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DEUTERIUM-ISOTOPE EFFECT IN THE BIOTRANSFORMATION OF 4-ETHYNYLBIPHENYLS TO 4-BIPHENYLYLACETIC ACIDS BY RAT HEPATIC MICROSOMES

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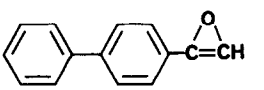
SUMMARY

In vitro studies using hepatic microsomal homogenate and 4-(1-deuteroethynyl) biphenyls have shown that there is an isotope effect in conversion of 4-ethynylbiphenyls to 4-biphenylylacetic acids. This effect is consistent with mechanisms involving either (1) direct hydroxylation of the ethynyl moiety or (2) oxirene formation followed by a rate-determining hydride shift to form the intermediate ketene.

INTRODUCTION

In 1979 we showed that 4-ethynyl-2'-fluorobiphenyl was converted in excellent yields both *in vivo* and *in vitro* to a 2'-fluoro-4-biphenylacetic acid (1). A speculative mechanism for this conversion involving direct hydroxylation via oxygen insertion of the acetylenic C-H bond to yield initially an ethynol intermediate was proposed (Figure 1). In the same year, Wade and associates (2) reported that 4-ethynylbiphenyl underwent the identical reaction and was readily converted both *in vivo* and *in vitro* to 4-biphenylylacetic acid. They proposed that the initial reaction intermediate had the oxirene configuration I which then rearranged to a ketene intermediate that upon subsequent addition of H_2O yielded the



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acid. Interest in acetylenic compounds in general has increased with the

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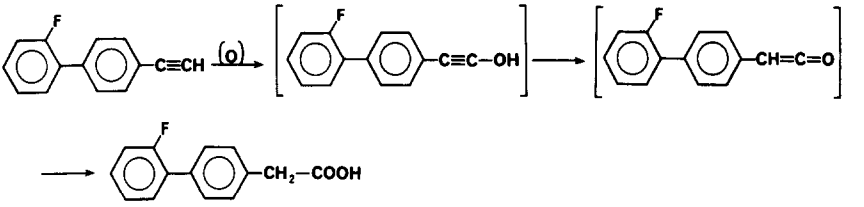


Figure 1. Proposed mechanism for the in vivo and in vitro conversion of 4-ethynyl-2'-fluorobiphenyl to 2'-fluoro-4-biphenylacetic acid.

discovery by White (3) that certain acetylenes interact with hepatic cytochrome P-450 to form a blue pigment much in the manner that alkyl compounds have been shown to react. Indeed, DeMatteis et al. (4) have demonstrated the direct reaction of an allylic double bond with the pyrrole nucleus of hemoglobin.

In order to further clarify which of the two postulated mechanisms, direct hydroxylation or oxirene formation, was responsible for the conversion of the arylacetylenes to arylacetic acids, two deuterium labeled 4-ethynylbiphenyls were synthesized and their in vitro conversion to the corresponding acetic acids was studied using the rat liver homogenate system.

Since the formation of oxirenes occurs without the breaking of a C-H bond, the reaction occurs without a deuterium isotope effect (5,6). Thus if the formation of an unstable epoxide intermediate is the rate-determining step in the conversion of 4-ethynyl-2'-fluorobiphenyl to 2'-fluorobiphenylacetic acid, the reaction would be anticipated to proceed without an isotope affect. Conversely, the mechanism we propose involving the hydroxylation of the acetylenic C-H bond to yield an acetylenic alcohol must produce an isotope effect since this C-H bond is broken. Indeed, microsomal hydroxylation reactions in general show isotope effects varying from approximately 1.3 to 2.0 (7). Results from in vitro studies using deuterium labeled 4-ethynylbiphenyls herein described give credence to this postulation.

MATERIALS AND METHODS

In Vitro Reaction. Reaction mixtures consisting of 3.9 ml 0.1 M phosphate, pH 7.4; 1.0 ml rat liver homogenate (250 mg of wet liver); 6.6×10^{-3}M $MgCl_2$; 2.8×10^{-3}M glucose-6-phosphate; 6.6×10^{-4}M $NADP^4$; 4×10^{-4}M 4-ethynylbiphenyl were incubated at 37° for 10 min. Following addition of 250 µl of 5N HCl, 100 µg of internal standard, 2'-fluoro-4-biphenylylacetic acid, was added and the mixture was extracted twice with 8 ml portion of ethyl acetate. When 2'-fluoro-4-ethynylbiphenyl was the substrate, the internal standard was 4-biphenylacetic acid. The combined ethyl acetate solution was concentrated to dryness under N_2 and the residual material derivatized by reaction with excess diazomethane. Samples were concentrated to dryness under N_2 and the residue dissolved in methanol for GC analysis.

Gas Chromatography Analysis. Analysis of concentrations of methyl esters of the biphenylylacetic acids were performed with a Hewlett-Packard Model 402B gas chromatograph equipped with a hydrogen flame ionization detector. A 4 ft glass column (I.D., 3.0 mm) packed with 3 percent OV-25 phenylmethyl silicon gum on Chromasorb G was heated isothermally at 215° while the detector and flask heater were maintained at 250°C for the analysis. Calculation of concentrations was performed using areas under the curve via an on-line computer for intergration and a standard curve.

Preparation of Deuterium-Labeled Substrates. Butyl lithium, 815 mg (12.80 mmol) in hexane (1.6M, Foote Chem. Co) was added dropwise to 20 ml of tetrahydrofuran in a 50 ml two-neck r.b. flask cooled to -20° in an isopropanol-dry ice mixture and under a stream of N_2. After complete addition, a solution containing 12.08 mmol of the appropriate 4-ethylnylbiphenyl in 5 ml of tetrahydrofuran was added dropwise over a period of 5 min. The reaction mixture was stirred for 1 hr at -20° whereupon 4.0 ml of D_2O (99.8 atom percent, Aldrich Chem.) was added dropwise. After stirring for 30 min, 10 ml of diethyl ether was added and stirring continued for 10 min. The organic phase was removed *in vacuo* to yield approximately 2.0 gm of the 4-(1-deuteroethynyl)biphenyl to be 84.9 percent isotopically pure while that of the 2'-fluoro-4-(1-deuteroethynyl) biphenyl was 86.6 percent. These analyses also revealed no deuterium substitution into the phenyl ring system of either compound.

Enzyme Preparation. Male Fisher rats, 180-220 g were obtained from Harlan Laboratories, Cumberland, IN. Livers were removed and homogenized in 3 vol. of 0.01 M phosphate buffer, pH 7.4 containing 1.15 KCL. Homogenates were centrifuged at 9,000 x g for 15 min in a Sorvall Model R-5C maintained at 10°. The 9000 x g supernate was stored at -90°C to await use.

RESULTS AND DISCUSSION

Initial experiments were designed to demonstrate that the deuterium atoms on the acetylenic carbons of these 4-ethynylbiphenyls would not exchange with H_2O under the pH 7.6 reaction conditions. This was accomplished in two ways. In the first experiment, unlabeled 4-ethynylbiphenyl was incubated for 1 hr under standard conditions in a

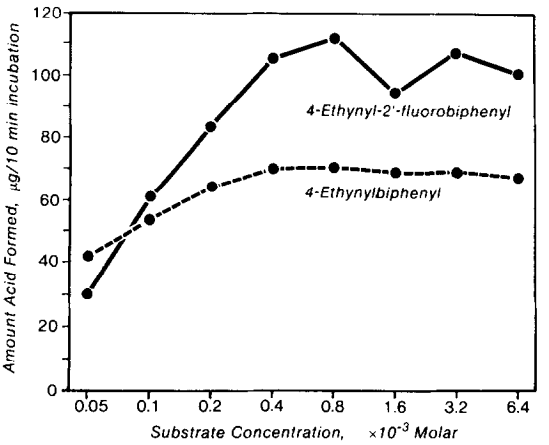


Figure 2. Effect of substrate concentrations on the in vitro conversion of two 4-ethynylbiphenyls to the corresponding 4-biphenylylacetic acids using rat liver homogenate.

medium in which the solvent was D_2O. Recovered 4-ethynylbiphenyl, isolated by extraction at pH 7.6 with ethyl acetate, was found to be devoid of deuterium. In a second experiment, 4-(1-deuteroethynyl)biphenyl was incubated for 1 hr under standard conditions. The unreacted substrate was recovered by extraction into ethyl acetate at pH 7.6 and was found not to have lost deuterium by exchange.

Subsequent in vitro substrate concentration studies using these 4-ethynylbiphenyls showed that the microsomal oxidative enzymatic activity was saturable and that maximum activity was attained at a 4×10^{-4}M substrate concentration (Figure 2). All incubations were carried out using this substrate concentration.

Since the deuterated 4-ethynylbiphenyls were not 100 percent isotopically pure, we resorted to the method we had developed earlier for the determination of isotope effects of compounds that contained less than 100 percent label (7). Thus, various mixtures of unlabeled and labeled substrate were incubated and the amount of the corresponding 4-biphenylyl acetic acid formed determined. The molar percent of deuterium in the substrate was plotted against the amount of the corresponding acid formed.

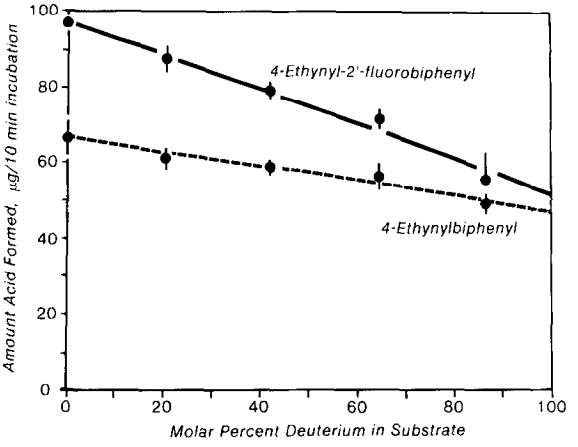
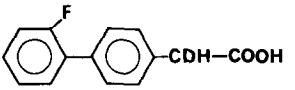


Figure 3. Deuterium isotope effect in the conversion of two 4-ethynylbiphenyls to their corresponding 4-biphenylylacetic acids.

Results obtained for both labeled 4-ethynylbiphenyls are shown in Figure 3. Using linear regression analysis, the k_H and k_D values of the two compounds were determined (7). Employing these k values, the resulting apparent isotope effect, k_H/K_D, in the conversion of 4-ethynyl-2'-fluorobiphenyl to its acetic acid was 1.95, while that of the conversion of 4-ethynylbiphenyl was 1.42. The fluorine substitution in the 2'-position obviously was responsible for the observed difference in this effect.

The observation that oxidation of these 4-ethynylbiphenyls occurs with an obvious isotope effect indicated that the C-H bond was broken in the rate-determining step, presumably by direct hydroxylation of the ethynyl moiety. If our initial assumption is correct, these results eliminate the oxirene configuration as a viable intermediate for this reaction. Mass spectrographic analysis of the product isolated from the reaction showed it to have the following structure, II. Although the extent of the deuterium



retention appeared to be almost quantitative, experiments designed to determine the absolute amount of retention in this rearrangement were not conducted. This retention of deuterium in the product, the arylacetic acid, could be explained by either of the proposed mechanisms. If the rate-determining step in Wade's proposed mechanism was not the formation of the oxirene intermediate but rather its tautomerization to the ketene intermediate, then a deuterium isotope effect would be anticipated. On the other hand, ethynols similar to our proposed intermediate have never been isolated as such and have been considered too thermodynamically unstable with respect to rearrangement to the corresponding ketene to make isolation possible. If the rate of this rearrangement was more rapid than the rate of exchange of the hydroxyl deuterium with hydrogen ions then the deuterium retention in the product is compatable with our proposed mechanisms. It is apparent from these results that deuterium isotope-effect studies will not completely resolve this mechanistic dilemma.

REFERENCES

1. Sullivan, H. R., Roffey, P., and McMahon, R. E. (1979) Drug Metab. Dispos. 7(2), 76-80.
2. Wade, A., Symons, A. M., Martin, L., and Parke, D.V. (1979) Biochem. J. 184, 509-517.
3. White, I.N.H. (1978) Biochem. J. 174, 853-861.
4. DeMatteis, F., Gibbs, A. H., and Unseld, A. (1977) Biochem. J. 168, 417-422.
5. Tomasyewski, J. E., Jerina, D. M., Kaufman, S., and Milstein, S. (1975) J. Chem. Soc. London, Chem. Commun. 881-882.
6. Smith, J.R.L., Jerina, D. M., Kaufman, S., and Milstein, S. (1975). J. Chem. Soc. London Chem. Commun. 881-882.
7. McMahon, R. E. and Culp, H. W. (1979) J. Med. Chem. 22, 1100-1103.