cogenolysis (i.e. phenylephrine, isoproterenol, glucagon, and vasopressin) are relatively insensitive to arsenicals. These findings are consistent with the proposal of Datta *et al.* [12] that trivalent arsenicals can alter the function of the hepatic vascular endothelium. Furthermore, higher doses of PhAsO (100 μM) alone increased substantially and irreversibly (over 30 min) portal vein pressure (0.9 ± 0.3 mm Hg) and glucose production (80 ± 15 μmol·hr^{-1}·g^{-1}).

The precise mechanism(s) by which trivalent arsenicals inhibit autacoid-mediated hepatic responses remains unknown. However, the close correlation between hepatic vasoconstriction and glycogenolysis found for several autacoids suggests that vasoconstriction-induced ischemia increases glycogenolysis in these situations [16, 19–21]. In such a scenario, trivalent arsenicals would inhibit hepatic glycogenolysis primarily by preventing agonist-induced vasoconstriction. Although arsenical toxicity has been attributed generally to their energy-depleting action [1], we observed effects on hepatic autacoid-stimulated vasoconstriction and glycogenolysis at arsenical doses which have only minor effects on hepatic oxygen consumption and which do not alter hepatic adenine nucleotide concentrations [23]. Thus, a primary locus for arsenical action may be proteins involved in the coupling of agonist binding with various cellular responses. Although the nature of such proteins remains unknown, Bernier *et al.* [25] have identified a 15,000 kD protein whose phosphorylation increases specifically when cells are stimulated with hormone (insulin) in the presence of PhAsO. Therefore, it seems appropriate to speculate that a major result of toxic arsenical exposure is a diminished responsiveness of cells to various autacoid signals. Such cellular desensitization may participate in the development of the pathological lesions that characterize arsenical toxicosis.

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Presence of the toxic metabolite *N*-hydroxy-norcocaine in brain and liver of the mouse

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The appearance of *N*-hydroxy-norcocaine (NHNC) in the liver has been associated with the hepatotoxic effect of cocaine [1]. The hepatotoxicity arises with production of a nitroxide free radical, a product of NHNC. This metabolic pathway is associated with the microsomal cytochrome P-450 system [2]. The metabolic sequence whereby cocaine is demethylated to norcocaine and then metabolized to NHNC and to a nitroxide free radical is active in the liver [2, 3]; however, the presence, or metabolic formation, *in vivo*, of NHNC in the central nervous system has not been established. Conversion of cocaine to norcocaine in rat brain after intracisternal injection has been reported [4], and the presence of norcocaine in brain after i.v. or s.c. administration of cocaine in rats has been demonstrated [5]. Since this pathway is toxic in liver, it was of interest to determine if NHNC is also present in brain.

Materials and methods

The animals used in these experiments were male BALB/cBy mice, 2 months of age, weighing 18–23 g, from the breeding colony of our institute. They were kept on a 12-hr light/dark cycle with food and water available *ad lib*.

Cocaine-HCl was purchased from the Mallinckrodt Chemical Corp. or the Sigma Chemical Co., St. Louis, MO. Chloramphenicol was also purchased from the Sigma Chemical Co. Diazinon [*O*,*O*-diethyl-*O*-(2-isopropyl-4-methylpyrimidyl)-thiophosphate] was donated by Ciba Geigy (Agricultural Division, Greensboro, NC). *N*-Hydroxy-norcocaine was a gift from L. Shuster, Tufts University School of Medicine, Boston, MA. Both diazinon and chloramphenicol were dissolved in saline containing polyethylene glycol.

Mice were injected i.p. with cocaine-HCl (30 mg/kg) in saline. The metabolic inhibitors diazinon (15 mg/kg) and chloramphenicol (50 mg/kg) were injected i.p. 30 min before cocaine administration. At various time points after cocaine injection animals were decapitated. The cerebral cortex and portions of the liver (150–250 mg) were removed, placed in 2-ml polypropylene conical tubes, and weighed, and 1 ml of ethanol was added. Sonication (brain) or homogenization (liver) of samples was followed by centrifugation in a microfuge for 3 min; the supernatant fractions were decanted into a second conical tube and stored at −20°. Before analysis by HPLC, samples were again centrifuged to remove any additional precipitate.

Aliquots (20 μl) of samples were analyzed by reversed-phase high-performance liquid chromatography (HPLC) on a Versapack C-18, 10 μm column, 250 mm × 4.1 mm (Alltech Associates, Deerfield, IL). Elution was performed with a solvent system consisting of 75% 0.3–0.4 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, Richmond CA). The retention times for norcocaine, cocaine and NHNC were 7.2, 7.9 and 10.6 min respectively. Quantitation was achieved by comparison of peak height or area with external standards of cocaine, norcocaine and NHNC, using the Hewlett–Packard 3392A integrator. Minimal levels of detection of these three compounds in brain and liver were near 0.5 μg/g, but variability was high at these low concentrations. Reproducibility was good when levels of these compounds reached 1 μg/g or higher in brain or liver. Recovery of 1 μg amounts of cocaine, norcocaine and NHNC added to liver or brain tissue in ethanol and carried through this procedure was greater than 95%.

Results and discussion

With reversed-phase HPLC, NHNC eluted as a distinct peak after norcocaine and cocaine, and could be measured at a concentration of 0.1 μg/ml. No interference from UV absorbing materials in the tissue extracts was observed (Fig. 1). A previous study by Shuster *et al.* [3] indicated that one can enhance the sensitivity by using an electrochemical detector. However, in our hands, UV detection was as sensitive, with less background noise. UV detection also enabled us to measure cocaine, norcocaine, and NHNC simultaneously.

An important pathway of cocaine metabolism in liver involves esterase activity, producing benzoylecgonine, ecgonine and ecgonine methyl ester [6]. We used diazinon, an esterase inhibitor, to increase the formation of norcocaine (Fig. 2). Without diazinon, injection of cocaine in mice led to levels of cocaine in liver of 20 μg/g within 2 min, and in brain, of 7.3 μg/g at 10 min. The amount of NHNC detected in the liver under these conditions was rather low, less than 0.8 μg/g at 5 and 10 min. In contrast, in the brain, NHNC reached levels of approximately 2 μg/g within 10 min; during this period, the concentration of NHNC was higher than that of norcocaine.

Levels of cocaine, norcocaine and the hydroxy derivative NHNC were measured at various time intervals after cocaine injection in animals pretreated with diazinon and

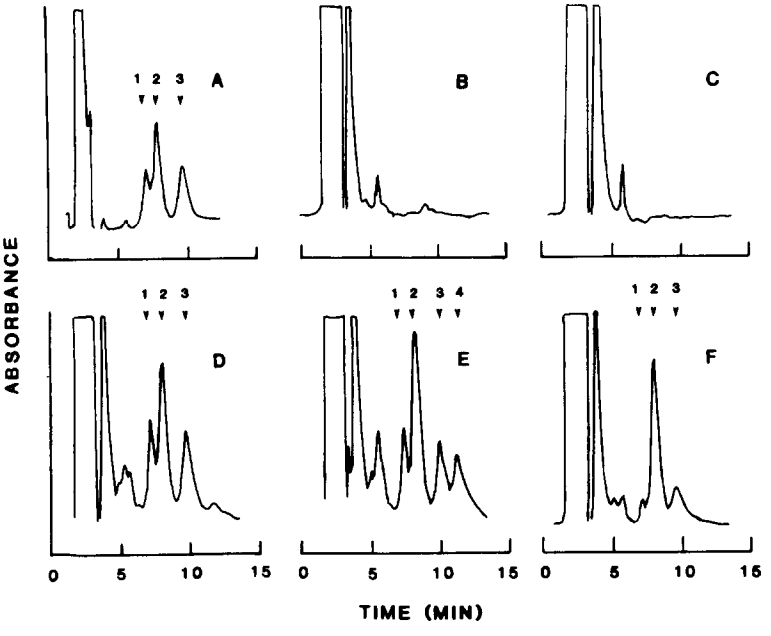

Fig. 1. Chromatograms of standards and sample extracts from brain and liver. Norcocaine (1), cocaine (2) and NHNC (3) had retention times of 7.2, 7.9, and 10.6 min respectively. Key: (A) standards alone; (B) liver extract of uninjected animal; (C) brain extract of uninjected animal; (D) liver extract from animal injected with cocaine and diazinon; (E) liver extract from animal injected with cocaine, diazinon and chloramphenicol (4); and (F) brain extract from animal injected with cocaine and diazinon.

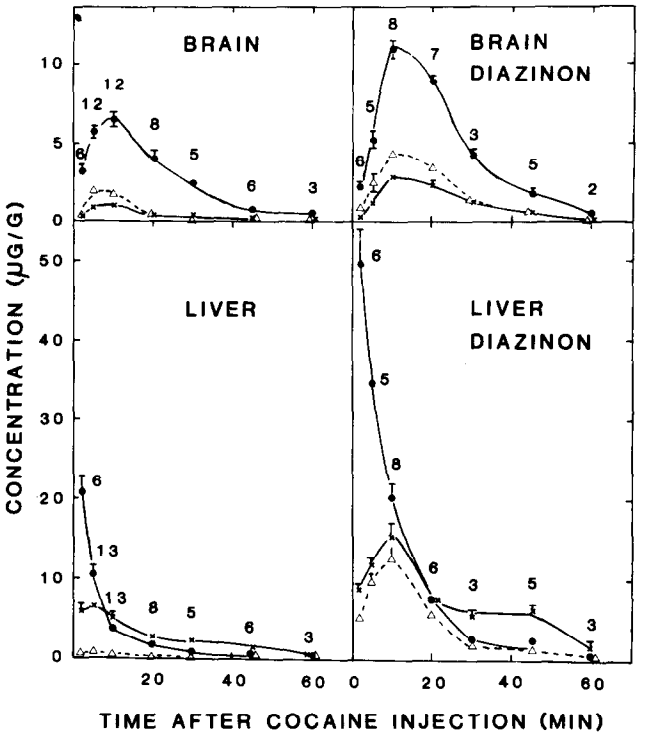

Fig. 2. Time courses for cocaine, norcocaine and NHNC in liver and brains of mice after i.p. administration of 30 mg/kg cocaine, with and without pretreatment with diazinon, as described in Materials and Methods. Each point is the average for the number of animals indicated. A standard error (vertical bar) is shown only if greater than the point itself. Two-way ANOVA indicates significant differences between the times of injection [$F(6,71-76) > 17$, $P < 0.005$] and between control and diazinon-treated animals [$F(1,71-76) > 44$, $P < 0.005$]. Key: (●—●) cocaine, (△—△) NHNC, and (×—×) norcocaine.

compared with levels in liver and brain of mice without diazinon. With diazinon, levels of cocaine, norcocaine and NHNC were higher in both brain and liver (Fig. 2). In liver, levels of cocaine were 2-fold higher within the first 2 min, and remained higher up to the last time point measured, 60 min. In brain, levels were approximately 2-fold higher from 10 min after injection until 45 min after injection. Under these conditions, high levels of NHNC were detected in liver, similar to the results reported by Shuster *et al.* [3]. Both metabolites, norcocaine and NHNC, were at levels of 10–15 μg/g at 5 and 10 min after cocaine injection. Levels of these two metabolites were also raised in the brains of the diazinon-pretreated animals. At 10 and 20 min after cocaine injection, levels of these compounds reached 2–4 μg/g in contrast to the animals not injected with the esterase inhibitor, where levels of both compounds reached approximately 1 μg/g at 10 min and were barely detectable at 20 min.

The increase in the metabolic products norcocaine and NHNC in liver in the presence of diazinon was as expected. A major metabolic pathway of cocaine in liver is via esterase conversion to benzoylecgonine, ecgonine, and ecgonine methyl ester [6], and if this pathway is inhibited one would expect an increase in the levels of norcocaine and the hydroxy derivative. However, in brain this pathway seems to be less active as judged from the relatively lower amounts of benzoylecgonine and benzoylnorecgonine after systemic administration of cocaine [7–9]. Whether the higher levels of norcocaine and NHNC found in the brain of diazinon-treated mice are caused by metabolic transformation in brain itself, following the higher levels of cocaine present, or whether these are a reflection of the higher levels of the metabolites in the peripheral circulation, and subsequent transport into brain, cannot be answered from these experiments. Possible contribution from occluded blood in these experiments would be low, since levels in plasma of all three compounds were lower than, or similar to, that in brain and liver (data not shown). The lack of norcocaine formation by brain homogenates as described by Estevez *et al.* [7] is not sufficient evidence to exclude the possibility that norcocaine and NHNC are produced in brain *in situ*. Evidence in support of the latter possibility comes from a study by Mulé *et al.* [4] demonstrating the appearance of norcocaine in the brain within 3 min after intracisternal injection of cocaine into rats.

To further investigate the metabolism of cocaine, animals were injected with chloramphenicol, an inhibitor of the microsomal cytochrome P-450 oxidase system. The microsomal P-450 system is present in brain, albeit at a low level of 10% or less of that in liver [10–12]. In these experiments, chloramphenicol was injected with or without diazinon. With chloramphenicol alone, the level of cocaine in liver was increased 81% at 10 min after cocaine injection (Table 1). However, no difference was found in brain. In experiments using both inhibitors, levels of cocaine in both brain and liver were increased by chloramphenicol, and a small but significant decrease in norcocaine was noted in liver. The results in liver can readily be interpreted as inhibition of the P-450 system: blockade of the *N*-demethylation pathway by chloramphenicol leads to an increase in the precursor cocaine. In this context, it should be emphasized that the concentrations of NHNC in liver of mice not pretreated with diazinon were very low and were difficult to quantify since the NHNC peaks in the chromatograms were close to the baseline. As in liver of diazinon-pretreated mice, increased levels of cocaine were observed in brain as

Table 1. Levels of cocaine, norcocaine, and NHNC in brain and liver of mice injected with cocaine

Tissue and pretreatment	Time after cocaine (min)	Cocaine (μg/g)	Norcocaine (μg/g)	NHNC (μg/g)
Brain				
None	10	6.40 ± 0.44 (12)	0.94 ± 0.11 (12)	1.56 ± 0.18 (12)
Chloramphenicol	10	7.02 ± 0.55 (6)	1.06 ± 0.16 (6)	1.87 ± 0.19 (6)
Diazinon	20	7.83 ± 0.77 (21)	2.17 ± 0.27 (21)	3.09 ± 0.18 (21)
Diaz. + chloram.	20	10.6 ± 0.9* (22) (+36%)	2.41 ± 0.25 (22)	2.99 ± 0.24 (21)
Liver				
None	10	3.57 ± 0.42 (13)	4.96 ± 0.40 (13)	0.63 ± 0.23 (11)
Chloramphenicol	10	6.46 ± 1.59* (6) (+81%)	5.21 ± 0.59 (6)	0.41 ± 0.18 (5)
Diazinon	20	9.00 ± 1.11 (21)	9.82 ± 0.65 (21)	7.76 ± 0.70 (21)
Diaz. + chloram.	20	13.8 ± 1.60† (22) (+54%)	8.18 ± 0.44* (22) (−17%)	7.60 ± 0.51 (21)

Cocaine and its metabolites were estimated by HPLC and doses of administered chloramphenicol (chloram.), diazinon (diaz.) and cocaine were as described in Materials and Methods. Treatment with inhibitors preceded the injection of cocaine by 30 min. Times indicated represent the intervals between cocaine injection and decapitation. Values are means ± SE for the number of animals indicated in parentheses.

* $P < 0.05$ two-tailed Student's t-test.

† $P < 0.02$ two-tailed Student's t-test.

a result of chloramphenicol administration (Table 1). Most likely, this is a reflection of the increase of cocaine levels in liver and plasma. The lack of effect of chloramphenicol on brain levels of norcocaine and NHNC along with the increase of cocaine in brain is consonant with the concept that norcocaine and NHNC measured centrally are derived from the periphery. Recent work using rat brain microsomes has shown that *in vitro* conversion of norcocaine to NHNC and to the norcocaine nitroxide takes place with the generation of lipid peroxyl radicals [12]. Although the amount of free radicals generated in brain would be much lower than that of liver, this is a source of potential CNS toxicity. Further work is needed to determine whether the NHNC found *in vivo* is produced in the brain from its cocaine precursor.

Regardless of its source, the presence of NHNC in brain tissue of cocaine-treated mice is of considerable interest. It should be emphasized that this metabolite was quickly observed in the brain, with peak levels appearing at the same time as those of cocaine and norcocaine (Fig. 2). NHNC in brain accumulated rapidly in the absence of an esterase inhibitor, unlike the formation of NHNC in liver, which was almost undetectable in the absence of diazinon (Fig. 2). It is tempting to speculate that frequent central exposure to NHNC could lead to CNS toxicity involving a nitroxide free radical.

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