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BENZO[*a*]PYRENE METABOLISM IN MOUSE LIVER

ASSOCIATION OF BOTH 7,8-EPOXIDATION AND COVALENT BINDING OF A METABOLITE OF THE 7,8-DIOL WITH THE *Ah* LOCUS

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Abstract—The 7,8-epoxidation of benzo[*a*]pyrene, and the 9,10-epoxidation of benzo[*a*]-pyrene *trans*-7,8-dihydrodiol coupled with covalent binding of the highly reactive diol-epoxide, are two key P-450-mediated reactions believed to be important in cancer initiation, mutagenesis and teratogenesis. New assays for these two reactions were developed with mouse liver microsomes. These two activities have apparent K_m values (~6 μM) similar to that of aryl hydrocarbon hydroxylase activity. Twenty-six individual 3-methylcholanthrene-treated Ah^b/Ah^d and Ah^d/Ah^d progeny of the (C57BL/6N)(DBA/2N) F_1 × DBA/2N backcross were studied. Both of the newly described activities appear to represent P-450 protein(s) that are responsible for aryl hydrocarbon hydroxylase activity and that are coordinately controlled by the Ah^b allele.

Multiple forms‡ of cytochrome P-450 exhibit characteristic but overlapping substrate specificities in the metabolism of polycyclic hydrocarbons to their proximate and ultimate carcinogenic metabolites (reviewed in refs. 2–5). The selective regulation of the P-450 proteins by genetic and environmental factors is an important area of carcinogenesis [6, 7], mutagenesis [8–11], and teratogenesis [12, 13].

The number of BP metabolites, especially the concept of a second oxygenation by P-450 enzymes on the same BP molecule [14], came to be appreciated with the advent of the hplc assay [15, 16]. Stereoselectivity of different P-450 proteins has been noted: liver microsomes from phenobarbital-treated and control animals give a predominance of BP 4,5-diol; microsomes from 3-methylcholanthrene-treated animals give a predominance of BP metabolites oxygenated in the 7,8- and 9,10-positions [17]. Cells in culture display a predominance of 7,8- and 9,10-oxygenated products, compared with microsomes from the intact animal [18].

The standard AHH assay [19] measures BP phenol formation. The 3-hydroxy and 9-hydroxy metabolites are particularly fluorescent [20]. Mouse P_1-450, a P-450 induced by polycyclic hydrocarbons and governed by the *Ah* receptor (reviewed in Ref. 21), is defined [1] as that enzyme having the highest turnover number for BP.

Most of this benzo[*a*]pyrene metabolism work has been carried out with the relatively nonspecific AHH assay.

The pathway by which BP becomes activated to the ultimate carcinogen or mutagen (Fig. 1) involves first a 7,8-epoxidation, then hydration of the arene oxide to form a *trans*-7,8-diol, and finally 9,10-epoxidation of the 7,8-diol [5]. Following covalent interaction of the diol-epoxide with the exocyclic N-2 of guanine [23], as well as other cellular nucleic acids and proteins [24], initiation of tumorigenesis is believed to occur in some manner.

Is P_1-450 therefore involved principally in the important metabolic pathway leading to the ultimate carcinogen/mutagen (Fig. 1) in detoxification, in the formation of toxic metabolites (phenols, quinones), or in some combination of all three? Several lines of indirect evidence would suggest some combination of all three. B6 inbred mice (Ah^b/Ah^b) having the high-affinity form of *Ah* receptor have been crossed with D2 inbred mice (Ah^d/Ah^d) having the poor-affinity form of receptor. 3-Methylcholanthrene-treated B6 mice and the B6D2F_1 (Ah^b/Ah^d) possess the high-affinity *Ah* receptor responsible for the P_1-450 induction process to proceed; 3-methylcholanthrene-treated D2 mice exhibit negligible P_1-450 induction [21]. Studies with polycyclic hydrocarbon-

‡ The term "P-450" is used to designate any or all forms of microsomal cytochrome P-450 (multisubstrate monooxygenases). Mouse "P_1-450" and "P_3-450" are defined as those forms of 3-methylcholanthrene-induced P-450 (controlled by the *Ah* receptor) with the highest turnover numbers for induced aryl hydrocarbon hydroxylase and acetanilide 4-hydroxylase activity, respectively [1]. Other abbreviations include: BP, benzo[a]pyrene; [7-^{3}H]BP, benzo[*a*]pyrene specifically tritiated in the 7-position; hplc, high-performance liquid chromatography; AHH, aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase (EC 1.14.14.1); B6, the inbred C57BL/6N mouse strain; D2, the inbred DBA/2N mouse strain; BP 7,8-diol, *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene; BP diol-epoxide, 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (it is realized that the diol-epoxide exists as a racemic mixture in which enantiomers are possible *in vivo*).

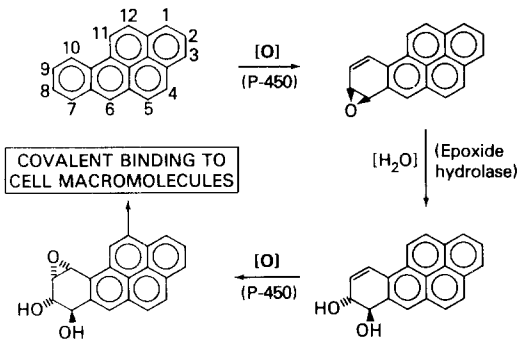

Fig. 1. Three coordinate steps in conversion of the precarcinogen BP to the ultimate carcinogenic diol-epoxide that binds covalently to nucleic acids and proteins. The first and third reactions involve P-450-mediated monooxygenations. In the second reaction, the 7,8-oxide is changed to the *trans*-7,8-dihydrodiol by epoxide hydrolase [22].

treated Ah^b/Ah^d and Ah^d/Ah^d progeny from the B6D2F$_1$ × D2 backcross have shown a high correlation between presence of the Ah^b allele and tumorigenesis [25], mutagenesis [26], teratogenesis [12], or covalent binding of metabolites [12, 27] and other toxicological events [28].

P-450 metabolism can be highly stereoselective. For example, 7-hydroxylation of warfarin predominates in control rat liver microsomes, whereas 6- and 8-hydroxylations predominate in polycyclic hydrocarbon-treated rats [29]. The major route of microsomal metabolism of BP 7,8-diol is different from that of the 9,10-diol [30]. It has been shown that the 7,8-epoxidation of BP (Fig. 1) is the rate-limiting step; once the 7,8-oxide forms, 7,8-diol formation and the diol-epoxide formation readily occur [4, 5]. Does P$_1$-450 participate in the 7,8-epoxidation, the 9,10-epoxidation, or both? Indirect *in vivo* studies with genetically different mice would suggest that both 7,8- and 9,10-monooxygenation are controlled by the Ah^b allele during tumorigenesis [31], marrow toxicity [32], and teratogenesis [13]. The purpose of this report is to prove this point directly by carrying out two newly designed BP metabolism assays in Ah^b/Ah^d and Ah^d/Ah^d mice.

EXPERIMENTAL PROCEDURES

Materials. [7-^{3}H]BP was custom-synthesized [33] and purchased from Midwest Research Institute (Kansas City, MO). Briefly stated, the 7-keto-8,9,10-trihydro-BP starting material was converted to the 7-ol-7-[^{3}H] in the presence of NaB[^{3}H]$_4$ and NaOH in ethanol; extraction of H_2O was carried out in acetic acid and HCl; [7-^{3}H]9,10-dihydrobenzo[*a*]-pyrene was then oxidized to the final product in the presence of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone. The crude product was purified by preparative hplc (μBondapak C$_{18}$, 70% acetonitrile, 30% water, U.V. detector at 254 nm, flow rate 2.4 ml/min, retention volume 30 ml). Specific activity of the 12.4 mg of pure [7-^{3}H]BP was 3.28 Ci/mmole; comparisons of the ultraviolet absorption and melting point with a pure unlabeled standard, and the chemical and radiochemical purity of ≥95%, were confirmed by thin-layer chromatography and radiochromatogram-scanning (SiO_2/hexanes : acetone, 4 : 1) (R_f = 0.65).

Generally tritiated BP 7,8-diol (416 mCi/mmole) and nonlabeled BP 7,8-diol were bought from the National Cancer Institute Chemical Repository (Bethesda, MD); purity is greater then 95%. Nonlabeled BP and 3-methylcholanthrene were purchased from Fluka A.G. (Buchs, Switzerland); glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NAD and NADP from Boehringer (Mannheim, Germany); Picofluor 30® from Packard (Downer Grove, IL); and Lumasolve® and Lipoluma® from Lumac (Basel, Switzerland). All other chemicals and solvents were of the highest analytical grade and were obtained from Merck (Darmstadt, Germany). B6 and D2 inbred mice (weanling, either sex) were bought from the Veterinary Resources Branch, National Institutes of Health (Bethesda, MD). Progeny of the B6D2F$_1$ × D2 backcross were generated in the Laboratory of Developmental Pharmacology mouse colony. Sexually immature mice of either sex were found to have no BP metabolic differences and were used interchangeably in this study.

Treatment of animals and preparation of liver microsomes. 3-Methylcholanthrene (200 mg/kg) was given intraperitoneally in peanut oil (25 ml/kg) 3 days before killing; controls received the oil alone. Livers were individually removed, washed as free as possible of hemoglobin, and homogenized in 8 ml of 0.1 M Tris chloride buffer (pH 7.4) and 0.25 M sucrose. Individual liver homogenates were centrifuged at 13,500 *g* for 20 min, the supernatant centrifuged at 105,000 *g* for 60 min. Individual liver microsomal pellets were resuspended in 2 ml of 0.1 M Tris chloride (pH 7.4), separated into three or more aliquots, and stored at −80° until assay.

Enzyme assays. The same incubation reaction mixture (1 ml) was used for all three assays and consisted of: 0.37 mM NADP, 0.43 mM NAD, 2.5 mM glucose-6-phosphate, glucose-6-phosphate dehydrogenase (0.15 I.U.), 50 mM Tris chloride buffer (pH 7.6), substrate, and microsomes. Bovine serum albumin (0.8 mg) was present in the AHH and 7,8-monooxygenase assays but was not included in the covalent binding assay. Protein determinations were made by the method of Lowry *et al.* [34] with bovine serum albumin as standard. All product formation was converted to nmoles/min/mg microsomal protein. All three activities were assayed in duplicate three times; values shown represent the means with a coefficient of variance (standard deviation ÷ mean) always less than 0.08.

AHH activity. The usual spectrophotofluorometric method [19] was used. BP substrate concentration was 80 μM; microsomal protein content was 0.2 mg in the 1-ml reaction mixture; incubation time was 10 min. One unit of activity is defined as that amount of enzyme catalyzing in 1 min at 37° the formation of hydroxylated product causing fluorescence equivalent to that of 1.0 nmole of the 3-hydroxybenzo[*a*]pyrene recrystallized standard.

BP 7,8-monooxygenase activity. The release of tritiated water into the reaction mixture was determined during incubation with [7-^{3}H]BP. This stereospecific method has been used extensively for ster-

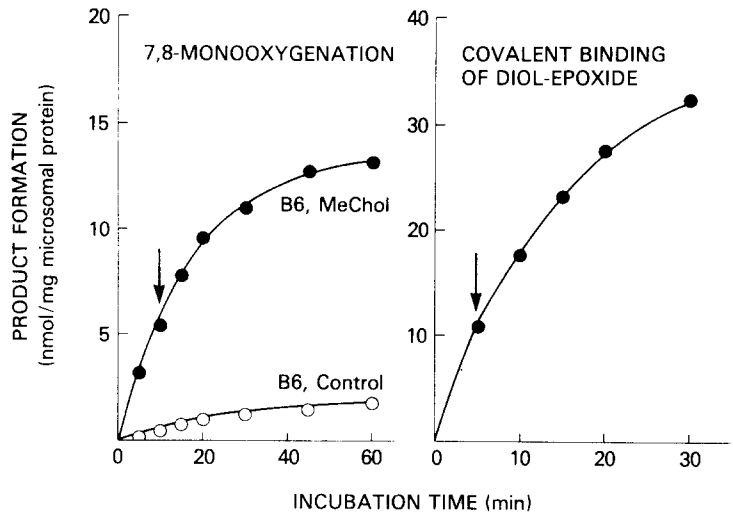

Fig. 2. Product formation as a function of incubation time. Control (○) and 3-methylcholanthrene (MeChol)-treated (●) B6 liver microsomes were used. BP 7,8-monooxygenation (left) was measured by tritium release, and covalent binding of the diol-epoxide to microsomal protein (right) was followed during incubation of generally tritiated BP 7,8-diol and microsomes in the reaction mixture. Arrows denote the times chosen for optimization of these two assays. Further details are described under Experimental Procedures.

oid metabolism in our laboratory [35, 36]. Unless otherwise indicated, the substrate [7-^{3}H]BP concentration was 80 μM and was diluted with nonlabeled BP to a specific activity of 30.8 μCi/μmole; microsomal protein was 0.4 mg in 1 ml; incubation time was 10 min. The reaction mixture was stopped with 1 vol. of 0.25 M NaOH and incubated again at 37° for 60 min. This second incubation facilitates the exchange of tritium remaining on the epoxide. Microsomal protein was then precipitated with 1 vol. of 2 M trichloroacetic acid, without removing any significant amount of tritium from the nonmetabolized [7-^{3}H]BP. Following centrifugation of the mixture at 2000 *g* for 5 min, the supernatants were transferred to appropriate tubes in which water was distilled under reduced pressure. The distilled water was trapped in a tube cooled to −50°; aliquots (1.0 ml) diluted with Picofluor® were counted by liquid scintillation spectrometry. Radioactivity background (substrate and microsomes in the reaction mixture kept at 0°; microsomes not added until after the 0.25 M NaOH had been added to the reaction mixture) were subtracted from the experimental radioactivity determinations. Radioactivity backgrounds were always less than 600 disintegrations/min. One nmole of released tritium was equivalent to 6840 disintegrations/min.

Covalent binding of BP 7,8-diol. We wanted to measure the covalent binding of the 7,8-diol. This reaction seems most relevant to cancer initiation (Fig. 1). [9-^{3}H]BP could have been custom-synthesized, but it would have been expensive. Moreover, a tritium exchange assay for [9-^{3}H]BP would not distinguish among 9,10-diols being metabolized at positions other than the 7,8-position [30], 7,8,9,10-tetraol formation [2–5], and the highly reactive, 7,8-diol-9,10-epoxide binding covalently to cellular macromolecules. We therefore used generally tritiated BP 7,8-diol in the reaction mixture, because more than 99% of the diol further metabolized and covalently bound is known to be the 7,8-diol-9,10-epoxide [2–5]. Unless otherwise indicated, the diol substrate concentration was 40 μM and was diluted by nonlabeled BP 7,8-diol to a specific activity of 38.6 μCi/μmole; microsomal protein content was 0.2 mg in 1 ml; incubation time was 5 min.

The reaction was terminated with the addition of 5 vol. of cold acetone, and the tubes were held at 0° for 30 min to facilitate protein precipitation. After centrifugation at 2000 *g* for 5 min, the supernatant was discarded and the pellets were washed twice with cold acetone. The washed precipitate was resuspended in a mixture of Lumasolve®/isopropanol (1:2) and transferred quantitatively into a counting vial; the tube was rinsed with 4 ml of Lipoluma®, and this washing was added to the scintillation vial. In order to eliminate chemiluminescence, we added concentrated acetic acid (20 μl) into each vial before counting. Radioactivity from the same controls was subtracted from the experimental radioactivity determinations. Radioactivity backgrounds were always less than 900 disintegrations/min. One nmole of covalently bound 7,8-diol was equivalent to 85,800 disintegrations/min.

RESULTS AND DISCUSSION

Optimization of assay conditions

Optimization of the AHH assay had been carried out previously [37]. For the 7,8-epoxidation and the diol-epoxide covalent binding assays, we chose optimal reaction times (Fig. 2), substrate concentrations (Fig. 3), and microsomal protein content (Fig. 4) in the linear portions of the curves. Hepatic microsomes from 3-methylcholanthrene-treated B6 mice and, in some cases, control B6 mice were chosen for study.

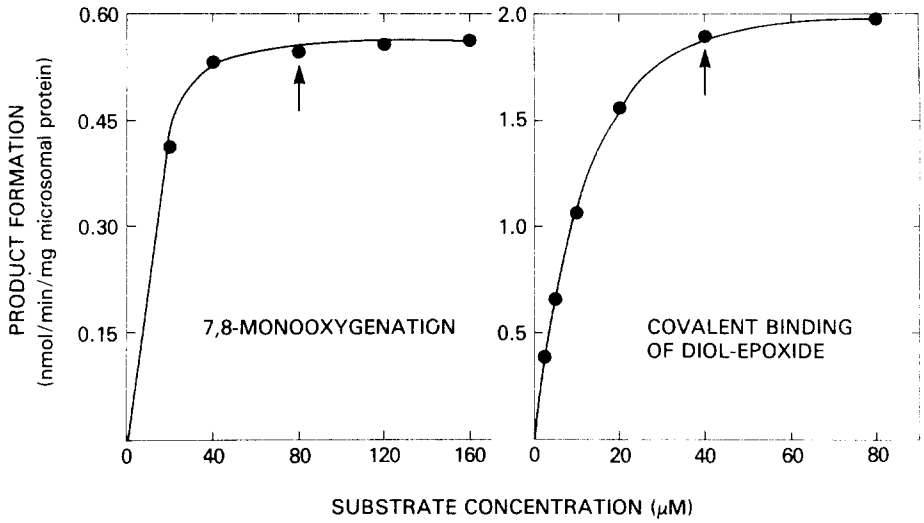

Fig. 3. Product formation as a function of substrate concentration. 3-Methylcholanthrene-treated B6 microsomes were used. BP 7,8-monooxygenation (left) and covalent binding of the diol-epoxide (right) were carried out as described in the legend to Fig. 2 and under Experimental Procedures. Arrows denote the times chosen for optimization of these two assays.

Apparent K_m values of ~6 μM were determined for the 7,8-epoxidation activity, the diol-epoxide covalent-binding activity, and aryl hydrocarbon hydroxylase activity in 3-methylcholanthrene-treated B6 liver microsomes. This finding suggests that all three activities may be associated with the same induced form(s) of P-450.

Genetic comparison of AHH activity with the 7,8-monooxygenase and the covalent binding of the diol-epoxide

All three activities were induced 5- to 15-fold by 3-methylcholanthrene in the four individual inbred B6 (Fig. 5), and there were no detectable increases in the 3-methylcholanthrene-treated D2 inbred strain. 3-Methylcholanthrene treatment of the progeny from the B6D2F_1 × D2 backcross elicited two responses for all three activities: about half exhibited induction not different ($P > 0.05$) from that seen in 3-methylcholanthrene-treated B6 mice; the remaining half of the animals were not different ($P > 0.05$) from 3-methylcholanthrene-treated D2 mice.

Each of the three activities was compared with each other (Fig. 6). When AHH activity was plotted as a function of the 7,8-monooxygenase (left) or covalent binding of the diol-epoxide (middle), eleven

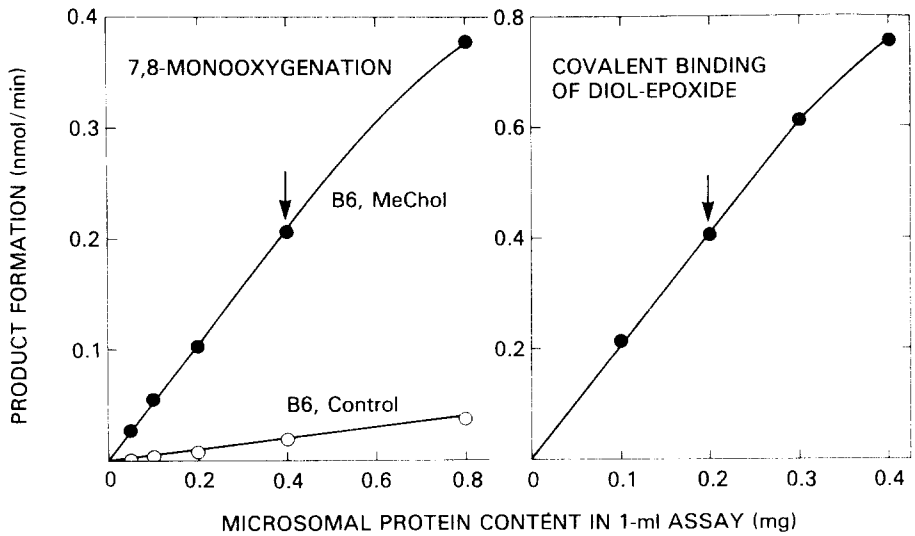

Fig. 4. Product formation as a function of microsomal protein content in the reaction mixture. Control (○) and 3-methylcholanthrene (MeChol)-treated (●) B6 microsomes were used. BP 7,8-monooxygenase activity (left) and covalent binding of the diol-epoxide (right) are described under Experimental Procedures. Arrows denote the times chosen for optimization of these two assays.

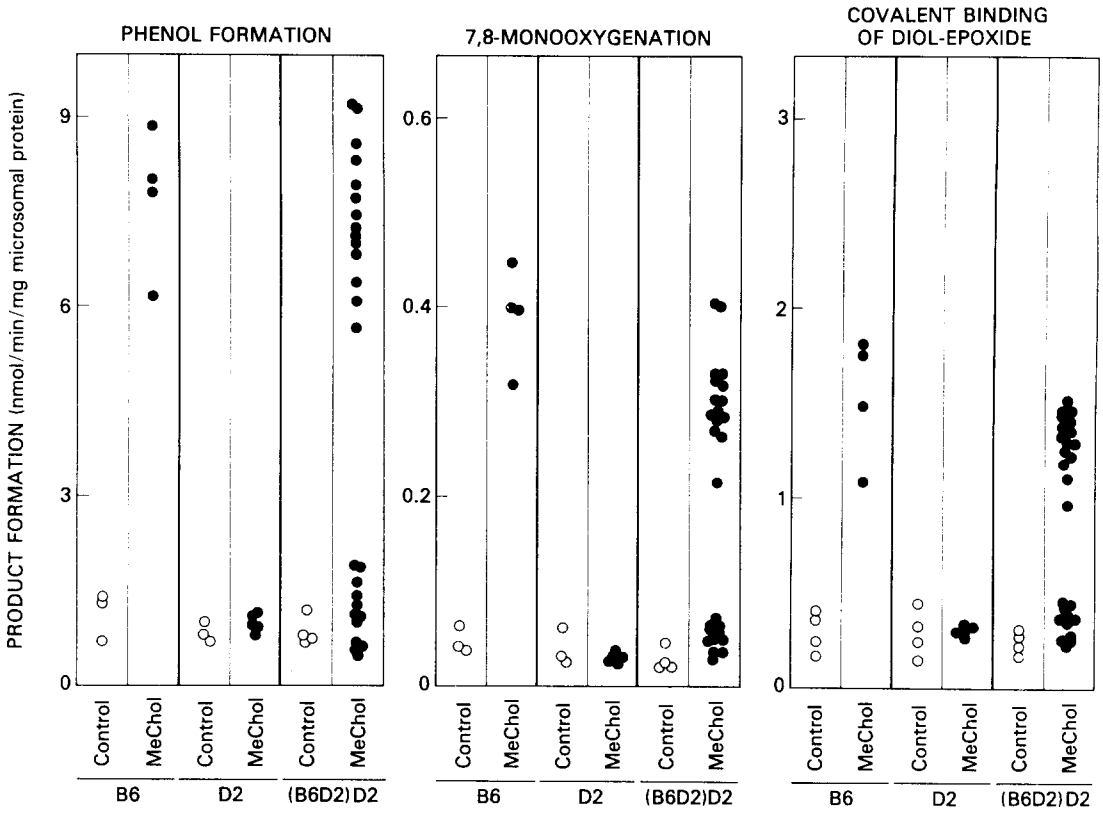

Fig. 5. Product formation of individual B6 and D2 inbred mice and 26 individual progeny [(B6D2)D2] from the B6D2F$_1$ × D2 backcross. AHH activity (phenol formation) and the assays for 7,8-monooxygenation and covalent binding of the diol-epoxide are described under Experimental Procedures. MeChol, 3-methylcholanthrene.

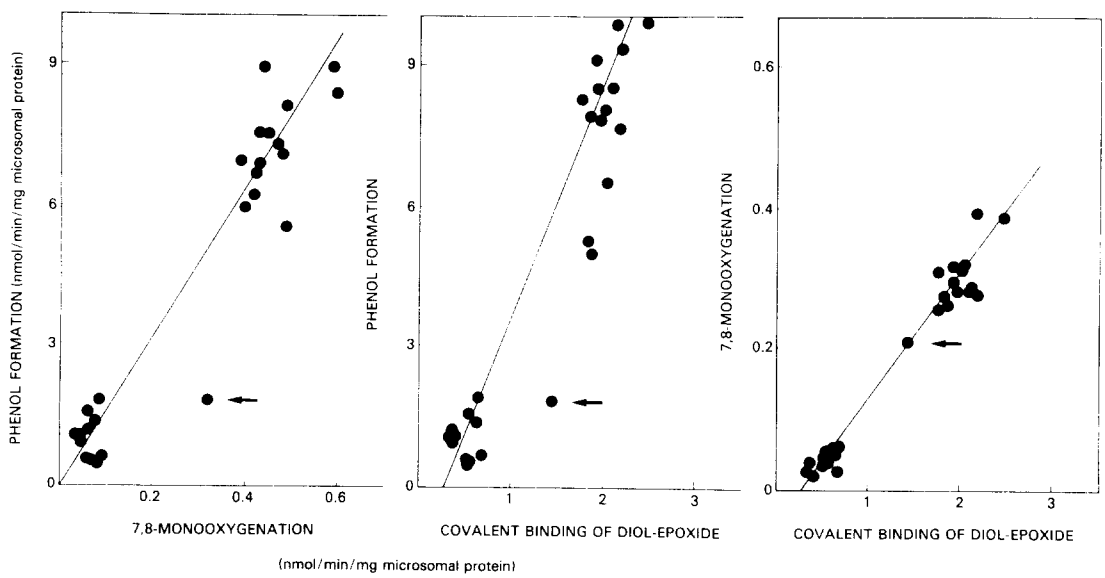

Fig. 6. Comparison of AHH activity (phenol formation), 7,8-monooxygenase activity, and covalent binding of the diol-epoxide among 26 individual 3-methylcholanthrene-treated progeny of the B6D2F$_1$ × D2 backcross. An arrow denotes the same unusual individual in all three comparisons. Further details are described in the text and under Experimental Procedures.

3-methylcholanthrene-treated backcross offspring clustered in the area identical to that expected for 3-methylcholanthrene-treated D2, and fourteen 3-methylcholanthrene-treated backcross offspring clustered in the area identical to that expected for 3-methylcholanthrene-treated B6. One individual, which had noninducible AHH activity on several repeated assays, displayed intermediate activities for both the 7,8-monooxygenase and covalent binding of the diol epoxide. This finding was reproducible and is not understood at present. When the two latter activities were compared (Fig. 6, right), the activities of this individual were on the low side of the induced activities but fell within two standard deviations of the means. Although this individual would have been phenotyped as Ah^d/Ah^d by the AHH assay, his susceptibility toward polycyclic hydrocarbon-induced tumorigenesis or mutagenesis might have been found to be unexpectedly high.

Conclusions

These data provide direct proof that the induced P-450 associated with the *Ah* locus is correlated not only with increased 7,8-monooxygenation of BP but also correlated with further metabolism of the 7,8-diol resulting in covalent binding of the diol-epoxide to cellular macromolecules. A control form of P-450, or one regulated by something other than the *Ah* receptor, has no major role in either of these two monooxygenations [17, 18]. These *in vitro* studies are consistent with the numerous observations in which an association in the intact animal has been shown between the *Ah* locus (the high-affinity *Ah* receptor in Ah^b/Ah^b or Ah^b/Ah^d mice) and cancer, mutagenesis, or teratogenesis.

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