*Biochemical Pharmacology*, Vol. 32, No. 20, pp. 3045–3051, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00


# METABOLISM OF COCAINE AND NORCOCAINE TO *N*-HYDROXYNORCOCAINE

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(*Received* 4 *January* 1982; *accepted* 28 *February* 1983)

**Abstract**—The mixed function oxidase system of mouse liver microsomes converts norcocaine to *N*-hydroxynorcocaine (NHNC). This metabolite can be measured by high performance liquid chromatography (HPLC) using an electrochemical detector. Experiments with inducers and inhibitors suggested that the cytochrome P-450 system was responsible for most of the formation of NHNC. NHNC was relatively unstable under physiological conditions, with a $T_{1/2}$ of 17 min at pH 7.4 and 37°. HPLC with the electrochemical detector was also used to demonstrate the formation of NHNC *in vivo* when mice were injected with norcocaine or cocaine.

*N*-Hydroxynorcocaine (NHNC) is a potent hepatotoxic agent in the mouse [1]. We have suggested that in mouse liver the cytochrome P-450 system converts cocaine, through norcocaine, to *N*-hydroxynorcocaine. To produce liver damage, the *N*-hydroxy compound has to be metabolized further, presumably to a free radical [1]. Evidence for the enzymatic conversion of NHNC to a nitroxide free radical has been presented by Evans and Johnson [2] and by Rauckman *et al.* [3]. A somewhat similar metabolic sequence has been postulated for the hepatotoxic drug acetaminophen [4], although the role of an *N*-hydroxy derivative has been disputed [5].

In this paper, we present evidence for the metabolic formation of *N*-hydroxynorcocaine *in vivo* and *in vitro*.

## MATERIALS AND METHODS

The mice used in these experiments were males, 3 to 5-months-old, purchased from the Jackson Laboratory, Bar Harbor, ME. They were housed in plastic cages on corncob or pine bedding in an air-conditioned room maintained at 22° with a lighting cycle of 12 hr light and 12 hr dark.

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.* [6], effectively preserved the activity of the cytochrome P-450 system for at least 3 months. We also found that, as Leadbeater and Davies have shown, a suspension of microsomes in buffer could be stored at −90° for several months with little loss in activity [7].

The high performance liquid chromatography (HPLC) apparatus used in these experiments consisted of a model U6K injector, model M-6000A pump, model 450 variable wavelength ultraviolet detector, a 25 cm × 4.6 mm reversed phase C18 column (all from Waters 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.* [8]. *N*-Hydroxynorcocaine was prepared from norcocaine as described previously [1]. The purity of these compounds was checked by determination of melting points, by thin-layer chromatography, and by HPLC. Cocaine–HCl (Merck & Co.) was purchased from Gilman Bros., Boston, MA. NADP, NAPDH, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from the Sigma Chemical Co., St. Louis, MO. Benzoyl ecgonine, benzoyl norecgonine, ecgonine and ecgonine methyl ester were supplied by the Research Technology Branch of the National Institute on Drug Abuse, Rockville, MD.

The protein content of microsomes was determined colorimetrically according to Lowry *et al.* [9].

For *in vitro* assays of 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; norcocaine·HCl, 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°. At various times 100 μl of the incubation mixture was removed into 300 μl of cold 95%

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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.

The formation of NHNC *in vivo* was determined after the injection of diazinon [*O,O*-diethyl-*O*-(2-isopropyl-4-methylpyrimidyl)-thiophosphate] in order to inhibit serum and tissue esterases. Diazinon, 15 mg/kg, was injected i.p. 30 min before cocaine · HCl or norcocaine · HCl, 50 mg/kg, i.p. The mice were killed 1 hr after the cocaine or norcocaine injection. The liver was homogenized with 19 vol. of cold 95% ethanol. The homogenate was centrifuged at 15,000 *g* for 10 min at 0°. The supernatant solution was evaporated under vacuum at 30° in a rotating evaporator. The residue was taken up in 5 ml of distilled water and extracted with 5 ml of ethyl acetate. The ethyl acetate was separated from the aqueous phase, dried with anhydrous sodium sulfate, and evaporated to dryness under vacuum. The residue was taken up in 300 μl of 95% ethanol. The ethanolic solution was clarified by centrifugation at 12,000 *g* for 1 min and then analyzed by HPLC-EC. The recovery of known amounts of NHNC added to liver homogenates and carried through this procedure was between 90 and 95%.

## RESULTS

*HPLC of* N-*hydroxynorcocaine*. After reversed phase chromatography on octadecyl silica (C18), *N*-hydroxynorcocaine (NHNC) could be measured by its absorption of ultraviolet light. However, as shown in Fig. 1, an electrochemical detector provided considerably more sensitivity and specificity. The least sensitive setting of the electrochemical detector (100 nA/V) gave as strong a signal as the most sensitive setting of the ultraviolet detector (0.01 optical density units full scale). Under the same conditions, cocaine, norcocaine, benzoyl ecgonine, benzoyl norecgonine, ecgonine methyl ester and ecgonine, each injected into the column in 10 μl of a 0.1 mg/ml solution, did not produce an electrochemical signal.

In vitro *formation of* N-*hydroxynorcocaine*. The aerobic incubation of norcocaine with mouse liver microsomes and NADPH or an NADPH-generating system resulted in the formation of substantial amounts of NHNC. The extent of conversion in 15 min was 10–15% of the amount of NHNC that was added (Fig. 2). The rate of formation was linear during the first 15 min of incubation, after which there was no further increase. By 60 min there was appreciable disappearance of the NHNC. When cocaine was substituted for norcocaine, the rate of NHNC formation was considerably less, but remained linear for 30 min.

The NHNC produced enzymatically from norcocaine had the same half-wave potential (1.03 V) as authentic NHNC.

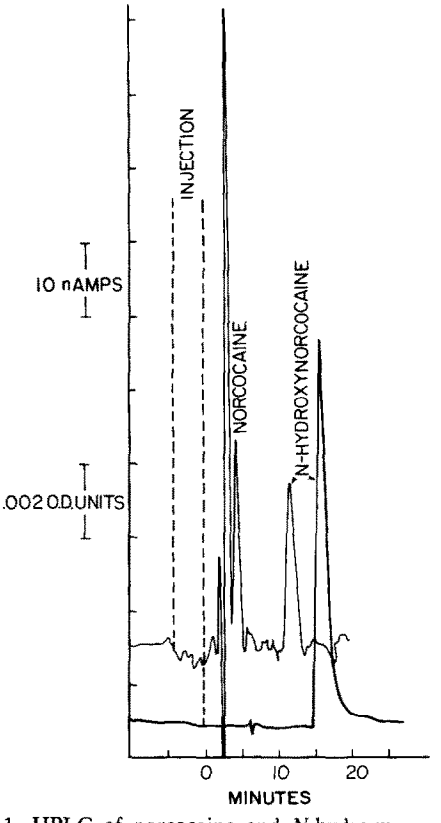


Fig. 1. HPLC of norcocaine and *N*-hydroxynorcocaine. The sample consisted of 10 μl of 75% ethanol containing 150 ng of norcocaine HCl and 150 ng of *N*-hydroxynorcocaine. The solvent was 45% 0.1 M $KH_2PO_4$ and 55% methanol, pH 4.8, pumped at a rate of 0.5 ml/min. The reference electrode was set at 0.8 V and sensitivity was 100 nA/V. The lower tracing (heavy line) is from the electrochemical detector. The upper tracing (lighter line) is from the u.v. detector set at 235 nm.

*Requirements for* N-*hydroxynorcocaine formation*. As shown in Table 1, the components needed for the production of NHNC from norcocaine are those associated with the cytochrome P-450 system, i.e. microsomes, oxygen and an NADPH-generating system.

Figure 3 shows that the amount of NHNC produced during 15 min of incubation was directly proportional to the amount of microsomal protein added up to 1.7 mg protein/ml. Heating the microsomes for 2 min at 100° destroyed their ability to make NHNC.

Storage of the microsomes for 3.5 months at −90° resulted in a loss of 7% of their original activity. Washing microsomes by resuspending them in phosphate buffer and recentrifuging at 100,000 *g* did not alter their specific activity (41.0 ± 3.3 nmoles NHNC per 15 min per mg protein vs 42.2 ± 3.4 nmoles/per 15 min per mg protein for unwashed microsomes, six mice per group). Microsomes prepared in 0.02 M Tris · HCl buffer, pH 7.4, containing 1.15% KCl had the same activity (40.5 ± 3.0 nmoles per 15 min per mg protein).

The inclusion of sodium fluoride to inhibit esterase activity increased the yield of NHNC. The concentration of fluoride sufficient for this purpose was

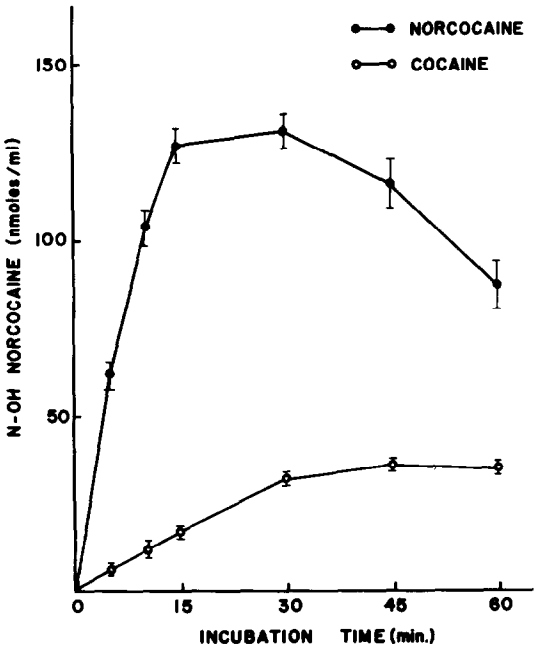


Fig. 2. Formation of *N*-hydroxynorcocaine from cocaine and norcocaine by liver microsomes. Liver microsomes were prepared from B6AF$_1$/J mice after they had been drinking sodium phenobarbital in water (0.8 mg/ml) for 6 days. Each point represents the mean value obtained from six mice. Bars represent S.E.M. Conditions were those described under Materials and Methods.

found in separate experiments to be as low as 5 mM (Fig. 4). We routinely used 0.01 M sodium fluoride in our incubations.

*Effect of inhibitors*. Table 2 shows the sensitivity of the microsomal *N*-hydroxylation of norcocaine to various inhibitors. Known inhibitors of the cytochrome P-450 system, such as SKF-525A, chloramphenicol, metyrapone, *n*-octylamine and iproniazid produced 50% inhibition at concentrations of about $10^{-4}$–$10^{-3}$ M. Carbon monoxide also inhibited strongly (Table 1).

In vivo *formation of* N-*hydroxynorcocaine*. By using the electrochemical detector we were able to demonstrate the *in vivo* conversion of cocaine and norcocaine to *N*-hydroxynorcocaine (Table 3). For this purpose it was necessary to inject the mice with diazinon in order to inhibit serum and tissue esterases. Preliminary experiments indicated that *in vivo* levels of NHNC reached a peak about 1 hr after the injection of cocaine.

When diazinon was not used, the amount of NHNC recovered after the injection of cocaine was only 2% of that obtained with the esterase inhibitor, and 10% after norcocaine. Pretreatment of the animals with phenobarbital did not increase the *in vivo* formation of NHNC, and the amount produced, either with or without diazinon, was less than that obtained from uninduced mice (Table 3).

In vitro *disappearance of* N-*hydroxynorcocaine*. The total amount of NHNC produced from norcocaine by an active microsomal system seemed to decrease after 30 min of incubation (Fig. 2). Added NHNC also disappeared under the same conditions, with a half-time of 17 min. There was little change in this half-time when microsomes and the NADPH-generating system were omitted. Adding EDTA ($10^{-3}$ M) or incubating under nitrogen instead of in air did not alter the rate of disappearance of NHNC.

The rate of disappearance of NHNC was influenced markedly by pH. There was a negligible decrease in the amount of NHNC after 2 hr at 37° in an aqueous solution (1.1 μmoles/ml, pH 5.4). In phosphate buffer, the *in vitro* half-life was inversely proportional to pH between pH 6.7 and 8.0. The NHNC was stable for at least several hours when the pH 7.4 solution was diluted with 3 vol. of 95% ethanol and kept at 4°.

HPLC of the incubation mixture with a u.v. detector set at 235 nm showed the appearance of a new peak with a retention time of 4.7 min as compared to 7.8 min for NHNC. The appearance of the new peak paralleled the disappearance of NHNC (Fig. 5).

The following procedure was used to isolate enough of the product for analysis. NHNC·HCl (20 mg) was dissolved in 20 ml of water. Twenty milliliters of 0.1 M potassium phosphate buffer, pH 7.5, was added and the mixture was incubated at 37° for 4 hr. As determined by HPLC, all of the NHNC

Table 1. Requirements for *N*-hydroxynorcocaine formation *in vitro**

| Conditions | NHNC formation (% of maximum) |
|---|---|
| Complete | 100 |
| Minus G-6-P dehydrogenase | 0 |
| NADPH, 1.0 μmoles/ml, in place of regenerating system | 100 |
| NADH, 1.0 μmoles/ml, in place of regenerating system | 25 |
| Minus NaF | 42 |
| Plus carbon monoxide | 20 |

* Conditions are those described in Materials and Methods. The pooled microsomes were prepared from B6AF$_1$/J mice that had been pretreated with phenobarbital for 6 days. The amount of NHNC formed in the complete incubation mixture was 157 nmoles per mg protein per 15 min. For addition of carbon monoxide, the phosphate buffer was saturated by bubbling with carbon monoxide, and incubation was carried out in a sealed 50-ml Erlenmeyer flask under air.

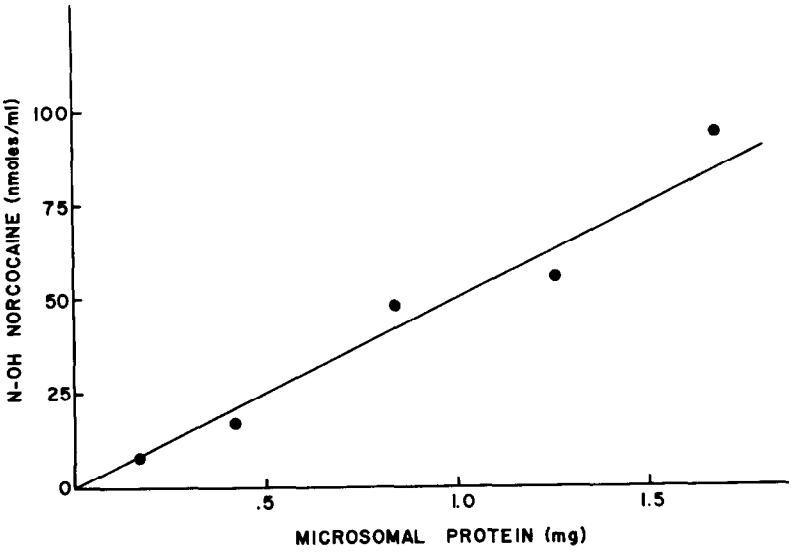


Fig. 3. Effect of varying amounts of microsomal suspension on the production of NHNC during a 15-min incubation.

appeared to have been converted to product at that time. As the reaction progressed, a fine precipitate appeared. Water was removed under vacuum with a rotating evaporator. The residue was extracted with three 15-ml portions of ethyl acetate, and the extract was dried with anhydrous sodium sulfate. Removal of the solvent under vacuum left 11 mg of a white solid. This material was recrystallized from ethanol to yield a substance with a melting point of 175–176°. Mass spectrometry showed that the molecular weight was 273 (*m/e*). Analysis by infrared spectrometry and nuclear magnetic resonance suggested the structure shown on the right-hand side of Fig. 6.

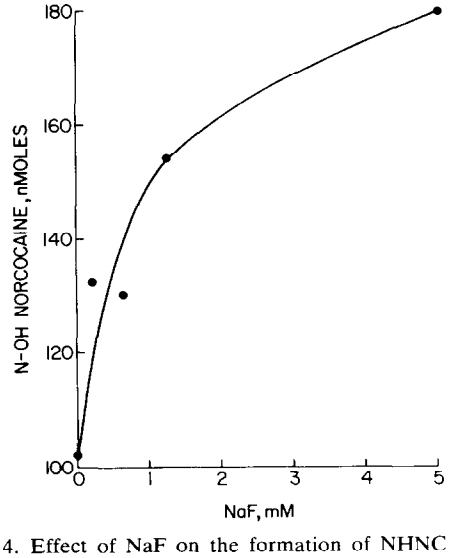


Fig. 4. Effect of NaF on the formation of NHNC from norcocaine. Conditions were those described under Materials and Methods. A single microsomal preparation was used, and incubation time was 15 min.

The formation of the five-membered dihydro-isoxazole ring presumably results from a base-catalyzed reaction between the hydroxylamine residue of NHNC and the methyl ester at position 2. Molecular models show that such an interaction is sterically possible.

## DISCUSSION

The results presented here provide evidence that cocaine or norcocaine can be metabolized to *N*-hydroxynorcocaine both *in vivo* and *in vitro*. Smaller amounts of NHNC are obtained *in vitro* from cocaine than from norcocaine, presumably because this substrate has to undergo prior *N*-demethylation. Because the *N*-hydroxy derivative is the only cocaine metabolite, among those that we tested, which gives a signal in the electrochemical detector, the use of this detector has proven very useful for monitoring HPLC.

There are at least two systems in liver that can catalyze the *N*-hydroxylation of secondary amines. One is a flavin-containing monooxygenase that uses either NADH or NADPH and is not inhibited by SKF-525A or octylamine [10]. The other is the cytochrome P-450 system that also carries out many other reactions of drug metabolism [11]. Our results support that conclusion that the *N*-hydroxylation of norcocaine is carried out mainly by the cytochrome P-450 system. The substitution of NADH for NADPH decreased the formation of NHNC by 75%. The hydroxylation of norcocaine was inhibited by chloramphenicol, iproniazid, metyrapone, *n*-octylamine, carbon monoxide and SKF-525A. These substances inhibit the cytochrome P-450 system, but not the flavin monooxygenase system. In fact, the latter system is actually stimulated by *n*-octylamine [10]. Furthermore, the flavin monooxygenase system is not induced by pretreatment with phenobarbital, while the P-450 system is. The cytochrome P-450-

Table 2. Inhibitors of the *in vitro* metabolism of norcocaine to *N*-hydroxynorcocaine*

| Inhibitor | Concentration for 50% inhibition (mM) |
|---|---|
| Chloramphenicol | 1.4 |
| Iproniazid phosphate | 4.7 |
| Metyrapone | 0.2 |
| *n*-Octylamine | 1.2 |
| SKF-525A | 0.1 |

* Conditions are those described under Materials and Methods. Microsomes were prepared from male B6AF$_1$/J mice that had been pretreated with sodium phenobarbital for 6 days. The incubation mixture contained 0.67 mg microsomal protein and various concentrations of inhibitor, together with the usual additions, in a total volume of 1 ml. After incubation for 15 min at 37°, the reaction was stopped with ethanol, and the amount of NHNC formed was determined by HPLC-EC.

Table 3. Formation of *N*-hydroxynorcocaine from cocaine and norcocaine *in vivo**

| Injection | Pretreatment | N | NHNC at 1 hr after injection (g/g liver) |
|---|---|---|---|
| Cocaine–HCl, 50 mg/kg | | 7 | 0.16 ± 0.04 |
| Cocaine–HCl, 50 mg/kg and diazinon, 15 mg/kg | | 7 | 10.0 ± 0.4 |
| Norcocaine–HCl, 50 mg/kg | | 8 | 0.53 ± 0.04 |
| Norcocaine–HCl, 50 mg/kg, and diazinon, 15 mg/kg | | 6 | 6.7 ± 0.7 |
| Norcocaine–HCl, 50 mg/kg | Phenobarbital | 5 | 0.327 ± 0.06 |
| Norcocaine–HCl, 50 mg/kg, and diazinon, 15 mg/kg | Phenobarbital | 5 | 1.35 ± 0.16 |

* All the animals in these experiments were B6AF$_1$/J mice kept on corncob bedding. Phenobarbital-pretreated mice received 0.8 mg/ml in drinking water for 6 days. Values are means ± S.E.M.

catalyzed *N*-hydroxylation of phentermine is inducible by pretreatment with phenobarbital [12]. We found that pretreatment with phenobarbital increased both the amount of cytochrome P-450 and the extent of NHNC formation.

The flavin-containing monooxygenase activity of liver microsomes is quite thermolabile in the absence of NADPH, having a $T_{1/2}$ of 6 min at 37° [13]. We observed no loss in the ability of liver microsomes to convert norcocaine to NHNC after they had been preincubated in phosphate buffer for 15 min at 37°.

On the other hand, Kloss *et al.* [14] have claimed recently that there is significant involvement by FAD monooxygenase in the oxidation of norcocaine to NHNC. This claim is based on qualitative analysis by TLC of incubation mixtures containing purified monooxygenase or liver microsomes. There was no quantitation. Indeed, meaningful quantitation would have been difficult to carry out because, at the pH of 8.3 used in these experiments, the half-time for non-enzymatic cyclization of NHNC should be less than 2 min.

There is still a possibility that a minor part of the activity which we observed with liver microsomes may be attributable to FAD monooxygenase. The amount of activity observed when NADH was substituted for NADPH, and the amount that remained in the presence of carbon monoxide or high concentrations of SKF-525A and *n*-octylamine, were each about 25%. This figure suggests a maximal level for the contribution of the flavin monooxygenase system to the *N*-hydroxylation of norcocaine. The situation in other organs may be different. In preliminary experiments with kidney microsomes, we have observed NHNC formation which was not inhibited by either SKF-525A or *n*-octylamine.

We added sodium fluoride to our incubation mixtures in order to inhibit esterase activity. Hinson *et al.* [15] have reported that fluoride also preferentially stimulates *N*-hydroxylation of *p*-chloracetanilide by the cytochrome P-450 system of liver microsomes. We have observed, like Stewart *et al.* [16], that fluoride inhibits the hydrolysis of cocaine and norcocaine by microsomal esterases, but have not found evidence for stimulation of *N*-hydroxylation. Direct measurement of the hydrolysis of cocaine by microsomal esterases suggests that the effect of fluoride in our experiments can be attributed completely to the inhibition of benzoyl esterase activity*.

*N*-Hydroxynorcocaine is not very stable under conditions of physiological pH and temperature. However, we were able to detect appreciable amounts of NHNC in the livers of mice that had been injected with cocaine or norcocaine. The

* J. A. Amador and L. Shuster, *Abstracts of the Twelfth Annual Meeting of the New England Pharmacologists* (1983).

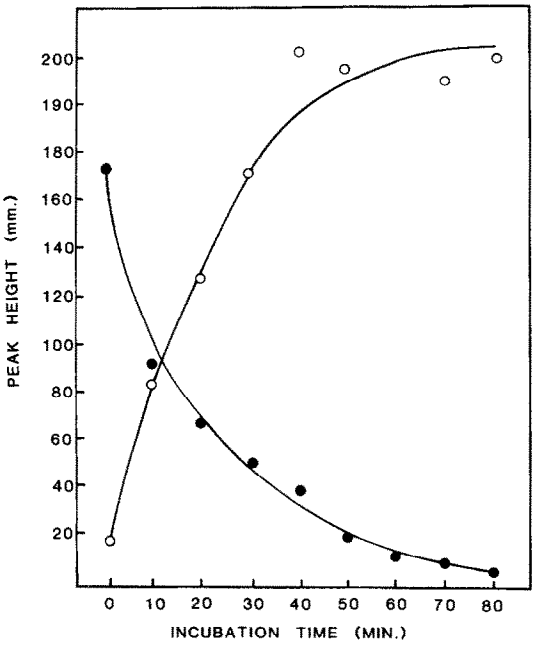


Fig. 5. Conversion of NHNC to a breakdown product *in vitro*. NHNC (1.1 μmoles) was incubated in 1 ml of 0.1 M potassium phosphate buffer, pH 7.4, at 37°, and 100 μl samples were taken into 300 μl of 95% ethanol at 0°. The mixture was centrifuged at 12,000 *g* for 10 min, and 10 μl of the supernatant fraction was injected into the HPLC. Peaks were quantitated with a u.v. detector set at 235 nm. Key: (●) NHNC; and (○) the product.

amount of NHNC recoverable from the liver was increased about 10-fold by pretreating the animals with an esterase inhibitor.

There were some striking differences between the *in vivo* results and those obtained *in vitro*. The increased yield of NHNC from cocaine *in vivo* compared to norcocaine may be related to the activity of microsomal and plasma esterases. Stewart *et al.* [16] have reported that the hydrolysis of norcocaine by these esterases is two to three times more rapid than that of cocaine. The injection of diazinon into our mice was found to inhibit serum esterases by about 50%. For these reasons cocaine yields more NHNC in diazinon-treated mice than does norcocaine. Without diazinon, the rapid hydrolysis of norcocaine as it is formed from cocaine by *N*-demethylation may limit the extent of subsequent *N*-hydroxylation. The finding that pretreatment with phenobarbital decreased the amount of NHNC recovered from *in vivo* experiments may be related to our preliminary observations that such pretreatment can produce a 2-fold increase in plasma and microsomal esterase activity.*

The fate of NHNC *in vivo* is not clear. Experiments with inhibitors and inducers suggest that further metabolism of NHNC by the cytochrome P-450 system is required before liver damage can occur [1]. There is evidence for the metabolism of NHNC to a nitroxide free radical [2, 3]. The rate of disappearance of NHNC *in vitro* did not seem to increase when microsomes and an NADPH-regenerating system were added to the incubation medium. Injection of the dihydroisoxazole formed non-enzymatically from NHNC (50 mg/kg, i.p.), did not produce any liver damage in $B6AF_1/J$ mice, as indicated by the measurement of serum glutamic-oxaloacetic transaminase (SGOT) levels.

It has been suggested that the metabolism of norcocaine to a nitroxide free radical may be responsible for some of the toxic effects of cocaine. Evans and Johnson recently observed that incubation of norcocaine or NHNC with a mouse liver mixed function oxidase system produced a metabolite with the electron spin resonance spectrum of a nitroxide free radical. They suggested that the free radical could bind covalently to proteins and other cellular macromolecules [2].

Rauckman *et al.* [3] have concluded that a direct reaction between norcocaine nitroxide and cellular proteins is highly unlikely. Instead, they favor the oxidation of glutathione secondary to depletion of NADPH by cyclic reduction of the nitroxide to *N*-hydroxynorcocaine followed by re-oxidation. The extent of glutathione depletion in the livers of cocaine-injected mice is only about 25% [1], an amount which by itself is unlikely to lead to hepatic necrosis.

An additional explanation which has now been proposed by Rosen *et al.* [17] is that lipid peroxides can be formed during the metabolism of *N*-hydroxynorcocaine and/or norcocaine nitroxide by liver microsomes, and that these lipid peroxides are responsible for liver damage.

One problem that arises in attempting to relate the formation and metabolism of NHNC and norcocaine nitroxide to liver damage produced by

* M. L. Thompson, L. Shuster and E. Casey, manuscript submitted for publication.

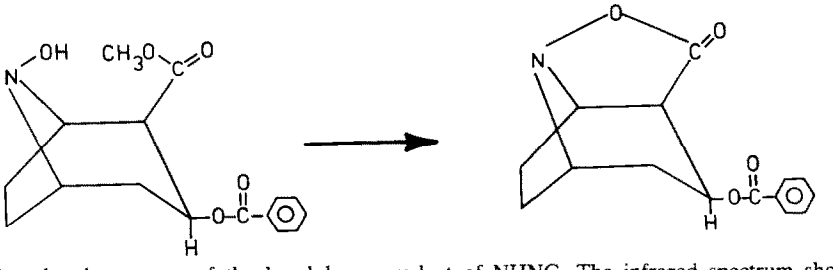


Fig. 6. Postulated structure of the breakdown product of NHNC. The infrared spectrum showed carbonyl bonds at 1725 $cm^{-1}$ (—O—CO—$C_6H_5$) and at 1810 $cm^{-1}$ (lactone), but not at 1705 $cm^{-1}$ ($COOCH_3$). The nuclear magnetic resonance spectrum showed signals at 2.1 (S, 4 h, protons at C6 and C7), 2.2 (S, 2 h, protons at C4), centered at 3.0 (m, 1 h, proton at C2), 3.95 (S, 1 h, proton at C5), 4.35 (S, 1 h, proton at Cl), 5.45 (m, 1 h, proton at $C_3$), and aromatic protons between 7.5 and 8.2 (5 h).

cocaine *in vivo* is that these reactions are also carried out by liver microsomes from animals which are not susceptible to cocaine-induced hepatic necrosis. For example, we have found that microsomes from the livers of BALB/c mice produce significant amounts of NHNC, even under conditions where they show little liver damage from cocaine.* Rosen and his collaborators have reported that microsomes from rats and hamsters, species showing little liver damage from cocaine, also make norcocaine nitroxide and lipid peroxides when incubated with NHNC [3, 17]. These observations suggest that, in examining the basis for strain and species differences in cocaine-induced hepatotoxicity, it may be wise to look more closely at some of the initial steps of cocaine metabolism, as well as the role of esterases.

*Acknowledgements*—We wish to thank Dr. M. L. Thompson for helpful discussions, and the NIH mass spectrophotometric facility at the Massachusetts Institute of Technology for running high resolution mass spectra. This work was supported by Grant DA 01885 from the National Institute on Drug Abuse.

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