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Branchpoint for Heme Alkylation and Metabolite Formation in the Oxidation of Arylacetylenes by Cytochrome P-450*

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Phenylacetylene and biphenylacetylene are oxidized by cytochrome P-450 to the corresponding arylacetic acids. The acetylenic hydrogen shifts to the adjacent carbon and one atom of molecular oxygen is incorporated into the carboxylic acid group in these transformations, which are subject to a large kinetic isotope effect when the acetylenic hydrogen is replaced by deuterium. The same products and isotope effects are observed when the two arylacetylenes are oxidized by *m*-chloroperbenzoic acid rather than by the enzyme. In contrast, the inactivation of cytochrome P-450 that occurs during the oxidation of phenylacetylene is insensitive to deuterium substitution. The partition ratio between metabolite formation and enzyme inactivation consequently changes from 26 to 15 in going from phenylacetylene to the deuterated analogue. Metabolite formation therefore diverges from heme alkylation very early in the catalytic process.

The epoxidation of olefins by cytochrome P-450 could involve simultaneous formation of bonds between the activated oxygen and the two carbons of the π-bond, or formation of the two bonds in discrete steps separated by an ionic or radical intermediate. Retention of the olefin stereochemistry in the epoxides favors a concerted epoxidation mechanism (1–4), but the fact that the prosthetic heme of cytochrome P-450 is alkylated during the oxidation of terminal olefins favors a nonconcerted mechanism. Heme[1] alkylation, which also occurs with retention of the olefin stereochemistry (4), is observed when the activated oxygen is transferred to the internal carbon of the π-bonds (5–9). Additional support for a nonconcerted mechanism is provided by the reports that 1,2-chlorine migration precedes rather than follows epoxide formation from halogenated olefins (10, 11), that aldehydes and ketones are formed as trace metabolites from certain olefins in a process that does not involve the epoxide (11, 12), and that the oxidation of styrene is subject to a secondary isotope effect when deuterium is located on the internal but not the terminal carbon of the π-bond (13).

The cytochrome P-450-catalyzed oxidation of a carbon-carbon triple bond, a reaction formally related to olefin epoxidation, has only been unambiguously demonstrated in the metabolism of biphenylacetylenes (14–19) but is implied by the excretion of phenylacetic acid when animals are treated with phenylacetylene (20) and by rearrangements observed in the metabolism of 17-ethynyl sterols (9). The catalytic oxidation of biphenylacetylene yields biphenylacetic acid in a reaction subject to a kinetic isotope effect of 1.4 when the acetylenic hydrogen is replaced by deuterium (19). The possibility that oxygen is inserted into the acetylenic carbon-hydrogen bond, suggested by the isotope effect, is ruled out by quantitative shift of the acetylenic hydrogen to the vicinal carbon during the oxidation (17–19) and by the observation of a similar intramolecular hydrogen shift in the chemical oxidation of biphenylacetylene (17, 18). The enzymatic oxidation of acetylenes, like that of olefins, thus involves reaction of the activated oxygen with the π-bond. In agreement with this, the turnover of acetylenes by cytochrome P-450 results in alkylation of the prosthetic heme group (21–23) and the formation of heme adducts similar to those obtained with terminal olefins (7, 24, 25). We describe here a mechanistic investigation of the oxidation of π-bonds by cytochrome P-450 that exploits the hydrogen shift associated with triple bond oxidation as an experimental probe.

MATERIALS AND METHODS[2]

RESULTS

Incorporation of Oxygen into the Biphenylacetylene Metabolite—Biphenylacetylene was incubated with hepatic microsomes from phenobarbital-pretreated rats under an $^{18}O_2$ atmosphere, and the resulting biphenylacetic acid was isolated and methylated with diazomethane. Mass spectrometric analysis (Fig. 1) of the esterified metabolite established that approximately 75% of one oxygen in the carboxylic acid moiety derived from labeled molecular oxygen. This fractional incorporation of label points to catalytic incorporation of one atom of molecular oxygen because some dilution of the label by oxygen not removed in the purging operations is unavoidable. The second oxygen in the carboxyl group, by implication, derives from the medium.

Kinetic Isotope Effect in the Metabolism of Biphenylacetylene—The kinetic isotope effect for the oxidation of biphenylacetylene was remeasured to confirm the value reported earlier (k_H/k_D = 1.42) (19). Labeled and unlabeled biphen-

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[1] Heme is used in this paper for iron protoporphyrin IX regardless of the oxidation state of the iron or the porphyrin.

[2] Portions of this paper (including "Materials and Methods," Tables 1 and 2, and Figs. 1, 3, 4, and 6) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-2538, cite the authors, and include a check or money order for $4.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

ylacetylene were incubated with hepatic microsomes from phenobarbital-induced rats and aliquots were taken from the incubation mixtures at 10-min intervals. Biphenylacetylene and biphenylacetic acid were extracted into methylene chloride, and their concentrations were determined spectroscopically. This spectroscopic assay is feasible because the absorbance maximum of biphenylacetylene is at 273 nm whereas that of biphenylacetic acid is at 254 nm, although direct spectroscopic analysis of the incubation mixture is not possible because an interfering chromophore is present in the assay mixture that is eliminated in the extraction procedure. Metabolite formation is limited to the first 10 min of the incubation because the same amount of metabolite is present at 30 as at 10 min. The isotope effect calculated from the data is $k_H/k_D = 1.38$ (Table 1), a value within experimental error of that obtained earlier by a gas chromatographic assay (19).

Isotope Effects on the Destruction of Cytochrome P-450 by Arylacetylenes—Biphenylacetylene causes NADPH- and time-dependent loss of cytochrome P-450 when incubated with hepatic microsomes from phenobarbital-pretreated rats (Table 2). The enzyme loss determined by spectroscopic quantitation of the ferrous-carbon monoxide complex, however, does not exceed 4–5% of the total microsomal enzyme. This loss occurs within the first 10 min and is not increased if the incubation is prolonged a further 20 min. Essentially identical results are obtained when the incubations are carried out with [1-^{2}H]biphenylacetylene (Table 2). The quantitative reliability of these values, however, is compromised by their small magnitude and by the fact that they reflect a 4% correction for the enzyme lost in the absence of substrates.

The completion of both metabolite formation and enzyme inactivation within the same 10 min period suggests that one isozyme, representing no more than 4–5% of the cytochrome P-450 in microsomes from phenobarbital-induced rats, is involved in both processes. This result, unexpected in view of the report that phenobarbital and 3-methylcholanthrene stimulate biphenylacetylene metabolism (14), led us to investigate whether the cytochrome P-450 loss could be amplified by pretreatment with clofibrate, an inducer of cytochrome P-450 enzymes involved in fatty acid hydroxylation (27, 28), or Arochlor 1254, an inducer of multiple isozymes (29, 30). However, microsomal enzyme loss from Arochlor 1254-treated rats is the same as that from phenobarbital-treated rats while enzyme loss from clofibrate-treated rats is negligible (Table 2). These results suggest that biphenylacetylene is a specific substrate for a minor, phenobarbital-inducible, isozyme.

Phenylacetylene was found earlier to cause time- and NADPH dependent loss of cytochrome P-450 when incubated with hepatic microsomes from phenobarbital-pretreated rats (22). Losses of 18, 25, and 27% were observed after 10, 20, and 30 min of incubation with 10 mM phenylacetylene. A hepatic pigment with the absorption spectrum of an *N*-alkylprotoporphyrin IX derivative was furthermore isolated from the livers of rats injected with phenylacetylene (22). The larger amplitude of the cytochrome P-450 loss and the evidence that phenylacetylene alkylates the prosthetic heme of cytochrome P-450 led us to use it as an alternative probe of the oxidative mechanism. The rates of inactivation of cytochrome P-450 by phenylacetylene and [1-^{2}H]phenylacetylene appear, within experimental error, to be identical (Fig. 2).

The Metabolism of Phenylacetylene—No information is available on the metabolism of phenylacetylene except for the report that phenylacetic acid is excreted by rabbits injected with phenylacetylene (20). Phenylacetylene therefore was incubated with hepatic microsomes from phenobarbital-pretreated rats and the metabolites were isolated by extraction

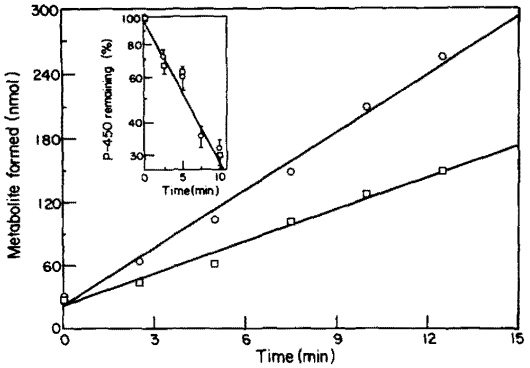


FIG. 2. **The metabolism of phenylacetylene to phenylacetate (○) and [1-^{2}H]phenylacetylene to [2-^{2}H]phenylacetate (□) by hepatic microsomes from phenobarbital-induced rats.** The loss of cytochrome P-450 caused by each of these two compounds (*symbols* as in the main panel) as a semilog function of time, assuming that the 30% maximum loss of cytochrome P-450 observed in long term incubations is equal to 100% of the vulnerable enzyme, is given in the *inset*.

into diethyl ether. The diethyl ether fraction, after esterification with diazomethane, was analyzed by gas chromatography. The only quantitatively significant (>5%) metabolite detected in the extracts is the methyl ester of phenylacetic acid (Fig. 3). A specific search was made for acetophenone, but none was detected. The metabolite was identified as phenylacetic acid by direct gas chromatographic and mass spectrometric comparison with an authentic sample (not shown).

The pattern of metabolites obtained with [1-^{2}H]phenylacetylene is the same as that obtained with the unlabeled substrate, but the molecular ion of the methyl phenylacetate metabolite is 1 mass unit higher (Fig. 4). The peak for the fragment obtained by decarboxylation of the molecular ion retains the difference of 1 mass unit. It is thus evident that the acetylenic hydrogen of phenylacetylene, like that of biphenylacetylene, shifts quantitatively to the vicinal carbon on oxidation of the triple bond.

Kinetic Isotope Effect in the Metabolism of Phenylacetylene—The formation of phenylacetic acid from phenylacetylene and [1-^{2}H]phenylacetylene in incubations with hepatic microsomes from phenobarbital-pretreated rats was quantitated as a function of time by gas chromatographic analysis (Fig. 2). Deuterium substitution significantly retards enzymatic oxidation of the triple bond and gives rise to a kinetic isotope effect $k_H/k_D = 1.80$ (Table 1).

The isotope effect for phenylacetylene metabolism was independently measured by incubating a 1:1 mixture of phenylacetylene and [1-^{2}H]phenylacetylene with hepatic microsomes and quantitating the ratio of the deuterated to undeuterated phenylacetic acid metabolites by gas chromatography-mass spectrometry. The kinetic isotope effect obtained by this internal competition method ($k_H/k_D = 1.60$) (Table 1) confirms that the oxidation of phenylacetylene by cytochrome P-450 is subject to a major isotope effect.

Isotope Effects on the Chemical Oxidation of Arylacetylenes—The oxidation of biphenylacetylene by *m*-chloroperbenzoic acid in methylene chloride with a trace of methanol yields methyl 2-biphenylacetate as the only detectable product. The ratio of the rates of reaction with biphenylacetylene and [1-^{2}H]biphenylacetylene reflects an isotope effect $k_H/k_D = 1.38$ (Table 1). Attempts to study the oxidation of phen-

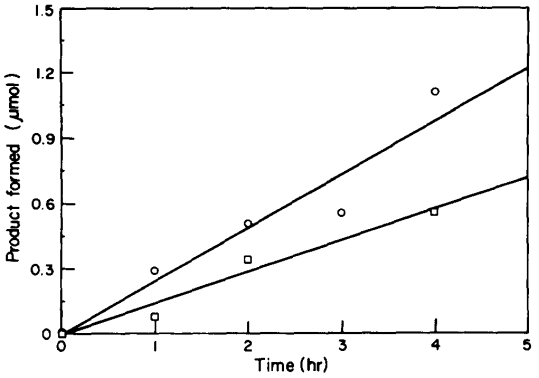
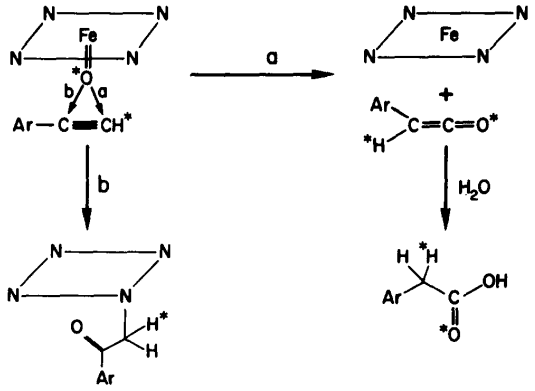


FIG. 5. **The oxidation of phenylacetylene to phenylacetate (○) and [1-^{2}H]phenylacetylene to [2-^{2}H]phenylacetate (□) by *m*-chloroperbenzoic acid in anhydrous benzene.**



SCHEME 1. The two consequences of cytochrome P-450-catalyzed arylacetylene oxidation. The *square of nitrogens* stands for protoporphyrin IX. The *stars* denote labeled atoms.

ylacetylene under the same conditions, however, were unsuccessful due to the low reactivity of this substrate. The oxidation of phenylacetylene was therefore examined in anhydrous benzene in the absence of proton sources as described in an earlier study (31). A kinetic isotope effect $k_H/k_D = 1.73$ is obtained from the rates of product formation from phenylacetylene and [1-^{2}H]phenylacetylene under these conditions (Fig. 5). Addition of methanol to the reaction mixture altered the kinetics and attenuated the isotope effect, but these changes were not investigated.

DISCUSSION

The oxidation of [1-^{2}H]phenylacetylene by both cytochrome P-450 and *m*-chloroperbenzoic acid yields phenylacetic acid with the acetylenic deuterium atom shifted to the benzylic carbon. The oxidation of phenylacetylene thus proceeds with a 1,2-hydrogen shift identical to that shown to occur previously in the oxidation of biphenylacetylene (17, 18). The oxidation of carbon-carbon triple bonds with concomitant shift of the acetylenic hydrogen thus appears to be a general, if frequently only minor, metabolic process.[3] The analogy between the enzymatic and chemical processes is strengthened by the incorporation of a labeled oxygen atom into the carboxylic acid function when biphenylacetylene is incubated under $^{18}O_2$ because this labeling pattern is that expected if biphenylacetylene is oxidized to biphenylketene which then reacts with a molecule of water. The chemical oxidation of acetylenes is believed to proceed through such a ketene mechanism (32).

The oxygen atom is bound to the terminal carbon in the arylacetylene metabolites but to the internal carbon in the heme adducts (Scheme 1) (7, 24, 25). The terminal carbon in the heme adducts is the site to which the protoporphyrin IX prosthetic group is bound. The reaction regiochemistry observed in the metabolites is favored by rate-determining oxygen addition to the π-bond because any electron deficiency that develops in the transition state can be stabilized by conjugation with the phenyl ring. The absence of acetophenone among the metabolites in the chemical and enzymatic reactions supports the view that metabolites stem from delivery of the oxygen to the terminal carbon. The fact that hydrogen migration is coupled to metabolite formation but not heme alkylation makes the observation of differential isotope effects highly informative. Olefins are not suitable for such studies because the regiochemistry of oxygen addition, which is masked in the epoxide products, can only be extracted by kinetic studies of secondary isotope effects. The isotope effects measured here for conversion of biphenylacetylene to biphenylacetic acid ($k_H/k_D = 1.38$) and phenylacetylene to 2-phenylacetic acid ($k_H/k_D = 1.80$), on the other hand, establish that enzymatic triple bond oxidation is subject to quite large *primary* isotope effects. The observed isotope effects approximate the maximum possible values for transition states in which the hydrogen moves in a sidewise rather than linear fashion (see Scheme 3) between the donor and acceptor atoms. Theoretical calculations suggest that the maximum isotope effect, barring tunneling effects, for a linear transition state (C—H—C angle = 180°) is approximately $k_H/k_D = 7.9$ whereas the corresponding values for angles of 120°, 90°, and 60° are 3.0, 1.7, and 0.9, respectively (33). Experimental isotope effects for reactions known to involve bent transition states conform to these theoretical predictions (*e.g.* pinacol rearrangement ($k_H/k_D = 2.7$–3.3) (34), amine oxide pyrolysis ($k_H/k_D = 2.7$–3.2) (35), insertion of carbenes into C—H bonds ($k_H/k_D = 0.9$–2.5) (36, 37), 1,2-shift of a hydrogen to a vicinal carbene center ($k_H/k_D = 1.7$) (38), and epoxide ring cleavage concerted with 1,2-shift of a hydrogen ($k_H/k_D = 1.59$) (39)). The secondary isotope effects on a reaction in which the carbons undergo *sp* to *sp*^{2} rehybridization are, on the contrary, expected to be inverse and of much smaller magnitude (40). The primary isotope effects observed in this study are therefore, if anything, slightly larger than the cited values if allowance is made for secondary isotope effects. Proportionately large intrinsic isotope effects have been demonstrated for cytochrome P-450-catalyzed carbon hydroxylations by experiments which measure the competition between deuterated and undeuterated sites in the same molecule (43, 44), but the present V_{max} isotope effects are among the largest (relative to the theoretical maximum) so far observed for cytochrome P-450.

Oxidation of the biphenylacetylene triple bond with *m*-chloroperbenzoic acid parallels the biological reaction in all respects including, as shown here, the magnitude of the kinetic isotope effect associated with replacement of the acetylenic hydrogen by deuterium. The isotope effect for the chemical oxidation of biphenylacetylene ($k_H/k_D = 1.82$) is larger than

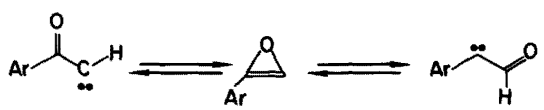
[3] Enzymatic oxidation of the triple bond in an *alkyl* acetylene (4-phenylbutyne) with concomitant 1,2-shift of the hydrogen has been demonstrated (P. R. Ortiz de Montellano, C. R. Wheeler, and E. Komives, unpublished results).

that for the enzymatic oxidation (k_H/k_D = 1.38) but is comparable to that for the enzymatic (k_H/k_D = 1.80) and chemical (k_H/k_D = 1.73) oxidations of phenylacetylene. The isotope effect in the chemical reaction requires that oxygen transfer from the peracid to the triple bond occur in concert with hydrogen migration. The alternative explanation, formation of a quasi-stable oxirene intermediate that decomposes to the ketene product in a second, rate-limiting, step, is incompatible with the failure to detect (much less isolate) oxirene intermediates even in frozen matrixes at cryogenic temperatures (32, 43, 44). The isotope effect data essentially rule out the formation of oxirenes in the perbenzoic acid reaction and consequently resolve the long standing question of whether oxirenes are formed in the chemical oxidation (32). The data substantiate the theoretically predicted asymmetry of the transition state for the oxidation of acetylene by peroxyformic acid (45). The parallel reaction course and isotope effects for the chemical and enzymatic reactions strongly argue, in turn, that oxirenes are also not intermediates in the enzymatic oxidation of acetylenes. Oxirene intermediates would, if anything, be destabilized by the interactions available within the cytochrome P-450 active site (*e.g.* metal coordination, hydrogen bonding) and would therefore be even less likely to accumulate prior to a rate-limiting step coupled to hydrogen migration.

The strong analogy between the enzymatic and *m*-chloroperbenzoic acid reactions contrasts with the reaction of acetylenes with hydroxyl radicals. Hydroxyl radicals readily add to terminal acetylenes, including acetylene, propargyl alcohol, and 3-hydroxy-3-methyl-1-butyne, but the corresponding acids are not obtained and products compatible with hydrogen migration are not observed (46). Products analogous to those obtained in the enzymatic reaction are not obtained even if Cu^{2+} or Fe^{+3} is added to the Fenton system. The Fenton oxidation of phenylacetylene differs in that it gives a trace of phenylacetic acid (Fig. 6), but is generally consistent with the earlier work in that phenylacetic acid is but a very minor component of a complex mixture of products. Clearly, the two-electron oxidation of acetylenes mediated by *m*-chloroperbenzoic acid, which exclusively yields the product obtained in the enzymatic reaction, better models cytochrome P-450 catalysis than the corresponding reaction with hydroxyl radicals.

The sharp contrast between the large isotope effects on metabolite formation and the absence of a detectable isotope effect on cytochrome P-450 destruction requires the oxidative trajectories that result in metabolite formation and enzyme inactivation to diverge prior to the point where the hydrogen shifts in the former pathway. This conclusion specifically rules out destruction of the enzyme by the ketene or any other species subsequent to migration of the hydrogen. The differential isotope effects are consistent with the fact that the oxygen finishes on the terminal carbon in the metabolite and the internal carbon in the heme adduct (7, 24, 25).

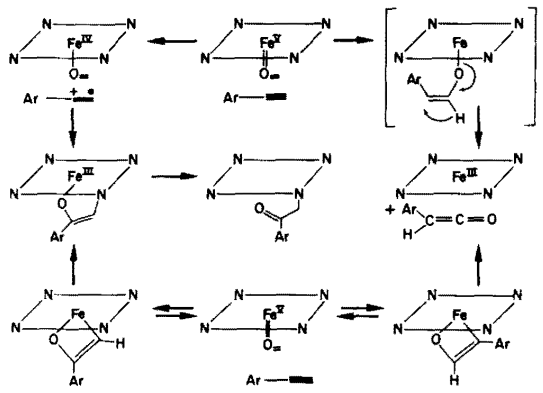
The oxidation of asymmetrically substituted acetylenes can, in principle, yield two α-ketocarbenes that are formally related to a common oxirene (Scheme 2) (32). If the α-ketocarbenes are not interconvertible *via* the oxirene, one α-ketocarbene isomer could give rise to the ketene metabolite and the other could alkylate the prosthetic heme. The α-ketocarbenes, however, can only be involved in the enzymatic reaction if α-ketocarbene formation is followed by a rate-determining hydrogen shift in the pathway to metabolites. The required formation of a relatively stable carbene intermediate is feasible in view of the chemical synthesis of stable carbene-iron complexes (47–50) and of the fact that such structures have been postulated for the stable complexes formed in the reactions of cytochrome P-450 with methylenedioxyphenyl compounds and halocarbons (51–53). Carbene complexes are readily detected in microsomal incubations because they have a characteristic absorption in the 440–490 nm range. We have recently demonstrated that fairly stable complexes with an absorption maximum at 445 nm are formed in the reactions of cytochrome P-450 with ethyl diazoacetate and diazoacetophenone.[4] The carbene from diazoacetophenone is identical to that expected if a carbene intermediate is generated by oxygen addition to the internal carbon of phenylacetylene. The microsomal oxidation of phenylacetylene, however, is not accompanied by the detectable formation of a species that absorbs in the 445 nm region.[5] The absence of such a chromophore, in view of the fact that the chromophore is readily detected when the expected carbene is generated by an alternative procedure (from the diazoketone), makes the intervention of α-ketocarbene intermediates unlikely.



SCHEME 2. The theoretical relationship between the two carbenes and the oxirene that could result from oxidation of an arylacetylene.

The mechanism for enzymatic carbon-carbon triple bond oxidation must adhere to the following constraints: (*a*) concurrent ketene formation and heme alkylation, (*b*) quantitative shift of the acetylenic hydrogen in the ketene pathway, (*c*) large primary isotope effects on metabolite formation but not heme alkylation, (*d*) incorporation of one atom of molecular oxygen into the metabolite, (*e*) location of the oxygen on the terminal carbon in metabolites but on the internal carbon in heme adducts, and (*f*) strong parallels in the reactions of acetylenes with cytochrome P-450 and peracids but not hydroxyl radicals. These results require heme alkylation and metabolite formation to diverge prior to (or during) transfer of the oxygen to the terminal carbon for metabolite formation and the internal carbon for heme alkylation. The preference for oxygen transfer to the terminal carbon is shown by the absence of products from oxygen addition to the internal carbon in the analogous oxidation by *m*-chloroperbenzoic acid. The substrate could, however, be bound a fraction of the time in an orientation that forces oxygen transfer to the internal carbon. The relatively loose binding of substrates by cytochrome P-450 required for the observation of high intramolecular isotope effects (41, 42) suggests that a more complicated mechanism may be involved. One alternative is for enzymatic electron transfer from the π-bond to precede carbon-oxygen bond formation in the heme alkylation pathway (Scheme 3, *upper mechanism*). The partitioning of substrates between metabolism and heme alkylation would be determined by the ratio of electron transfer to direct oxygen addition. A second alternative is for the iron-oxo complex to add to the phenylacetylene π-bond to form the two possible metallooxocyclobutene isomers (Scheme 3, *lower mechanism*). Rearrangement of the isomer with the phenyl vicinal to the iron would result in ketene formation whereas internal ligand transfer in the isomer with the phenyl vicinal to the oxygen would result in heme alkylation. The isotope effect on metabolite formation requires, however, reversible formation of the

[4] P. R. Ortiz de Montellano and E. A. Komives, unpublished results.
[5] A search for long wavelength absorption in incubations of phenylacetylene with liver microsomes from phenobarbital-induced rats has been fruitless.



SCHEME 3. Two mechanisms for triple bond oxidation consistent with the available information. In the *upper half* of the scheme is a mechanism in which heme alkylation results from initial electron transfer and metabolite formation from direct oxygen transfer. In the *bottom half* is a mechanism involving the reversible formation of isomeric metallooxocyclobutenes.

metallooxocyclobutene isomers followed by rate-determining hydrogen migration in the pathway to metabolites. Metallooxocyclobutane intermediates, first proposed by Sharpless *et al.* (54) to explain the oxidation of olefins by chromyl chloride, have been observed by NMR in the reaction of osmium tetroxide with 1,1-diphenylethylene (55) and have been invoked by Collman *et al.* (56) to explain olefin oxidation by an oxo-manganese complex. No precedent is yet available, however, for the formation of metallooxocyclobutene intermediates with iron porphyrins or from acetylenes. The factors that control the regiochemistry of oxygen addition thus remain to be defined.

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