase added to 220 µg/ml, 37 /2 hours

DNA SOLUTION

-adjust to 0.9 M NaCl with 4 M NaCl (in Tris)
-extract 2X with $CHCl_3$ solution in culture tubes
-centrifuge in Tefzel centrifuge tubes

AQUEOUS

-ppt. DNA with i.c. ethanol (3 vol.)
-spool on glass rod and wash in ethanol

DNA PPT.

-dry in vacuo over P_2O_5
-hydrolyze in 0.5 N PCA, 80 /15 min.

HYDROLYZED DNA

radioactivity determinations or diphenylamine reaction

APPENDIX II

DIPHENYLAMINE REACTION FOR THE COLORIMETRIC ESTIMATION OF DNA

Standard DNA Solution

Use highly polymerized calf-thymus DNA

1. Stock Solution
   -Dissolve DNA at approximately *0.4 mg/ml in 5 mM NaOH
   -No deterioration in refrigerator up to 6 months

2. Working Standards
   -prepare every 3 weeks

   measured volume of stock standard
   +
   equal volume 1.0 M $HClO_4$
   15 min. ↓ 70°C
   store in refrigerator

*Standardization, 1 O.D. = 50 ug/ml in 0.01 M NaCl(@ 260 nm)

Diphenylamine Reagent

1. Dissolve 1.5 g steam distilled dephenylamine in 100 ml redistilled acetic acid *
2. Add 1.5 ml of concentrated H_2SO_4
3. Store in dark (Make daily if possible)
4. On day to be used, add 0.10 ml aqueous acetaldehyde (16 mg/ml) for each 20 ml of reagent

*Most batches of A.R. acetic acid are suitable without distillation

Method of Color Development

1. Dilute DNA in 0.5 M $HClO_4$ to a final concentration of between 0.02 and 0.25 ug atoms DNA-P/ml (20-60 ug DNA/ml)
2. 1 or 2 ml is mixed with 2 vol. diphenylamine reagent c/n acetald.
3. Standard tubes with known amts. of DNA and a blank with 0.5 M $HClO_4$ are also prepared
4. Color is developed by incubation @ 30°C/16-20 hours
5. Measure O.D. @ 600 nm against blank, compare with standards

APPENDIX III

PRONASE SOLUTION

Want 10 mg/ml Pronase
in 0.1 M NaCl
0.1 M Tris
0.01 M EDTA, pH 8.0
heat 70°/1 min.
then 37°/1 hour
store frozen