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Genetic Differences in 2-Acetylaminofluorene Mutagenicity *in vitro* Associated with Mouse Hepatic Aryl Hydrocarbon Hydroxylase Activity Induced by Polycyclic Aromatic Compounds

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SUMMARY

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The genetically mediated difference in aromatic hydrocarbon-inducible hepatic aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase activity is associated with the metabolic activation of 2-acetylaminofluorene to a mutagen *in vitro* by liver fractions. With the use of "responsive" C57BL/6N and "nonresponsive" DBA/2N inbred strains and offspring from the appropriate crosses, the aromatic hydrocarbon-inducible hydroxylase activity appears to be expressed as an autosomal dominant trait, whereas 2-acetylaminofluorene mutagenicity *in vitro* appears to be expressed additively. With *N*-hydroxy-2-acetylaminofluorene added *in vitro*, no metabolic activation is necessary for mutagenesis to occur; however, mutagenicity is enhanced 20-40-fold in the presence of liver fractions. The metabolic activation of *N*-hydroxy-2-acetylaminofluorene to a frameshift mutagen *in vitro* is not associated with the genetically mediated difference in aromatic hydrocarbon responsiveness. We therefore suggest that the rate-limiting step of 2-acetylaminofluorene mutagenesis is its activation by cytochrome P_1450 to the *N*-hydroxy derivative, which is metabolized further to a much more mutagenic intermediate by a reaction independent of cytochrome P_1450—possibly a deacetylation reaction.

INTRODUCTION

A sensitive test *in vitro* for the detection of chemical compounds as mutagens has been recently developed by Ames and coworkers (2, 3). With this experimental system, numerous carcinogens, including 2-acetylaminofluorene and 2-aminofluorene, were shown (4, 5) to require metabolic activation by rat liver homogenates in order to form potent frameshift mutagens.[1] Other

Portions of this work were presented at the meeting of the Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, April 1975 (1).

[1] The mechanism of mutation by compounds which intercalate in the DNA base-pair stack is thought to be that the intercalation distorts the DNA backbone so that a mispairing during DNA replication, repair, or recombination causes the addition or deletion of a base. These chemicals are called *frameshift mutagens* because the reading frame of the mRNA is shifted, resulting in synthesis of a different peptide. In the case of each *Salmonella* tester strain (2-5), a frameshift mutation has already been introduced into one of the genes of the

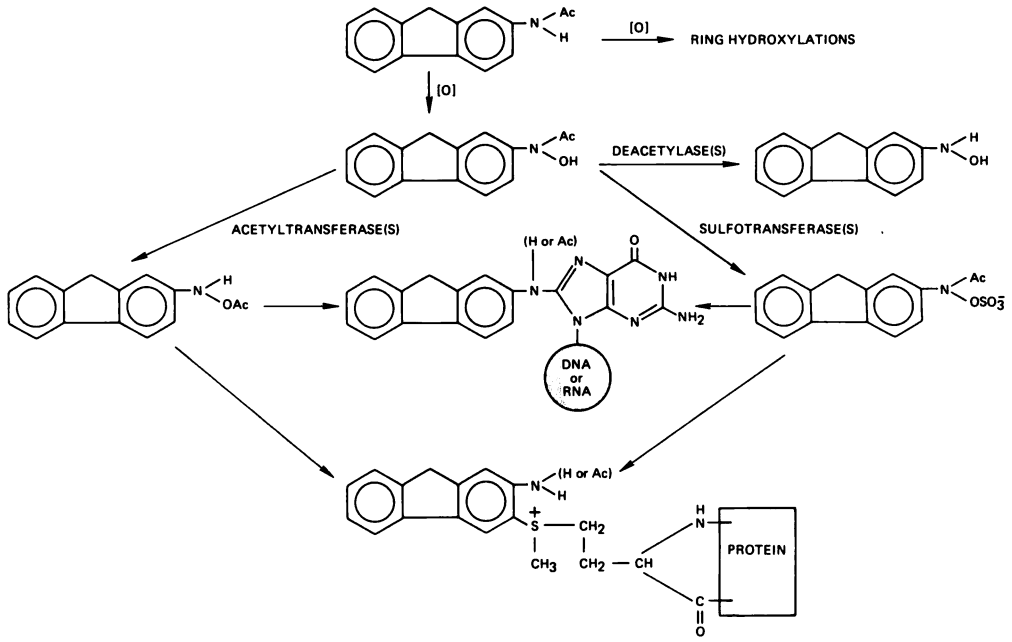

FIG. 1. *Metabolic pathways for 2-acetylaminofluorene*

compounds in the metabolic pathways for 2-acetylaminofluorene (Fig. 1), such as *N*-hydroxy-2-acetylaminofluorene and 2-nitrosofluorene and *N*-acetoxy-2-acetylaminofluorine, were shown (2, 3) to be mutagenic in the absence of liver fractions *in vitro*, indicating that these compounds themselves are actively mutagenic without requiring further metabolism. The occurrence of ring hydroxylations (Fig. 1) of 2-acetylaminofluorene is increased in phenobarbital-treated rats (7), whereas the *N*-hydroxylation of 2-acetylaminofluorene is increased markedly by 3-methylcholanthrene treatment of rats (8). The hepatotoxicity and carcinogenicity of 2-acetylaminofluorene have been shown (9–12) to result from reactive intermediates arising via cytochrome P450-dependent *N*-oxidation and therefore correlate well with the mutagenicity results *in vitro* (2–5, 8).

The induction of new cytochrome P_1450 formation (13) and numerous monooxygenase activities, including aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase (13) and *N*-acetylarylamine (2-acetylaminofluorene) *N*-hydroxylase (14), has been shown to be closely associated with the genetic locus for aromatic hydrocarbon "responsiveness" in mice (15). The mutagenicity of 2-acetylaminofluorene *in vitro* has recently been shown (1, 16) to be increased by liver postmitochondrial fractions from 3-methylcholanthrene-treated, genetically responsive C57BL/6N inbred mice, but not from 3-methylcholanthrene-treated, genetically nonresponsive DBA/2N inbred mice. One advantage of comparing genetically responsive and nonresponsive mice is that all animals receive the same amount of aromatic hydrocarbon inducer *in vivo* and that the liver fractions contain similar amounts of bound aromatic hydrocarbon (17). The major advantage of this experimental model system, however, is that the aromatic hydrocarbon "responsiveness" which is correlated with cytochrome P_1450 induction appears to be expressed as a single autosomal dominant gene when C57BL/6N and DBA/2N inbred strains are crossed (13, 15).

In this report we therefore show that whatever gene(s) is responsible for aro-

histidine operon; it is the intercalation of the frameshift mutagens that corrects the reading frame so that a functional protein in the histidine-biosynthetic pathway is now synthesized.

matic hydrocarbon "responsiveness" in the mouse cosegregates with the gene(s) responsible for increased mutagenicity of 2-acetylaminofluorene *in vitro*. Furthermore, we demonstrate that, although *N*-hydroxy-2-acetylaminofluorene is mutagenic without requiring metabolic activation, the *N*-hydroxy derivative is far more mutagenic after further metabolism by postmitochondrial or microsomal fractions, and that this activation of the *N*-hydroxy derivative is independent of genetic differences in aromatic hydrocarbon "responsiveness."

MATERIALS AND METHODS

Sources of reagents. Benzo[*a*]pyrene, NADPH, NADH, $NADP^+$, and glucose 6-phosphate were obtained from Sigma Chemical Company. 3-Methylcholanthrene and 2-acetylaminofluorene were purchased from J. T. Baker Chemical Company and Eastman Kodak Company, respectively. *N*-Hydroxy-2-acetylaminofluorene was a generous gift from Dr. Elizabeth Weisburger.

Source of animals. All mice used in the study either were provided by the National Institutes of Health animal supply or were the result of crosses made in our laboratory animal facility, using the NIH stocks C57BL/6N and DBA/2N as progenitors. All mice used in either the mutagenesis experiments or the aryl hydrocarbon hydroxylase studies were sexually immature mice of either sex, 4–6 weeks old.

Treatment of mice. The conditions for the housing of the mice have been described previously (13, 18). 3-Methylcholanthrene treatment consisted of a single intraperitoneal dose (80 mg kg^{-1}) in corn oil 40 hr before death; controls received corn oil alone.

Preparation of liver fractions. All steps were carried out at 0–4° with cold sterile solutions and glassware. Mouse liver, after being washed twice with phosphate-buffered 0.85% NaCl (pH 7.2), was minced and washed three more times with phosphate-buffered NaCl and finally was suspended in 3 volumes of buffer. Homogenization was carried out in a sterile Potter-Elvehjem homogenizer, using 8–10 strokes. The homogenate was centrifuged for 10 min at 9000 × *g*, and the supernatant (the S-9 fraction) was removed with a sterile pipette and saved. For the assay of aryl hydrocarbon hydroxylase, a portion of the S-9 fraction was centrifuged at 15,000 × *g* for 15 min and decanted, and the supernatant fraction was then centrifuged at 105,000 × *g* for 60 min. The resulting microsomal pellet was resuspended in ice-cold 250 mM potassium phosphate–KCl buffer, pH 7.4, and homogenized at the lowest setting with a Willems Polytron homogenizer.

Mutagenesis test with S-9 fraction. Mutagenesis was carried out according to Ames and co-workers (4). To 2 ml of molten top agar at 45° were added 0.1 ml of the bacterial tester strain TA1538 (2–3 × 10^9 bacteria/ml), 0.1 ml of dimethyl sulfoxide containing the chemical to be tested, and 0.5 ml of the S-9 mixture, containing 0.3 ml of S-9 fraction, 8 μmoles of $MgCl_2$, 33 μmoles of KCl, 5 μmoles of glucose 6-phosphate, 4 μmoles of NADP, and 100 μmoles of sodium phosphate buffer, pH 7.4, per milliliter. The colonies on each plate (histidine revertants) were counted after a 2-day incubation at 37°.

Hydroxylase assay. The hydroxylase activity and protein determination were carried out as previously described (13, 18). One unit of aryl hydrocarbon hydroxylase activity is defined as that amount of enzyme activity catalyzing, per minute at 37°, the formation of hydroxylated product equivalent to 1 pmole of the recrystallized 3-hydroxybenzo[*a*]pyrene standard.

RESULTS

Characterization of mutagenesis assay in vitro. Figure 2 shows the histidine revertant rate as a function of 2-acetylaminofluorene concentration *in vitro*. With 10 μg of 2-acetylaminofluorene and 2.5 mg of liver S-9 protein per plate, the 3-methylcholanthrene-treated, responsive C57BL/6N mouse showed an approximately 5-fold increase in the number of mutations, compared with the 3-methylcholanthrene-treated, nonresponsive DBA/2N mouse. Even when much less protein (0.45 mg/plate) was used, the S-9 fraction from 3-

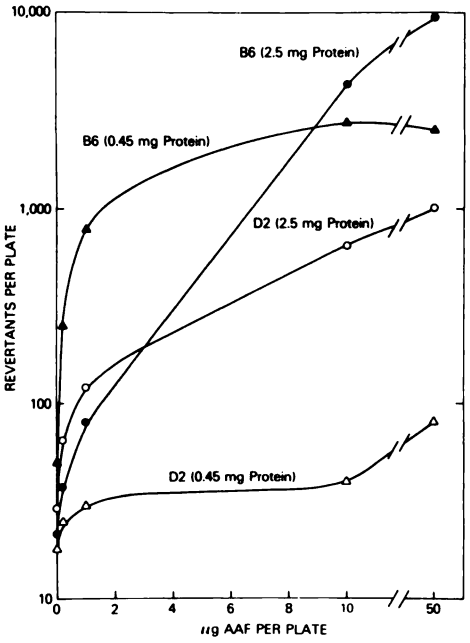

FIG. 2. *Dependence of histidine revertant rate on concentration of 2-acetylaminofluorene (AAF) in vitro*

▲ and ●, the responsive C57BL/6N (B6) mouse; △ and ○, the nonresponsive DBA/2N (D2) mouse. The liver S-9 fractions were from mice which had received prior treatment with 3-methylcholanthrene *in vivo*. Two S-9 protein concentrations (milligrams per plate) for each inbred strain are shown.

methylcholanthrene-treated C57BL/6N mice caused a more than 5-fold higher mutagenesis rate than that from 3-methylcholanthrene-treated DBA/2N mice. We consistently found suppression of mutagenesis at high protein and low 2-acetylaminofluorene concentrations when fractions from 3-methylcholanthrene-treated C57BL/6N mice were used. Most likely, mutagens formed by the liver monooxygenase system(s) or other enzymes are unable to react with the bacteria and "score" as a mutation in the presence of sufficiently quenching amounts of liver protein relative to the amount of parent compound added *in vitro*, or, alternatively, induction by 3-methylcholanthrene of "detoxification" pathways for *N*-hydroxy-2-acetylaminofluorene could lead to nonmutagenic products. Similar observations and conclusions were reported with benzo[*a*]pyrene and rat liver S-9 fractions *in vitro* (19).

The revertants per plate shown in Fig. 2 are the averages of duplicate determinations, which always varied in a given experiment by less than 10%. Experiments performed on different weeks gave the same relative genetic differences. However, the absolute colony counts sometimes varied as much as several fold. Because $NADP^+$, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase gave results equal to those when NADPH was used *in vitro*, the former system was used routinely because of lower cost.

A metabolite that is more toxic than mutagenic to the bacterial tester strain would appear as a smaller number of histidine revertants simply because death of the bacteria had intervened. This possibility was ruled out by assessing the total number of bacteria able to grow on histidine-enriched agar following exposure of the bacteria, which had been serially diluted to either 10,000 or 1000 bacteria/ml, to 1–50 μg of 2-acetylaminofluorene per plate. No more than a 5% death rate in tester strain TA1538 was found with the highest (50 μg/plate) concentration of 2-acetylaminofluorene. We thus feel that toxicity is not an important factor in the mutagenesis assay under the conditions employed in this study.

Figure 3A and B shows the dependence of the revertant rate on the S-9 and the microsomal protein concentration, respectively, for both C57BL/6N and DBA/2N 3-methylcholanthrene-treated mice. With both the S-9 and microsomal fractions in the presence of 10 μg of 2-acetylaminofluorene per plate, there was an increased revertant rate when C57BL/6N mice were compared with DBA/2N mice. At lower 2-acetylaminofluorene concentrations, there was also a large difference between C57BL/6N and DBA/2N mice, especially at protein concentrations below 0.5 mg/plate. At higher protein concentration, the suppression effect described above for Fig. 2 was again clearly evident.

N-Hydroxy-2-acetylaminofluorene vs. 2-acetylaminofluorene mutagenesis in vitro. Table 1 shows the effects of prior treatment of the mice *in vivo* and the requirement for metabolic activation by liver

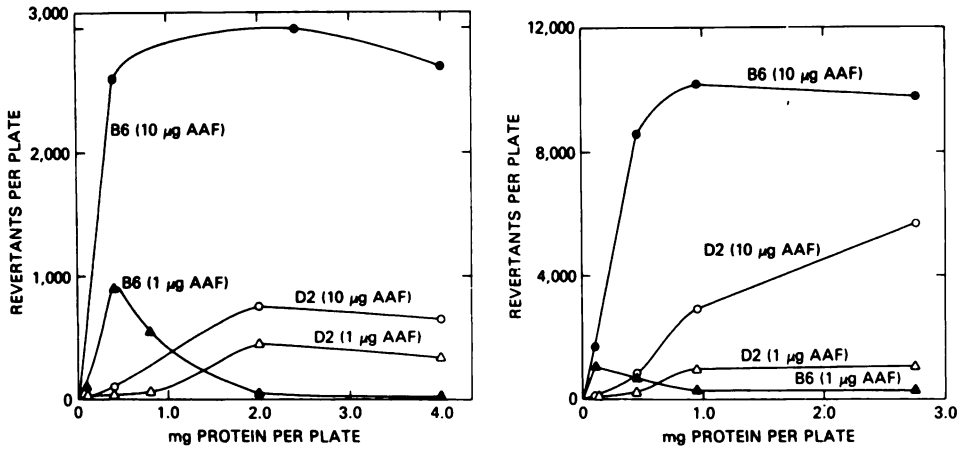

FIG. 3. *2-Acetylaminofluorene (AAF) mutagenicity in vitro as a function of S-9 protein concentration (A) and microsomal protein concentration (B)*

▲ and ●, the responsive C57BL/6N (B6) mouse; △ and ○, the nonresponsive DBA/2N (D2) mouse. The mice had previously been treated with 3-methylcholanthrene *in vivo*. Two concentrations of 2-acetylaminofluorene per plate are shown.

TABLE 1

Effect of prior aromatic hydrocarbon treatment on 2-acetylaminofluorene and N-hydroxy-2-acetylaminofluorene mouse liver activated mutagenesis

Treatment of mice, preparation of liver homogenates, and the bacterial assay are described in MATERIALS AND METHODS. All values are histidine-positive revertants per plate. Each plate contained approximately 2 × 10^8 bacteria and approximately 2.5 mg of protein. All values are the means from two separate experiments.

Strain	Prior treatment	Liver fraction	Test substance added *in vitro*[a]				
			AAF, 1 μg	AAF, 10 μg	*N*-OH-AAF, 1 μg	*N*-OH-AAF, 10 μg	DMSO alone
DBA/2N	Corn oil	S-9	191	495	905	4,560	46
C57BL/6N	Corn oil	S-9	171	540	2,630	4,100	41
DBA/2N	3-Methylcholanthrene	S-9	180	685	960	5,745	56
C57BL/6N	3-Methylcholanthrene	S-9	61	5,200	281	3,185	49
DBA/2N	Corn oil	Microsomes	368	354	925	8,360	56
C57BL/6N	Corn oil	Microsomes	382	540	890	7,320	48
DBA/2N	3-Methylcholanthrene	Microsomes	710	2,600	845	8,650	60
C57BL/6N	3-Methylcholanthrene	Microsomes	75	10,000	262	7,355	53
		None	35	42	44	242	39
DBA/2N	Corn oil	S-9 without NADPH		46		3,360	
C57BL/6N	Corn oil	S-9 without NADPH		56		4,515	
DBA/2N	3-Methylcholanthrene	S-9 without NADPH		39		6,735	
C57BL/6N	3-Methylcholanthrene	S-9 without NADPH		43		10,065	

[a] The abbreviations used are: AAF, 2-acetylaminofluorene; *N*-OH-AAF, *N*-hydroxy-2-acetylaminofluorene; DMSO, dimethyl sulfoxide.

postmitochondrial or microsomal fractions on the mutagenicity *in vitro* of 2-acetylaminofluorene, *N*-hydroxy-2-acetylaminofluorene, or the solvent dimethyl sulfoxide alone. The spontaneous revertant rate with dimethyl sulfoxide alone was approximately 50 colonies/plate. With the S-9 fraction, 1 μg of 2-acetylaminofluorene per plate produced a suppression of the histidine revertant rate, whereas 10 μg of the chemical per plate produced a more than 7-fold increase in mutagenicity, when 3-methylcholanthrene-treated C57BL/6N mice were compared with 3-methylcholanthrene-treated DBA/2N mice or with either strain treated with corn oil alone. A similar result was found when the microsomal rather than the S-9 fraction was used. Ten micrograms of *N*-hydroxy-2-acetylaminofluorene caused 242 revertants in the absence of any liver fraction, confirming (2, 3) that this compound does not necessarily require metabolic activation in order to cause frameshift mutations. However, the mutagenesis rate was at least 20 times higher in the presence of the S-9 fraction and about 40 times higher in the presence of microsomes. Of particular interest is the lack of any genetic difference in *N*-hydroxy-2-acetylaminofluorene mutagenicity between 3-methylcholanthrene-treated C57BL/6N and DBA/2N mice. In addition, the number of revertant colonies did not depend on the presence of an NADPH-regenerating system, as both microsomal C57BL/6 and DBA/2 fractions still showed more than 5000 colonies. This suggests that the activation of *N*-hydroxy-2-acetylaminofluorene does not depend on the cytochrome P450 monooxygenase enzyme system; rather, a different microsomal enzyme(s) is probably responsible for the activation. We therefore conclude that the *N*-hydroxy metabolite, although slightly mutagenic without metabolic activation by liver fractions *in vitro*, is far more mutagenic when metabolized by some enzymatic step other than cytochrome P_1450.

The 2630 mutants/plate caused by 1 μg of *N*-hydroxy-2-acetylaminofluorene in the presence of the S-9 fraction from control C57BL/6N mice decreased to 281 mutants/plate following 3-methylcholanthrene treatment *in vivo*. This effect is consistent with the suppression seen in Figs. 2 and 3.

Association of 2-acetylaminofluorene mutagenicity in vitro with aromatic hydrocarbon responsiveness. Figure 4 demonstrates aromatic hydrocarbon-inducible aryl hydrocarbon hydroxylase activity as a function of 2-acetylaminofluorene mutagenesis *in vitro* for the inbred C57BL/6N and DBA/2N parents, the F_1 heterozygote generated from both reciprocal crosses, offspring from the backcross of the F_1 hybrid to the nonresponsive DBA/2N parent, and F_2 progeny. The F_1 offspring showed the expected expression of a dominant gene for aryl hydrocarbon hydroxylase activity; however, there was the suggestion of additive inheritance for 2-acetylaminofluorene mutagenicity *in vitro*, because the F_1 animals were intermediate between the two parents. Offspring from the (C57BL/6N)(DBA/2N)F_1 × DBA/2N backcross segregated with 19 animals in the low class (low hydroxylase activity and low revertant rate) and 23 in the high class (high hydroxylase activity and high revertant rate). Since we found no recombinants (i.e., low hydroxylase activity and high revertant rate, or vice versa) and since the segregation was in approximately a 1:1 ratio, it appears that increased 2-acetylaminofluorene mutagenicity *in vitro* segregates in a Mendelian manner as a single gene—the same as, or closely linked to, the gene(s) for aromatic hydrocarbon responsiveness.

The F_2 generation also segregated in a manner consistent with this conclusion; approximately a 3:1 ratio occurred, with the high class having about three-fourths and the low class about one-fourth of the total animals tested. Theoretically, if 2-acetylaminofluorene mutagenicity is additively inherited, the F_2 generation should contain three groups: one-fourth low, one-half intermediate, and one-fourth high revertant rates. This relative distribution may in fact occur in the lower right portion of Fig. 4, but is difficult to state with certainty because of the overlap caused by the

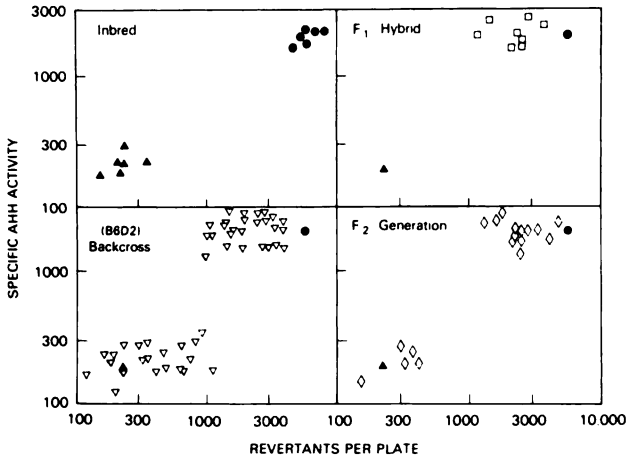

FIG. 4. *Relationship between 2-acetylaminofluorene mutagenesis in vitro and microsomal aryl hydrocarbon hydroxylase (AHH) activity in liver fractions from inbred C57BL/6N (●) and DBA/2N (▲), the (C57BL/6N) (DBA/2N)F_1 hybrid (□), offspring from the (C57BL/6N) (DBA/2N)F_1 × DBA/2N backcross (▽), and the F_2 generation (◇)*

Each symbol represents the result from an individual mouse, except that the ● and ▲ in the upper right and two lower graphs represent the means of six values found for the C57BL/6N and DBA/2N inbred mice, respectively. All mice in this study had been treated with 3-methylcholanthrene *in vivo*. The abscissa and ordinate are both plotted on logarithmic scales so that the mean ± standard deviation of the nonresponsive group is equal, in terms of linear distance, to the mean ± standard deviation of the responsive group. In this study 10 μg of 2-acetylaminofluorene and 0.45 mg of protein per plate were used.

greater variance at the higher revertant rates.

Since there was the suggestion that F_1 hybrids may show additive inheritance for 2-acetylaminofluorene mutagenicity *in vitro* (Fig. 4), additional F_1 animals were screened in a separate experiment (Fig. 5). The observed mean revertant rate for the F_1 heterozygotes is almost identical with the arithmetic mean revertant rate of the two inbred parents, suggesting additive inheritance.

DISCUSSION

We have previously shown (14) that the *N*-hydroxylation of 2-acetylaminofluorene is linked to aromatic hydrocarbon responsiveness and to new cytochrome P_1450 formation in mice. This report demonstrates that the metabolic activation of 2-acetylaminofluorene to a frameshift mutagen *in vitro* is controlled in the mouse by the same (or closely linked) gene(s) as that regulating aromatic hydrocarbon responsiveness. The *N*-hydroxylating step is thus judged to be important in causing mutagenicity, as well as carcinogenicity (9, 10) and hepatotoxicity (11, 12, 14).

Our data also demonstrate that, although *N*-hydroxy-2-acetylaminofluorene is actively mutagenic in the absence of liver fractions *in vitro*, its mutagenicity is enhanced 20–40-fold in the presence of liver fractions from either 3-methylcholanthrene-treated or control C57BL/6N or DBA/2N mice. These results indicate that the steady-state concentration of the *N*-hydroxy metabolite is critical in causing differences in the rate of mutagenesis and that the cytochrome P_1450-mediated *N*-hydroxylation is a rate-limiting step in the metabolic activation of 2-acetylaminofluorene to its mutagenic form. Hence, if equal amounts of the *N*-hydroxy metabolite are added to liver fractions from 3-methylcholanthrene-treated or control C57BL/6N or DBA/2N mice, the mutagenesis rates no longer show the genetic difference associated with cytochrome P_1450. The activation of *N*-hydroxy-2-acetylaminofluorene is independent of NADPH, indicating that enzymes other than monooxygenases are

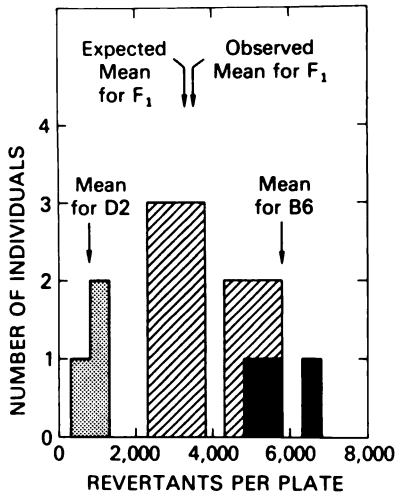

FIG. 5. *Histogram showing distribution of 2-acetylaminofluorene mutagenesis rates in vitro when liver S-9 fractions from three DBA/2N (D2) and three C57BL/6N (B6) inbred parents and 13 (C57BL/6N) (DBA/2N)F_1 hybrids of the same age were examined on the same day*

For each plate, 10 μg of 2-acetylaminofluorene and 0.45 mg of protein were used. The expected mean for a gene-dose (additive) relationship is shown by the arrow exactly midway between the mutation rates of the two inbred parents.

involved. The most likely "activation" enzyme(s) for *N*-hydroxy-2-acetylaminofluorene is the microsomal deacetylase(s), since the revertant rate is about doubled when the microsomal fraction is used instead of the S-9 fraction. Deacetylation of *N*-hydroxy-2-acetylaminofluorene results in the product *N*-hydroxy-2-aminofluorene, which has been shown to be 380 times more mutagenic than *N*-hydroxy-2-acetylaminofluorene in the TA1538 strain (3). Experiments designed to examine genetic differences in the specific activities of the *N*-hydroxy-2-acetylaminofluorene deacetylase(s), transacetylase(s), and sulfotransferase(s) in these mice should provide further insight into understanding the mechanism of *N*-acetylarylamine mutagenicity, carcinogenicity, and hepatotoxicity.

How can 2-acetylaminofluorene mutagenesis *in vitro* exhibit additive inheritance, whereas 3-methylcholanthrene-inducible aryl hydrocarbon hydroxylase activity exhibits apparent autosomal dominant inheritance? Mutagenesis presumably reflects the steady-state concentration of whatever chemical intermediate(s) react with DNA. Aryl hydrocarbon hydroxylase "activity," on the other hand, represents the quantity of phenolic metabolites of benzo[*a*]pyrene formed (15); these phenols may be formed either by a two-step process via an arene oxide or by a direct oxygen-insertion, one-step hydroxylation (20, 21). For these reasons it is perhaps not surprising that 2-acetylaminofluorene mutagenesis *in vitro* appears to be inherited additively, whereas the formation of phenolic benzo[*a*]pyrene metabolites *in vitro* by these same liver microsomal fractions appears to be inherited in an autosomal dominant fashion. Another possibility to explain the additive inheritance of 2-acetylaminofluorene mutagenesis *in vitro* would be that enzymes subsequent to formation of the *N*-hydroxy metabolite become rate-limiting in the F_1 hybrids. This possibility is unlikely because (a) no recombinants are seen in offspring from the (C57BL/6N) (DBA/2N)F_1 × DBA/2N backcross or in the (C57BL/6N) (DBA/2N)F_2 generation and (b) no effects other than the expected additivity are found when S-9 fractions from responsive mice are mixed *in vitro* with S-9 fractions from nonresponsive mice.

Studies *in vitro* with 1,1,1-trichloropropene 2,3-epoxide, an inhibitor of microsomal epoxide hydrase (22), and diethyl maleate, a compound which depletes glutathione content in liver (23), indicated recently that whereas benzo[*a*]pyrene mutagenesis *in vitro* is closely associated with arene oxide formation, the mutagenicity of 3-methylcholanthrene, 6-aminochrysene, and 2-acetylaminofluorene *in vitro* is apparently not associated with an arene oxide (16). The data in this report support this hypothesis. We conclude that genetically controlled formation of the *N*-hydroxy derivative of 2-acetylaminofluorene by cytochrome P_1450, rather than ring oxygenation, is the critical rate-limiting step and that subsequent metabolism of the *N*-hydroxy derivative of 2-acetylaminofluorene by cytochrome P_1450, rather than ring oxygenation is the critical rate-limit-

ing step and that subsequent metabolism of the *N*-hydroxy derivative by enzymes other than cytochrome P_1450 results in the highly mutagenic intermediate(s).

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