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SIMILARITY OF NUCLEAR AND MICROSOMAL CYTOCHROMES P-450 IN THE *IN VITRO* ACTIVATION OF AFLATOXIN B_1*

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Abstract—The metabolism of the hepatocarcinogen aflatoxin B_1 (AFB_1) by rat liver nuclear and microsomal preparations was examined using covalent binding of AFB_1 to nucleic acids as an index of activation. Nuclear activation of AFB_1 was induced by treatment of rats with either phenobarbital or 3-methylcholanthrene. The nuclear enzymes catalyzed AFB_1 binding to endogenous nucleic acids (principally DNA) but the binding was considerably enhanced over a wide concentration range of AFB_1 (2–500 μM) by the addition of microsomal enzymes. Antibodies, prepared to microsomal cytochromes P-450, inhibited metabolism of both AFB_1 and the model substrate 7-ethoxycoumarin in microsomal and nuclear preparations; antigen–antibody specificity was observed with regard to induction of the animals used for preparation of the enzymes. Immunodiffusion studies also indicated that microsomal and nuclear cytochromes P-450 are similar. The AFB_1–DNA adducts produced by nuclei and microsomes were identical as judged by high pressure liquid chromatography of hydrolysates.

Aflatoxin B_1 (AFB_1) is an extremely potent and environmentally important hepatocarcinogen [1, 2]; this compound has been widely used as a model for chemical carcinogenesis [3]. Metabolic activation of AFB_1 is required for covalent binding to tissue nucleophiles and presumably for expression of its tumorogenic activity [1, 4–6], as is the case with many other carcinogens [3].

The activation of AFB_1 appears to be carried out by cytochrome P-450 containing mixed-function oxidases that produce the transient intermediate AFB_1-2, 3-oxide. Evidence for the role of this epoxide comes from (1) the identification of the 2, 3-dihydrodiol derivative of AFB_1 as a hydrolysis product of AFB_1–RNA complexes formed *in vivo* or *in vitro* [5–7], (2) identification of 2, 3-dihydro-2-(N^7-guanyl)-3-hydroxy AFB_1 as the major modified base of DNA resulting from incubation with AFB_1 *in vivo* or *in vitro* [8–11], and (3) structure–function relationships with the 2,3-dichloro derivative of AFB_1, aflatoxin B_2, and various metabolites and analogs of AFB_1 [1, 5, 12]. Purified liver microsomal cytochromes P-450 (P-450s) have been shown to activate AFB_1 to metabolites covalently bound to DNA [13] and toxic to *Bacillus cereus* [14]. However, the role of microsomal P-450s in AFB_1 binding to nucleic acids remains unclear. Phenobarbital (PB) induction elevates microsomal P-450 as well as *in vitro* AFB_1 binding [15] and mutagenic activity in *Salmonella typhimurium* tester strains [16], but decreases the *in vivo* covalent binding [5, 17], toxicity [18] and carcinogenicity [5, 19] of AFB_1. Also, AFB_1-2, 3-oxide is believed to be a very unstable compound [5] and might not be able to migrate from the endoplasmic reticulum to nuclear DNA, a major target of AFB_1 *in vivo* [4].

Recently, P-450s have been identified in highly purified preparations of rat liver nuclei and nuclear membranes [20–25]. The nuclear P-450s appear to be capable of activating benzo(a)pyrene to metabolites covalently bound to nuclear proteins, DNA, and RNA [26–29]; microsomal P-450s appear to play the major role in benzo(a)pyrene binding to nuclear components at high concentrations of this carcinogen, while the role of the nuclear P-450s becomes more important at low substrate concentrations [30]. More recently, nuclear enzymes have been shown to metabolize other compounds and to be capable of activating the carcinogens N-acetylaminofluorene [31] and N-nitrosopyrrolidine [32]. Nuclei have been demonstrated to convert AFB_1 to aflatoxins M_1 and Q_1 and metabolites covalently bound to DNA [33]: nuclear activation of AFB_1 has been offered as an explanation for the discrepancy between the *in vivo* and *in vitro* microsomal alterations of AFB_1–DNA binding observed after PB induction [34]. Because of its extreme hepatocarcinogenicity and well-defined interactions with nuclei acids, AFB_1 appears to be a useful model for extrapolation of hepatic studies to the general field of chemical carcinogenesis. The roles of nuclear and microsomal P-450s in AFB_1 activation were examined in this work to better understand the similarities and differences between these enzymes, particularly with regard to activation of carcinogens to metabolites covalently bound to nuclear components.

MATERIALS AND METHODS

Materials. [G-^{3}H]AFB_1 was obtained from the

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Nuclear Dynamics Corp., El Monte, CA, and diluted with carrier AFB_1 to the desired specific activity (2.0 mCi/μmole when used at substrate concentrations > 50 μM; 20 mCi/μmole for concentrations < 50 μM). All work with solutions (in dimethylsulfoxide) of AFB_1 was carried out in dim light, and appropriate safety precautions were taken [10]. AFB_1 solutions were repurified every 2 days using thin-layer chromatography on 1 mm layers of silica gel G [$CHCl_3$–acetone (9:1, v/v); two developments; elution with acetone]. 7-Ethoxycoumarin was synthesized and purified as described previously [35]; 7-Hydroxycoumarin was purchased for the Aldrich Chemical Co., Milwaukee, WI, and recrystallized from hot water (m.p. 220–222°, uncorr.). AFB_1 and calf thymus DNA were purchased from Calbiochem-Behring, San Diego, CA, and bovine pancreatic RNase from the Sigma Chemical Co., St. Louis, MO. All other commercial chemicals were of reagent quality and were used without further purification.

Enzyme preparations and antibodies. Male Sprague–Dawley rats (Harlan, Cumberland, IN.), weighing 100–125 g, were used in all experiments. For PB induction, the rats drank a 0.1% solution for 5 days while eating a commercial diet *ad lib.* 3-Methycholanthrene (3-MC) induction was achieved by i.p. injection (25 mg/kg; dissolved in corn oil) daily for 3 days [13].

Microsomes were prepared from rat liver as described [13, 36]. Preparations were inactivated by dialysis against 50 volumes of 0.1 M K_3PO_4 (pH 12) for 6 hr followed by dialysis against 50 volumes of 10 mM Tris–acetate buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol (normal storage buffer). Spectral analysis indicated that all P-450 was converted to cytochrome P-420.

P-450s were purified from liver microsomes of PB- and 3-MC-treated rats as described [13, 37]. These P-450s were homogeneous, as judged by polyacrylamide gel electrophoresis in six different systems, sedimentation velocity and equilibrium measurements, immunochemical analyses, N-terminal analyses, steady-state kinetics of 7-ethoxycoumarin de-ethylase, and specific contents of P-450 (18 and 17 nmoles/mg of protein) [37]. Antibodies to the P-450 "B" preparations were raised in female New Zealand white rabits and immunoglobulin G (IgG) fractions were isolated [37, 38].

Nuclei were prepared from rat liver by sedimentation through sucrose as first described by Chauveau *et al.* [39] and modified by Chiu *et al.* [40]. Microscopic examination confirmed the apparent purity and integrity of the nuclei prepared by these procedures; specific P-450 contents were not altered by repeated centrifugation through the 2.2 M sucrose buffer. The use of biochemical analyses to estimate microsomal contamination in nuclei is difficult, as low levels of common marker enzymes may be constitutive parts of nuclei [41]. One approach is examination of the inducibility of certain mixed-function oxidative activities in the nuclear preparations—work in three different laboratories [20–22, 42, 43] indicates that nuclear benzo(a)pyrene hydroxylase is induced by 3-MC but not PB, while the microsomal activity is induced by either compound. In this work, the respective activities found with nuclei derived from untreated, PB-treated and 3-MC-treated rats were 12.0 ± 1.8, 12.5 ± 1.7, and 123 ± 17.8 pmoles of 3-hydroxybenzo(a)pyrene formed/min/mg of protein (the corresponding rates found with microsomes were 220 ± 10, 1060 ± 80 and 2690 ± 498). Kasper [21] and Sagara *et al.* [42] have also indicated that, while microsomal NADPH-cytochrome *c* reductase is induced by PB, the nuclear activity is not. Nuclear preparations from untreated and PB- and 3-MC-treated rats had specific activities of 40.5 ± 4.3, 33.8 ± 2.9 and 31.4 ± 2.9 nmoles of cytochrome *c* reduced/min/mg respectively (corresponding rates found with the microsomal preparations were 151 ± 21, 491 ± 36 and 100 ± 9).

If one assumes that microsomal activity is induced but corresponding nuclear activity is not, then the following equation can be written, where sp. act. indicates the measured specific activity in nmoles of product/min/mg of protein:

(uninduced apparent nuclear sp. act.) (x mg) + (induced apparent microsomal sp. act.) ([100 − x]mg) = (induced apparent nuclear sp. act.) (100 mg).

The derived value x is the percentage of protein in the nuclear preparation derived from actual nuclei and (100−x) is the percentage of microsomal contamination on a protein basis. The degree of purity of the nuclei based on enzyme activity can be estimated using the following equations after solving for x:

[(x) (apparent nuclear sp. act.) − (100−x) (apparent microsomal sp. act)]/100 = y

100 − [y/(apparent nuclear sp. act.)] = % microsomal contamination

The above equations were used to assess the limits of possible microsomal contamination of the nuclei; standard deviations were added to the induced nuclear specific activities in the first equation. This technique indicates that less than 1 per cent of the nuclear protein and less than 15 per cent of either nuclear NADPH-cytochrome *c* reductase or benzo(a)pyrene hydroxylase were due to microsomal contamination.

Binding of [^3H]AFB_1 to nucleic acids. The covalent binding of [^{3}H]AFB_1 to calf thymus DNA was assayed as described previously [13]; microsomes were added to incubates on the basis of P-450 content. Covalent binding to liver nuclear DNA and RNA was estimated using the procedure of Vaught and Bresnick [28]; only in the preliminary and the chromatographic experiments were DNA and RNA separated from each other. In all of these assays, radioactive counting was done in 10 ml of ACS mixture (Amersham-Searle, Arlington Heights, IL); corrections were made using [^{3}H]toluene internal standards. Nucleic acid concentrations (expressed on a per residue basis) were based on A_{260} measurements (ε_{260} = 6.6 $mM^{-1}cm^{-1}$) [15]. Each data point presented on a chart represents a single determination.

Chromatography. DNA–AFB_1 complexes were prepared in the manner described for binding experiments; the AFB_1 concentration was 200 μM and the AFB_1 specific activity was 20 mCi/μmole. The isolated complexes were hydrolyzed in 90% HCO_2H [9] and lyophilized. The hydrolysates were chromatographed using a Varian 8500 high pressure liquid chromatography system. A Varian CH-10 reverse phase column was used at ambient temperature with a linear 100% water to 100% CH_3OH gradient (flow rate 60 ml/hr); 1.0 ml fractions were collected and counted in 4.0 ml of ACS mixture.

Other assays. P-450 was assayed according to the

Table 1. Induction of P-450 and AFB_1 binding activity in microsomes and nuclei*

Enzyme source	Inducer	P-450 (nmoles/mg protein)	AFB_1 (μM)	AFN_1 bound (pmoles/μmole nucleic acid/hr)	
				Per nmole P-450	Per mg protein
Microsomes		0.47 ± 0.05	5	67 ± 3	30 ± 1
			200	1480 ± 70	700 ± 30
Microsomes	3-MC	1.87 ± 0.10†	5	81 ± 15‡	151 ± 3†
			200	1300 ± 180‡	2420 ± 340†
Microsomes	PB	2.71 ± 0.12†	5	126 ± 19†	342 ± 50†
			200	2260 ± 590†	6120 ± 160†
Nuclei		0.018 ± 0.002	5	50 ± 10	0.93 ± 0.15
			200	1430 ± 790	26 ± 14
Nuclei	3-MC	0.040 ± 0.003†	5	220 ± 50†	8.8 ± 2.2†
			200	5090 ± 290†	204 ± 12†
Nuclei	PB	0.077 ± 0.002†	5	360 ± 120†	28 ± 10†
			200	5180 ± 2330†	390 ± 180†

* P-450, protein, and AFB_1 binding were assayed as described under Materials and Methods. All values given are means of three experiments ± S.D. (each enzyme preparation was pooled from livers of at least twelve rats). Microsomes (0.3 μM P-450) were incubated with thymus DNA for 60 min, while nuclei (0.3 μM P-450) were incubated for 20 min, with the indicated concentrations of ^{3}H-labeled-AFB_1.

† Significantly greater than value obtained with uninduced microsomes ($p < 0.05$).

‡ Not significantly greater than value obtained with uninduced microsomes ($p > 0.10$).

method of Omura and Sato used previously [13, 44]; protein was estimated according to the method of Lowry *et al.* [45]. Benzo(a)pyrene hydroxylase activity was measured fluorimetrically as described [46]; assays were carried out at three different protein concentrations to insure that enzyme was rate-limiting. NADPH-cytochrome *c* reductase activity was estimated as described [36]. 7-Ethoxycoumarin de-ethylase activity was measured as described [37, 47]; 0.1 nmole of P-450 was used in all assays and the substrate concentration was 0.3 mM. All IgG inhibition experiments utilized the general procedure of Thomas *et al.* [38, 48]; immunodiffusion analyses were carried out according to the same reference with 0.2% (w/v) Emulgen 913 in the gels.

RESULTS

Determination of optimal AFB_1 binding conditions. At AFB_1 concentrations of 5 and 200 μM, binding to calf thymus DNA was linear for at least 60 min in the presence of microsomes. Nuclear activation of AFB_1 to bound metabolites was linear for only about 20 min at either AFB_1 concentration. Binding of AFB_1 to calf thymus DNA was a linear function of added microsomal P-450 to at least 1.6 μM P-450 when the AFB_1 concentration was 200 μM; however, at an AFB_1 concentration of 5 μM, the assay was only linear to 0.8 μM P-450. In the nuclear experiments, the ratio of bound AFB_1 to endogenous nucleic acid was constant to a P-450 concentration of at least 1.5 μM. Therefore, all subsequent experiments were carried out with 0.3 to 0.8 μM P-450 for 20 min (nuclei) or 60 min (microsomes) so that initial enzyme velocities could be considered.

The *in vitro* nuclear system resembled the *in vivo* situation, in which AFB_1 is more readily bound to nucleic acids than to protein [4]; binding of AFB_1 to nuclear protein was not statistically significant under these conditions at any time points. The level of AFB_1 bound to DNA in the nuclear system was about ten times that bound to RNA.

Induction of P-450s and AFB_1 binding activity. Administration of PB or 3-MC to rats increased the specific content of P-450 in both liver microsomes and nuclei (Table 1). On a protein basis, both PB and 3-MC induced microsomal AFB_1 activation; on a P-450 basis, the activity was induced only marginally, in accord with relative activities of purified microsomal P-450s toward AFB_1 [13]. Nuclear activity toward AFB_1 was induced by both PB and 3-MC more substantially than was microsomal activity. The induction patterns were similar at both high and low AFB_1 concentrations. In both microsomes and nuclei, PB was somewhat more effective than 3-MC in induction of AFB_1 activation.

Microsomal enhancement of AFB_1 binding to nuclear components. PB rat microsomes enhanced the binding of AFB_1 to endogenous nucleic acids of intact nuclei: the effect was nearly linear with regard to added microsomal P-450 to a 6:1 ratio of microsomal to nuclear P-450 and appeared to reach saturation at a ratio of 16:1. The ratio 10:1 was used arbitrarily in an experiment designed to examine the generality of this effect over a wide AFB_1 concentration range (Fig. 1).* The addition of microsomes was found to enhance the level of binding to endogenous nucleic acids at concentrations of 2–500 μM AFB_1.

Immunological comparison of nuclear and microsomal P-450s. Thomas *et al.* [48] prepared antibodies to rat liver microsomal P-450s and demonstrated the abilities of such preparations to inhibit P-450-catalyzed reactions carried out with microsomal preparations; inhibition patterns were dependent upon both the source of the microsomes and the substrate under

* The ratio of nuclear protein to microsomal protein was 3.5:1.

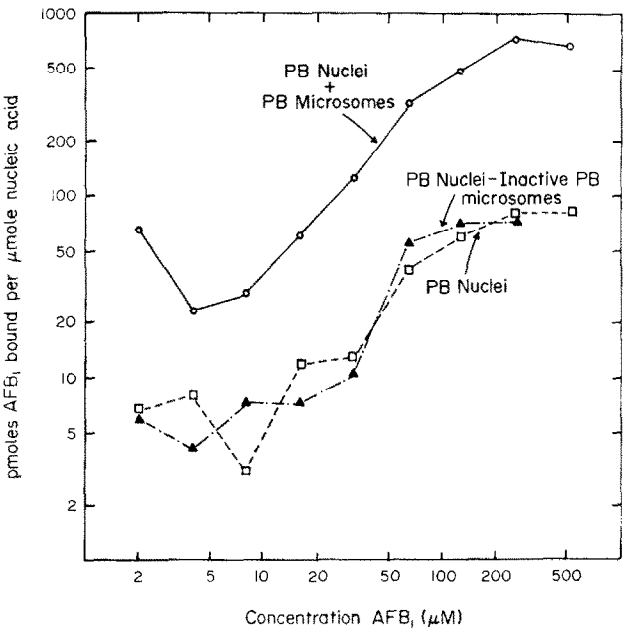

Fig. 1. Nuclear and microsomal activation of [^{3}H]AFB_1 to metabolites covalently bound to endogenous nucleic acids as functions of AFB_1 concentration. Incubations included 0.3 μM PB nuclear P-450, 3 μM PB-treated rat liver microsomal P-450, and 3 μM inactive PB-treated rat liver microsomal P-450 (cytochrome P-420) in the combinations presented in the chart. Standard Assay conditions were used; the dimethylsulfoxide concentration (2%, v/v) was the same in all experiments.

consideration. Such an immunological approach was utilized in this work to examine the similarity of the nuclear and microsomal P-450s. IgG preparations raised to microsomal P-450s inhibited 7-ethoxycoumarin de-ethylation catalyzed by either microsomes or nuclei. At a ratio of 12 mg IgG/nmole of P-450, the activity of PB-treated rat liver microsomes was inhibited 58 per cent by PB-IgG and 24 per cent by 3-MC-IgG, while the activity of 3-MC-treated rat liver microsomes was inhibited 20 per cent by PB-IgG and 81 per cent by 3-MC-IgG. The de-ethylase activity of PB-treated rat liver nuclei was inhibited 70 per cent by PB-IgG and 5 per cent by 3-MC-IgG; the activity of 3-MC-treated rat liver nuclei was inhibited 15 per cent by PB-IgG and 92 per cent by 3-MC-IgG.

The antibody inhibition experiments were repeated with AFB_1 as a substrate (Figs. 2 and 3). The specificity and extents of inhibition were not as definitive as in the case of 7-ethoxycoumarin; 3-MC-IgG was not particularly effective in inhibiting the microsomal activity. However, the nuclear activation of AFB_1 (to metabolites covalently bound to endogenous nucleic acids) was definitely inhibited by antibodies raised to microsomal P-450s. Preferential inhibition of 3-MC-treated rat liver nuclei with 3-MC-IgG and PB-treated rat liver nuclei and microsomes with PB-IgG was noted; however, 3-MC-treated rat nuclei were also inhibited by PB-IgG and PB-treated rat liver microsomes were also inhibited by 3-MC-IgG.

Double-diffusion analysis was also used to examine the immunological similarity of microsomal and nuclear P-450s (Fig. 4). No precipitin lines were observed when any of the nuclei or microsomes were tested against IgG prepared from pre-immune serum. All of the nuclear and microsomal preparations reacted with both PB-IgG and 3-MC-IgG, although the most intense bands were observed for the reactions of 3-MC-IgG with 3-MC-treated rat liver microsomes and nuclei and PB-IgG with PB-treated rat liver microsomes and nuclei.*

Chromatography of hydrolysates of AFB_1–DNA complexes. Both Essigmann *et al.* [9] and Lin *et al.* [10] have identified 2, 3-dihydro-2-(N^7-guanyl)-3-hydroxy AFB_1 as the major AFB_1 metabolite isolated from hydrolysates of AFB_1–DNA complexes formed by the microsomal activation of AFB_1 in the presence of naked DNA. High pressure liquid chromatography of hydrolysates of DNA isolated from PB-treated rat liver microsomes–thymus DNA–AFB_1 incubates gave a single major peak, presumed to be 2,3-dihydro-2-(N^7-guanyl)-3-hydroxy AFB_1 based upon its migration in a system similar to that used by Essigmann *et al.* [9] (Fig. 5). The same major chromatographic peak was found in hydrolysates of DNA isolated from incubation of AFB_1 with intact nuclei.

DISCUSSION

Nuclei were found to be capable of activating AFB_1 to metabolites covalently bound to endogenous nucleic acids, particularly DNA. This binding was induced by PB or 3-MC and was considerably enhanced by the addition of microsomal enzymes (i.e. P-450s), suggest-

* The two antibodies show a high degree of specificity with respect to ability to inhibit metabolism; however, neither antibody is monospecific and some cross-reaction occurs with other P-450s, as judged by double diffusion analysis [37, 38].

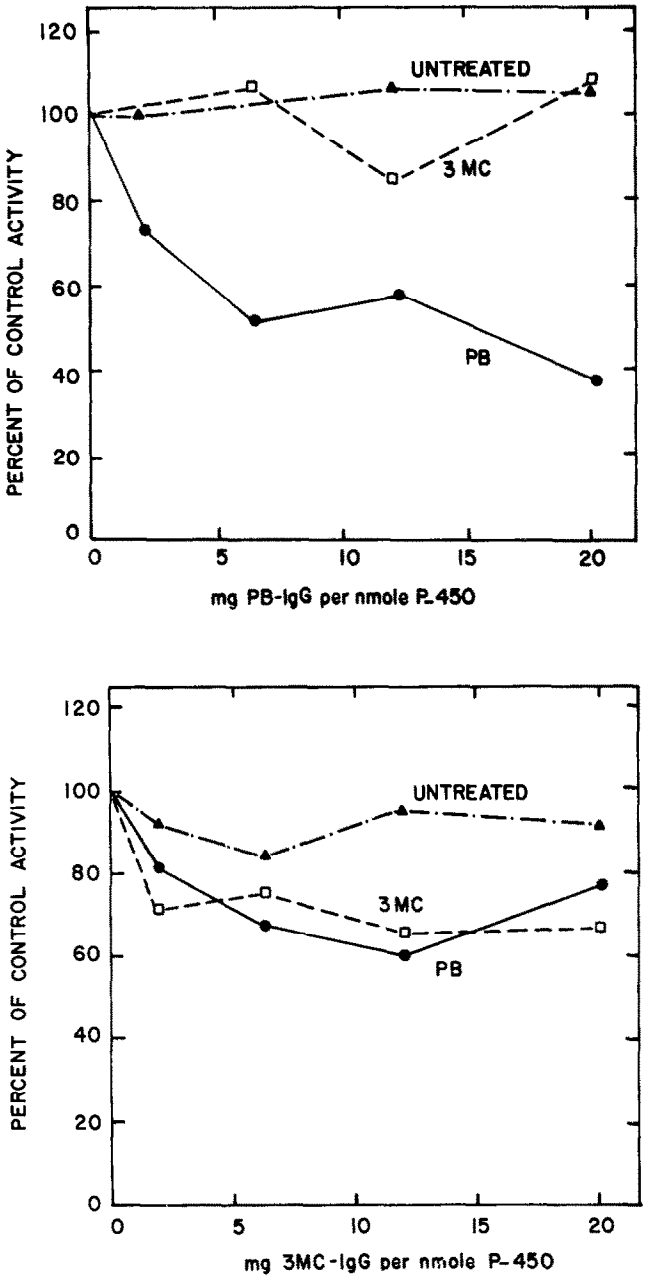

Fig. 2. Panel A: inhibition of microsomal activation of AFB_1 by IgG prepared to PB-treated rat liver microsomal P-450. Sources of rat liver microsomes (0.3 μM P-450 present) are shown. The AFB_1 concentration was 200 μM and activation to calf thymus DNA adducts was assayed as described under Materials and Methods. Panel B: inhibition of microsomal activation of AFB_1 by IgG prepared to 3-MC-treated rat liver microsomal P-450. Sources of microsomes (0.3 μM P-450 present) are shown. The AFB_1 concentration was 200 μM.

ing that most of the AFB_1 activation is catalyzed by microsomal, not nuclear, enzymes in the cell. In contrast to the case with the lung carcinogen benzo(a)pyrene [30], the binding of AFB_1 was enhanced by microsomes over a wide substrate concentration range.

Nuclear and microsomal P-450s appear to be similar with regard to AFB_1 products formed [33] (Fig. 5). These enzymes are also similar immunochemically as judged by double-diffusion analysis and antibody inhibition curves, using both AFB_1 and 7-ethoxycoumarin as substrates (Figs. 2 and 3). Recent reports from two other laboratories also indicate immunological similarity of nuclear and microsomal P-450s as judged by immunodiffusion analysis [49], binding of microsomal IgG to nuclei [49], and inhibition of nuclear 7-ethoxy-

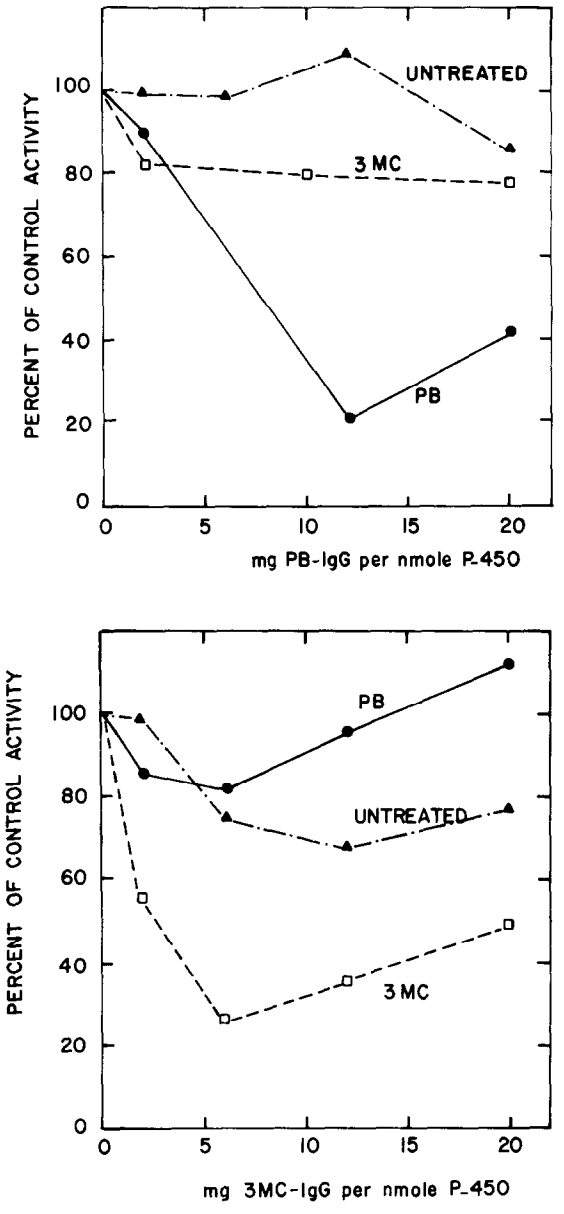

Fig. 3. Panel A: inhibition of nuclear activation of AFB_1 by IgG prepared to PB-treated rat liver microsomal P-450. Sources of nuclei are shown. The AFB_1 concentration was 200 μM and P-450 was present at 0.3 μM. Assays were as in Table 1. Panel B: inhibition of nuclear activation of AFB_1 by IgG prepared to 3-MC-treated rat liver microsomal P-450. Sources of nuclei are shown. The AFB_1 concentration was 200 μM and P-450 was present at 0.3 μM.

coumarin de-ethylase by microsomal IgG [42]. Thus, these studies indicate that the microsomal P-450s, the metabolic importance of which seems to vary depending upon the substrate under consideration. The P-450s in both the nuclear and microsomal membranes must be exposed sufficiently to permit the inhibition of mixed-function oxidase activities by the antibodies.

In making these conclusions, one must consider that all microsomal contamination of the nuclear preparations cannot be ruled out, as in any experiment involving subcellular organelles. The criteria used here suggest that at least 85 per cent of the nuclear mixed-function oxidase components were not derived from the endoplasmic reticulum. If the nuclear and microsomal P-450s were considerably different immunologically or with regard to their activities toward AFB_1, differences should have been apparent in this work.

The data on P-450 induction were consistent with those of several other workers [24, 42, 43], although P-450 was not found to be elevated by PB treatment in nuclear membrane preparations in another laboratory [20–22]. The possibility exists that P-450s are located within the nucleus [29], although removal of the nuclear membrane with detergent removes much of the P-

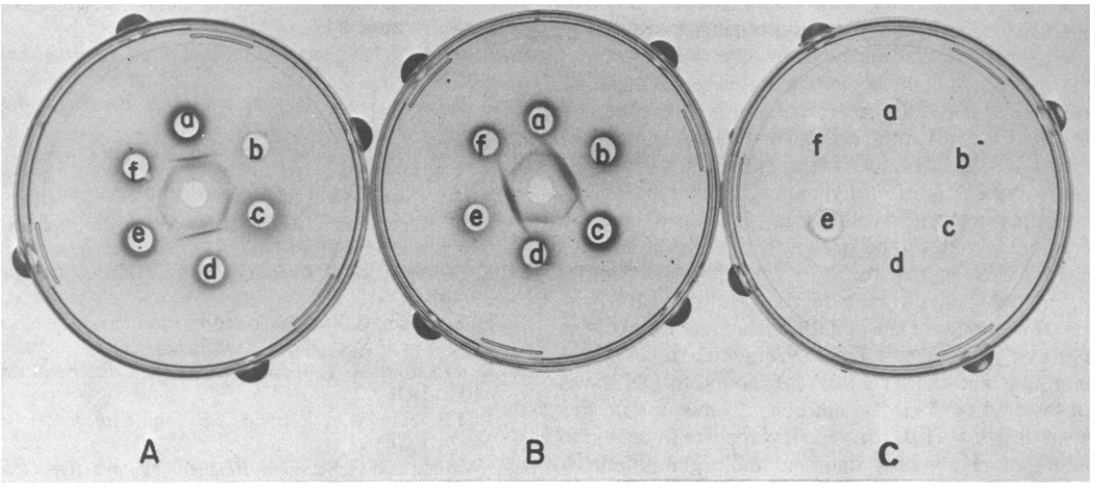

Fig. 4. Ouchterlony double diffusion analysis of microsomal and nuclear preparations using IgGs prepared to liver microsomal P-450s isolated from PB- and 3-MC-treated rats. Sample wells contained 13 μl of each solution. Microsomes and nuclei (1.3 μM P-450) were solubilized in 10mM tris–acetate (pH 7.4) buffer containing 1 mM EDTA, 20% glycerol, and 0.5% Emulgen 913. The outer wells in each case contained (a) PB-treated rat microsomes, (b) 3-MC-treated rat microsomes, (c) untreated rat microsomes, (d) PB-treated rat nuclei, (e) 3-MC-treated rat nuclei and (f) untreated rat nuclei. The center wells contained either (A) PB-IgG, (B) 3-MC-IgG, or (C) pre-immune IgG at 25 mg/ml [in 10 mM potassium phosphate (pH 8.0) buffer].

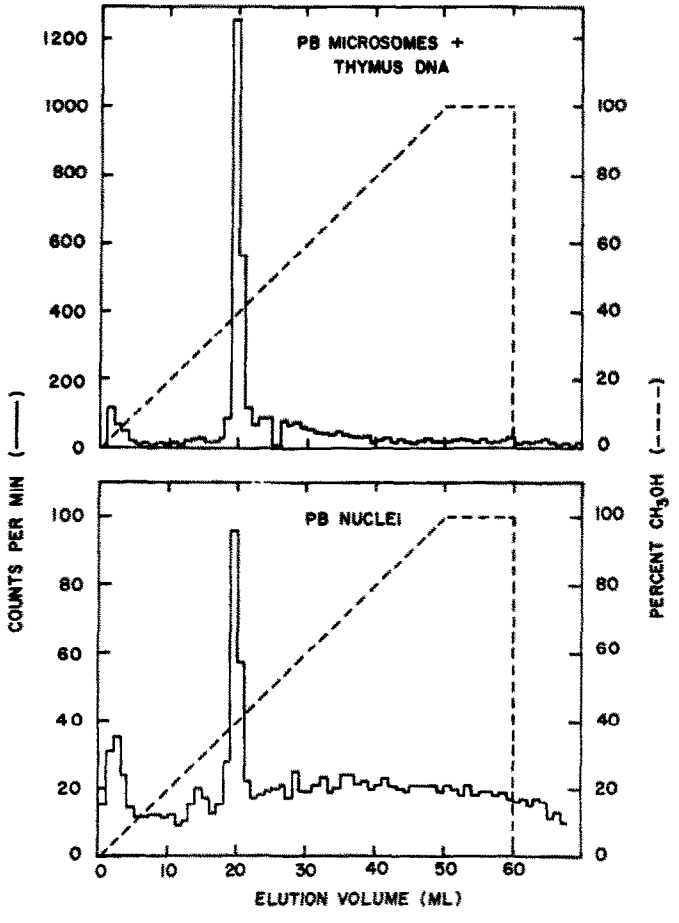

Fig. 5. High pressure liquid choromatography (h.p.l.c.) of hydrolysates of [^{3}H]AFB_1–DNA complexes prepared from incubations of [^{3}H]AFB_1 with either PB-treated rat liver microsomes (plus calf thymus DNA) or PB-treated rat liver nuclei. Details of preparations and h.p.l.c. are described under Materials and Methods. (Background counts were not subtracted from the values shown.)

450 associated activity [25]. Further work is required to distinguish whether these discrepancies are due to differences in actual purity or to other variables.

As discussed at the beginning of this paper, there is a discrepancy between the *in vitro* and *in vivo* effects of PB on AFB_1 activation, and the data presented here do not resolve the dilemma. One possibility for the difference is that PB-inducible cytosolic glutathione-*S*-transferases protect critical celllular targets from damage by AFB_1 metabolites. The presence of such an enzyme activity, as suggested by Raj *et al.* [50], was demonstrated and shown to be induced 10-fold by PB. However, the amount of the glutathione adduct formed was only one-sixth that of the DNA adduct under these conditions and the level of AFB_1 bound to DNA was not lowered by 5 mM glutathione.* Thus, the *in vivo* versus *in vitro* AFB_1 discrepancy appears to be due to factors more complex than inducible glutathione-*S*-transferases or nuclear P-450s.

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