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MECHANISM OF THE METABOLISM OF 1,3-BENZODIOXOLES TO CARBON MONOXIDE

M. W. ANDERS* and JOAN M. SUNRAM
Department of Pharmacology, University of Minnesota, Minneapolis, MN 55455, U.S.A.
and
CHRISTOPHER F. WILKINSON
Department of Entomology, Cornell University, Ithaca, NY 14853, U.S.A.

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Abstract—Carbon monoxide is a minor product formed during the cytochrome P-450-catalyzed oxidation of 1,3-benzodioxoles. Studies with [2-^{13}C]methylene 1,3-benzodioxoles established that the methylenic carbon of the 1,3-benzodioxole ring is the source of the carbon atom in the carbon monoxide, and an isotope effect of 1.7 to 2.0 was observed with [2-$^{2}H_2$]methylene derivatives. Incubations conducted in the presence of [^{18}O]dioxygen and [^{18}O]water showed that the oxygen atom in carbon monoxide arises from both oxygen and water. A mechanism consistent with these data has been proposed for carbon monoxide formation. It involves initial monooxygenation of the 1,3-benzodioxole to a 2-hydroxy derivative that subsequently forms a 2-hydroxyphenyl formate intermediate, which yields either carbon monoxide or formate. The proposed mechanism is discussed in terms of its possible relationship to the inhibitory activity of 1,3-benzodioxoles toward microsomal oxidation.

Numerous studies have established the metabolism of a variety of 1,3-benzodioxoles (methylenedioxyphenyl compounds) both *in vivo* and *in vitro* [1–3]. Although many of the metabolic pathways involve the action of microsomal mixed function oxidases or conjugating enzymes, or both, on specific functional groups or substituents in the aromatic ring, the major pathway common to all 1,3-benzodioxoles results from oxidative demethylenation of the methylenedioxy ring to yield the corresponding catechol [4–6]. Early metabolic studies with [2-^{14}C-*methylene*]1,3-benzodioxole established that the methylenic carbon atom was liberated as formate in *in vitro* incubations with NADPH-fortified microsomal fractions from mammalian liver and insect tissues and as carbon dioxide *in vivo* in the same species [5–8]. It was suggested that the reaction proceeded through a 2-hydroxy-1,3-benzodioxole intermediate resulting from a cytochrome P-450-catalyzed oxidation [7].

More recently it has been established that a variety of substituted 1,3-benzodioxoles are metabolized to carbon monoxide during incubation with NADPH-fortified microsomal fractions from rat liver [9] and armyworm midguts [10]. The rate of carbon monoxide production was well correlated with the Hammett sigma constants of the aromatic substituents in the 5 or 6 positions of the ring [9] and was greatest in compounds containing electron-withdrawing substituents.

There is currently a great deal of interest in the mechanism through which 1,3-benzodioxoles exert their inhibitory effects on microsomal drug oxidation, and this inhibition is generally thought to involve oxidative metabolism to an active species [11], possibly a carbene [12], that forms an inhibitory complex with cytochrome P-450. It was, therefore, of interest to investigate further the enzymatic reaction mechanism through which carbon monoxide is formed.

MATERIALS AND METHODS

Chemicals. 5,6-Dichloro-1,3-benzodioxole was prepared as described previously by the chlorination of piperonylic acid [13, 14]. [2-^{13}C]4,5-Dichloro-1,3-benzodioxole (m.p. 75–76°, 87.8 atom% ^{13}C as measured by gas chromatography/mass spectrometry) was synthesized in 70% yield by methylation of 4,5-dichlorocatechol with [^{13}C]dichloromethane (90 atom% ^{13}C; Merck Sharp & Dohme, Montreal, Canada) [15]; the 4,5-dichlorocatechol (m.p. 110–112°) was prepared by demethylenation of 5,6-dichloro-1,3-benzodioxole with anhydrous aluminum chloride in dry chlorobenzene [16, 17]. 5-Nitro-1,3-benzodioxole was prepared as described previously [13], and 4-nitrocatechol was purchased from the Sigma Chemical Co., St. Louis, MO. [2-$^{2}H_2$]5-Nitro-1,3-benzodioxole and [2-$^{2}H_2$]5,6-dichloro-1,3-benzodioxole were prepared by methylenation of the corresponding catechols with [$^{2}H_2$] dichloromethane (99+% atom% ^{2}H) purchased from the Aldrich Chemical Co. [15]. The [2-$^{2}H_2$]5-nitro- and [2-$^{2}H_2$]5,6-dichloro-1,3-benzodioxoles were 80 and 95% enriched in deuterium, respectively, as measured by FT ^{1}H-NMR analysis with a Bruker 270 mHz spectrometer; structural assignments were verified by homonuclear decoupling. [^{18}O]Dioxygen and [^{18}O]water were purchased from the Monsanto Research Corp., Miamisburg, OH, and Bio-Rad Laboratories, Richmond, CA,

* Present address: Department of Pharmacology, University of Rochester Medical Center, 601 Elmwood Ave., Rochester, NY 14642, U.S.A. All correspondence and reprint requests should be addressed to Prof. M. W. Anders at this address.

respectively, and all other chemicals were of analytical or reagent grade.

Animals. Male Sprague–Dawley rats (250–300 g) were used. Hepatic microsomal fractions were isolated as described previously [18], and protein concentrations were measured by the method of Lowry *et al.* [19].

Metabolism studies. Incubation mixtures contained, unless otherwise stated, Tris–EDTA buffer (150 μmoles Tris and 0.6 μmole EDTA, pH 7.4), 15 μmoles magnesium chloride, 10 μmoles DL-isocitric acid, 1 μmole $NADP^+$, 1 unit pig heart isocitric acid dehydrogenase (Sigma), various amounts of substrate, and 3.0 mg of microsomal protein in a final volume of 3.0 ml. The incubation mixtures were contained in 10-ml Erlenmeyer flasks closed with sleeve-type serum stoppers. The reactions were started by the addition of enzyme, and the reaction mixtures were incubated for 15 min at 37°. The reactions were stopped by placing the flasks in an ice bath, when carbon monoxide was the product that was quantified, or by the addition of 0.1 ml of 70% perchloric acid, when 4-nitrocatechol was the product that was quantified.

Carbon monoxide concentrations were measured by gas chromatography [20], and [^{13}C]carbon monoxide enrichments were measured by gas chromatography/mass spectrometry [21]; [^{18}O]carbon monoxide formation was determined by gas chromatography/mass spectrometry [22]. 4-Nitrocatechol concentrations were measured in supernatant fractions obtained by centrifuging the incubation mixtures after the addition of perchloric acid. Two milliliters of the supernatant fraction was mixed with 0.3 ml of 5 N potassium hydroxide solution, and the samples were centrifuged to remove the potassium perchlorate. The absorbance of the clear supernatant fraction was measured at 512 nm. A standard curve was prepared by measuring the absorbances of reaction mixtures containing various concentrations of 4-nitrocatechol.

RESULTS

Initial studies were directed toward establishing the relative amounts of catechol and carbon monoxide formed during the metabolism of 5-nitro-1,3-benzodioxole. When 0.2 mM 5-nitro-1,3-benzodioxole was incubated with hepatic microsomal fractions, the ratio of 4-nitrocatechol to carbon monoxide was 14.6 ± 2.2 (mean ± S.D., N = 8). Thus, carbon monoxide is a relatively minor metabolite of 1,3-benzodioxoles.

The effect of deuterium substitution in the methylene position of the 1,3-benzodioxole ring was also investigated in relation to carbon monoxide production. Carbon monoxide generation was decreased in compounds containing deuterium; isotope effects of 2.03 ± 0.01 (mean ± S.D., N = 3) and 1.79 ± 0.27 (mean ± S.D., N = 3), respectively, were observed when [2-$^2H_2$]5-nitro-1,3-benzodioxole (0.2 mM) and [2-$^2H_2$]5,6-dichloro-1,3-benzodioxole (0.2 mM) were the substrates.

Attempts to establish the source of both the carbon and the oxygen atoms in the carbon monoxide formed during the metabolism of 1,3-benzodioxoles by hepatic microsomal fractions were conducted with [2-^{13}C]1,3-benzodioxoles as well as with [^{18}O]dioxygen and [^{18}O]water. Incubation of [2-^{13}C]5,6-dichloro-1,3-benzodioxole (87.8 atom% ^{13}C) with NADPH-fortified microsomal fractions yielded [^{13}C]carbon monoxide containing the same enrichment of ^{13}C (86.5 atom%) as the substrate. When hepatic microsomal fractions from phenobarbital-treated rats were incubated with 5-cyano-1,3-benzodioxole in the presence of [^{18}O]dioxygen (92–95 atom% ^{18}O), [^{18}O]carbon monoxide containing only 5–12 atom% ^{18}O was formed. A similar experiment conducted in the absence of [^{18}O]dioxygen and in the presence of [^{18}O]water (11.8 atom% ^{18}O) yielded [^{18}O]carbon monoxide containing about 12 atom% ^{18}O.

DISCUSSION

The results obtained in the present study confirm the earlier observations that 1,3-benzodioxoles are metabolized to carbon monoxide by hepatic cytochrome P-450-dependent monooxygenases [9].

The finding that [2-^{13}C]1,3-benzodioxoles are metabolized to [^{13}C]carbon monoxide with the same degree of enrichment as the substrate shows clearly that the carbon monoxide is derived from the 1,3-benzodioxole and does not arise from heme breakdown or lipid peroxidation.

The observation of a primary deuterium isotope effect in the formation of carbon monoxide from 1,3-benzodioxoles indicates that C—H bond cleavage is, at least, partially rate limiting. Similar deuterium isotope effects have been found in, for example, the cytochrome P-450-dependent metabolism of dihalomethanes to carbon monoxide [22] and in the benzylic hydroxylation of [1,1-$^2H_2$]1,3-diphenylpropane [23].

Oxygen atoms from both [^{18}O]dioxygen and [^{18}O]water were incorporated into the carbon monoxide formed as a metabolite of the 1,3-benzodioxoles. The incorporation of oxygen from [^{18}O]dioxygen is consistent with the involvement of cytochrome P-450-dependent monooxygenase activity, although the enrichment observed in incubations conducted in the presence of [^{18}O]dioxygen was low. This observation, combined with the incorporation of ^{18}O from [^{18}O]water, suggests the formation of an intermediate that exchanges with the medium; the incorporation of [^{18}O]oxygen from water approximated the enrichment of the water.

A possible reaction mechanism incorporating such an intermediate is shown in Fig. 1. According to this mechanism, initial monooxygenase-catalyzed hydroxylation of the 1,3-benzodioxole would yield an intermediate is shown in Fig. 1. According to this mechanism, initial monooxygenase-catalyzed 2-hydroxyphenyl formate (II, Fig. 1). Decarbonylation of the 2-hydroxyphenyl formate would yield carbon monoxide and catechol, and hydrolysis of II would yield formic acid and catchol. This mechanism is consistent with the observation of a deuterium isotope effect and with the finding that [2-^{13}C]1,3-benzodioxoles give rise to [^{13}C]carbon monoxide. It is also consistent with earlier observations [5–8] that the methylenic carbon atom is metabolized to

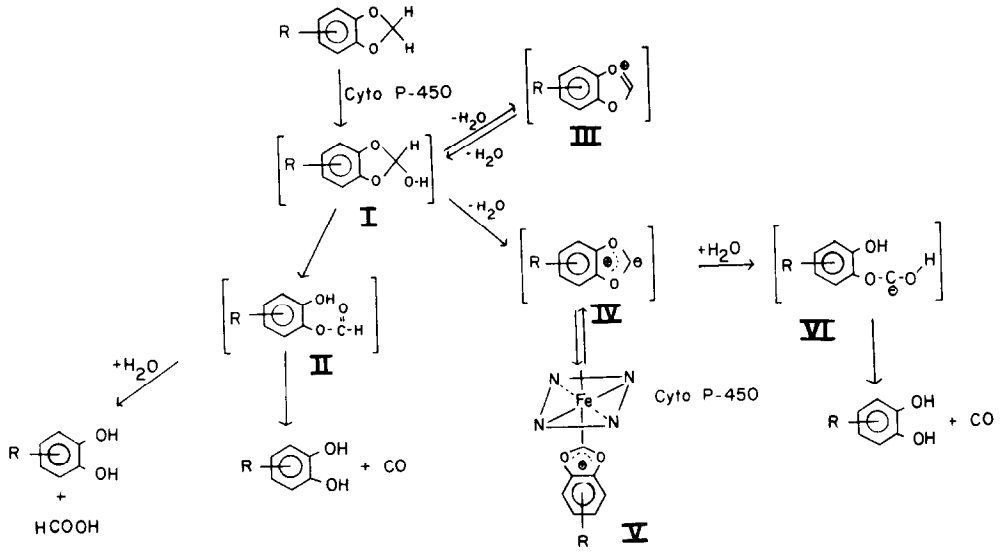

Fig. 1. Proposed reaction mechanism for the metabolism of 1,3-benzodioxoles. Postulated intermediates are shown in brackets. Cyto P-450 = cytochrome P-450; CO = carbon monoxide.

formate *in vitro* in NADPH-fortified microsomal fractions from mammalian liver and insect tissues. Indeed, in view of the 15:1 product ratio of catechol to carbon monoxide observed during the metabolism of 4-nitro-1,3-benzodioxole, it is probable that the pathway leading to formate and catechol is quantitatively more important than that leading to carbon monoxide and catechol.

The 2-hydroxy-1,3-benzodioxole intermediate (I, Fig. 1) is analogous to an *ortho*-ester (RO_3CH); such compounds readily lose alcohol, or water in the case of I, to form an oxonium ion (III, Fig. 1), and this reaction is readily reversible [24]. This would explain the formation of [^{18}O]carbon monoxide when the reaction was carried out in the presence of either [^{18}O]dioxygen or [^{18}O]water.

A carbene (IV, Fig. 1) has been suggested as an intermediate in the metabolism of 1,3-benzodioxoles [12, 25]. The carbene (IV, Fig. 1) may arise from 2-hydroxy-1,3-benzodioxole (I, Fig. 1). Base-catalyzed proton abstraction would yield a carbanion that could undergo an α-elimination reaction to form the carbene. The conversion of 2-hydroxy-1,3-benzodioxole to the carbene may also be subject to bifunctional catalysis, as has been proposed for the conversion of 4-hydroxycyclophosphamide to aldophosphamide [26]. This would involve the concerted proton abstraction from the methylene carbon atom and the protonation of the methylene hydroxyl of 2-hydroxy-1,3-benzodioxole. Alternatively, it has been proposed that the carbene may arise from an oxygenated cytochrome P-450–substrate complex rather than by α-elimination of water from I [12]. The present data do not allow these possibilities to be distinguished. Finally, the carbene may undergo hydrolysis to yield carbon monoxide and catechol (VI, Fig. 1).

The possible relationship of the metabolic reactions shown in Fig. 1 to the well-established ability of 1,3-benzodioxoles to inhibit cytochrome P-450-catalyzed monooxygenase activity is of considerable interest. Metabolism is required for inhibition [3, 11, 27], and it is generally accepted that this involves the formation of an active metabolite, possibly a carbene [12], that forms a stable complex with ferrocytochrome P-450 through the sixth axial ligand (V, Fig. 1). The complex can be observed as the so-called type III optical difference spectrum with dual Soret region peaks at 455 and 427 nm [3, 27]. Several interesting observations have been made that suggest a close relationship between the formation of the 1,3-benzodioxole metabolite–cytochrome P-450 complex and the generation of carbon monoxide.

The ability of 1,3-benzodioxoles to form type III spectral complexes with cytochrome P-450 and, as a result, to inhibit monooxygenase activity both *in vitro* and *in vivo* [1] is decreased on substitution of the methylenic carbon atoms with deuterium and is almost absent in 2-methyl and 2,2-dimethyl-substituted derivatives [3, 28, *]. On the other hand, 2-ethoxy-1,3-benzodioxole, which presumably can undergo hydrolysis to the 2-hydroxy-intermediate, forms a spectral complex with cytochrome P-450 [29], thus supporting the view that the 2-hydroxy derivative might be a common intermediate in the metabolic pathway leading to both inhibitory complex formation and carbon monoxide production.

This suggestion is further supported by the finding that there appears to be an inverse relationship between formation of the type III complex and production of carbon monoxide [9]; 1,3-benzodioxoles with electron-donating substituents in the aromatic ring lead mainly to complex formation and generate little carbon monoxide, and those with electron withdrawing substituents produce mainly carbon monoxide and show little complex formation. This suggests that electron donating substituents favor

* E. Hodgson, personal communication.

complex formation with cytochrome P-450, either through formation of a carbene intermediate from the 2-hydroxy-1,3-benzodioxole, or directly through a concerted reaction mechanism occurring at the active center of the cytochrome. A recent report indicates that the isosafrole metabolite–ferrocytochrome P-450$_{LM4}$ complex decomposes spontaneously to carbon monoxide [30], although it is not known whether this occurs through hydrolysis to the 2-hydroxy intermediate, as suggested in Fig. 1, or by another mechanism.

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