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SEX AND STRAIN DIFFERENCES IN RESPONSE TO COCAINE

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Abstract—After pretreatment with phenobarbital, female B6AF$_1$ mice showed considerably higher serum glutamic oxaloacetic transaminase (SGOT) elevations and more periportal necrosis from a single injection of cocaine than males. This sex difference was androgen dependent. Castration or treatment with flutamide made males respond like females, while testosterone made females behave like males. There was no significant sex difference in enzymes of cocaine metabolism. When the mice were induced by exposure to pine bedding, males showed higher SGOT elevations and more centrilobular necrosis after cocaine than females. In this case, the sex difference could be attributed to increased levels of cytochrome P-450 and cocaine *N*-demethylase in liver microsomes. BALB/cBy mice on pine bedding showed much less liver damage from cocaine than B6AF$_1$ mice, but they were more sensitive to norcocaine and *N*-hydroxynorcocaine. This difference was correlated with low levels of cocaine *N*-demethylase in the BALB/cBy mice. Liver microsomes from phenobarbital-induced BALB/cBy mice had less norcocaine *N*-hydroxylase activity than those from B6AF$_1$ mice. These studies demonstrate that the pattern of sex and strain differences in liver damage from cocaine depends on the inducing agent and can be related to a large extent to the microsomal enzymes induced by that agent.

Recently, we reported that cocaine is a strong hepatotoxin in mice, producing fatty infiltration, midzonal and periportal necrosis, and marked elevation of serum glutamic oxaloacetic transaminase (SGOT) [1]. In a subsequent paper, we demonstrated that the hepatotoxicity of cocaine is due to the action of a metabolite of cocaine, rather than cocaine itself. Our results showed this bioactivation to be a multistep process, most likely carried out by the cytochrome P-450 microsomal mixed-function oxidase system. The metabolites norcocaine and *N*-hydroxynorcocaine (NHNC) were found to be at least as hepatotoxic as cocaine, and it was suggested that a metabolite of *N*-hydroxynorcocaine was responsible for producing liver damage [2]. In the present paper, we report that significant sex and strain differences exist in the hepatotoxic effects of cocaine. The direction of the sex difference depends on the type of inducing agent used to stimulate the bioactivation of cocaine, and is under androgenic control. Strain differences are correlated with *in vitro* measures of cocaine metabolism.

METHODS AND MATERIALS

Subjects. The mice used in these experiments were B6AF$_1$ hybrids (C57B1/6J X A/J) or BALB/cBy mice, obtained from Jackson Laboratories, Bar Harbor, ME, at 4–6 weeks of age, and were between 3 and 12 months old at the time of testing. Unless specified, all mice were housed on corncob bedding in clear plastic cages, three to six in a cage, in an air-conditioned room maintained at 22° with a light–dark cycle of 12/12 hr, not reversed.

Procedures. Injections were given at 5:00 p.m. and blood samples were drawn at 9:00 a.m. on the following day. Determination of SGOT values was according to the procedure described previously [3]. Phenobarbital induction of mice was carried out by administering phenobarbital (0.8 g/l) in drinking water for 4–6 days. Pine bedding induction was achieved by housing the mice on pine bedding for at least 1 week prior to cocaine administration. β-Ionone (60 mg/ml in olive oil) was injected s.c. in a volume of 0.10 ml 72 and 48 hr prior to cocaine. 3-Methylcholanthrene (3-MC) was dissolved in olive oil and injected i.p. at a dose of 25 mg/kg per day for 6 days. Diazinon was suspended in water and injected i.p. at a dose of 20 mg/kg 30 min prior to cocaine.

Hormonal studies. Mice were castrated under ether anesthesia and allowed to recover for 10 days prior to testing. Testosterone propionate was dissolved in sesame oil, and 1 mg in a volume of 0.05 ml was injected daily s.c. for 5 days. Flutamide was dissolved in sesame oil, and 0.3 mg in a volume of 0.05 ml was injected daily s.c. for 5 days. Control mice were injected with vehicle.

Drugs. Sodium phenobarbital was purchased from Gilman Bros., Boston, MA. Testosterone propionate and flutamide were gifts from the Schering Corp.,

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Bloomfield, NJ. Diethyl-*O*-(2-isopropyl-4 methylpyrimidyl) thiophosphate (diazinon) and β-ionone (BI) were purchased from Pfaltz & Bauer, Flushing, NY. 3-Methylcholanthrene (3-MC) was purchased from Eastman, Rochester, NY.

In vitro *studies*. For the preparation of microsomes, livers were homogenized in a teflon–glass homogenizer with 4 vol. of cold 0.1 M potassium phosphate buffer, pH 7.4. The supernatant solution that was obtained by centrifuging the homogenate for 10 min at 10,000 *g* was centrifuged for 1 hr at 105,000 *g*. The resulting microsomal pellet was rinsed with a few milliliters of cold buffer. Enough buffer to reconstitute the original volume was layered over the pellet, and the pellet was stored at −90°. This method, described by Levin *et al.* [4], effectively preserved the activity of the cytochrome P-450 system for at least 3 months. A suspension of microsomes in buffer could also be stored at −90° for several months with little loss in activity [5]. The protein content of microsomes was determined colorimetrically according to Lowry *et al.* [6].

The high performance liquid chromatography (HPLC) apparatus used in these experiments consisted of a model U6K injector, a model M-6000A pump, a model 450 mm reversed phase C18 column (all from Water Associates, Milford, MA), and a model LC-2A controller plus a model TL7A glassy carbon detector cell from Bioanalytical Systems, West Lafayette, IN.

Norcocaine was synthesized from cocaine according to Borne *et al.* [7]. *N*-Hydroxynorcocaine was prepared from norcocaine as described previously. The purity of these compounds was checked by determination of melting points, by thin-layer chromatography, and by HPLC. The glutathione content of trichloroacetic acid extracts of liver was determined with Ellman's reagent according to the method of Kaplowitz [8]. The cytochrome P-450 content of microsomal preparations was assayed according to the method of Omura and Sato [9].

For *in vitro* assays of norcocaine or NHNC formation, the standard incubation mixture contained, in a volume of 1 ml: potassium phosphate buffer, pH 7.4, 65 μmoles; glucose-6-phosphate, 5.4 μmoles; glucose-6-phosphate dehydrogenase from Torula yeast, 0.26 units; $MgCl_2$, 4 μmoles, nicotinamide, 2 μmoles; fluoride, 10 μmoles; cocaine HCl (3 μmoles) or norcocaine (1 μmole) and 100 μl of a microsomal suspension, equivalent to 25 mg of whole liver, containing about 0.8 mg of microsomal protein. Incubation was carried out in a 50-ml Erlenmeyer flask on a rotating incubator-shaker at 37° for 15 min. Formaldehyde produced from the *N*-demethylation of cocaine was assayed according to the method of Chastril and Wilson [10]. The formation of *N*-hydroxynorcocaine from norcocaine was measured according to the method of Shuster *et al.* [11]. One hundred microliters of the incubation mixture was removed into 300 μl of cold 95% ethanol in a 1.5-ml conical polypropylene centrifuge tube. After centrifugation for 1 min at 12,000 *g* in a Brinkmann microfuge, the supernatant solution was decanted and kept on ice for assay by high performance liquid chromatography with an electrochemical detector (HPLC-EC). Quantitation of NHNC was done by peak-height comparison with an external standard of NHNC. Values obtained in this way were in good agreement with those calculated from areas under the curve.

FAD-monooxygenase activity was measured according to Cashman and Hanzlik [12]. Plasma esterase activity was measured by the method of Mendoza *et al.* [13] using indophenyl acetate as a substrate.

Histopathology. Animals were killed by cervical dislocation 16–18 hr after treatment with cocaine or its metabolites, norcocaine or *N*-hydroxynorcocaine. The livers were removed and fixed in 10% formal-saline solution, embedded in araldite, and 3 μm hematoxylin and eosin tissue sections examined.

Statistics. Results within the various studies were compared by either *t*-tests or analysis of variance using Student–Newman–Keuls tests for post-hoc testing. Significance testing was set at the $P < 0.05$ level.

RESULTS

Sex and strain differences in cocaine hepatotoxicity in phenobarbital-induced mice. Phenobarbital-induced males and females of two strains of mice were compared in terms of SGOT elevation following the injection of cocaine in doses of 50 or 100 mg/kg. Separate groups received equal doses of either norcocaine or *N*-hydroxynorcocaine.

The most obvious differences between the two strains and sexes was that $B6AF_1$ mice were much

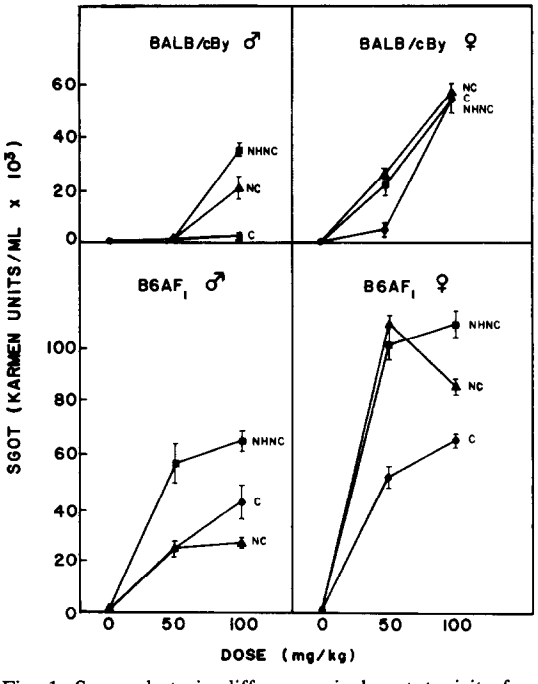

Fig. 1. Sex and strain differences in hepatotoxicity from cocaine (C), norcocaine (NC) or *N*-hydroxynorcocaine (NHNC) following phenobarbital pretreatment. Each point represents the mean (± S.E.M.) of SGOT determinations on at least five mice at 16–18 hr after the injection of cocaine or its metabolites.

more sensitive to the hepatoxic effects of cocaine and its two metabolites than were BALB/cBy mice. $B6AF_1$ mice showed no dose-related increase in SGOT response, being already near maximal at the lower dose. On the other hand, BALB/cBy mice did show a significant increase in SGOT response in going from the low dose to the high dose (Fig. 1).

The second most obvious finding was that, for both strains, females showed significantly larger SGOT responses after cocaine, norcocaine, or *N*-hydroxynorcocaine than did males. This was true for both doses. At the 50 mg/kg dose, regardless of the compound injected, females in both strains showed SGOT elevations more than twice as large as those found in males. For all compounds females still showed significantly greater SGOT elevations at the higher dose, but the magnitude of the difference was less in some instances.

The relative toxicity of cocaine, norcocaine, or *N*-hydroxynorcocaine varied with the dose, sex, or strain and the various combinations thereof. Small numbers of subjects in some groups, especially at the higher dose in $B6AF_1$ mice (due to deaths), and high variability in SGOT response, also made interpretation difficult. In male mice, significant differences between the three drugs were only found for BALB/cBy mice with the 100 mg/kg dose, where the order of toxicity was *N*-hydroxynorcocaine > norcocaine > cocaine. In $B6AF_1$ males, the order appeared to be *N*-hydroxynorcocaine > norcocaine = cocaine, but the difference just failed to reach significance. In female mice, the order of toxicity was *N*-hydroxynorcocaine = norcocaine > cocaine for both strains at the 50 mg/kg dose. At the higher dose, BALB/cBy females showed no difference in response to the three compounds, while in $B6AF_1$ females *N*-hydroxynorcocaine > cocaine, with the response to norcocaine intermediate between the other two.

The histologic pattern of tissue damage was similar regardless of sex, strain, or whether cocaine, norcocaine, or *N*-hydroxynorcocaine was injected. However, the degree of damage varied directly with the change in SGOT activity. Hepatocytes in the periportal zone were predominantly involved with an eosinophilic and granular type of cell necrosis. Most of the time one to three layers of cells immediately adjacent to the portal tracts were spared of injury; however, when necrosis was most severe (i.e. in $B6AF_1$ females), these cells were also damaged. Moderate fatty change was prominent in female mice of both strains, but inconsistently present in male $B6AF_1$ mice. Portal tracts in all mice showed no significant changes.

Sex and strain differences in cocaine hepatotoxicity after induction by pine bedding. Males and females of two strains of mice were housed on pine bedding for 7–10 days prior to the injection of cocaine (50 mg/kg, i.p.). Separate groups received equal doses of norcocaine or *N*-hydroxynorcocaine. The SGOT response was quite different in these mice (Table 1) compared to that observed in phenobarbital-induced mice. Only $B6AF_1$ males showed the typically large SGOT elevation in response to cocaine. $B6AF_1$ females and BALB/cBy males and females showed only a minimal response. However, males and females of both strains showed liver damage following injections of norcocaine or *N*-hydroxynorcocaine. BALB/cBy mice were much more sensitive than $B6AF_1$ mice, a finding just opposite to that observed in phenobarbital-induced mice. Female BALB/cBy mice showed significantly larger SGOT responses than did males, while in $B6AF_1$ mice the sex difference was reversed. In terms of order of toxicity, in $B6AF_1$ males cocaine = norcocaine = *N*-hydroxynorcocaine, while in $B6AF_1$ females and BALB/cBy males and females the order of toxicity was *N*-hydroxynorcocaine = norcocaine > cocaine.

Histological examination again showed the pattern of tissue damage to be the same regardless of sex, strain, or compound injected, and the degree of damage again reflected the change in SGOT activity. However, in contrast to the mainly midzonal-periportal necrosis found in phenobarbital-induced mice, pine bedding-induced mice showed necrosis in the midzonal-centrilobular areas.

Sex differences following chronic cocaine injection. Significant sex differences were also evident when liver damage was produced by chronic injection of cocaine. Male and female $B6AF_1$ mice, housed on corncob bedding, were injected daily for 5 days with 30 mg/kg cocaine i.p. Blood samples were drawn daily at 16–18 hr after each injection. This regimen produced a large increase of SGOT by day 3 in both males and females. Males showed much more of an increase than females (Fig. 2). Continuation of daily

Table 1. Liver damage in pine bedding-induced mice*

Strain	Drug	SGOT (Karmen Units/ml) Males (N)	Females (N)
$B6AF_1$	Cocaine	13,989 ± 4,689 (6)	353 ± 176† (6)
	Norcocaine	12,709 ± 3,769 (5)	45,656 ± 39,383 (5)
	N-Hydroxynorcocaine	9,882 ± 4,361 (6)	71,882 ± 42,595 (4)
BALB/cBy	Cocaine	201 ± 23 (6)†	313 ± 54 (6)
	Norcocaine	65,896 ± 13,259† (7)	142,151 ± 18,075†‡ (7)
	N-Hydroxynorcocaine	26,234 ± 9,651 (6)	100,629 ± 27,274† (6)

* SGOT values are expressed as mean ± S.E.M. All compounds were injected i.p. at a dose of 50 mg/kg.

† Indicates significant sex difference ($P < 0.05$).

‡ Indicates significant within sex strain difference ($P < 0.05$).

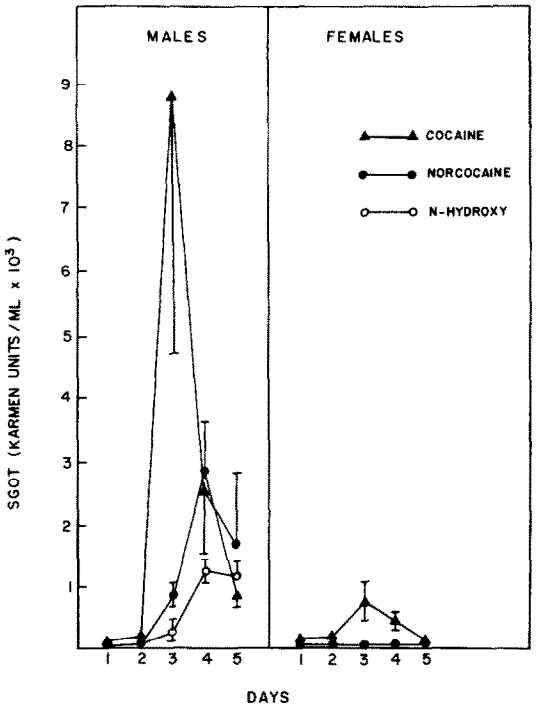

Fig. 2. Sex differences in SGOT elevation following chronic treatment with cocaine, norcocaine, or *N*-hydroxynorcocaine. B6AF$_1$ male and female mice housed on corncob bedding were injected i.p. once a day for 5 days with cocaine, norcocaine, or *N*-hydroxynorcocaine at a dose of 30 mg/kg. Blood samples for SGOT assay were taken 16–18 hr after each injection. Each point represents the mean (± S.E.M.) for a group of six mice.

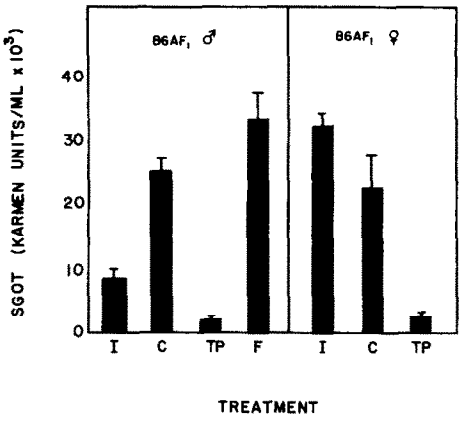

Fig. 3. Effect of hormonal manipulations on cocaine-induced SGOT elevation in phenobarbital-induced intact (I) or castrated (C) male and female B6AF$_1$ mice. Testosterone propionate (TP) (1 mg/day) to castrated males and females or flutamide (F) (0.3 mg/day) to intact males was given s.c. for 5 days. On day 6, cocaine (50 mg/kg) was injected. Bars represent the mean (± S.E.M.) for a group of six mice.

injections for 2 more days did not produce further increases in SGOT. Instead, SGOT values returned toward baseline levels. Similar treatment with norcocaine or *N*-hydroxynorcocaine also produced significant SGOT elevations in males, with the peak elevation occurring on day 4. The SGOT levels of females, on the other hand, remained normal throughout the 5 days of treatment with these cocaine metabolites. For histological examination, livers were removed from male mice after 3 days of cocaine treatment. This type of drug treatment resulted in mild to moderate necrosis, which again was correlated with the SGOT response. The necrosis started in midzonal regions and extended to the centrilobular area, with hepatocyte sparing around the central vein when the necrosis was not severe. In addition, there was chronic infiltration in the areas of necrosis, with the degree of infiltrate varying with the degree of necrosis.

Effect of hormonal manipulations of sex differences on cocaine hepatotoxicity. Figure 3 describes the result of various hormonal manipulations on cocaine hepatotoxicity in phenobarbital-induced B6AF$_1$ males and females. The previously observed sex differences were replicated once again in the intact, oil-injected groups, with females showing SGOT elevations after cocaine that were four times as great as those observed in males. Ovariectomized females had somewhat reduced SGOT levels, whereas castrated males showed SGOT changes three times greater than those observed in the intact males, and comparable to those observed in intact or ovariectomized females. Pretreatment of castrated males or females with testosterone propionate (TP) resulted in significantly less SGOT elevation after cocaine. One group of intact males was pretreated with flutamide, a nonsteroidal anti-androgen devoid of hormonal activity which inhibits androgen uptake and retention by the androgen receptors of the target tissues [14]. This group showed SGOT elevations similar to those of intact females and castrated males.

Analyses of variance of the data supported these conclusions. Males, castrated males injected with TP, and females injected with TP showed significantly less SGOT elevation than those groups (females, castrated males or intact males pretreated with flutamide) not exposed to TP. Groups within each of these two subgroups did not differ significantly from each other.

Effects of other inducing agents on cocaine-induced liver damage. The magnitude, as well as the direction, of sex and strain differences in the amount of SGOT elevation from cocaine varies with the inducing agent, as is obvious from a comparison of Fig. 1 and Table 1. Two other inducing agents were also examined in B6AF$_1$ mice, and Fig. 4 compares their effects to those of pine and phenobarbital (data repeated from Table 1 and Fig. 1 respectively). β-Ionone, an essential oil which has been shown to be a potent inducer of mixed-function oxygenases [15], induced cocaine hepatotoxicity in a pattern similar to pine bedding, with males responding more than females. Mice pretreated with 3-methylcholanthrene, which induces primarily cytochrome P-448, failed to show any elevation of SGOT.

Sex and strain differences in cocaine metabolism by the cytochrome P-450 system. In vitro determinations of cytochrome P-450 levels as well as

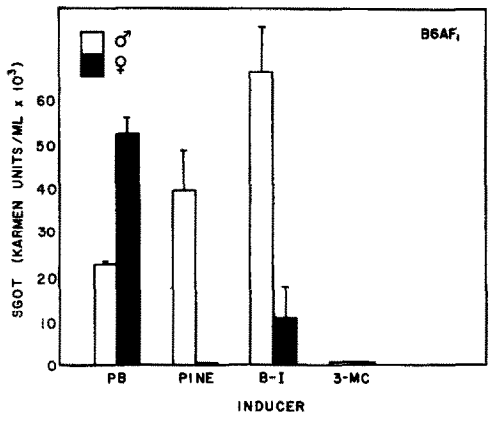

Fig. 4. Effect of pretreatment with different inducing agents on increases in SGOT produced by cocaine in male and female $B6AF_1$ mice. Each bar represents the mean (± S.E.M.) of SGOT determinations on at least five mice exposed to phenobarbital (PB) (0.8 g/l in water for 6 days—data repeated from Fig. 1), pine bedding (7 days housing in pine bedding), β-ionine (B-I) (6 mg/per mouse injected s.c. at 72 and 48 hr prior to cocaine) or 3-methylcholanthrene (3-MC) (25 mg/kg/day for 6 days). Cocaine was injected at a dose of 50 mg/kg i.p.

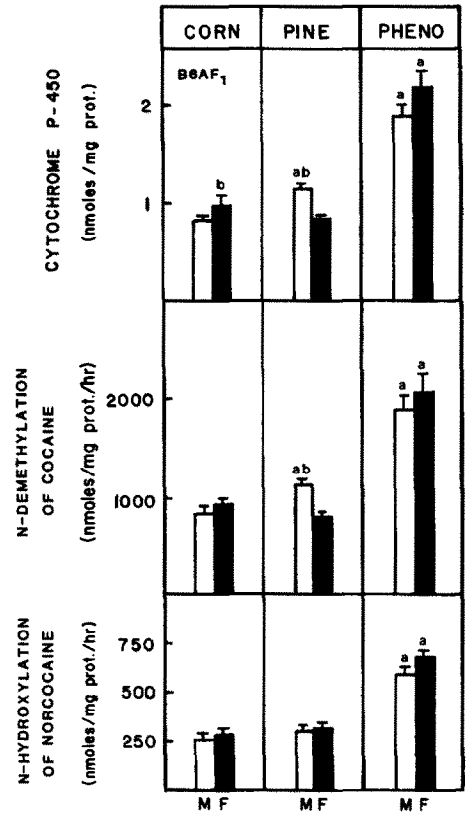

Fig. 5. Sex differences in mean (± S.E.M.) cytochrome P-450 levels, cocaine *N*-demethylase, and *N*-hydroxylation of norcocaine in liver microsomes from $B6AF_1$ mice. Male and female mice were either uninduced (housed on corncob bedding) or induced by exposure to pine bedding for 1 week or by phenobarbital (0.8 g/l in the drinking water 6 days). Each group had at least eight mice. Key: (a) significantly different from uninduced mice; (b) significant within strain sex difference for that particular condition.

microsomal cocaine *N*-demethylase and *N*-hydroxynorcocaine formation from norcocaine were carried out with microsomal preparations from the livers of mice that were not induced (corncob bedding) and of mice induced by exposure to pine bedding or by pretreatment with phenobarbital. Non-induced $B6AF_1$ females showed significantly higher cytochrome P-450 levels than $B6AF_1$ males (Fig. 5). Other baseline measures did not differ significantly. The responses of males and females to induction by pine bedding were quite different. Males showed significantly increased cytochrome P-450 levels and cocaine *N*-demethylase activities, while females showed no change on any measure. Induction by phenobarbital produced significant increases over control values in both males and females on all three measures. In contrast to mice kept on pine bedding, there were no significant sex differences on any of these measures, although females tended to exhibit higher values.

Non-induced BALB/cBy mice (Fig. 6) showed no significant sex differences. In general, baseline

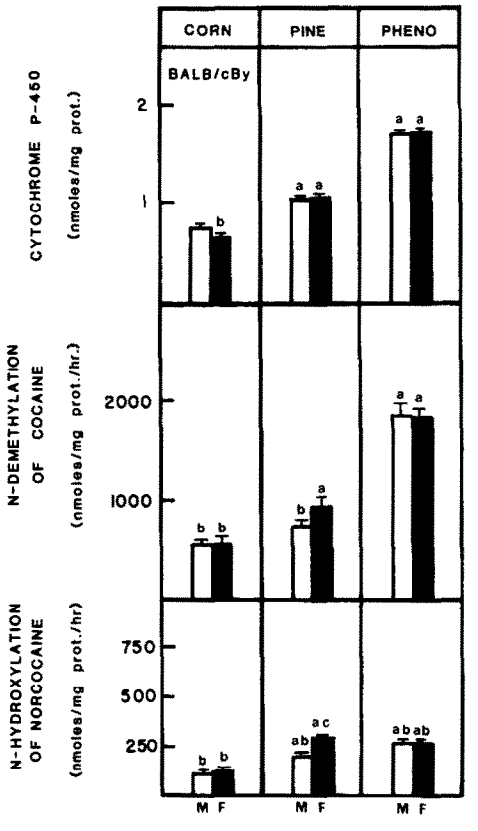

Fig. 6. Sex differences in mean (± S.E.M.) cytochrome P-450 levels, cocaine *N*-demethylase, and *N*-hydroxylation or norcocaine in liver microsomes from BALB/cBy mice. Male and female BALB/cBy mice were either uninduced (housed on corncob bedding) or induced by pine bedding for 1 week or by phenobarbital (0.8 g/l in the drinking water for 6 days). Each group had at least eight mice. Key: (a) significantly different from non-induced mice; (b) significant within sex strain difference for that particular condition; and (c) significant within strain sex difference for that particular condition.

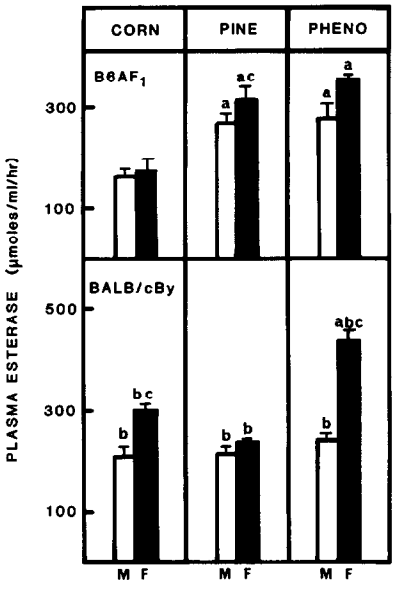

Fig. 7. Sex and strain differences in mean (± S.E.M.) plasma esterase activity. Male and female B6AF1 or BALB/cBy mice were either uninduced (housed on corncob bedding) or induced by exposure to pine bedding for 1 week or by phenobarbital (0.8 g/l in the drinking water for 6 days). Each group had at least six mice. Key: (a) significantly different from non-induced mice; (b) significant within sex strain difference for that particular condition; and (c) significant within strain sex difference for that particular condition.

enzyme activities and cytochrome P-450 levels were lower in this strain than they were in B6AF1 mice. In contrast to B6AF1 mice, enzyme activities and cytochrome P-450 levels were significantly induced in both sexes of BALB/cBy mice by exposure to pine bedding, reaching levels similar to those of B6AF1 females. Phenobarbital induction increased cytochrome P-450 and cocaine *N*-demethylase to levels equal to those obtained with B6AF1 mice. *N*-Hydroxylation of norcocaine, though significantly increased by phenobarbital, remained significant lower than that observed in B6AF1 mice.

Sex and strain differences in plasma esterase. Measurements of plasma esterase activity in males and females of both strains were carried out using indophenyl acetate as a substrate [13] (Fig. 7). In B6AF1 mice, esterase activities were increased significantly both by exposure to pine bedding and by phenobarbital. Females, in general, had more esterase activity than males, although this difference achieved significance only in the case of mice housed in pine bedding.

Non-induced BALB/cBy mice had significantly more esterase activity than B6AF1 mice. However, pine bedding did not increase esterase activity in BALB/cBy mice. Phenobarbital induction increased esterase activity in females but not in males. Females of this strain also had higher esterase activity than males, significantly so in uninduced and phenobarbital-induced mice.

Sex difference in cocaine-induced SGOT elevation after diazinon. As a further test of the involvement of esterases in sex and strain differences in response to cocaine, non-induced male and female B6AF1 mice and also BALB/cBy mice were injected with diazinon to inhibit esterase activity prior to cocaine administration. Glutathione levels were not affected by diazinon pretreatment. This combination resulted in significant liver damage (SGOT elevation) in both sexes and strains (Fig. 8). Surprisingly, B6AF1 females were affected the least. It might have been expected that, because they have higher cytochrome P-450 levels and *N*-hydroxynorcocaine activity than B6AF1 males, they would show the most damage. However, inspection of esterase levels following diazinon pretreatment revealed that these esterase activities in females were not inhibited by the diazinon to the same extent as in males of this strain or in males and females of the BALB/cBy strain. In BALB/cBy mice, both sexes showed large decreases (>65%) in esterase activity following diazinon pretreatment. In contrast, in B6AF1 mice only males showed a significant decrease (>50%) following diazinon pretreatment. Females were much less affected (20% decrease).

Sex differences in FAD-monooxygenase activity. *In vitro* FAD monooxygenase activity was also measured from B6AF1 mice. Non-induced females were found to have significantly more enzyme activity than non-induced males (Fig. 9). This enzyme activity was not increased by phenobarbital pretreatment. In contrast, induction by pine bedding did lead to a significant increase in enzyme activity, but only in males. As a result, there was no significant sex difference in enzyme activity in pine bedding-induced mice. Castration of non-induced males resulted in enzyme activity which was between that of males and females, but not significantly different from either.

Changes in liver glutathione. Sex differences in cocaine hepatotoxicity might result from a difference

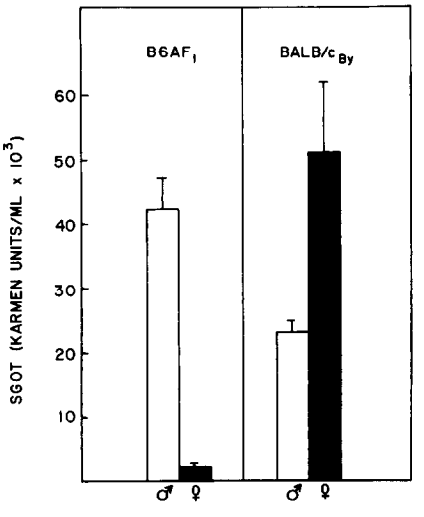

Fig. 8. Sex and strain differences in SGOT elevation following cocaine injection in mice pretreated with the esterase inhibitor diazinon. Diazinon (20 mg/kg) was injected i.p. 30 min prior to cocaine, 50 mg/kg i.p. Mice were housed on corncob bedding. Each point represents the mean (± S.E.M.) SGOT response of at least six mice. Blood samples for SGOT assay were taken 16–18 hr after cocaine.

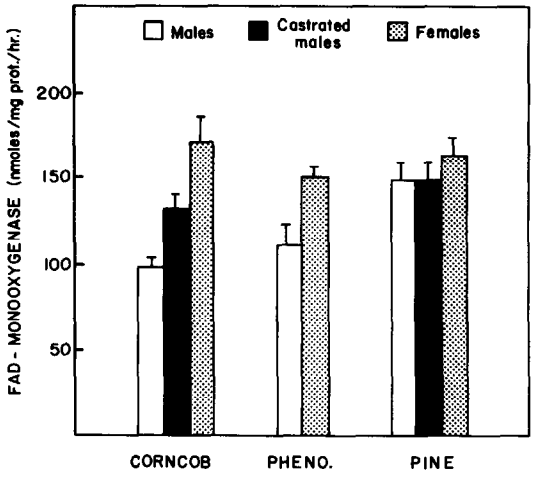

Fig. 9. Sex differences in FAD-monooxygenase activity in liver microsomes from B6AF$_1$ male and female mice. Mice were either uninduced (housed on corncob bedding) or induced by exposure to pine bedding for 1 week or by phenobarbital (0.8 g/l in the drinking water for 6 days). Each bar reflects the mean (± S.E.M.) of at least six mice.

in the amount of liver glutathione available to scavenge free radicals produced during the metabolism of cocaine. Accordingly, glutathione levels were compared in control and cocaine-injected male or female B6AF$_1$ mice. All groups were tested concurrently. The mice were induced by exposure to pine bedding [the condition which produces the largest sex differences in SGOT levels after cocaine (Fig. 3)].

As is evident from Fig. 10, although females have slightly higher baseline glutathione levels, both sexes showed equivalent decreases of 20–21% following cocaine. Under these conditions, only males showed liver damage.

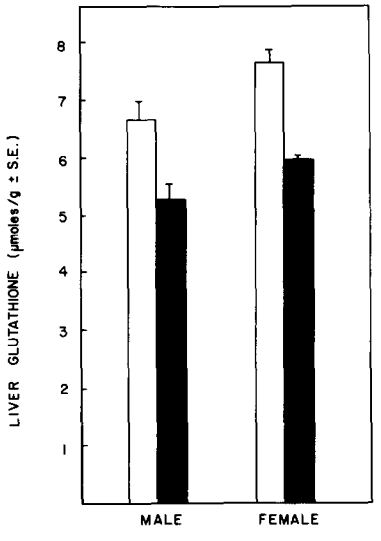

Fig. 10. Sex differences in glutathione levels before and after cocaine in liver homogenates from B6AF$_1$ mice housed on pine bedding. Mice were injected with either saline (white bars) or cocaine (black bars). Liver glutathione levels were determined at 60 min after injection. Each point represents the mean (± S.E.M.) liver glutathione level of six mice.

DISCUSSION

This report provides the first demonstration of significant sex differences in the hepatotoxicity of cocaine in mice. These sex differences are evident whether liver damage is produced by a single injection of cocaine or of its more proximal toxic metabolites (norcocaine, *N*-hydroxynorcocaine), by chronic injections in non-induced mice, or by pretreating with an esterase inhibitor prior to cocaine. The direction and magnitude of the sex difference were dependent upon the nature of the pretreatment. Phenobarbital induction prior to cocaine resulted in significantly greater SGOT elevations in female mice than in male mice. Induction by pine bedding or β-ionone prior to cocaine, or chronic treatment with cocaine or its metabolites, led to large SGOT elevations in males but only small increases, if any, in females.

In phenobarbital-induced B6AF$_1$ mice, this sex difference was found to be under androgenic control. Castration of males or testosterone treatment of females eliminated this difference. Furthermore, the suppressive effect of testosterone was blocked by pretreating males with flutamide, a nonsteroidal antiandrogen which acts at the receptor level [14]. This finding suggests that the sex difference is due to an androgen-receptor mediated stimulation of microsomal protein and liver weight [16].

While hormonal influences on the biochemical components of the microsomal drug-metabolizing enzyme system are well-documented in rats [17], attempts to define this relationship in mice have not met with great success. Noordhoek [8] found hormonally influenced sex differences in NADPH–cytochrome *c* reductase activity, and cytochrome P-450 content, but could not relate these to observed sex differences in the rate of metabolism of various substrates. Similarly, Davies *et al.* [19] and Brown *et al.* [20] also reported sex differences in cytochrome P-450 content (males higher than females) but again were not able to correlate these differences with observed differences in enzyme activity. The problem is further complicated by the finding that androgens may decrease [18] or increase [20] microsomal enzyme activity and cytochrome P-450 content, or have no effect on either measure, depending on the strain of mice used.

Haugen *et al.* [21] have shown recently that four different microsomal cytochromes can be induced, and that the proportions of these cytochromes vary with the inducing agent. The proportions of inducible cytochromes are also likely to be affected by sex and strain differences. It is no wonder that many attempts to relate these biochemical components to sex and strain differences in drug metabolism or response in mice have been successful.

We were able to find differences in P-450 content and *in vitro* microsomal enzyme activity that might be related to the sex and strain differences in SGOT levels following cocaine. When B6AF$_1$ mice housed

on pine bedding were injected with cocaine, males showed SGOT increases similar to those seen in phenobarbital-induced mice. $B6AF_1$ females, however, showed little SGOT response to cocaine, as did BALB/cBy mice. Our *in vitro* studies showed that cytochrome P-450 levels and cocaine *N*-demethylase activity in the livers of $B6AF_1$ males on pine bedding were significantly greater than in $B6AF_1$ females, or in BALB/cBy mice. This difference may explain why more cocaine than norcocaine or *N*-hydroxynorcocaine is required to produce liver damage in $B6AF_1$ females and BALB/cBy mice. The disparity in SGOT responses between $B6AF_1$ males and females on pine could also be related to the observation that exposure to pine bedding increased esterase activity more in females than in males. Esterases were not induced in BALB/cBy mice on pine bedding. This finding may account for the greater SGOT response of BALB/cBy mice to norcocaine and *N*-hydroxynorcocaine. Phenobarbital induction also led to significant increases in both cytochome P-450 levels and the activity of the three enzymes measured in both strains, to levels almost twice as high as those for pine bedding-induced mice. However, although phenobarbital-induced $B6AF_1$ females tended to be higher on all four measures, none of these proved to be significantly higher than the values for the somewhat less sensitive $B6AF_1$ males. The greater sensitivity of females to cocaine may be related to the fact that the phenobarbital exposure increases lipid synthesis in the liver. Females have been shown to be more susceptible to this effect than males [22]. Increased fatty infiltration is also one of the earliest changes observed in the liver following cocaine administration, and is most likely related to its pharmacological effects. This action is potentiated by small doses of epinephrine given in conjunction with cocaine. Lipid peroxidation has been proposed as one of the possible mechanisms of cell damage produced by cocaine [23], and it is likely that such an action would be potentiated in livers already possessing large concentrations of lipids to begin with. *N*-Hydroxylation of norcocaine was not stimulated as much by phenobarbital in BALB/cBy mice, and this finding may account for their being less sensitive to cocaine than $B6AF_1$ mice.

Esterase values in phenobarbital-induced mice did not correlate with the SGOT response, in contrast to what was observed in the pine bedding-induced mice. Both males and females of both strains showed increased esterase activity after induction. It is possible that, in phenobarbital-induced mice, the increased plasma indophenyl esterase activities do not relate to the metabolism of cocaine. A change in the nature of the esterase is suggested by the finding that after phenobarbital diazinon effectively inhibited esterase activity in $B6AF_1$ females. Alternatively, an enzyme needed for activating diazinon could have been induced by phenobarbital.

Part of the bioactivation of cocaine may be mediated by a FAD-containing monooxygenase [24, 25]. Female mice have been shown to have higher levels of this enzyme [26]. Furthermore, the finding that this enzyme is susceptible to manipulation of testosterone levels [27] might explain at least some of our data regarding the effects of castration on cocaine-induced SGOT levels. However, our measurements of *in vitro* FAD-monooxygenase activity in $B6AF_1$ mice suggest that this enzyme is not solely responsible for the observed sex differences in cocaine response. We also found that females have higher levels of this enzyme than males, and this difference was to some extent testosterone mediated. However, there was no sex difference in FAD-monooxygenase activity in pine bedding-induced mice, even though in this case males show greatly elevated SGOT levels and females do not.

Additional studies also showed that sex differences in $B6AF_1$ mice could not be attributed solely to differences in glutathione depletion following cocaine injection. This latter finding again raises questions about the involvement of glutathione as an important protective mechanism against the hepatotoxic action of cocaine, a conclusion also reached by Evans [28].

Our results suggest that males and females may differ in the metabolic processing which occurs after the conversion of norcocaine to *N*-hydroxynorcocaine, or by some mechanism that intervenes between the production of the proposed nitroxide free radical [2] and the final cytotoxic step, perhaps the binding of this free radical to cell membranes or lipid peroxidation [23]. Such differences may also account for the species differences in the hepatotoxicity of cocaine. Evans [29] has postulated that the failure to observe hepatotoxicity from cocaine in species other than mice is related to differences in the rate of norcocaine formation.

The results of the experiment on the effects of chronic administration of cocaine were interesting. Cocaine has been reported previously to be hepatotoxic when given daily for 1–3 weeks at doses from 10 to 30 mg/kg [30]. We also found that cocaine produced SGOT elevation when given chronically to $B6AF_1$ mice, with males showing much larger effects than females. Males also showed a similar response to norcocaine and *N*-hydroxynorcocaine, although these metabolites were less hepatotoxic. It may be that these metabolites are better substrates for esterases than cocaine [31]. Females did not respond to these metabolites.

The reason for the decline in SGOT response on days 4 and 5 of cocaine treatment is unclear. It may be that liver function was altered to the extent that cocaine was no longer being metabolized to the same degree or in the same fashion as on the first 3 days. Our preliminary experiments show cytochrome P-450 to be reduced significantly by 3 days of cocaine treatment.

It has been reported that the lesion from chronic cocaine is strikingly similar to that observed in phenobarbital-induced mice [30]. However, we found that, in terms of SGOT changes, phenobarbital-induced mice were equally, if not more, sensitive to norcocaine and *N*-hydroxynorcocaine, and that females were twice as sensitive as males. Perhaps chronic administration of cocaine may lead to a different bioactivation process than phenobarbital induction, at least under the conditions used in our laboratory.

Reports have varied as to the intralobular location in the liver of damage from cocaine. In our original

report, we described the necrosis as being centrilobular-midzonal in location [1]. Subsequently, other reports have shown the location to vary, depending upon inducing agent or whether cocaine or one of its metabolites is injected. Thus, Smith *et al.* [32] reported that ethanol induction resulted in cocaine producing centrilobular necrosis, while phenobarbital pretreatment resulted in cocaine inducing periportal necrosis. Freeman and Harbison [33] reported that norcocaine produced periportal necrosis in phenobarbital-induced mice but centrilobular necrosis in uninduced mice.

In our present study, we too find that the type of inducing agent used to stimulate metabolism of cocaine can shift the intralobular area of necrosis produced by cocaine. We confirmed our original report that pine bedding induction results in cocaine producing centrilobular-midzonal necrosis, and replicated the results of others that phenobarbital induction leads to periportal necrosis from cocaine. Regardless of the inducing agent, we did not observe any sex or strain difference in the pattern or location of damage. The extent of the necrosis correlated with the magnitude of the SGOT response. Furthermore, we saw no shift in the site of the lesion, at least in phenobarbital mice, when liver damage was produced by injection of norcocaine or *N*-hydroxynorcocaine, rather than cocaine. Both metabolites produced a periportal lesion similar to that produced by cocaine. Neither cocaine nor its metabolites produced SGOT elevations in uninduced mice, unless given repeatedly.

We found that chronic treatment with cocaine produced a centrilobular-midzonal pattern of necrosis. Freeman and Harbison [30] reported that chronic cocaine produced a periportal lesion very similar to that observed in phenobarbital-induced mice. Differences in treatment conditions may account for the discrepancies between the two studies. Our animals were killed after 3 days of treatment, while their treatment lasted from 1 to 3 weeks.

The locus of the necrosis apparently depends upon the intralobular distribution of oxidative enzymes and cytochrome P-450 in the liver. It has been shown that this distribution can be changed by exposing the animals to different inducers [34, 35]. Phenobarbital induction increases enzyme activities and cytochrome P-450 levels mainly in the centrilobular region. The fact that phenobarbital-induced mice show a periportal rather than a centrilobular necrosis from cocaine has led Freeman and Harbison [33] to speculate as to the exact role of the mixed-function oxidases in the bioactivation of cocaine. However, until enzyme distribution studies are done in mice, and the effects of inducing agents on these distributions in mice are known, this conclusion would seem premature, especially in light of all the available evidence supporting a role for the mixed-function oxidases.

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REFERENCES

1. L. Shuster, F. Quimby, A. Bates and M. L. Thompson, *Life Sci.* **20**, 1055 (1977).
2. M. L. Thompson, L. Shuster and K. Shaw, *Biochem. Pharmac.* **28**, 2389 (1979).
3. L. Shuster, A. Bates and C. A. Hirsch, *Analyt. Biochem.* **86**, 648 (1978).
4. W. Levin, A. Alvares, M. Jacobson and R. Kuntzman, *Biochem. Pharmac.* **18**, 883 (1969).
5. L. Leadbeater and D. R. Davies, *Biochem. Pharmac.* **13**, 1607 (1969).
6. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
7. R. F. Borne, J. A. Bedford, J. L. Buelke, C. A. Craig, T. C. Hardin, A. H. Kibbe and M. C. Wilson, *J. med. Chem.* **66**, 119 (1977).
8. N. Kaplowitz, *J. Pharmac. exp. Ther.* **200**, 479 (1977).
9. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
10. J. Chastril and J. Wilson, *Analyt. Biochem.* **63**, 202 (1975).
11. L. Shuster, E. Casey and S. Welankiwar, *Biochem. Pharmac.* **32**, 3045 (1983).
12. J. R. Cashman and R. P. Hanzlik, *Biochem. biophys. Res. Commun.* **98**, 147 (1981).
13. C. E. Mendoza, J. B. Shields and W. E. J. Phillips, *Comp. Biochem. Physiol.* **40**, 841 (1971).
14. E. A. Peets, M. F. Henson and R. Neri, *Endocrinology* **94**, 532 (1974).
15. D. V. Parke and H. Rahman, *Biochem. J.* **113**, 128 (1969).
16. T. R. Brown, R. E. Green and C. W. Baroin, *Endocrinology* **99**, 1353 (1976).
17. R. Kato, *Drug Metab. Rev.* **3**, 1 (1974).
18. J. Noordhoek, *Fedn. Eur. biochem. Soc. Lett.* **24**, 255 (1972).
19. O. S. Davies, P. L. Gigon and J. R. Gillette, *Life Sci.* **8**, 85 (1969).
20. T. R. Brown, C. W. Bardin and F. E. Greene, *Pharmacology* **16**, 159 (1978).
21. D. A. Haugen, M. J. Coon and D. W. Nebert, *J. biol. Chem.* **257**, 1817 (1976).
22. R. A. Salvador, C. Atkins, S. Haber and A. H. Conney, *Biochem. Pharmac.* **19**, 1463 (1970).
23. G. M. Rosen, M. W. Kloss and E. J. Rauckman, *Molec. Pharmac.* **22**, 529 (1982).
24. J. W. Gorrod, *Chem. Biol. Interact.* **7**, 289 (1973).
25. M. W. Kloss, J. Cavagnaro, G. M. Rosen and E. J. Rauckman, *Toxic. appl. Pharmac.* **64**, 88 (1982).
26. P. J. Wirth and S. S. Thorgeirsson, *Biochem. Pharmac.* **27**, 601 (1978).
27. M. W. Duffel, J. M. Graham and D. M. Ziegler, *Molec. Pharmac.* **19**, 134 (1981).
28. M. A. Evans, *J. Pharmac. exp. Ther.* **224**, 73 (1983).
29. M. A. Evans, *Pharmacologist* **20**, 182 (1978).
30. R. W. Freeman and R. D. Harbison, *Biochem. Pharmac.* **30**, 777 (1981).
31. D. J. Stewart, T. Inaba, M. Lucassen and W. Kalow, *Clin. Pharmac. Ther.* **25**, 464 (1979).
32. A. C. Smith, R. W. Freeman and R. D. Harbison, *Biochem. Pharmac.* **30**, 453 (1981).
33. R. W. Freeman and R. D. Harbison, *Pharmacologist* **20**, 193 (1978).
34. P. E. Gooding, J. Chayen, B. Sawyer and T. F. Slater, *Chem. Biol. Interact.* **20**, 299 (1978).
35. J. Baron, J. A. Redick and F. P. Guengerich, *Life Sci.* **23**, 2627 (1978).