Communications to the Editor

Enzymatic Hydroxylation of Ferrocene

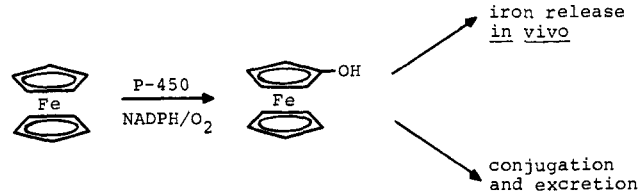
Sir:

The enzymatic hydroxylation of aromatic rings is a process of vital importance to the metabolism of endogenous as well as xenobiotic substances. It is generally believed that the process of "aromatic hydroxylation" involves a cytochrome P-450 dependent *epoxidation*, followed by rapid nonenzymatic rearrangement (NIH shift) of the resultant arene oxide to afford the phenolic metabolite.[1] However, it has recently been suggested, largely on the basis of kinetic deuterium isotope effects,[2] that in some cases direct hydroxylation of aromatic C-H bonds may also occur. In connection with the latter possibility, we have been investigating the in vivo and in vitro metabolism of transition metal π complexes of aromatic systems, such as ferrocene. In addition to theoretical interest in the interaction of oxygenase enzymes with the ferrocene nucleus, biological interest in ferrocene centers around its action as a hematinic agent[3-5] and the potential applications of ^{59}Fe ferrocene-based radiopharmaceuticals.[6-8] Early studies of ferrocene metabolism[3,4] in a variety of mammals including man[3] suggested the urinary excretion of iron in a form which was neither ferrocene nor inorganic iron; similar observations have been made recently with ruthenocene.[9] Although these observations suggest that aromatic π complexes indeed undergo biotransformation and excretion, none of the metabolites have been isolated and characterized. In this communication we wish to report the isolation and characterization of a hydroxylated metabolite of ferrocene in which the "sandwich" structure of the ferrocene nucleus has remained intact.

Urine collected from rats which had been given oral doses of ferrocene in sesame oil was acidified, filtered, and passed slowly over a column of XAD-2 nonionic resin. Washing the column with methanol removed materials giving a strong test[10] for organometallic iron. Evaporation of the methanol gave a yellow-brown gum which was dissolved in water and subjected to preparative reverse-phase HPLC.[11] Monitoring the column effluent by refractive index and for iron indicated a minor peak (A) and a major peak (B, ~26% of the administered dose) with retention volumes of 50–55 and 80–95 mL, respectively.

Metabolite B was acetylated (Ac_2O/*p*-TsOH catalyst) and methylated (CH_2N_2/Et_2O) to yield dark golden crystals of derivative C,[12] mp 213–216 °C dec. Both elemental analysis and the mass spectrum of C suggested that it was a diacetate methyl ester of ferrocenyl glucuronide. In addition to a molecular ion at *m/e* 476 ($C_{21}H_{24}FeO_9$), numerous fragment ions associated with the carbohydrate moiety (275, 215, 197, 173, and 155) as well as the hydroxyferrocene moiety (base peak 203; 202, 201, 185, 121) were observed. The NMR spectrum of C in acetone-d_6 showed an unsubstituted C_4H_5Fe unit (δ 4.22, s, 5 H), a C_5H_4 unit *bearing an electron-donating substituent* (3.86, t, 2 H ortho, and 4.18, t, 2 H meta), a methoxyl group (3.68, s, 3 H), two acetates ($CDCl_3$ solution: 2.17, s, 3 H, and 2.09, s, 3 H), and five sugar protons (4.36, d, J = 12 Hz, 1 H; 5.20–4.90, m, 4 H). Treatment of C with acetic anhdyride and pyridine gave in low yield a triacetate methyl ester, D,[12] mp 152–154 °C, which gave a satisfactory elemental analysis. The mass spectrum of D showed many parallels to that of C, including a molecular ion (*m/e* 518, $C_{23}H_{26}FeO_{10}$), a base peak at 202 ($C_5H_5FeC_5H_4OH$), carbohydrate fragments at 257, 215, 197, 173, and 155, and organometallic fragments at 185 and 121. The aglycone was also conclusively shown to be hydroxyferrocene by the conversion of B to methoxyferrocene (identical by TLC, GLC, and mass spectrum with an authentic standard[13]) by acid hydrolysis (5% H_2SO_4) followed by methylation ($NaOH/(MeO)_2SO_2$).

Scheme I



In vitro, the metabolism of ferrocene was observed to require viable liver microsomes, NADPH cofactor, and molecular oxygen. The process was stimulated (~7-fold!) by pretreatment of the rats with phenobarbital and was inhibited by CO. Ferrocenyl glucuronide (i.e., B) was formed in vitro if UDPGA was added to the incubations, but, in its absence, hydroxyferrocene, which is known to be rather unstable and oxygen sensitive,[13] was not observed. Ferrocene-d_{10}[14] was also metabolized in vitro, although at a much slower rate than ferrocene. In a number of parallel incubations at 0.1 mM initial concentrations of the organometallics, the observed isotope effect (k_H/k_D) was always in the range of 1.80–2.06, with an average value of 1.93.

The above observations indicate that the hydroxylation of ferrocene is a cytochrome P-450 dependent process. Moreover, they suggest that in vivo recovery of iron from ferrocene for hemoglobin synthesis may occur by spontaneous decomposition of enzymatically formed hydroxyferrocene which escapes conjugation with sulfate[15] or glucuronic acid (Scheme I). It is noteworthy that the deuterium isotope effect observed is much larger than those observed for the hydroxylation of simple aromatic substrates.[2] However, further experimental work will be required to determine if a direct hydroxylation mechanism is involved here. It is hoped that π-arene complexes, because of their unusual geometric and electronic properties, will provide a new set of probes for use in studying reaction mechanisms of P-450 enzymes. Information about the metabolism of this class of compounds will also be important in connection with their potential use as radiopharmaceutical diagnostic agents,[6-9] and with their widespread use (viz., methylcyclopentadienylmanganesetricarbonyl, MMT) as fuel additives.

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References and Notes

(1) For a recent review, see D. M. Jerina and J. Daly, *Science*, **185**, 573 (1974).
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(9) A. J. Taylor and M. Wenzel, *Naturwissenschaften*, **64**, 273 (1977).

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(10) S. I. Goldman, *Anal. Chem.*, **31**, 486 (1959).
(11) The separation was carried out with a Whatman Magnum-9 C-18 reverse phase column eluted with 10% MeCN in 0.1 M KH_2PO_4 at a flow rate of 7 mL/min.
(12) On silica TLC plates eluted with 40% ethyl acetate in hexane, derivative C had R_f 0.16 and derivative D had R_f 0.43.
(13) Methoxy- and hydroxyferrocene were synthesized according to A. N. Nesmeyanov, V. A. Sazonova, and V. N. Drozd, *Tetrahedron Lett.*, 13 (1959).
(14) Ferrocene-d_{10} was prepared by repetitive treatment of ferrocene with CF_3CO_2D. Mass spectral analysis indicated that several such exchanges afforded material of >98% isotopic purity.
(15) Metabolite A appears to be a sulfate ester of hydroxyferrocene, based on the observation that it is found to contain iron and tritium when rats are given tritiated ferrocene orally, and iron and ^{35}S when rats are given ferrocene orally and $Na_2{}^{35}SO_4$ intraperitoneally.

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Hemiacetal Buildup during Acetal Hydrolysis

Sir:

During the course of examining solvent effects, solvent isotope effects, and substituent effects on the general and specific acid catalyzed hydrolyses of acetals of benzaldehydes, we noticed a reproducible "induction period" as shown in Figure 1. This was recognized as classic behavior for the kinetic system A → B → products,[1] wherein it is necessary for B to attain a certain finite concentration prior to observing a first-order appearance of product.[2] For hydrolyses of acetals of benzaldehydes, where progress of the reaction is followed spectrophotometrically, Scheme I obtains. In principle, of course, the steps are all reversible; however, at the concentrations involved in the spectrophotometric rate studies (10^{-5} M), the v_{-1} and v_{-2} processes are negligible and thus the scheme essentially becomes that for consecutive *pseudo*-first-order reactions.

In order to determine what seemed to be the unlikely possibility that our observation of an induction period was actually due to the buildup of hemiacetal according to Scheme I, the following experimental procedure was devised, based on the well-known fact that v_1 is acid catalyzed whereas v_2 is both acid *and* base catalyzed: a known amount of acetal (20 μL of a 10^{-2} M solution in CH_3CN) was introduced into the aqueous acidic solution using a rapid injection syringe;[3] after the reaction had proceeded for the desired time (e.g., 10% reaction), the reaction was quenched by rapid injection of base (150 μL of 1 N KOH). Immediately upon injection of base, the absorbance due to benzaldehyde increased because of the rapidity of the v_2 process in aqueous base. A typical experimental result is presented in Figure 2, wherein the absorbance difference labeled A refers to benzaldehyde produced by acid catalyzed hydrolysis via Scheme I by time t_q, absorbance difference B refers to benzaldehyde produced by rapid base catalyzed hydrolysis of the hemiacetal present at time t_q, and the absorbance difference C refers to the benzaldehyde produced in the total hydrolysis of acetal (i.e., A_∞). Thus, B/(C − A − B) = [hemiacetal]/[acetal] at the time of quenching t_q. By varying t_q, a plot such as Figure 3 may be constructed and these data may be treated in one of two ways: most directly, the time at which d[hemiacetal]/dt = 0 can be measured (the time corresponding to the maximum in curve B of Figure 3) and at this time, t_{max}, [hemiacetal]/[acetal] = k_1/k_2. Thus, k_2 may be calculated, since [hemiacetal]/[acetal] can be calculated where $t_q = t_{max}$ (Figures 2 and 3) and k_1 is obtained from the slope of the usual pseudo-first-order semilog rate plot (Figure 1, line A). Alternatively, the data of curve A in Figure 3 can be plotted as in Figure 1 and the *difference* between the "early" points and the extrapolated line plotted vs. time (line B). The slope of line B produces k_2. Both procedures produce equivalent values of k_2 as shown in Table I; thus, while an exact kinetic treatment of consecutive first-order reactions is rather complex,[1a,c] the data can in fact be treated rather simply provided that the competitive reaction rates are different by at least a factor of 2.[2]

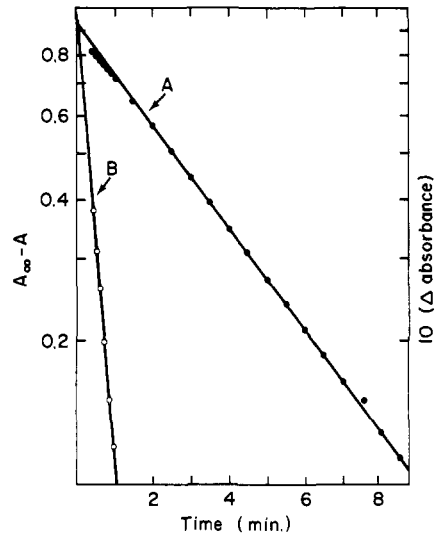

Figure 1. A plot of log ($A_\infty - A$) vs. minutes for *p*-methylbenzaldehyde diethyl acetal in acetate buffer (μ = 0.5, KCl) at 25 °C. Line A (●, left ordinate scale) represents experimental data, line B (○, right ordinate scale) represents the difference between line A (based data from the second and third half-lives of reaction time) and the experimental data recorded early in the reaction.

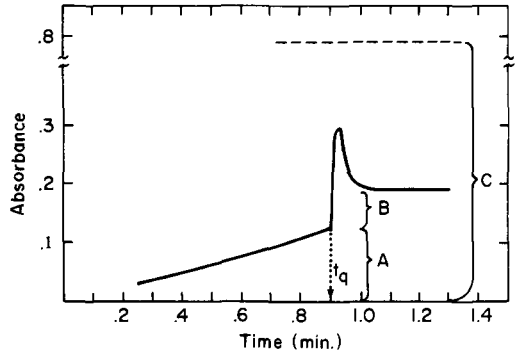

Figure 2. A plot of absorbance vs. minutes for *p*-methylbenzaldehyde diethyl acetal in acetate buffer at 25 °C. The time designated t_q corresponds to the time of injection of 150 μL of 1 N KOH. The dashed line represents the absorbances of an acetal solution of equal concentration which was not quenched (i.e., allowed to completely hydrolyze) and thus is the absorbance at $t = \infty$.

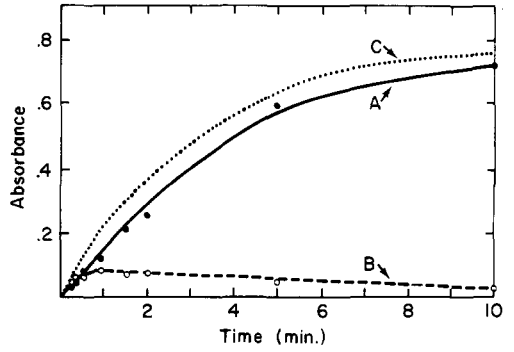

Figure 3. A plot of absorbance vs. minutes for *p*-methylbenzaldehyde diethyl acetal in acetate buffer (μ = 0.5, KCl) at 25 °C. The solid line A, is the appearance of *p*-methylbenzaldehyde, the dashed line B, is the appearance of hemiacetal (measured at various t_q; cf. Figure 2). The sum of A and B is the dotted line C.

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