Oxidative Metabolism of Cocaine: Comparison of Brain and Liver (42822)

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Abstract. **Norcocaine (NC) and *N*-hydroxynorcocaine (NHNC), products of the oxidative metabolism of cocaine, were examined in plasma, brain, and liver of mice injected intraperitoneally with cocaine. Plasma levels of NHNC were altered *in vivo* by inhibiting esterase activity with diazinon and chloral hydrate or activating esterase activity with phenobarbital, and activating the microsomal P-450 system with phenobarbital. Changes in plasma concentrations of NHNC resulted in similar changes in brain, which were often different from those in liver. After intracisternal administration of cocaine to mice, no appreciable amount of NC or NHNC could be detected in brain; the same results were obtained upon intracisternal and intraventricular administration to rats. Microsomal preparations from mouse brain were found to be considerably less active than those from liver in converting NC to NHNC. We conclude that the cerebral oxidative metabolism of cocaine is not appreciable and that most of the NC and NHNC found in the brain after systemic cocaine administration is derived from plasma rather than formed centrally by brain microsomal enzymes.** [P.S.E.B.M. 1989, Vol 190]

The major metabolic pathway of cocaine metabolism involves esterase activity cleaving the carbomethoxy group and the esteratic link between the tropane and phenyl rings (1–3). This pathway is an effective mechanism for eliminating the inhibitory effect of cocaine on dopamine uptake, which is implicated in its locomotor stimulatory activity (4), and other monoaminergic effects of cocaine, which are involved in various psychopharmacologic activities (5). In addition, there is oxidative metabolism centered around the nitrogen atom in the tropane ring yielding norcocaine (NC), *N*-hydroxynorcocaine (NHNC), and the free radical norcocaine nitroxide (6, 7). It is probably the latter pathway that is involved in the hepatotoxicity after cocaine administration to mice pretreated with phenobarbital to induce the microsomal P-450 system (1, 6, 8); the products may be toxic to central nervous tissue as well (1).

We previously reported that NHNC is present in the brain of mice after intraperitoneal administration of cocaine (9), but we did not examine whether this compound is formed endogenously in the brain or is derived via peripheral metabolism from plasma. Although brain microsomal preparations have been shown to be capable of oxidizing *in vitro* NC to NHNC and norcocaine nitroxide (10), it is still unclear whether the oxidative pathway of cocaine metabolism in the brain is functionally operative *in vivo*. In the present study four sets of experiments were carried out in addressing this question. In the first set, NHNC was measured in the brain after intraperitoneal administration of cocaine. In these experiments, changes in plasma levels of NHNC were introduced, and changes in NHNC concentrations in the brain and in the liver were compared. In an instance where changes in plasma and liver are not parallel, and the changes in brain resemble those in liver after activation of the microsomal P-450 system, oxidative metabolism by brain microsomal enzymes is a plausible mechanism; conversely, changes in brain parallel to plasma would support peripheral NHNC formation followed by penetration into the brain.

In the second set of experiments, cocaine was administered intracisternally in order to minimize the contribution of peripherally produced cocaine metabolites. In the third set of experiments, levels of glutathione in the brain were measured after cocaine administration; a reduction in glutathione would support centrally occurring activity of drug-metabolizing microsomal enzymes similar to the cocaine-induced decrease in glutathione observed in the liver of induced mice (8, 11). In the fourth set, the activity of brain and liver microsomal preparations in converting NC to NHNC under identical assay conditions was compared.

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Materials and Methods

Animals. The animals used in these experiments were 2-month-old male BALB/cBy mice, weighing 18–23 g, from the breeding colony of our institute, and 2-month-old male Wistar Kyoto rats, weighing 130–170 g, from Taconic Farms (Germantown, NY). Animals were kept on a 12-hr light/dark cycle with food and water available *ad libitum*.

Chemicals. Cocaine hydrochloride, phenobarbital, and chloramphenicol were purchased from Sigma Chemical Co., St. Louis, MO. NHNC was a gift from L. Shuster, Tufts University School of Medicine, Boston, MA. Diazinon (O,O-diethyl-O-(2-isopropyl-4-methylpyrimidyl)-thiophosphate) was donated by Ciba Geigy (Agricultural Division, Greensboro, NC). Diazinon and chloramphenicol were dissolved in saline containing polyethylene glycol (Carboxaan 6000; Union Carbide) (1 g/4 ml).

Drug Treatment and Sample Preparation. Cocaine hydrochloride (30 mg/kg body wt) was injected ip into mice. Chloral hydrate (380 mg/kg ip) was given 5 min and diazinon (15 mg/kg ip) 30 min, before cocaine administration. Phenobarbital was administered via the drinking water (0.8 g/liter) 5 days before cocaine injection or preparation of microsomes. At 5 and 10 min after cocaine administration, mice were decapitated. Blood was collected in 1.5-ml tubes containing 5–10 mg of sodium potassium oxalate and centrifuged. An aliquot (50–100 μl) of the plasma was added to 900 μl of ethanol. The cerebral cortex and portions of the liver (150–250 mg) were removed, weighed, and added to 2 ml of ethanol. Homogenization of samples was followed by centrifugation in a microfuge for 3 min; the supernatant was decanted into a microfuge tube and stored at −20°C. Before analysis by high-performance liquid chromatography (HPLC), samples were again centrifuged to remove any additional precipitate. For intracisternal injections, 10 μg of cocaine were administered in saline in a total volume of 5 μl. For rats the amount was 60 μg in 10 μl. After 1, 3, 5, and 10 min, animals were decapitated and the cerebral cortex was removed, weighed, and homogenized in 1–1.5 ml of ethanol. Animals were anesthetized with chloral hydrate (380 mg/kg ip) before intracisternal cocaine administration. For intraventricular injections into rats without anesthetic, guide cannulas were implanted aimed at a position 1 mm above the lateral ventricle. Two weeks later, 60 μg of cocaine were injected through an inner cannula, which extended 1 mm beyond the guide cannula into the lateral ventricle. The animals were decapitated 3 min after cocaine administration, and the cerebral cortex was removed for assay.

Studies with Microsomes. Microsomal fractions from brain or liver of the mouse were prepared as described by Shuster *et al.* (11) and Levin *et al.* (12) using 0.25 and 0.32 *M* sucrose for liver and brain, respectively. The microsomal fraction was resuspended in 0.1 *M* potassium phosphate buffer (pH 7.4) at a final concentration of 200 mg original wet weight of liver per ml or 400 mg original wet weight of brain per ml. The microsomal preparation was stored at −20°C until use. Incubations were performed at 37°C in 0.1 *M* potassium phosphate buffer (pH 7.4) as described by Shuster *et al.* (11), but without addition of sodium fluoride. Aliquots of 100 μl were removed from the incubation mixture at 0, 15, 30, and 60 min and added to 1 ml of ethanol to stop the reaction.

HPLC. Cocaine and its metabolites were analyzed by a modification of the method of Jatlow *et al.* (13) as described previously (2). Briefly, 20-μl aliquots were analyzed by reversed phase high-performance liquid chromatography on a Versapack C-18, 10-μm column, 250 × 4.1 mm (Alltech Assoc., Deerfield, IL). Elution was with a solvent system consisting of 75% 0.4 *M* potassium phosphate buffer (pH 2.7) and 25% acetonitrile at a flow rate of 1 ml/min. Elution of cocaine and its metabolites was monitored at 235 nm (Bio-Rad UV detector Model 1305A; Bio-Rad Laboratories, Richmond, CA). The retention times for norcocaine, cocaine, and NHNC were 7.2, 7.9, and 10.6 min, respectively. Quantitation was based on peak height or area relative to that of standards of cocaine, norcocaine, and NHNC, estimated with the Hewlett Packard 3392A integrator.

In the *in vivo* experiments with intraperitoneal administration of cocaine, the detection limit of all compounds in brain and liver was estimated to be 0.2 μg/g tissue (wet weight) and for plasma 0.5 μg/ml, under the conditions of tissue sampling and extraction. In the *in vivo* experiments with centrally administered cocaine, the detection limit was reduced to approximately 0.05 μg/g by concentrating the samples in ethanol. In the *in vitro* experiments with microsomes, the detection limit was less than 0.5 μg/ml of incubation assay (see Fig. 4). This would mean that in *in vitro* incubations with 300 μg/ml of substrate a conversion of as little as 0.2% could be detected. The recovery of 1-μg amounts of cocaine, norcocaine, or NHNC added to samples in 1 ml of ethanol and carried through this procedure was greater than 95%.

Glutathione assays on samples from liver and brain were performed by HPLC as described by Anderson (14).

Statistics. In all cases the accepted level of significance (two-tailed) was 0.05. Paired data were examined by Student's *t* test for the correlation coefficient. In the experiments of Figure 1 some values of NHNC were below the detection limit and were set at zero; therefore, a distribution-free test, the Mann-Whitney *U* test, was used to compare groups.

Results

Manipulation of Plasma Levels of NHNC after Intraperitoneal Administration of Cocaine. The anes-

thetic chloral hydrate strongly inhibited conversion of cocaine to benzoylecgonine and norbenzoylecgonine in the liver (data not shown), indicating inhibition of esterase activity. After pretreatment with chloral hydrate, the concentration of cocaine increased in plasma, brain, and liver as measured 10 min after cocaine administration (Fig. 1A). At 5 and 10 min, the level of NHNC showed little or no change in plasma and brain, whereas the NHNC concentration in the liver significantly increased. Thus, brain levels of NHNC appeared to follow those in plasma and did not respond in the same manner to chloral hydrate as the levels in liver, where NHNC is formed locally by the hepatic cytochrome P-450 system. There was a significant correlation between plasma and brain values of cocaine in the control group ($r = 0.75$, $n = 11$, $0.002 < P < 0.01$) and chloral hydrate group ($r = 0.87$, $n = 14$, $P < 0.001$), indicating accumulation in the brain above plasma levels (Fig. 2A). In contrast, the brain to plasma ratio of NHNC was close to unity for both control ($r = 0.74$, $n = 11$, $0.002 < P < 0.01$; Fig. 2B) and chloral hydrate-treated animals ($r = 0.99$, $n = 15$; $P < 0.001$, Fig. 2B).

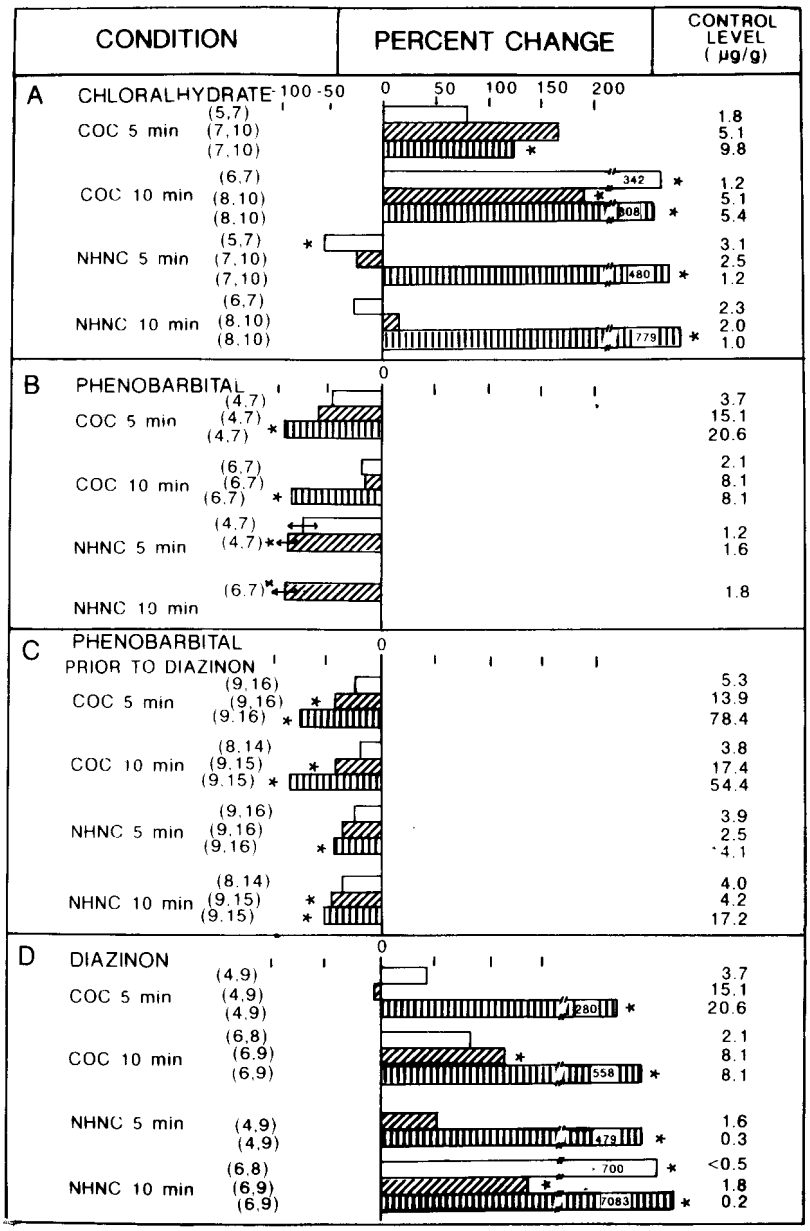

Figure 1. Changes in levels of cocaine (COC) and NHNC in plasma (□), brain (▨), and liver (▩) as a result of various pretreatments. Five or 10 min after injection of cocaine (30 mg/kg ip) mice were decapitated and samples were prepared for analysis. The bar graphs represent the percentage of change in the average level of the experimental group as compared with that of the control group with the group sizes (control, experimental) indicated in parentheses. The average level of cocaine or NHNC (μg/g) for the control animals is listed in the right column. Arrows are used in those cases where animals had levels of NHNC below the detection limit; a maximal estimate for the percentage of change was computed by assigning zero values to these animals and a minimal estimate for the change by assigning the value of the detection limit itself. In a few cases for NHNC, animals in both the control and experimental groups were below detection limit and no attempt was made to estimate the range of the change. For details on procedures see Materials and Methods. *$P < 0.05$ (Mann-Whitney U test).

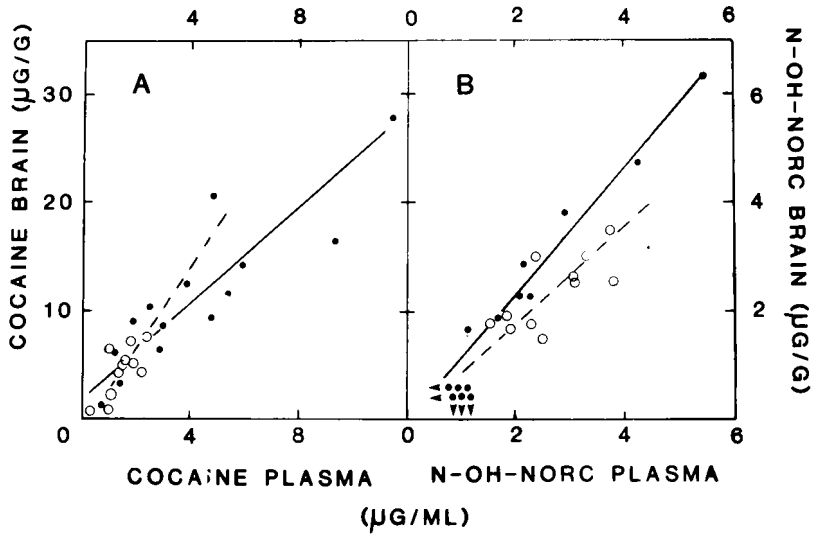

Figure 2. Effect of chloral hydrate. Mice were injected with cocaine (30 mg/kg ip) with or without pretreatment with chloral hydrate (380 mg/kg ip 5 min prior to cocaine administration). Five and 10 min after cocaine injection, animals were decapitated and levels of cocaine, norcocaine, and *N*-hydroxynorcocaine (N—OH—NORC) in plasma, brain, and liver were determined. Each point represents an individual animal. Points with arrowheads represent values that were below the detection limit. Straight lines are linear regression estimates. For details of the procedures, see Materials and Methods; for correlation coefficients see Results. O- - -O, Control; ●——●, chloral hydrate pretreated.

Phenobarbital pretreatment has been suggested to increase plasma and microsomal esterase activity (11). In agreement with this, cocaine concentrations in the liver were reduced at 5 and 10 min after cocaine administration in phenobarbital-pretreated animals (Fig. 1B). At both time points, NHNC levels were also significantly reduced in the brain. There was microsomal enzyme induction in the liver by phenobarbital, as judged by the increases in the ratios of NC to cocaine in the liver from 0.28 ± 0.04 (n = 4) to 1.22 ± 0.18 (n = 7) at 5 min after cocaine administration ($P < 0.01$) and from 0.80 ± 0.10 (n = 6) to 2.84 ± 1.08 (n = 7) at 10 min ($P < 0.01$) as expected for an activator of the P-450 system. In a separate group of animals, an attempt was made to counteract the phenobarbital-induced activation of esterases by diazinon, an esterase inhibitor (see (6)). However, the esterase activation of phenobarbital appeared to persist in diazinon-treated animals, as shown by the decreases in cocaine and NHNC concentrations in brain and liver (Fig. 1C). In addition, phenobarbital appeared to stimulate the P-450 system in the liver, as judged by the greater reduction in cocaine levels than in NHNC levels and by the increase observed in the NHNC to cocaine ratios from 0.14 ± 0.02 (n = 9) to 0.47 ± 0.06 (n = 15) at 5 min after cocaine administration ($P < 0.0001$) and from 0.29 ± 0.03 (n = 9) to 0.96 ± 0.16 (n = 15) at 10 min ($P < 0.0001$) as expected for an activator of the P-450 system. Under the same conditions, no significant changes were observed in brain (from 0.16 ± 0.03 to 0.16 ± 0.02 at 5 min and from 0.23 ± 0.03 to 0.14 ± 0.03 at 10 min) (data in Fig. 1C). Diazinon alone increased cocaine levels in both brain and liver (Fig. 1D). Moreover, diazinon increased the NHNC concentration in the liver far more than in plasma or brain (Fig. 1D), suggesting a different mechanism for the effects in brain and liver.

Intracisternal Administration of Cocaine. An attempt was made to minimize the contribution of peripherally generated cocaine metabolites by introducing cocaine directly into the central nervous system. At various times (1–10 min) after intracisternal administration of cocaine to mice, no appreciable amount of NC and NHNC was detected (data not shown). This was surprising in view of the reports showing the presence of [^{3}H]NC after intracisternally injected [^{3}H]cocaine in rats (15). Additional experiments were therefore carried out with rats receiving 60 μg of cocaine intracisternally (Table I). The larger brain samples gave us a lower detection limit than in the experiments with mice described above in terms of micrograms of detectable compound per gram of tissue under the conditions of extraction and sample size applied to the column; the detection limit was such that conversion of 10% or more of cocaine to NC or NHNC could have been easily detected at the 1- and 3-min time points when the cocaine concentrations were on the average 1.3 and 0.7 μg/g, respectively (Table I). However, no NC or NHNC was observed at those time points or at later times up to 10 min. An attempt at stimulating the P-450 system by phenobarbital pretreatment, or the omission of anesthesia during the intracisternal injection by the use of an intraventricular cannula, did not result in any significant amount of NC or NHNC (data not shown). In addition, no appreciable conversion to NC or NHNC was found in subcortical areas of these animals.

Glutathione Levels after Intraperitoneal Admin-

Table I. Measurement of Cocaine Metabolites in Rat Cerebral Cortex after Intracisternal Administration of Cocaine

Time (min)	*n*	Cocaine (μg/g)	Norcocaine (μg/g)	*N*-hydroxynorcocaine (μg/g)
1	2	1.3 (0.5–2.1)	<0.05	<0.05
3	7	0.7 (0.4–1.2)	<0.05	<0.05
5	4	0.5 (0.4–0.6)	<0.05	<0.05
10	2	0.4 (0.3–0.5)	<0.05	<0.05

Note. The number of animals is indicated by *n*. An amount of 60 μg of cocaine was injected intracisternally into each rat. Results are expressed as μg per g of wet tissue weight. Values for cocaine are the mean, with the range in parentheses. Values for norcocaine and *N*-hydroxynorcocaine are less than the detection limit (0.05 μg/g) under the conditions of these experiments.

Table II. Effect of Cocaine on Levels of Glutathione in Brain and Liver of the Mouse

Group	Time (min)	Glutathione Liver (% of control)	Glutathione Brain (% of control)
Cocaine			
30 mg/kg (6, 6)	10	93 ± 7.2	99 ± 3.1
(6, 6)	30	93 ± 5.6	89 ± 5.0
(10, 10)	60	91 ± 3.6	98 ± 3.1
50 mg/kg (8, 8)	60	90 ± 7.4	100 ± 5.7
Diazinon + cocaine			
30 mg/kg (6, 6)	60	82 ± 7.0	99 ± 4.7
Phenobarbital + cocaine			
30 mg/kg (8, 8)	60	102 ± 10.3	104 ± 2.6
Phenobarbital + cocaine			
50 mg/kg (9, 10)[a]	60	103 ± 8.3	100 ± 5.3
Buthionine sulfoximine (6, 6)	120	56 ± 5.6[b]	nd[c]

Note. Diazinon (15 mg/kg ip) was administered 30 min before i.p. cocaine injection. L-Buthionine-*S*,*R*-sulfoximine (2 mmol/kg ip) was given 2 hr before decapitation. Phenobarbital was administered in the drinking water (see Materials and Methods). Results are the mean ± SEM for the number of animals given in parentheses. Levels of glutathione in saline-treated animals were approximately 30 nmol/mg protein and 10 nmol/mg protein for liver and cerebral cortex, respectively. Control animals received saline instead of cocaine.

[a] Female mice.

[b] $P < 0.01$ (Student's *t* test).

[c] Not determined.

istration of Cocaine. Cocaine has been shown to reduce liver glutathione levels in mice with an induced P-450 system, presumably via oxidative metabolism depleting hepatic NADPH (1, 16). It was of interest to assess whether changes in glutathione levels in the brain occur upon cocaine administration because such changes would support the occurrence of central oxidative metabolism of cocaine. At all time points measured, a dose of 30 mg/kg of cocaine ip had no significant effect on liver or brain glutathione in uninduced mice (Table II). Increasing the dose of cocaine to 50 mg/kg, or inhibiting the esterase activity by diazinon, did not result in significant depletion. Likewise, pretreatment with phenobarbital and substitution of female for male mice did not change the lack of effect of cocaine. Diazinon or phenobarbital treatment by itself did not affect glutathione levels (data not shown). In contrast, buthionine sulfoximine, an inhibitor of glutathione synthesis, was shown to cause a 44% depletion in liver (Table II), in agreement with results reported by Griffith (17). Glutathione in the brain was not measured in these experiments but is known to be unresponsive to a single treatment with buthionine sulfoximine (18). The lack of effect of cocaine in altering glutathione levels in phenobarbital-pretreated BALB/cBy mice is in agreement with findings by Shuster's group (8) demonstrating important differences between strains of mice with $B6AF_1$ mice showing glutathione reductions upon cocaine administration.

***In Vitro* Formation of NHNC by Brain and Liver Microsomes.** As expected, pretreatment with phenobarbital increased the formation of NHNC from NC by liver microsomes (Fig. 3A). Chloramphenicol (0.14 *M*), an inhibitor of the cytochrome P-450 system, completely inhibited the conversion of NC to NHNC (data not shown). Under identical conditions, we compared the activity of brain and liver microsomal preparations in converting NC to NHNC by microsomes prepared from phenobarbital-treated animals (Fig. 3B). Whereas NHNC was produced in a linear fashion as a function of time with liver microsomes, no NHNC was observed at any time with microsomes from brain, even though the amount of microsomal protein in the brain assays was six times higher than that in the liver assays (Fig. 3B).

Discussion

Central vs Peripheral Formation of NC and NHNC. After intraperitoneal administration of cocaine, under various experimental conditions, NHNC levels in the brain appear to follow those in plasma, as judged both by the observed changes in group averages (Fig. 1) and by the brain to plasma ratios measured in separate animals (Fig. 2B). If cocaine were the precursor for NHNC formation in the brain, one would expect a correlation between cocaine and NHNC levels in the brain. In fact, many chloral hydrate-treated animals had very low concentrations of NHNC in the brain despite appreciable levels of cocaine (data not shown). In these animals a low concentration of plasma NHNC was found (Fig. 2B). This suggests that NHNC found in the brain is derived from plasma rather than formed centrally by brain microsomal enzymes. In agreement with this, significant differences were observed between the brain and liver in the way NHNC levels were affected by various experimental conditions. For instance, chloral hydrate treatment increased NHNC levels in the liver but had no such effect in brain or plasma (Fig. 1A). Furthermore, diazinon administration caused a much greater increase in NHNC concentrations in liver than in brain (Fig. 1D). Finally, in diazinon-treated

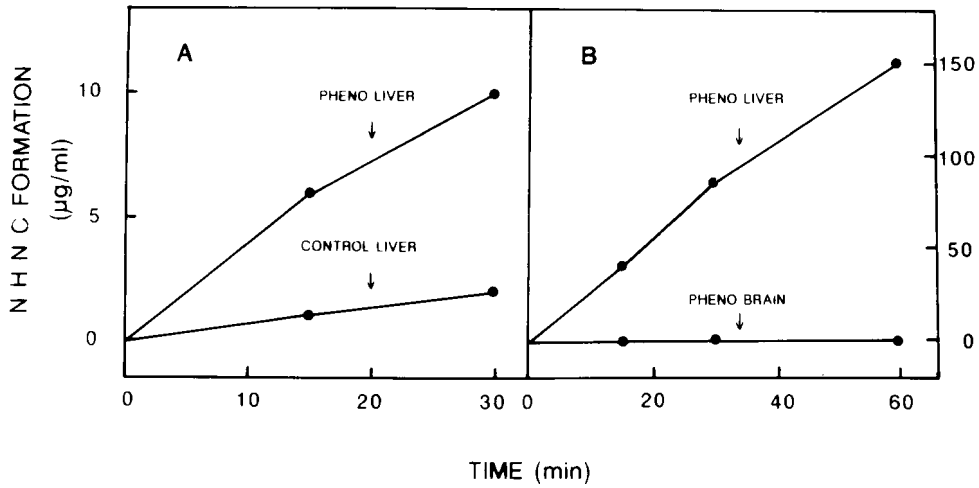

Figure 3. *In vitro* formation of NHNC by microsomal preparations. A, Liver microsomes (0.17 mg protein/ml) from phenobarbital (PHENO)-pretreated and control mice were incubated with norcocaine (3 μg/ml). B, Liver microsomes (0.17 mg protein/ml) or brain microsomes (0.96 mg protein/ml) from phenobarbital-pretreated mice were incubated with norcocaine (300 μg/ml). Values shown are the amounts of NHNC formed in μg per ml of incubation mixture.

animals, phenobarbital increased the NHNC to cocaine ratio in the liver but not brain (see Results). The present results do not rule out the possibility that NHNC is formed centrally by the brain cytochrome P-450 system and that this system differs from that in the liver in its responsiveness to inhibitors and activators. However, we regard this possibility as less likely because of i) the extremely low activity of *in vitro* brain microsomes as compared with liver microsomes in converting NC to NHNC (Fig. 3; see also (10)), and ii) the lack of appreciable amounts of NC or NHNC found in the brain after intracisternal administration of cocaine to both mice and rats (Table I).

The interpretation above agrees with that of Estevez *et al.* (19) based on the lack of formation of NC by brain homogenates in contrast to liver homogenates. However, it is at variance with conclusions advanced by Mulé *et al.* (15) from experiments in which rats were injected intracisternally with [^{3}H]cocaine and significant amounts of [^{3}H]NC were detected in the brain from 3 to 30 min later. At this time, no explanation can be given for the discrepancy between these observations and the present results with intracisternally administered cocaine in both mice and rats. There are differences in the techniques used by Mulé *et al.* (15) and by us, such as thin-layer chromatography vs HPLC and the use of radiolabeled cocaine vs unlabeled cocaine, but it is not clear how these could lead to the different results. The use of chloral hydrate as an anesthetic in the present intracisternal work did not appear to be a confounding factor, because i) chloral hydrate did not affect the ratio of NC to cocaine found in the brain after intraperitoneal administration of cocaine (data not shown), and ii) intraventricular administration of cocaine via a cannula without chloral hydrate anesthesia still did not result in appreciable concentrations of NC or NHNC in the brain.

Relevance of Centrally Occurring NHNC. It is important to consider the question of whether the cytochrome P-450 system in brain is active enough to locally produce NC and metabolites that originate from NC. The free radical norcocaine nitroxide is formed from NHNC by the cytochrome P-450 system. The present results show the presence of NHNC in the brain after intraperitoneal administration of cocaine but suggest that most of it is derived from the periphery and that the activity of the P-450 system in the brain is low. Nevertheless, it is possible that small amounts of NHNC and perhaps norcocaine nitroxide are formed in the brain, and we can only speculate on the effects that this might have. In a recent report, reductions in tyrosine hydroxylase activity in dopaminergic areas are described after chronic administration of cocaine (20). However, to our knowledge, there are no other reports on long-lasting changes in monoamine levels or markers for monoaminergic nerve terminals as a result of chronic treatment with cocaine. If oxidative metabolism of cocaine is potentially toxic to the brain, as it is to the liver, it is relevant to compare levels of NHNC, the precursor for the free radical norcocaine nitroxide, in the brain and liver of mice under conditions that are known to produce hepatotoxicity. In the present study, diazinon-treated mice had peak levels of NHNC of 4 μg/g in the brain at 10 min after cocaine administration, substantially lower than the peak levels of NHNC of 17 μg/g found in the liver at 10 min (data in Fig. 1). In this context it should be recalled that diazinon pretreatment renders uninduced mice susceptible to the hepatotoxic effect of cocaine (8). Of course, it cannot be ruled out that small amounts of NHNC can lead to

trace amounts of reactive metabolites that are toxic to the CNS. Further investigations are necessary to assess whether the presence of NHNC over extended periods of time can lead to reductions in glutathione levels in brain with resultant generation of free radicals and alterations in lipid peroxidation.

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