Identification of Crotonaldehyde as a Hepatic Microsomal Metabolite Formed by \( \alpha \)-Hydroxylation of the Carcinogen \( N \)-Nitrosopyrrolidine

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Crotonaldehyde (2-butenal), which reacts with DNA and is mutagenic and carcinogenic, was identified as a hepatic microsomal metabolite of the hepatocarcinogen \( N \)-nitrosopyrrolidine. Incubation mixtures of \( N \)-nitrosopyrrolidine, cofactors, and hepatic microsomes from Aroclor pretreated or control F344 rats were derivatized with (2,4-dinitrophenyl)hydrazine reagent and the resulting mixtures analyzed by high-performance liquid chromatography. Crotonaldehyde (2,4-dinitrophenyl)hydrazine was identified by its retention time in two different systems and by its ultraviolet and mass spectrum. The ratio of 4-hydroxybutyraldehyde, which has previously been identified as a metabolite of \( N \)-nitrosopyrrolidine, to crotonaldehyde was 1.5-2 over a range of substrate concentrations. The approximate values of \( K_m \) and \( v_{\text{max}} \) for crotonaldehyde were 5.8 mM and 0.6 nmol/min/mg of protein and for 4-hydroxybutyraldehyde 14.1 mM and 1.7 nmol/min/mg of protein, for substrate concentrations between 1 and 8 mM, with microsomes from Aroclor pretreated rats. The ratio of 4-hydroxybutyraldehyde to crotonaldehyde was 1.9 upon esterase-catalyzed solvolysis of \( \alpha \)-acetoxy-\( N \)-nitrosopyrrolidine, a stable precursor to the initial product of \( N \)-nitrosopyrrolidine \( \alpha \)-hydroxylation. These results demonstrate that crotonaldehyde is formed upon metabolic \( \alpha \)-hydroxylation of \( N \)-nitrosopyrrolidine and suggest that it may be involved in \( N \)-nitrosopyrrolidine–macromolecule interactions.

Introduction

\( N \)-nitrosopyrrolidine (NPYR) is a carcinogenic cyclic nitrosamine which has been detected in mainstream and sidestream tobacco smoke, unburned tobacco, cooked bacon, and some cured meat products (1-3). It causes liver tumors in rats and respiratory tract tumors in mice and hamsters (4). The mechanism of its metabolic activation is of interest not only because of its environmental occurrence and carcinogenicity but also because it is the structural parent of such commonly occurring nitrosamines as \( N \)-nitrosopropylene and \( N \)'-nitrosopropynicotine.

Previous studies have demonstrated that \( \alpha \)-hydroxylation is a major pathway of hepatic microsomal metabolism of NPYR (5). As illustrated in Figure 1, this results in formation of 4-hydroxybutyraldehyde (6), which exists primarily as the cyclic hemiacetal, 2-hydroxymethyltetrahydrofuran (6). This metabolic pathway was elucidated by using \( \alpha \)-AcO-NPYR (2) as a stable precursor to \( \alpha \)-HO-NPYR (3), the initial product of NPYR \( \alpha \)-hydroxylation (6). Investigations of the esterase-catalyzed reaction of \( \alpha \)-AcO-NPYR with deoxyguanosine showed that cyclic 1,\( \alpha \)^2-propanodeoxyguanosine adducts were major products and that they were identical with those formed upon reaction of crotonaldehyde (9) with deoxyguanosine (7). These results suggested that crotonaldehyde should be a hydrolysis product of \( \alpha \)-AcO-NPYR and a metabolite of NPYR and that it might be involved in the metabolic activation of NPYR. The object of this study was therefore to determine whether crotonaldehyde is formed from \( \alpha \)-AcO-NPYR and in the hepatic microsomal metabolism of NPYR.

Experimental Section

Apparatus. HPLC was carried out with a Waters Associates System (Millipore, Waters Division, Milford, MA), equipped with a Model 590 Photodiode Array Detector and either two 3.9 mm \( \times \) 30 cm \( \mu \)Bondapak C18 reverse-phase columns (Waters) in series or two Lichrsorb Si60 5\( \mu \) normal-phase columns (EM Reagents, Cincinnati, OH) in series. Detection was by UV absorbance at 340 nm. The reverse-phase columns were eluted isocratically with 60% acetonitrile in water, flow rate 1.5 mL/min, for analysis of crotonaldehyde (2,4-dinitrophenyl)hydrazine, or 30% acetonitrile in water, flow rate 1.0 mL/min, for 4-hydroxybutyraldehyde (2,4-dinitrophenyl)hydrazine and 5-hydroxybutyraldehyde (2,4-dinitrophenyl)hydrazine. The normal-phase columns were eluted isocratically with 40% solvent A in solvent B at 1 mL/min. Solvent A was 500 mL of isooctane and 5 mL of ethanol. Solvent B was 250 mL of isooctane, 100 mL of CH\(_2\)Cl\(_2\), 50 mL of acetonitrile, and 20 mL of methanol. MS were run on a Hewlett-Packard Model 5988A instrument. NMR spectra were obtained with a JEOL Model FX 90 Q spectrometer.

Materials. NPYR and crotonaldehyde (2-butenal) were obtained from Aldrich Chemical Co., Milwaukee, WI. Their structures were confirmed by NMR. Crotonaldehyde (2,4-di-

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2 Abbreviations: NPYR, \( N \)-nitrosopyrrolidine.
a-Hydroxylation of the Carcinogen N-Nitrosopyrrolidine

**Figure 1.** Intermediates and products involved in the α-hydroxylation of NPYR.

The structure of 3-hydroxybutyraldehyde (2,4-dinitrophenyl)hydrazone, 4-hydroxybutyraldehyde (2,4-dinitrophenyl)hydrazone, and 3-hydroxybutyraldehyde (2,4-dinitrophenyl)hydrazone were prepared as described (8-10). The melting points of crotonaldehyde (2,4-dinitrophenyl)hydrazone and 4-hydroxybutyraldehyde (2,4-dinitrophenyl)hydrazone were 195 [lit.(8) mp 190 °C] and 118 °C [lit.(9) mp 120 °C], respectively. The structure of 3-hydroxybutyraldehyde (2,4-dinitrophenyl)hydrazone was confirmed by its chemical ionization MS, m/e (relative intensity) 209 (M+1, 65), 194 (50), 87 (100). α-AcO-NPYR was synthesized (17). Hepatic microsomes were isolated from male F344 rats (230-280 g) which had been given an ip injection of 500 mg/kg Aroclor 1254 in corn oil 5 days prior to sacrifice or from untreated rats. Microsomes were prepared as described previously (6); protein content was determined by the method of Lowry (12). Glucose-6-phosphate dehydrogenase, type XII, and carboxyl esterase (EC 3.1.1.1) were obtained from Sigma Chemical Co., St. Louis, MO.

In Vitro Metabolism. Incubations were carried out by a slight modification of a previously described method (13). Assay mixtures in a volume of 1 mL contained 50 mM Tris buffer, pH 7.5, 5.0 mM glucose-6-phosphate, 0.4 units/mL of glucose-6-phosphate dehydrogenase, 1.0 mM EDTA, 1.0 mM NADP+, 5.0 mM semicarbazide hydrochloride, and the appropriate quantity of NPYR. Reactions were initiated by addition of microsomes (0.6-1.5 mg of protein) and were carried out in a shaking water bath at 37 °C for 30 min, unless noted otherwise. All incubations were protected from light. Reactions were quenched by addition of 1 mL of saturated Ba(OH)2 and 1 mL of 0.25M ZnSO4. The mixture was allowed to stand on ice for 1 h and then centrifuged at 10,000 rpm at 5 °C to pellet precipitated protein. A 1.5-mL portion of the supernatant was diluted to 10 mL with H2O. To this was added 0.4 mL of (2,4-dinitrophenyl)hydrazine reagent, freshly prepared each time by mixing 250 mg of (2,4-dinitrophenyl)hydrazine with 100 mL of 6 N HCl. After 1 h at room temperature, the solution was extracted three times with 15 mL of isooctane (for isolation of crotonaldehyde (2,4-dinitrophenyl)hydrazone), followed by CH2Cl2 (for isolation of 3- and 4-hydroxybutyraldehyde (2,4-dinitrophenyl)hydrazone). The combined organic extracts were evaporated in vacuo to dryness, the residue was redissolved in acetonitrile, and aliquots were analyzed by reverse-phase HPLC. Retention times were as follows: crotonaldehyde (2,4-dinitrophenyl)hydrazine, 11 min; 4-hydroxybutyraldehyde (2,4-dinitrophenyl)hydrazone, 35.2 min; 3-hydroxybutyraldehyde (2,4-dinitrophenyl)hydrazone, 36.8 min. Some samples were also analyzed by normal-phase HPLC, with retention times as follows: crotonaldehyde (2,4-dinitrophenyl)hydrazine, 8.8 min; 4-hydroxybutyraldehyde (2,4-dinitrophenyl)hydrazone, 8.5 min; 4-hydroxybutyraldehyde (2,4-dinitrophenyl)hydrazine, 27.4 min; 3-hydroxybutyraldehyde (2,4-dinitrophenyl)hydrazone, 21.2 min.

**Hydrolysis of α-AcO-NPYR.** A mixture of α-AcO-NPYR (58 μmol) and 11.2 units of hog liver esterase in 2 mL of pH 7 phosphate buffer was incubated with shaking for 4 h at 37 °C. The resulting mixture was analyzed for crotonaldehyde and 3- and 4-hydroxybutyraldehyde, as described above, except that 16 mL of (2,4-dinitrophenyl)hydrazine reagent was used.

**Quantitation.** HPLC peaks were quantified by comparison to standard curves. The recovery of the procedure for analysis of microsomal metabolism was estimated by adding crotonaldehyde to a mixture of microsomes and cofactors as described above; crotonaldehyde was added after 15 min of incubation and the reaction was allowed to continue for an additional 15 min. Analysis of crotonaldehyde (2,4-dinitrophenyl)hydrazone showed that the recovery was 35%; all values reported for incubations with microsomes were corrected accordingly. The recovery of 4-hydroxybutyraldehyde has previously been shown to be greater than 90% (14). The values reported for the incubation of α-AcO-NPYR with esterase are uncorrected.

**MS Analysis.** For MS characterization of crotonaldehyde (2,4-dinitrophenyl)hydrazone, incubations were carried out on 10 times the scale described above. The isooctane extracts were concentrated, redissolved in acetonitrile, and applied to 20 cm × 20 cm × 0.25 mm silica gel TLC plates (E. Merck, Darmstadt, FRG). The plates were eluted with benzene/ethyl acetate (7/3) and the band corresponding to crotonaldehyde (2,4-dinitrophenyl)hydrazone (Rf 0.57) was isolated and eluted from the silica gel with methanol. It was purified further by reverse-phase HPLC, using the system described above, except with elution by 50% acetonitrile in H2O, flow rate 1 mL/min. The retention time of crotonaldehyde (2,4-dinitrophenyl)hydrazine was 32.2 min.

**Results**

After incubation of NPYR with cofactors and hepatic microsomes, (2,4-dinitrophenyl)hydrazine reagent was added to derivatize crotonaldehyde and 4-hydroxybutyraldehyde for HPLC analysis. A typical reverse-phase HPLC chromatogram illustrating the crotonaldehyde (2,4-dinitrophenyl)hydrazine peak, as formed with hepatic microsomes from Aroclor pretreated rats, is shown in Figure 2. The indicated peak coeluted with standard crotonaldehyde (2,4-dinitrophenyl)hydrazine in this system as well as in the normal-phase HPLC system. This
peak was not present in control incubations, carried out with boiled microsomes or without cofactors. Its UV spectrum was identical with that of a standard, with λ max 382, which was 15 nm longer than that of 4-hydroxybutyraldehyde (2,4-dinitrophenyl)hydrazone due to the α,β-unsaturated system. This material was purified from large-scale incubations, and identified as crotonaldehyde (2,4-dinitrophenyl)hydrazone by comparison of its MS to that of a standard, as illustrated in Figure 3. 4-Hydroxybutyraldehyde (2,4-dinitrophenyl)hydrazone was also detected, as described previously (6, 14).

The formation of crotonaldehyde and 4-hydroxybutyraldehyde increased over a 90-min period as shown in Figure 4. A double-reciprocal plot of crotonaldehyde and 4-hydroxybutyraldehyde formation from NPYR is illustrated in Figure 5, for substrate concentrations between 1 and 8 mM, with microsomes from Aroclor pretreated rats. The approximate values of $K_m$ and $v_{max}$ for crotonaldehyde were 5.8 mM and 0.6 nmol/min/mg of protein, respectively; the corresponding values for 4-hydroxybutyraldehyde were 14.1 mM and 1.7 nmol/min/mg of protein. The amount of 4-hydroxybutyraldehyde was 1.5–2-fold greater than that of crotonaldehyde at each substrate concentration. At a substrate concentration of 6 mM, the rates of formation of crotonaldehyde and 4-hydroxybutyraldehyde were 4.7 and 6.5 times less, respectively, when microsomes from untreated rats were used, compared to those from rats pretreated with Aroclor.

Analysis of incubation mixtures of NPYR, cofactors, and hepatic microsomes from Aroclor pretreated rats demonstrated that the yield of 3-hydroxybutyraldehyde, if formed, was less than 10% of that of 4-hydroxybutyraldehyde. Similar results were obtained upon esterase-catalyzed hydrolysis of α-AcO-NPYR.

As shown in Figure 1, a likely mechanism for formation of crotonaldehyde is through the carbonium ion 6. This carbonium ion could also yield 3-hydroxybutyraldehyde (10). To investigate this possibility, HPLC systems were developed to separate 3-hydroxybutyraldehyde (2,4-dinitrophenyl)hydrazone and 4-hydroxybutyraldehyde (2,4-dinitrophenyl)hydrazone. Analysis of incubation mixtures of NPYR, cofactors, and hepatic microsomes from Aroclor pretreated rats demonstrated that the yield of 3-hydroxybutyraldehyde, if formed, was less than 10% of that of 4-hydroxybutyraldehyde. Similar results were obtained upon esterase-catalyzed hydrolysis of α-AcO-NPYR.

**Discussion**

The results of this study have clearly demonstrated that crotonaldehyde is a hepatic microsomal metabolite of NPYR and that it is formed in the esterase-catalyzed solvolysis of α-AcO-NPYR. One likely mechanism for its production is illustrated in Figure 1. α-HO-NPYR, the initial product of α-hydroxylation of NPYR or of hydrolysis of α-AcO-NPYR, should undergo spontaneous ring opening to 4-oxobutanediazohydroxide (4). This diazohydroxide could react with H₂O to give 4-hydroxybutyraldehyde (5). Alternatively, 4 could decompose to the carbonium ion 6, which could rearrange to 7. Loss of a proton from 7 gives crotonaldehyde (9), and hydration...
gives 3-hydroxybutyraldehyde (10). Another likely mechanism would be loss of a proton from 6 or loss of H₂O and N₂ from 4, to give 3-butenal, which could rearrange to crotonaldehyde. It is also possible that some of the crotonaldehyde (2,4-dinitrophenyl)hydrazone could have been formed from 3-hydroxybutyraldehyde. The central involvement of α-HO-NPYR as a precursor to both 4-hydroxybutyraldehyde and crotonaldehyde is supported by the observation that the ratios of the yields of these two products were similar in the metabolism studies and in the esterase-catalyzed hydrolysis of α-AcO-NPYR.

The formation of crotonaldehyde as a solvolysis product of α-AcO-NPYR is consistent with the observation that cyclic 1,N²-propanodeoxyguanosine adducts are formed when the solvolysis is carried out in the presence of deoxyguanosine (7). As previously discussed, these adducts could arise either directly by reaction of crotonaldehyde with deoxyguanosine, as demonstrated in our earlier study, or by initial reaction of 4 with deoxyguanosine followed by carbonium ion rearrangement and ring closure (7). The present results provide support for the formation of the cyclic adducts via crotonaldehyde. The detection of crotonaldehyde as a metabolite of NPYR thus strongly suggests that the cyclic 1,N²-propanodeoxyguanosine adducts should also be formed during NPYR metabolism, if the crotonaldehyde produced reaches DNA prior to further metabolism, or reaction with protein (15). Since α,β-unsaturated aldehydes react readily with thiols, cellular amino acids and proteins may have a significant protective role against DNA modification by NPYR (16).

Previous studies have shown that crotonaldehyde is a direct acting mutagen in S. typhimurium and is carcinogenic to rat liver (17–19). Its carcinogenic activity was less than that of NPYR, suggesting that it is not a major ultimate carcinogen of NPYR (19). However, the effects of crotonaldehyde generated intracellularly from NPYR may be significantly different from those observed upon its administration in the drinking water as in the carcinogenicity study. In this respect, it is interesting to note that benzo[a]pyrene-7,8-diol 9,10-epoxide is a less effective carcinogen on mouse skin than is benzo[a]pyrene, despite strong evidence that it is a major ultimate carcinogen. Metabolism studies have shown that benzo[a]pyrene-7,8-diol 9,10-epoxide is rapidly cleared from mouse epidermis following topical application, in contrast to benzo[a]pyrene, and binds less effectively to DNA under these conditions (20). It would therefore be important to measure the levels of adducts formed in hepatic DNA upon treatment of rats with either NPYR or crotonaldehyde, if sensitive enough methods can be developed. This would provide a better indication of the potential role of crotonaldehyde as an ultimate carcinogen of NPYR.

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References


