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INTRAMOLECULAR ISOTOPE EFFECTS ASSOCIATED WITH META-HYDROXYLATION OF BIPHENYL CATALYZED BY CYTOCHROME P-450

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SUMMARY: Intramolecular isotope effects and the degree of deuterium retention were determined for the meta-hydroxylation of biphenyl as catalyzed by microsomal cytochrome P-450 obtained from rats pretreated with phenobarbital. The percent deuterium retention after meta-hydroxylation of 3,5,3',5'-[$^2H_4$]-biphenyl was found to be 77.3% ± 1.9. The intramolecular isotope effects associated with 3,3'-[$^2H_2$]-biphenyl and 3,5-[$^2H_2$]-biphenyl were found to be 0.90 ± .05 and 1.05 ± .06, respectively. These data demonstrate conclusively that a direct insertion or abstraction mechanism is not operable in the meta-hydroxylation of biphenyl and suggest the possibility of an addition-rearrangement mechanism as opposed to initial and direct arene oxide formation.

Introduction: The intermediacy of arene oxides in the oxidation of aromatic substrates to phenols by cytochrome P-450 is well established (1). Because of their electrophilic nature their involvement in various chemical toxicities such as mutagenisis and carcinogenisis via covalent binding to critical biomacromolecules is equally well accepted (2).

The evidence supporting the involvement of arene oxides in aromatic hydroxylation is compelling. The oxides of certain substrates, e.g., naphthalene, have actually been isolated from microsomal preparations. They have been trapped by nucleophilic reagents such as glutathione to yield addition products and isolated as dihydrodiols (3). Moreover, when deuterium is substituted for protium at the site of hydroxylation, the lack of a significant isotope effect (4) and the observation of the NIH shift (5) are taken as strong evidence in support of the arene oxide mechanism.

These criteria have been met in most of the systems studied. However, the question as to whether or not arene oxides are the first and obligatory intermediates in such reactions is unknown as are the substrate structural parameters

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contributing to their formation. The first inconsistancies in the proposed arene oxide mechanism were noted by Jerina's laboratory while investigating the cytochrome P-450 mediated hydroxylation of chlorobenzene (6). These investigators found that the relative ratios of the ortho, meta and para chlorophenols that were obtained as products varied significantly as a function of the specific enzyme preparation used. The results clearly indicated that multiple isozymes were involved in the oxidative reactions. Moreover, significant quantities of m-chlorophenol were obtained particularly from whole liver preparations. The dilemma this observation presented was that due to the fact that synthetic epoxides such as 3,4 or 2,3 oxo-chlorobenzene were known to open exclusively to form either the para or ortho phenol respectively (7). Because of these facts, the authors concluded that meta-hydroxylation in contrast to either ortho or para arises via direct formation of phenol. This conclusion was further strengthened by the finding of significant isotope effects associated with the meta-hydroxylation of nitrobenzene and methyl phenyl sulfone (8). Of the four proposed pathways leading to phenol formation the authors felt that the direct insertion pathway, i.e., insertion of the equivalent of a singlet oxygen atom into a carbon hydrogen bond best fit the data. The abstraction-recombination pathway in which a triplet like oxygen abstracts a hydrogen atom and recombines was excluded, on energetic grounds; while the direct epoxidation (arene oxide) pathway was excluded on the basis of the observed isotope effects and the known regioselectivity of oxirane ring opening. Finally, the addition-rearrangement pathway in which a triplet oxygen atom adds to form a tetrahedral intermediate which then can rearrange to phenol in 3 ways, was excluded since the initial addition of oxene would have to be reversible in order to observe an isotope effect. Such reversibility was thought to be unrealistic.

If meta-hydroxylation proceeds by direct insertion and if deuterium is substituted for a proton at the site of hydroxylation no deuterium will be found in the product because of fascile exchange of the OD that is produced with the media. Conversely, if an epoxide or addition-rearrangement mechanism is operative the NIH shift would be expected with a concommittant high degree of

deuterium retention in the product. Unfortunately, the experimental design utilized in the chlorobenzene, nitrobenzene and methyl phenyl sulfone studies precluded obtaining the necessary data.

To investigate this problem we studied the 7-hydroxylation of selectively deuterated 7-^{2}H-warfarin by microsomes from phenobarbital pre-treated rats (9). The 7 position in warfarin corresponds to the meta position in substituted benzenord systems and it is the major site of biotransformation in both man and the rat. The study indicated that the 7-hydroxylation of 7-^{2}H-warfarin proceeded with greater than 75% deuterium retention in the product, a result clearly inconsistent with a direct insertion mechanism.

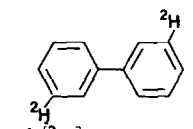
To clarify potential isotope effects associated with meta-hydroxylation we examined the microsomal hydroxylation of selected deuterated biphenyls. McMahon and Billings had reported that the meta-hydroxylation of biphenyl was associated with a primary isotope effect of 1.29 (10). Thus, to hopefully maximize the effect and obtain unambiguous results the deuterated substrates were chosen so as to take advantage of the bond breaking isolation characteristics of experiments utilizing the intramolecular isotope effect design.

Materials and Methods: Reagents and chemicals were of the highest commercial grade available . NADP+(monosodium salt), D-glucose-6-phosphate, D-glucose-6-phosphate dehydrogenase, and bovine serum albumen were purchased from Sigma Biochemicals. PFPA was obtained from Pierce Chemical Company and sodium phenobarbital from the Eli Lilly Company.

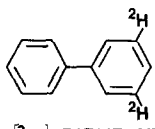
Synthesis: 3,3'-Dibromobiphenyl was prepared according to the method of Synder, Weaver, and Marshall (11), while 3,5-dibromobiphenyl was prepared by the method of Scarborough and Waters (12). 3,5,3',5'-[$^{2}H_4$]-biphenyl was synthesized according to the method of Akawie et. al. (13). 3,3'-[$^{2}H_2$]-Biphenyl and 3,5-[$^{2}H_2$]-biphenyl were synthesized from the corresponding bromobiphenyls by reduction with $NaBD_4$ over 10% Pd/charcoal, the details of these procedures will be published elsewhere. The percent deuterium incorporation was 91.16% d_4 for 3,5,3',5'-[$^{2}H_4$]-biphenyl, 87.01% d_2 for the 3,5-[$^{2}H_2$]-biphenyl and 92.95% d_2 for the 3,3'-[$^{2}H_2$]-biphenyl as determined on a VG 7070 mass spectrometer at low electron voltage. The exact method will be published elsewhere.

Metabolite Analyses: Liver microsomes were prepared from phenobarbital induced male Sprague-Dawley rats (160-190 gm) were as previously reported (14). Protein concentrations were determined according to the modified Lowry method (15). P-450 concentrations were measured according to the method of Omura and Sato (16). Incubations, 5 ml, 30 min, with 1 mM substrate in 25 µl of methanol were carried out as previously reported (9). Incubations were terminated with 5 ml of pentane, the pentane was separated, combined with a second 5 ml pentane extraction, dryed over $NaSO_4$, and evaporated to dryness. The pentafluoropropionic esters of the hydroxybiphenyls were prepared by reaction with pentafluoropropionyl anhydride in pyridine and ethyl acetate. Pyridine was necessary in order to neutralize the pentafluroproprionic acid generated in the reaction in order to prevent loss of deuterium by exchange. GCMS analysis was performed on a VG 7070 mass spectrometer in the selected ion mode interfaced to a HP-5710A

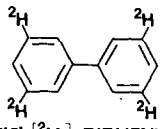
GC. A J&W DB-1 thick film narrow bore fused silica capillary column was used to separate the pentafluoropropionic esters of the hydroxybiphenyls. The derivatized metabolites were cold trapped on the column at 40°C and eluted at 1°C/min with ballistic ramping to 190°C.



3,3'-[$^2H_2$]-BIPHENYL



3,5-[$^2H_2$]-BIPHENYL



3,5,3',5'-[$^2H_4$]-BIPHENYL

Results and Discussion: The intrinsic isotope effect associated with C-H(D) bond cleavage is rarely observed in enzymatically mediated reactions because steps other than the catalytic step can enter the rate equation in such a way as to reduce the observed isotope effect. Thus, an intermolecular (sequential use of proteo vs deutero substrates) isotope experiment is unlikely to give a true measure of the isotope effect (17). Hjelmeland and others (18) have demonstrated that a much closer approximation to the intrinsic isotope effect can be obtained by employing the strategy of intramolecular isotope experiments, which isolate the rate sensitive steps after initial substrate binding and before the first irreversible step. In such experiments the substrate is designed to have two chemically equivalent sites within the molecule, one of which is substituted with deuterium. Provided equilibration between the two equivalent sites on the enzyme surface is rapid with respect to subsequent reactions a truer measure of the magnitude of the isotope effect prior to the first irreversible step can be obtained by product analysis.

A survey of Table 1 reveals that 77.3 ± 0.19 of the deuterium is retained after meta-hydroxylation of 3,5,3',5'-[$^2H_4$]-biphenyl. Clearly this result eliminates either the direct insertion or abstraction pathways as viable mechanisms. Thus either direct epoxidation or addition rearrangement are the probable routes of formation of the meta phenol of biphenyl.

Since direct epoxidation as a first irreversible step in the reaction cannot be associated with a positive isotope effect the observation of such an effect would be compelling evidence for the addition-rearrangement mechanism (Figure 1). In contrast to the results of McMahon and Billings, we find no isotope effect associated with the meta-hydroxylation of 3,5-[$^2H_2$]-biphenyl

TABLE 1 Isotope Effects and Deuterium Retention for Meta-hydroxylation of Biphenyl Catalyzed by Cytochrome P-450 in Microsomes obtained from Phenobarbital Pretreated Rats

Compound	K_H/K_D	% retention
3,5,3',5'-[$^2H_4$]-biphenyl[a]		77.3 ± .019
3,3'-[$^2H_2$]-biphenyl[b]	0.896 ± 0.049	
3,5-[$^2H_2$]-biphenyl[c]	1.052 ± 0.620	
Biphenyl-d_{10}[d]	1.290 ± 0.009	

[a] n = 12, % retention determined by r = (1/i) (% d_4) where r equals % retention, i equals % d_4 in substrate and % d_4 is the % d_4 in product.

[b] n = 16, K_H/K_D was determined by $K_H/K_D = \frac{d_2}{\left(\frac{r}{1-r}+1\right)\left(\frac{i[d_2+d_1+d_o]-d_2}{}\right)} - r$ where r is the precent of deuterium retained after migration, i is the percent deuterium in substrate, d_2 the amount of d_2-hydroxybiphenyl after correction for natural isotopic abundance, d_1 the amount of d_1-hydroxybiphenyl and d_o the amount of d_o-hydroxybiphenyl. The derivation will be published elsewhere.

[c] n = 13, K_H/K_D determined as in b.

[d] see ref 10.

(1.052 ± 0.62) and an apparent inverse isotope effect associated with the meta-hydroxylation of 3,3'-[$^2H_2$]-biphenyl (.896 ± 0.49).

Unfortunately, these results do not allow an unambiguous assignment of either the direct epoxidation or the addition rearrangement mechanism. However, the directional opening of the synthetic 2,3 and 3,4 chlorobenzeneoxides (ortho and para respectively) coupled with the fact that 7 hydroxylation is the major

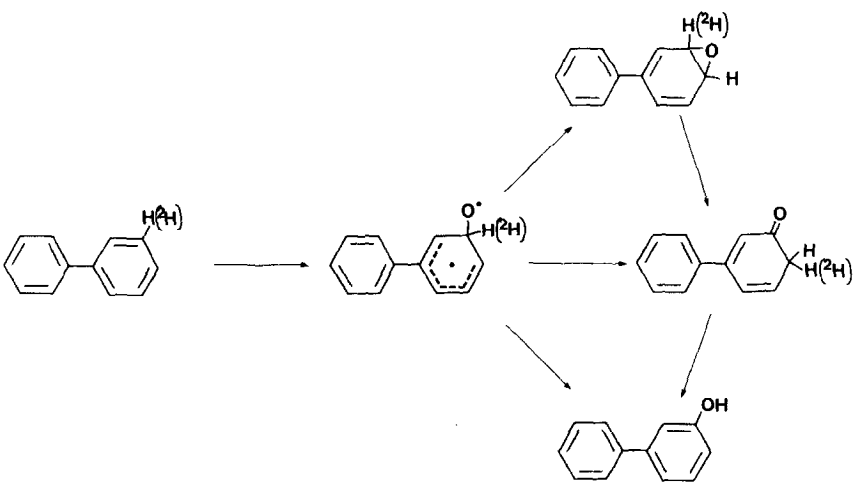


Figure 1: Addition-rearrangement mechanism.

route of the P-450 mediated metabolism of warfarin, argue strongly in favor of the later. If true, this would mean that transfer of oxygen to substrate in the first step of the reaction is irreversible or that no significant isotope effect is associated with subsequent events and that the subsequent events (which of the three possible routes of rearrangement occur) are probably largely dictated by substrate structure.

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References:

1. Jerina, D. M., Kaubisch, N. and Daly, J. W., (1971) PNAS 68,2545-2548; Kaubisch, N., Daly, J. W., and Jerina, D. M., (1972) Biochem., 11, 3080-3088.
2. Jerina, D. M. and Daly, J. W., (1974) Science 185, 573-582; Daly, J. W., Jerina, D. M., and Witkop, B., (1972) Experentia 28, 1129-1150.
3. Jerina, D. M., Daly, J. W., Witkop, B., Zaltzman-Nirenberg, D., and Udenfriend, S., (1968) JACS 90, 6525-6527; (1970) Biochemistry 9, 147-155.
4. Kasperek, G. J., Bruice, T. C., Yagi, H., and Jerina, D. M., (1972) J. Chem. Soc. D., 784-785.
5. Guroff, G., Daly, J. W., Jerina, D. M., Renson, J., Witkop, B., and Udenfriend, S., (1967) Science 157, 1524-1530.
6. Selander, H. G., Jerina, D. M., and Daly, J. W., (1975) Arch. Biochem. Biophys. 168, 309-321.
7. Selander, H. G., Jerina, D. M., Piccolo, D. E., and Berchtold, G., (1975) J. Amer. Chem. Soc. 97, 4428-4430.
8. Tamaszewski, J. E., Jerina, D. M., and Daly, J. W., (1975) Biochemistry 14, 2024-2031.
9. Bush, E. D., and Trager, W. F. (1982) Biochem. Biophys. Res. Comm. 104, 626-632.
10. Billings, R. E., and McMahon, R. E., (1978) Mol. Pharm. 14, 145-154.
11. Snyder, H. R., Weaver, C., and Marshall, C. D., (1949) J. Amer. Chem. Soc. 71, 289-291.
12. Scarborough, H. A., and Waters, W. A., (1926) J. Chem. Soc., 557-562.
13. Akawie, R. I., Scarborough, J. M., and Burr, J. G., (1959) J. Org. Chem. 24, 946-949.
14. Porter, W. R., Wheeler, C., and Trager, W. F., (1981) Biochem. Pharm. 30, 3099-3104.
15. Miller, G. L., (1959) Anal. Chem. 31, 964.
16. Omura, T., and Sato, R., (1964) J. Biol. Chem. 239, 2370-2378.
17. Northrup, D. B., (1971) in "Isotope Effects on Enzymes Catalyzed Reactions," Cleland W. W., O'Leary, M. H., and Northrup, D. B., eds. University Park Press, Baltimore, p. 122-152.
18. Foster, A. B., Jarman, M., Stevens, J. D., Thomas, P., and Westwood, J. H., (1974) Chem-Biol. Inter. 9, 327-340; Hjelmeland, L. M., Aronow, L., and Trudell, J. R., (1977) Biochem. Biophys. Res. Comm. 76, 541-549; Miwa, G. T., Garland, W. A. Hodshom, B. J., Lu, A. Y. H., and Northrup, D. B., (1980) J. Biol. Chem. 255, 5049-6054.