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Multiple Aflatoxin B_1 Binding Proteins Exist in Rat Liver Cytosol (42310)

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Abstract. The *in vitro* binding of aflatoxin B_1 to rat liver cytosolic proteins was investigated. Aflatoxin B_1 binding activity was assayed with protein purified by gel permeation chromatography, ammonium sulfate fractionation, and DEAE-cellulose chromatography. Twenty-five percent of the total binding activity was associated with proteins eluted by 0 and 0.1 *M* NaCl. Over 50% of the total binding activity was associated with protein present in the 0.2 *M* NaCl fraction. Glutathione *S*-transferase activity was also monitored and found only in the low salt (<0.2 *M* NaCl) fractions. The proteins eluted by 0.2 *M* NaCl were further purified by hydroxylapatite column chromatography and binding was found predominantly in a single fraction. The protein purification steps resulted in a 20-fold increase in the specific binding activity over that initially observed in the cytosol. These results indicate that multiple proteins are capable of binding aflatoxin B_1 in rat liver cytosol.

Aflatoxin B_1 (AFB_1) is hepatotoxic and hepatocarcinogenic in a wide variety of animal species (1, 2). Aflatoxins are dihydrofurans, produced by the fungus *Aspergillus flavus*, found as contaminants in food supplies such as peanuts and grains. In intact animals, AFB_1 is converted to an activated epoxide form by mixed function oxidases that are found in microsomal, mitochondrial, and nuclear membranes (3–5). AFB_1-2,3-epoxide is a highly reactive species that readily forms covalent bonds to DNA, RNA, and proteins in the cell (6). The formation of covalent AFB_1 adducts with macromolecules has been shown to be influenced by hormonal, nutritional, and other factors (7). It remains unclear, however, how the AFB_1 is translocated through the aqueous cytosol to the sites of activation, or how the activated carcinogen is translocated to target sites within the hepatocyte. Previous studies have indicated that binding proteins for AFB_1 may exist in rat liver cytosol (8). Such binding proteins are known to exist for other lipophilic carcinogens such as aminoazo dyes (9), benzo[*a*]pyrene (10, 11), 3-methylcholanthrene (12, 13), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (14, 15). Glutathione *S*-transferases are known to bind to a wide variety of organic compounds as well (16, 17). It has also been proposed that such binding proteins may translocate these carcinogens into the nucleus (9–14), similar to the models proposed for steroid receptor proteins (18). In this paper, we report that several species of proteins in rat liver cytosol are capable of binding noncovalently to AFB_1 *in vitro*.

Materials and Methods. *Materials.* Sephadex G-25 was obtained from Pharmacia (Piscataway, N.J.); hydroxylapatite from Bio-Rad (Richmond, Calif.); sucrose and ammonium sulfate (ultrapure grade) from Schwarz/Mann (Orangeburg, N.Y.); DEAE-cellulose, tris(hydroxymethyl)-aminomethane (Tris), 1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), and phenylmethylsulfonyl fluoride (PMSF) from Sigma (St. Louis, Mo.); NCS tissue solubilizer and OCS scintillation fluid from Amersham (Arlington Heights, Ill.); [^{3}H]AFB_1 (12 Ci/mmole) from Moravek Biochemicals (Brea, Calif.). Animals were obtained from the West Jersey Farm Division of Parco Scientific Company. They were fed water and Ziegler Laboratory chow *ad libitum* and were maintained on a diurnal cycle of 12 hr light/dark.

Partial purification of binding protein. Two male Wistar rats, weighing 250–300 g each, were used in each experiment. Preparation of cytosol began by anesthetizing the animals with Ketaset, incising the abdomen, and perfusing the liver *in situ* via the hepatic portal vein with 0.1 *M* sodium phosphate buffer (pH 7.4). The livers were immediately excised and rinsed in ice-cold 0.25 *M* sucrose in 0.1 *M* sodium phosphate buffer (pH 7.4). Homogenization was performed in a motorized glass-

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Teflon homogenizer. The homogenate was centrifuged twice at 9770*g* for 15 min and the pellets discarded. The supernatant was centrifuged at 105,000*g* for 60 min. The clear supernatant was withdrawn without disturbing the top lipid layer. Further purification was carried out by applying sample to a Sephadex G-25 column (2.0 × 40 cm) to remove low molecular weight materials. Ammonium sulfate fractionation was carried out and the 0–70% pellet kept. The pellet was resuspended in 10 m*M* Tris–HCl buffer (pH 8.0) and dialyzed to remove ammonium sulfate. All of the above steps were conducted at 4°C. Anion-exchange column chromatography (2.0 × 40 cm), using DEAE-cellulose, was performed and the salt-eluted fractions were dialyzed overnight at 4°C in the 10 m*M* Tris–HCl buffer. These fractions were assayed and desired fractions were applied to a hydroxylapatite column (2.5 × 6.0 cm) that was previously equilibrated with 0.01 m*M* sodium phosphate buffer (pH 7.4). Fractions were eluted off with increasing ionic strength and were dialyzed overnight at 4°C in the 10 m*M* Tris–HCl buffer. All of the dialysis buffers contained 0.1 m*M* PMSF.

Assays. Protein peaks during column chromatography were followed by monitoring absorbance at 280 nm on an ISCO UA-4 absorbance monitor. Protein concentration was determined by the method of Lowry *et al.* (19). Glutathione *S*-transferase activity was measured by the method of Habig *et al.* (20), using CDNB and GSH as substrates. Immunodiffusion was carried out by a modified version of the method of Ouchterlony (21). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was conducted as described previously (22).

AFB_1 binding activity was measured by incubating samples containing 0.5 mg protein in 1 mL 0.1 *M* sodium phosphate buffer (pH 7.4) with 0.2 μCi [^{3}H]AFB_1 for 16–20 hr at room temperature on a mixer. 0.8 mL was applied to Sephadex G-25 columns (0.9 × 11.0 cm) and 0.5 mL fractions were collected. A_{280} and radioactivity were determined for each fraction and pmoles AFB_1 bound per mg of protein were calculated. Radioactivity was measured by adding 0.1 mL of sample to a scintillation vial, adding 1.0 mL of NCS, and allowing to stand for 30 min. This was followed by addition of 2 drops of glacial acetic acid and 10 mL of OCS. Counting was done on a Beckman LS-3100 liquid scintillation system. Cpm were converted to dpm using ESR. The counting error was 5% or less.

Results. AFB_1 radioactivity present in the void volume, as measured in the *in vitro* binding assay, was found to be proportional to the amount of protein added to the column, through 0.4 mg (Fig. 1). Samples without protein demonstrated a flat line through fraction 8, as did samples treated with 0.1% SDS. This latter fact indicates binding is noncovalent.

In initial experiments, proteins were eluted from the DEAE-cellulose column by sodium chloride gradient (Fig. 2). The A_{280} absorption profile shows a small peak of cationic proteins that elute in the flow-through, or 0 *M* NaCl, fraction; a larger peak, consisting of proteins of increasing anionic nature, was eluted by the gradient up to 0.3 *M* NaCl. Selected fractions were assayed for [^{3}H]AFB_1 binding, which was found throughout. When samples were assayed for glutathione *S*-transferase activity, positive results were obtained in fractions eluting in the flow-through (the cationic forms) and in the low salt range (the anionic forms). Negligible enzyme activity was noted at high salt concentrations. Similar results were obtained with stepwise elution (Table I). The 0 and 0.1 *M* NaCl eluted fractions demonstrate both glutathione *S*-transferase and [^{3}H]AFB_1 binding activities. The 0.2 *M* NaCl eluted fractions, however, had the highest binding activity with negligible enzyme activity. Fractions were also eluted at 0.3 and 0.5 *M* NaCl. While both contained protein, binding and transferase activity were minimal or not detectable. In immunodiffusion using antibody to the anionic forms of the transferase (Y_b and $Y_{b'}$) strong percipitin bands were noted against the 0 and 0.1 *M* NaCl eluted fractions and extremely faint bands were found against the 0.2 *M* NaCl eluted fraction.

The next step of purification was to apply the 0.2 *M* NaCl eluted sample to a hydroxylapatite column and elute with increasing ionic strength, specifically at 0.01, 0.06, 0.08, 0.10, 0.15, and 0.20 *M* sodium phosphate (pH 7.4). Sodium chloride-containing solutions were also washed through the column. While there was some binding associated with many of the fractions (data not shown), the 0.06 *M* sodium

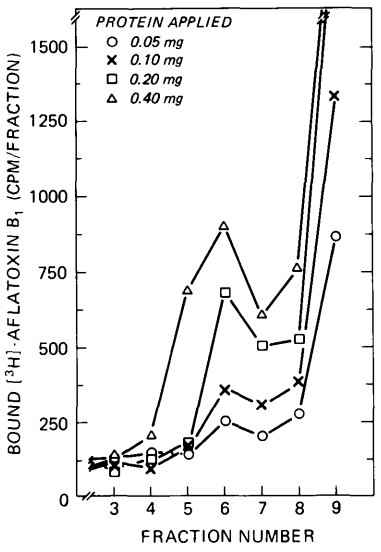

FIG. 1. Effect of sample volume on assay efficiency. The incubation mixture contained 0.5 mg protein and 0.2 μCi [^{3}H]AFB_1 per 1 ml total volume. Various amounts of the incubation mixture were added to different Sephadex G-25 columns and assays were performed as detailed under Materials and Methods. The sample used was the 0.2 *M* NaCl eluted fraction from DEAE-cellulose (see Table I and text).

phosphate eluted fraction consistently demonstrated a significantly higher specific binding activity.

A summary of the purification scheme is shown in Table II. It can be noted that early steps result in little purification of binding activity. However, anion-exchange and hydroxylapatite column chromatography steps resulted in a 20-fold increase in the specific binding activity. Recovery was 30% of the total cytosol activity, and represents approximately 2% of the cytosolic protein.

Discussion. In the present study, we have demonstrated that multiple proteins isolated from rat liver cytosol can bind noncovalently to AFB_1 *in vitro*. Proteins eluted from a DEAE-cellulose column with a wide range of ionic strengths show varying degrees of specific binding activity (pmole of AFB_1/mg protein). Values were 0.64, 1.70, and 2.25 pmole/mg for the 0, 0.1, and 0.2 *M* NaCl eluted fractions, respectively (Table I). Total binding activities (pmole of AFB_1 bound) were 5.6, 71.5, and 161.0 pmole in these fractions. One major difference between the fractions was the presence of glutathione *S*-transferase activity in fractions eluted at 0.1 *M* NaCl or less and the absence of enzyme activity in fractions eluted at higher salt. This distribution of glutathione *S*-transferase activity was verified by SDS-PAGE and immunodiffusion studies.

Our results show that the 0.1 *M* NaCl eluted fraction contains the anionic form of glutathione *S*-transferase and has approximately 24% of the total AFB_1-binding activity. The existence of an anionic glutathione *S*-transferase has been reported by others (23) and shown to reversibly bind to lipophilic molecules such as steroids and bile acids (24). In addition, previous studies have investigated the conjugation of glutathione to activated AFB_1 by the glutathione *S*-transferases *in vivo* and *in vitro* (25, 26). It is likely that one or more isozymes of glutathione *S*-transferase are

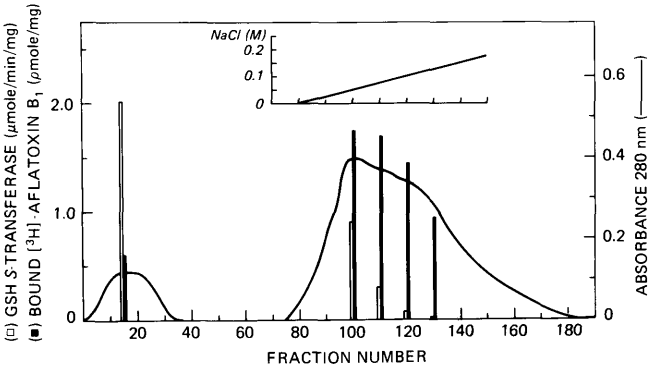

FIG. 2. Comparison of the [^{3}H]AFB_1 binding and glutathione *S*-transferase activities of samples eluted from DEAE-cellulose column by NaCl gradient. Activities were assayed at five fractions, corresponding to 0, 0.05, 0.08, 0.11, and 0.13 *M* NaCl elution. Fraction size was 2 ml each.

TABLE I. COMPARISON OF GLUTATHIONE *S*-TRANSFERASE AND [^{3}H]AFB_1 BINDING ACTIVITIES OF SAMPLES ELUTED FROM DEAE-CELLULOSE BY STEPWISE ADDITION OF NaCl

	Enzyme activity		Binding activity		
Step	μmole/min/mg	μmole/min	pmole/mg	pmole	% of total
0 *M* NaCl	2.29 ± 0.41 (2)	20.2 ± 4.2 (2)	0.64 ± 0.03 (2)	5.6 ± 0.4 (2)	1.8
0.1 *M* NaCl	1.27 ± 0.31 (5)	56.4 ± 37.9 (5)	1.70 ± 0.80 (3)	71.5 ± 59.5 (3)	23.5
0.2 *M* NaCl	0.05 ± 0.07 (4)	2.3 ± 3.2 (4)	2.25 ± 0.48 (3)	161.0 ± 122.0 (3)	52.9
0.3 *M* NaCl	N.D. (2)	N.D. (2)	0.31 (1)	66.4 (1)	21.8
0.5 *M* NaCl	N.D. (2)	N.D. (2)	—	—	—

Note. N.D., not detectable; data are given as means ± SD; number of experiments is given in parentheses.

capable of binding noncovalently to AFB_1. This may explain the earlier finding by Mainigi and Sorof (8) in which a 45- to 50-kDa protein–carcinogen complex eluted from a Sephadex G-200 column after an *in vitro* incubation of radioactive carcinogen and rat liver cytosol.

The 0.2 *M* NaCl eluted fraction does not contain glutathione *S*-transferase activity, but demonstrates a higher specific and total AFB_1-binding activity. This fraction was further purified using hydroxylapatite column chromatography and specific binding activity was 2.97 pmole/mg protein in the fraction eluted with 0.06 *M* sodium phosphate. Since the *in vitro* binding assay employed in our study is free of activating enzymes, and since there is an increase of specific binding activity during purification (Table II), the interaction between AFB_1 and partially purified protein is noncovalent and specific. Preliminary studies of this fraction using Sephadex G-200 and SDS–PAGE indicate that three protein bands are enhanced by the purification procedure. Subunit molecular weights are 32–33 kDa, 45–50 kDa, and 67–69 kDa. The 45- to 50-kDa protein elutes prior to the 67- to 69-kDa protein suggesting that the protein exists as a dimer with a molecular weight of 90–100 kDa. The 67 to 69 kDa protein could be liver albumin, as albumin in the blood is known to bind AFB_1 (27). The major band at 32–33 kDa may be the bile acid binding Y proteins recently described by Sugiyama *et al.* (28) that resemble the glutathione *S*-transferases in several respects, yet lack transferase activity.

The physiological role of these proteins remains unclear. Translocation of other lipophilic carcinogens has been shown to be due to specific cytosolic proteins in target tissues

TABLE II. PARTIAL PURIFICATION OF AFB_1 BINDING PROTEIN

			Binding activity	
Step	Volume (ml)	Protein (mg)	pmole/mg	pmole
Cytosol	54	1174 ± 128 (10)	0.147 ± 0.017 (2)	244.9 ± 138.7 (2)
Sephadex G-25	61	1142 ± 193 (8)	0.203 ± 0.047 (2)	225.1 ± 124.7 (2)
$AmSO_4$ 0–70%	54	772 ± 243 (8)	0.283 ± 0.070 (6)	218.6 ± 53.9 (6)
DEAE-cellulose 0.2 *M* NaCl	48	117 ± 42 (4)	2.25 ± 0.48 (3)	161.0 ± 122.0 (3)
Hydroxylapatite 0.06 *M* sodium phosphate	22	25 ± 5 (2)	2.97 ± 0.25 (2)	72.8 ± 9.5 (2)

Note. Data are given as means ± SD; number of experiments is given in parentheses.

(9–14). Recent studies have indicated that one or more proteins may be involved in the translocation of aflatoxin B_1 in isolated hepatocytes (29). Such translocation would, necessarily, be the first step toward adduct formation. Further purification and characterization should aid our understanding of the importance of these proteins in the pathogenesis of AFB_1.

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