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SOURCE OF OXYGEN IN CYTOCHROME P-450 CATALYZED CARBINOLAMINE FORMATION

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N-Methylcarbazole was incubated in H_2O^{18} and under an ^{18}O atmosphere. N-Hydroxymethylcarbazole, 1-OH- and 3-OH-N-methylcarbazole were isolated by HPLC and analyzed for ^{18}O content. In incubations containing ^{18}O, all three metabolites showed >95% ^{18}O incorporation. In incubations containing H_2O^{18}, the N-hydroxymethyl metabolite showed ^{16}O incorporation equal to control incubations in 100% H_2O. These data demonstrate that the sole source of oxygen in cytochrome P-450 catalyzed, NADPH supported N-hydroxymethylcarbazole formation is atmospheric oxygen.

The mechanism of cytochrome P-450 catalyzed N-dealkylations is generally accepted to procede via intermediate carbinolamine formation (1). The instability of most carbinolamines results in their fragmentation into a carbonyl compound and the dealkylated amine, precluding their direct examination. In this report, we have taken advantage of the unusual stability of N-hydroxymethylcarbazole to determine the source of the carbinolamine oxygen in the NADPH supported, cytochrome P-450 catalyzed N-dealkylation of N-methylcarbazole (2). By microsomal incubation of N-methylcarbazole in H_2O^{18} and under an ^{18}O atmosphere, we have determined that atmospheric oxygen is the sole source of the carbinolamine oxygen.

METHODS

N-methylcarbazole and N-hydroxymethylcarbazole were purchased from Alfred Bader Chemicals. NADP+, glucose-6-phosphate and glucose-6-

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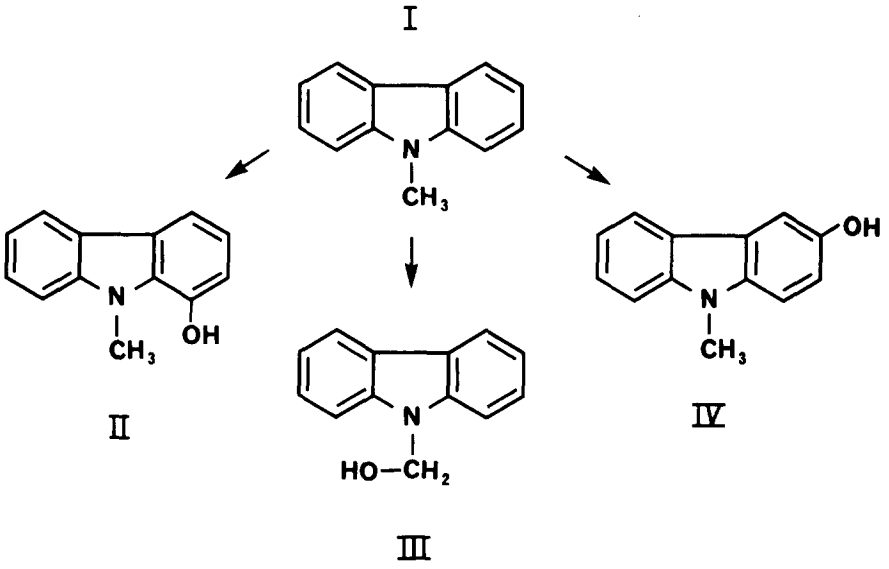

Figure 1. Structure of N-methylcarbazole (I) and three of its metabolites, 1-OH-N-methylcabazole (II), N-hydroxymethylcarbazole (III), and 3-OH-N-methylcarbazole (IV)

phosphate dehydrogenase were purchased from Sigma Chemical. N-methylcarbazole was incubated in rat liver microsomes containing the following: 0.1 M phosphate buffer (pH 7.4), 0.5 mM NADP+, 3.0 mM glucose-6-phosphate, 2 units of G-6-P dehydrogenase, 3.0 mM $MgCl_2$, 2 mg microsomal protein and 1.5 mM N-methylcarbazole in final volume of 1 ml. Thirty minute incubations at 37°C were terminated by extraction with ethyl acetate. Extracts were combined and metabolites separated on a Waters HPLC using a silica column. The flow rate of the hexane: isopropanol (100:2.2) mobile phase was 2 ml/min.

^{18}O atmosphere experiments were carried out in a specially designed apparatus consisting of a manifold vented via a three way stopcock and connected to four incubation flasks. The apparatus was purged repeatedly with nitrogen and the reactions initiated by the addition of ^{18}O gas. Percent of ^{18}O in the atmosphere was determined by the co-incubation of acetanilide and subsequent mass spectral analysis of the 4-OH metabolite, which has been shown to arise solely from atmospheric oxygen incorporation (4). The H_2O^{18} experiments were performed in incubations enriched with H_2O^{18} to fifty percent levels. Percent of H_2O^{18} enrichment was confirmed by mass spectral analysis following termination of incubations.

Isolated metabolites were analyzed on a VG-7070 Mass Spectrometer with direct probe insertion, isobutane CI, selected ion recording mode. Percent ^{18}O incorporation was determined by the ratio of M, M+1, and M+2 masses for the ^{16}O (197,198,199) and ^{18}O (199,200,201), corrected for ^{13}C contributions.

RESULTS

Following incubation of N-methylcarbazole (I),(Fig. 1) we isolated N-hydroxymethylcarbazole (III) and 1-OH(II)- and 3-OH(IV)-

Table I

O^{18} Atmosphere Incubation of N-Methylcarbazole

Metabolite Percent Incorporation of Oxygen-18

Incubation	N-MeOH	1-OH	3-OH
I	92.98	93.07	96.33
	93.02	97.31	96.95
	91.07		94.32
	92.36(1.11)	95.19(2.99)	95.87(1.38)
II	101.71	94.62	97.74
	105.72	96.81	95.62
	106.25	96.32	98.29
	104.56(2.48)	95.92(1.15)	97.22(1.41)
III	89.15	94.67	94.94
	85.82	95.05	88.71
	87.74		88.27
	87.57(1.67)	94.86(0.27)	90.64(3.73)
Totals	94.83(7.75)	95.41(1.48)	94.57(3.67)

N-methylcarbazole. The results of the mass spectral analysis of the ^{18}O and H_2O^{18} incubations are shown in Tables I and II, respectively. Under an ^{18}O atmosphere, all metabolites showed >95 precent ^{18}O incorporation. The greater variability in the N-hydroxymethyl metabolite was due to its more complex fragmentation pattern, in which significantly more of the M+ ion was formed than in the phenolic metabolites. In the H_2O^{18} incubations, the N-hydroxymethyl metabolite showed ^{16}O incorporation equal to that of control incubations in 100 percent H_2O^{16}. The phenolic metabolites also had >97% ^{16}O incorporation (data not shown). These data clearly demonstrate that the sole oxygen source in the N-hydroxymethyl metabolite is atmospheric oxygen.

DISCUSSION

Griffin and co-workers have shown that the cumene hydroperoxide dependent P-450 catalyzed demethylation of aminopyrene occurs via an aminium cation-radical intermediate (3,4). This cation radical undergoes a second one electron oxidation to form an iminium ion, which

Table 2

H_2O^{18} Incubation of N-Methylcarbazole

N-MeOH Metabolite Percent of Oxygen-16 Incorporation

Incubation	Control(a)	50% H_2O^{18}
I	98.13	98.30
	97.37	98.22
	91.96	97.88
	95.82(3.36)	98.13(0.22)
II	97.30	97.65
	97.64	97.73
	97.18	97.59
	97.37(0.24)	97.66(0.07)
III	96.79	96.25
	96.17	95.96
	96.06	94.23
	96.34(0.39)	95.48(1.09)
Totals	96.51(1.83)	97.09(1.35)

(a) Control incubations were run in 100% H_2O

is proposed to be hydrated to form the carbinolamine intermediate (Figure 2, path b). If such a mechanism was occuring in the NADPH supported dealkylation of N-methylcarbazole, the source of the carbinolamine oxygen would be water. Since we have shown that this is not the case, hydration of the iminium ion is ruled out. If the iminium ion is formed, then it must be followed by rapid attack by the heme iron bound hydroxide ion, generating a carbinolamine with atmospheric oxygen

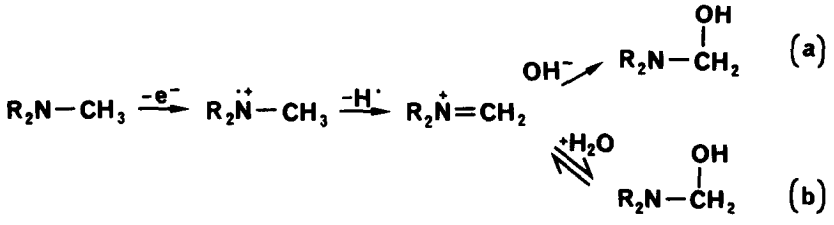

Figure 2. Mechanism of carbinolamine formation involving a nitrogen radical cation intermediate. Path (a) represents attack by a heme iron bound hydroxide ion, resulting in incorporation of atmospheric oxygen. Path (b) represents hydration of the iminium ion with resultant incorporation of oxygen from water

incorporation (Figure 2, path a). The total lack of ^{18}O in the H_2O^{18} incubated N-hydroxymethyl metabolite also rules out the equilibrium between the iminium ion and carbinolamine that has been proposed for other carbinolamines (5,6). The absence of any oxygen exchange in the carbinolamine is most likely due to extensive delocalization of the nitrogen lone pair into the aromatic system of the carbazole.

These data are also consistent with other mechanisms of carbinolamine formation (1). Direct insertion of oxygen into an alpha C-H bond or abstraction by oxygen of a hydrogen radical, followed by rapid recombination, would also generate a carbinolamine with atmospheric oxygen incorporation. In our laboratory, we are working to determine the mechanism of cytochrome P-450 catalyzed N-dealkylation of several substrates and to examine closely any differences between peroxide and NADPH supported oxidations.

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