# Theory for the Observed Isotope Effects on the Formation of Multiple Products by Different Kinetic Mechanisms of Cytochrome P450 Enzymes

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ABSTRACT: Cytochrome P450 systems are unusual in that many of them can convert a substrate to a number of different metabolites. Several kinetic mechanisms may be envisioned by which the metabolites may be formed. In each of the mechanisms, the substrate combines with the enzyme in different orientations to form a set of (ES) complexes that then are activated to a set of (EOS) complexes. The fate of these (EOS) complexes determines the kinetic mechanism. In the "parallel pathway" mechanism, the (EOS) complexes are so stable and rigid they cannot be converted either directly or indirectly to complexes with different orientations; the orientation of the (ES) complexes thus determines which metabolite will be formed. In the "nondissociative" mechanisms, the complexes are not rigid; instead they undergo interconversion while the substrate remains in the active site of the enzyme. In the "dissociative" mechanisms, the (EOS) complexes dissociate to (EO) and (S), but recombine to form (EOS) complexes with either the same or different orientations. Steady-state equations describing the deuterium isotope effects for these kinetic mechanisms have been derived and solved for competitive experiments, in which equal concentrations of both deuterated and nondeuterated substrates are present in incubation mixtures, and for noncompetitive experiments, in which only one of the substrates is present. The equations reveal that comparisons of the isotope effects on the formation of a metabolite by a pathway that does not involve the abstraction of a deuterium from a deuterated substrate (the non-deuterium abstraction pathway) in both experiments can differentiate between the kinetic mechanisms. A value of 1.0 for $(v)_H/(v)_D$ in the competitive experiment, but <1.0 to >1.0 for the value of $(V_{max}/K_m)_H/(V_{max}/K_m)_D$ in the noncompetitive experiment, is diagnostic for the "dissociative" mechanisms. Values of 1.0 in both kinds of experiments are diagnostic for the "parallel pathway" mechanism. Values of <1.0 in both types of experiments are diagnostic for the "nondissociative" mechanisms. The equations also predict possible unusual substrate–inhibitor interactions when the "dissociative" mechanism is operative.

The enzymes comprising the superfamily of cytochrome P450 are known to catalyze the oxidation of many substrates, including steroids, fatty acids, drugs, and environmental chemicals, to several different metabolites. During the past decade many of the enzymes have been purified, their amino acid sequences determined, and their cDNAs constructed. Studies with the purified enzymes and expressed enzymes from transfected cDNA clones have made it obvious that some of the enzymes have narrow substrate specificities, whereas others have broad specificities.

Despite the plethora of substrates that are metabolized by the cytochrome P450s, the general mechanism for the formation of the "active oxygen complex", (EOS), appears to be identical for nearly all of the reactions (Porter & Coon, 1991). Most investigators believe that the general mechanism of cytochrome P450 enzymes involves the following intermediate reactions. The enzyme (E) combines with the substrate to form an (ES) complex. An electron is introduced into the complex to form a reduced complex (ESred) which then reacts with molecular oxygen to form a monoreduced oxygenated complex ($monoEO_2S$). Another electron is introduced into the complex to form a direduced oxygenated complex ($diEO_2S$). The complex then decomposes to form water and a monooxygen complex (EOS) (Figure 1).

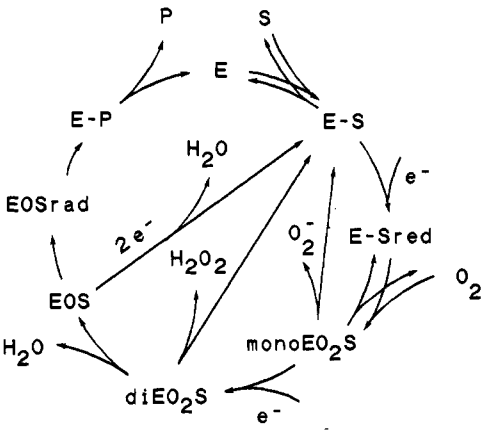


FIGURE 1: The cytochrome P450 cycle for the hydroxylation of alkyl groups and the oxidative dealkylation of ethers.

Several mechanisms have been proposed to describe the fate of (EOS) complexes. In aliphatic hydroxylation and ether cleavage reactions, a hydrogen atom is abstracted from the substrate to form a carbon-centered radical (EOSrad) and a heme-stabilized hydroxyl radical; these then "rebound" to form the enzyme–product complex (EP) (Groves et al., 1978; Linsay Smith & Sleath, 1983). The mechanisms of N-dealkylation and N-hydroxylation of amines and amides and the hydroxylation of aromatic rings are less certain. For example, the (EOS) complex may possibly abstract an electron from amines, heterocyclic compounds (Ortiz de Montellano, 1986), or aromatic rings to form radical cations followed by oxygen rebound to form the enzyme–product complex. It is also

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possible that the oxygen in the (EOS) complexes of aromatic compounds may combine with a $\pi$-orbital of an aromatic ring to form a tetrahedral charge-transfer complex (Hanzlik & Shearer, 1978; Ortiz de Montellano & Correia, 1983; Korzekwa et al., 1989a), which ultimately may rearrange to a phenol, either directly or by way of a ketone, or to an epoxide.

In some systems, the (mono$EO_2S$) may dissociate to form superoxide and (ES), the (di$EO_2S$) may dissociate to form hydrogen peroxide and (ES), and the (EOS) may be reduced to form water and (ES) (Gorsky et al., 1983; Atkins & Sligar, 1987). Thus the relationships for the consumption of substrate, NADPH, and oxygen are not always 1:1:1.

A given form of cytochrome P450 may convert a substrate to several different products, but the kinetic mechanisms by which several metabolites may be formed by a single enzyme are not always obvious. Clearly, the substrate must be able to assume several different orientations, relative to the oxygen, at crucial phases of the reactions. Several mechanisms may be envisioned.

In some kinetic mechanisms, which may be called "post-abstraction mechanisms", the formation of multiple products depends on the rigidity of the (EOSrad) complexes formed after the hydrogen atom is abstracted from a methyl or methylene group. For example, the unpaired electron in the substrate in the complex may migrate from its original position to different positions, and the complex may have sufficient flexibility that the heme-stabilized hydroxyl group may rebound with the unpaired electron in the new position. This kind of mechanism can account for the isomerization of trideuteriomethyl pulelone during its oxidation to menthofuran (McClanahan et al., 1988) and for the formation of both hydroxybicyclopentane and hydroxycyclopentene from bicyclopentane (Ortiz de Montellano & Sterns, 1987). Another postabstraction kinetic mechanism is the double abstraction mechanism, which has been proposed for the formation of $\Delta^6$-testosterone as well as 7$\alpha$-hydroxytestosterone from testosterone by cytochrome P450 2A1 (Korzekwa et al., 1990).

In many kinetic mechanisms, the different orientations that account for multiple product formation may be achieved before the abstraction of a hydrogen and thus may be called "preabstraction mechanisms". In these mechanisms the substrate may combine with the enzyme in several different orientations to form a set of (ES) complexes. These complexes may be then activated to form a set of (EOS) complexes. But the kinetic mechanism depends on the properties of these (EOS) complexes.

In one group of kinetic mechanisms, the different orientations of the substrate in the (EOS) complexes are interconvertible while the substrate remains within the active site of the enzyme before the abstraction of the hydrogen atom occurs; these may be called "nondissociative mechanisms". The mechanisms depend on the rate constants for the interconversion relative to the rate constants that describe the abstraction of the hydrogen from the substrate and the reduction of the complexes to (ES) and water. When the rate constants for the interconversions approach infinity (which we call the "rapid interconvertible mechanism"), the mechanism approaches the "branched pathway mechanism" described elsewhere (Jones et al., 1986; Korzekwa et al., 1989b).

In another group of kinetic mechanisms, suggested by Harada et al. (1984), the orientations of the substrate in the (EOS) complexes are not interconvertible while the substrate is in the active site of the enzyme. Instead, the substrate may dissociate from the monooxygenated complexes and recombine to form complexes in either the same or different orientations; we call these "dissociative mechanisms". These mechanisms lack rate constants for interconversion, but depend on the rate constants for the dissociation and reassociation of the (EOS) complexes relative to the rate constants for the abstraction of the hydrogen from the substrate and the reduction of the (EOS) complexes to (ES) and water. When the rate constants for dissociation and reassociation are very large, the mechanism becomes the "rapid equilibrium mechanism".

In another mechanism, the (EOS) complexes are sufficiently rigid and stable that they are not interconvertible while the substrate is in the active site of the enzyme, nor do they dissociate. Such mechanisms may be envisioned as special cases of the other two mechanisms, in which the rate constants for the reorientation and dissociation of the (EOS) complexes are virtually zero. The equations for this kind of mechanism, which we call the "parallel pathway mechanism", were first derived by Grimshaw and Cleland (1980).

In the present study, we have developed the theoretical basis for designing deuterium isotope experiments that can differentiate between these preabstraction kinetic mechanisms.

## THEORY

Because of the complexity of the mechanisms we envisioned, several simplifying assumptions were made, which do not invalidate the general conclusions. We have assumed that the concentrations of (ESred) and (EOSrad) were negligible compared with the other intermediates; this would occur if the concentration of oxygen is very high and if the rate constant for the "rebound" step is much larger than the rate constant for the abstraction of the hydrogen. We also assumed that we could use a single intermediate ($EO_2S$) to describe both (mono$EO_2S$) and (di$EO_2S$). This would be valid when either, but not both, is negligible; in most cytochrome P450 systems the rate constant for the decomposition of (di$EO_2S$) appears to be larger than that for the introduction of the second electron, and thus the steady-state concentration of (di$EO_2S$) is negligible. We further assumed that the conversions of (ES) to ($EO_2S$) and ($EO_2S$) to (EOS) were irreversible reactions; although the validity of the first of these assumptions may be questionable, it is highly doubtful that (EOS) could be converted back to ($EO_2S$).

The rate constant for the abstraction of a deuterium from a given carbon–deuterium group in a deuterated substrate is invariably smaller than the rate constant for the abstraction of a hydrogen from the corresponding carbon–hydrogen group in the nondeuterated substrate. When several metabolites are formed from a deuterated substrate, some metabolites may be formed by "deuterium abstraction pathways", which involve the abstraction of the deuterium, whereas others may be formed by "non-deuterium abstraction pathways", which may involve the abstraction of a hydrogen from the deuterate substrate or the hydroxylation of an aromatic ring. There will be virtually no difference between deuterated and nondeuterated substrates in the rate constants associated with reactions that do not involve the breaking of carbon–hydrogen bonds. In the different kinetic preabstraction mechanisms for multiple metabolite formation, the substrate may exist in different orientations, which must be represented as different entities. Intermediate forms leading directly to the metabolite, $P_w$, by a deuterium abstraction pathway may be designated ($ES_w$), ($EO_2S_w$), ($EOS_w$), and ($EP_w$), whereas those leading directly to the formation of a metabolite, $P_x$, by a non-deuterium abstraction pathway may be designated ($ES_x$), ($EO_2S_x$), ($EOS_x$), and ($EP_x$) (Figure 2).

Studies of isotope effects on the metabolism of substrates are performed by two different kinds of experiments. In

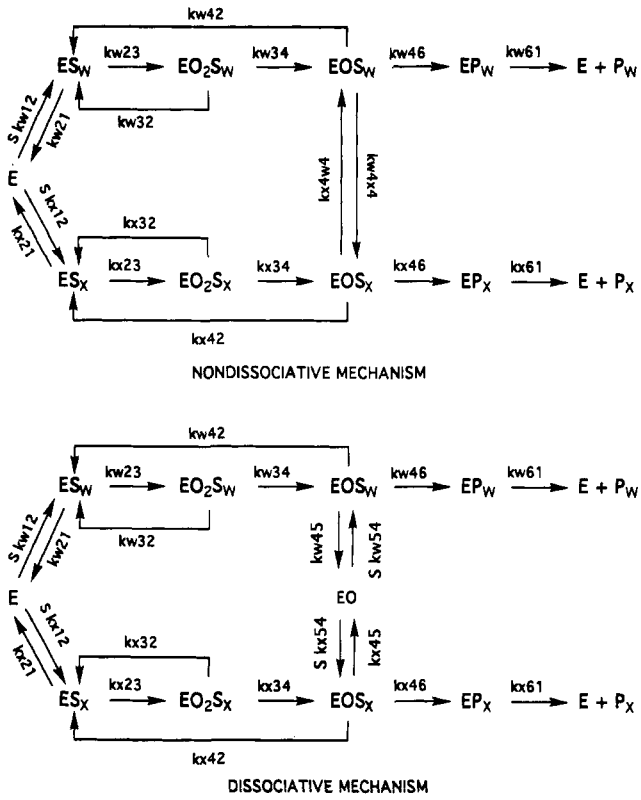


FIGURE 2: The nondissociative and dissociative mechanisms in noncompetitive experiments.

noncompetitive experiments the deuterated and nondeuterated substrates are incubated with the enzyme in separate experiments. Since only one substrate is present, the metabolism of various concentrations of the each substrate by the enzyme provides the information necessary to calculate the $V_{max}$ for each metabolite of each substrate and, when appropriate, the $K_m$ for each substrate. In competitive experiments, both the deuterated and the nondeuterated substrates are present in the incubation mixture, and the metabolites from each substrate are measured. In some competitive experiments the concentration of one of the substrates is held constant and that of the other substrate is varied; such experiments may be viewed as typical competitive inhibitor experiments and, when appropriate, may be analyzed by any of several plotting techniques for competitive inhibitors. In most competitive experiments, however, the deuterated and nondeuterated substrates are present in equal concentrations. Both competitive and noncompetitive experiments are necessary to differentiate the preabstraction kinetic mechanisms.

The schemes for the nondissociative and dissociative mechanisms in noncompetitive (Figure 2) and competitive (Figures 3 and 4) experiments have been developed in ways in which the various reactions and the appropriate rates constants are kept as analogous as possible. For example, in both of the mechanisms, the immediate precursors of the deuterium abstraction reaction for the nondeuterated and deuterated substrates are $(EOS_w)_H$ and $(EOS_w)_D$, the rate constants for this reaction are $k_{w46H}$ and $k_{w46D}$, and the products formed from the two substrates by the pathways are $(P_w)_H$ and $(P_w)_D$. The rate constant for the formation of a metabolite by the non-deuterium abstraction pathway is $k_{x46}$, the enzyme–product complexes are $(EP_x)_H$ and $(EP_x)_D$, and the products from the two substrates are $(P_x)_H$ and $(P_x)_D$. Indeed, the only differences between the two schemes are that the nondissociative models contain $k_{w4x4}$ and $k_{x4w4}$ for the interconversion of the orientations, whereas the dissociative models contain $k_{w45}$, $Sk_{w54}$, $k_{x45}$, and $Sk_{x54}$ to describe the dissociation and reassociation of the (EOS) complexes.

It is noteworthy that analogous schemes for intramolecular isotope effects will be special cases of those described in Figures 2–4. In intramolecular isotope experiments the substrate must have two symmetrical, catalytically susceptible sites; one of the sites contains the deuterium and the other site contains the hydrogen. Thus, the equations for the intermediate forms of the intramolecular isotope effects would be the same as those for the deuterated substrate, but the $k_x$ rate constants would equal their corresponding $k_w$ rate constants, except $k_{x46}$ would equal $k_{w46H}$. In the nondissociative mechanism, the $k_{x4w4}$ would equal $k_{w4x4}$, and in the dissociative mechanism $k_{x45}$ and $k_{x54}$ would equal $k_{w45}$ and $k_{w54}$, respectively.

The seminal work of Northrop (1975, 1978, 1981) and Cleland (1982) provided the theoretical basis for evaluating the differences between "observed" and "intrinsic" deuterium isotope effects. An "observed" isotope effect is defined as the ratio of a kinetic parameter (usually $V_{max}$ or $V_{max}/K_m$) obtained with an nondeuterated substrate to that obtained with a deuterated substrate. An "intrinsic" deuterium isotope effect is defined as the isotope effect on the rate constant for the abstraction of a deuterium from a carbon–deuterium bond in a deuterated substrate. In the present paper, the intrinsic isotope effect is defined as $k_{w46H}/k_{w46D}$. The "observed" isotope effect for the metabolite formed by the deuterium abstraction pathway depends not only on the intrinsic isotope effect but also on the isotope effect on the steady-state concentration of the immediate precursor of the deuterium abstraction reaction. In the models described in this paper, the observed isotope effect on the metabolite formed by the deuterium abstraction pathway would be $[(EOS_w)_H k_{w46H}]/[(EOS_w)_D k_{w46D}]$. By contrast, the observed isotope effect on a metabolite formed by the pathway that does not involve the abstraction of a deuterium, the non-deuterium abstraction pathway, will depend solely on the isotope effect upon the concentration of its immediate precursor. In the models described in this paper, the observed isotope effect will formally be $[(EOS_x)_H k_{x46H}]/[(EOS_x)_D k_{x46D}]$. But $k_{x46D}$ equals $k_{x46H}$, and therefore, the observed isotope effect on the nondeuterium abstraction pathway depends solely on the ratio $(EOS_x)_H/(EOS_x)_D$.

For any enzymatic mechanism, we may obtain these relationships indirectly by writing the equation as the total enzyme concentration times the fraction of the total that exists as a given intermediate under steady-state conditions times the appropriate rate constant for the initial rate of formation of a product. Thus,

$$v = \frac{E_t(EP_i)k_i}{(\text{denominator})} \qquad (1)$$

in which $E_t$ is the total concentration of the enzyme, $(EP_i)$ is the steady-state concentration of the enzyme–product complex, $k_i$ is the rate constant of dissociation of the complex, and the denominator is the sum of the steady-state concentrations of the free enzyme and each of the intermediate forms of the enzyme. The total metabolism of the substrate is given by the sum of such equations. The steady-state equations for (E) and each of the intermediate forms may be solved by the determinant method described by King and Altman (1956).

After the appropriate substitutions into eq 1 are made, the equation may be then inspected to determine whether it fits the general form of $v = AS_H/(B + CS_H)$ in the equation for a noncompetitive experiment and $v = AS_H/(B + CS_H + DS_D)$ in a competitive experiment. When the equations fit these

forms, they may be converted to Michaelis–Menten equations for noncompetitive and competitive experiments, respectively. For example, for a noncompetitive experiment for the nondissociative mechanism, we may write

$$(V_{max})_i(S/K_m) = E_t(EP_i)k_i/(E) \quad (2n)$$

$$(V_{max})_i = E_t(EP_i)k_i/[(ES_w) + (EO_2S_w) + (EOS_w) + (EP_w) + (ES_x) + (EO_2S_x) + (EOS_x) + (EP_x)] \quad (3n)$$

$$K_m/S = (E)/[(ES_w) + (EO_2S_w) + (EOS_w) + (EP_w) + (ES_x) + (EO_2S_x) + (EOS_x) + (EP_x)] \quad (4n)$$

For the noncompetitive experiment for the dissociative mechanism, we may write

$$(V_{max})_i(S/K_m) = E_t(EP_i)k_i/[(E) + (EO)] \quad (2d)$$

$$(V_{max})_i = E_t(EP_i)k_i/[(ES_w) + (EO_2S_w) + (EOS_w) + (EP_w) + (ES_x) + (EO_2S_x) + (EOS_x) + (EP_x)] \quad (3d)$$

$$K_m/S = [(E) + (EO)]/[(ES_w) + (EO_2S_w) + (EOS_w) + (EP_w) + (ES_x) + (EO_2S_x) + (EOS_x) + (EP_x)] \quad (4d)$$

*Ratio of Metabolites.* When the enzyme catalyzes the formation of two or more metabolites from the same substrate, the ratio of the rates of formation of the metabolites in the same incubation mixture may be calculated solely from the numerators of eq 1, because the denominators of eq 1 will be the same for both metabolites. Thus, in noncompetitive experiments,

$$v_{(pw)}/v_{(px)} = [(EP_w)k_{w61}]/[(EP_x)k_{x61}] \quad (5)$$

In competitive experiments isotope effects may be calculated for other ratios of metabolites. Thus,

$$(v_{(pw)})_H/(v_{(pw)})_D = [(EP_w)_Hk_{w61}]/[(EP_w)_Dk_{w61}] \quad (6)$$

$$(v_{(px)})_H/(v_{(px)})_D = [(EP_x)_Hk_{x61}]/[(EP_x)_Dk_{x61}] \quad (7)$$

$$\frac{(v_{(pw)})_H + (v_{(px)})_H}{(v_{(pw)})_D + (v_{(px)})_D} = \frac{(EP_w)_Hk_{w61} + [(EP_x)_Hk_{x61}]}{(EP_w)_Dk_{w61} + [(EP_x)_Dk_{x61}]} \quad (8)$$

Since the isotope-sensitive rate constant, $k_{w46}$, is the only difference between the equations for the deuterated and nondeuterated substrates, only that constant need be distinguished, i.e., $k_{w46H}$ and $k_{w46D}$. After separation of the terms that contain the isotope-sensitive rate constant in the various parameters from those that do not, the equation for the nondeuterated substrate is divided by the equation for the deuterated substrate. Northrop (1975) pointed out that the resulting equations for the isotope effects on $V_{max}/K_m$ and $V_{max}$ may be frequently rearranged to the following general form for a normal isotope effect:

$$\frac{(\text{parameter})_H}{(\text{parameter})_D} = \frac{C + k_{w46H}/k_{w46D}}{C + 1} \quad (9)$$

where the $k_{w46H}/k_{w46D}$ term is the "intrinsic" isotope effect and $C$ is a "masking" term. In the mechanisms developed here, however, many of the masking terms comprise several hundred terms, and thus it is impractical to publish the complete list. Nevertheless, the direction of the isotope effects may be frequently determined by inspection of eqs 2–4 after appropriate substitutions are made. For example, the following may be shown:

*Case 1.* When the equation for the parameter does not contain the isotope-sensitive rate constant, the ratio of the equations will be 1.0, and therefore there will be no isotope effect.

*Case 2.* The equation of the parameter has the following form:

$$\text{parameter} = \frac{bk_{w46}}{c + dk_{w46}}; \quad \frac{(\text{parameter})_H}{(\text{parameter})_D} = \frac{(d/c)k_{w46H} + (k_{w46H}/k_{w46D})}{(d/c)k_{w46H} + 1} \quad (10)$$

where $k_{w46}$ is the isotope-sensitive rate constant. Thus, the ratio of the equations for the nondeuterated and deuterated substrates can be rearranged to the Northrop form for a normal isotope effect; i.e., the observed isotope effect will be greater than 1.0.

*Case 3.* The equation of the parameter has the following form:

$$\text{parameter} = \frac{a}{c + dk_{w46}}; \quad \frac{(\text{parameter})_H}{(\text{parameter})_D} = \frac{c/(dk_{w46H}) + (k_{w46D}/k_{w46H})}{c/(dk_{w46H}) + 1} \quad (11)$$

Thus, the ratio of the equations can be rearranged to the Northrop form for an inverse isotope effect; i.e., the observed isotope effect will be less than 1.0.

*Case 4.* When the equation of the parameter has the following form:

$$\text{parameter} = \frac{a + bk_{w46}}{c + dk_{w46}}; \quad \frac{(\text{parameter})_H}{(\text{parameter})_D} = \frac{(a + bk_{w46H})(c + dk_{w46D})}{(a + bk_{w46D})(c + dk_{w46H})} \quad (12)$$

the ratio of the equations cannot be modified to a Northrop form. Indeed, some of the terms would tend to make the isotope effect normal, and other terms would tend to make it inverse; the net effect thus depends on the relative concentrations of the intermediate forms that contribute to the isotope effects.

Since by definition of a normal isotope effect the parameter for the nondeuterated substrate is greater than the parameter for the deuterated substrate, subtraction of the denominator of the equation for the ratio of the parameters from the numerator should be positive for a normal isotope effect and negative for an inverse isotope effect. This procedure sometimes can aid in identifying what parts of the system govern the direction of the isotope effect.

*Case 5.* Some models predict that the ratio of the rates of formation obtained from the nondeuterated substrate divided by that obtained from the deuterated substrate will give the "intrinsic" isotope effect, i.e.,

$$(v_{(pw)}/v_{(px)})_H/(v_{(pw)}/v_{(px)})_D = k_{w46H}/k_{w46D} \quad (13)$$

This will occur, for example, when the equation for $(EP_x)$ does not contain the isotope-sensitive rate constant, $k_{w46}$.

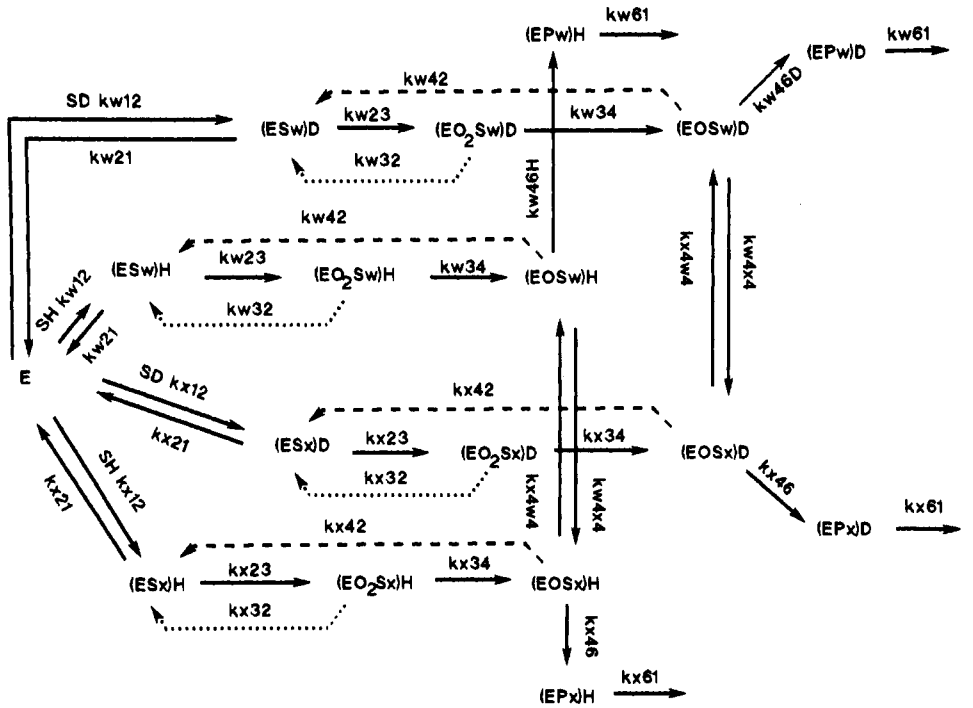


FIGURE 3: The nondissociative mechanisms in competitive experiments. The rate constants shown in the figures have been used in the derivation of the equations in Tables 1–3.

Table 1: Nondissociative Mechanism, Competitive Experiment: Steady-State Solutions for the Intermediate Forms of the Enzyme[a,b]

$(E) = (\mathbf{W5_n})(\mathbf{Y5_n})$
$(ES_w)_H = (S_H)(\mathbf{Y5_n})[k_{w12}k_{x21}(k_{w46H} + k_{w42})X2_n + A_{(ESw)}1]$
$(EO_2S_w)_H = (ES_w)_H k_{w23}/(k_{w32} + k_{w34})$
$(EOS_w)_H = (S_H)(\mathbf{Y5_n})(A_{(EOSw)}1)$
$(EP_w)_H = (EOS_w)_H(k_{w46H}/k_{w61})$
$(ES_x)_H = (S_H)(\mathbf{Y5_n})[k_{w21}k_{x12}(k_{w46H}W1 + k_{w42})X3_n + A_{(ESx)}1]$
$(EO_2S_x)_H = (ES_x)_H k_{w23}/(k_{w32} + k_{w34})$
$(EOS_x)_H = (S_H)(\mathbf{Y5_n})[A_{(EOSx)}1 + F_{x34}k_{w21}k_{x12}k_{x23}\mathbf{W2_n}]$
$(EP_x)_H = (EOS_x)_H k_{x46}/k_{x61}$
$(ES_w)_D = (S_D)(\mathbf{W5_n})[k_{w12}k_{x21}(k_{w46D} + k_{w42})X2_n + A_{(ESw)}1]$
$(EO_2S_w)_D = (ES_w)_D k_{w23}/(k_{w32} + k_{w34})$
$(EOS_w)_D = (S_D)(\mathbf{W5_n})(A_{(EOSw)}1)$
$(EP_w)_D = (EOS_w)_D k_{w46D}/k_{w61}$
$(ES_x)_D = (S_D)(\mathbf{W5_n})[k_{w21}k_{x12}(k_{w46D}W1 + k_{w42})X3_n + A_{(ESx)}1]$
$(EO_2S_x)_D = (ES_x)_D k_{x23}/(k_{x32} + k_{x34})$
$(EOS_x)_D = (S_D)(\mathbf{W5_n})[F_{x34}k_{w21}k_{x12}k_{x23}\mathbf{Y2_n} + A_{(EOSx)}1]$
$(EP_x)_D = (EOS_x)_D k_{x46}/k_{x61}$

[a] All of the equations have been divided by $(k_{w32} + k_{w34})$, $k_{w61}$, $(k_{x32} + k_{x34})$, and $k_{x61}$. [b] Boldfaced terms contain the isotope-sensitive rate constant, $k_{w46}$.

Table 2: Nondissociative Mechanism: Definitions[a]

Part A: General Nondissociative Mechanism

$F_{w34} = k_{w34}/(k_{w32} + k_{w34})$ $F_{x34} = k_{x34}/(k_{x32} + k_{x34})$
$W1 = (F_{w34}k_{w23}/k_{w21}) + 1$ $X1 = (F_{x34}k_{x23}/k_{x21}) + 1$
$\mathbf{W2_n} = (k_{w4x4} + k_{w46H})W1 + k_{w42}$ $X2_n = (k_{x4w4} + k_{x46})X1 + k_{x42}$
$\mathbf{W3_n} = k_{w4x4} + k_{w46H} + k_{w42}$ $X3_n = k_{x4w4} + k_{x46} + k_{x42}$
$\mathbf{Y2_n} = (k_{w46D} + k_{w4x4})W1 + k_{w42}$
$\mathbf{Y3_n} = k_{w46D} + k_{w4x4} + k_{w42}$
$\mathbf{W5_5} = k_{w21}k_{x21}[\mathbf{W2_n}(k_{x46}X1 + k_{x42}) + k_{x4w4}(k_{w46H}W1 + k_{w42})X1]$
$\mathbf{Y5_n} = k_{w21}k_{x21}[(k_{x46}X1 + k_{x42})\mathbf{Y2_n} + k_{x4w4}(k_{w46D}W1 + k_{w42})X1]$
$A_{(ESw)}1 = k_{w12}k_{w4x4}k_{x21}(k_{x46}X1 + k_{x42}) + F_{x34}k_{w42}k_{x12}k_{x23}k_{x4w4}$
$A_{(EOSw)}1 = F_{w34}k_{w12}k_{w23}k_{x21}X2_n + F_{x34}k_{w21}k_{x12}k_{x23}k_{x4w4}W1$
$A_{(ESx)}1 = k_{w21}k_{w4x4}k_{x12}(k_{x46} + k_{x42})W1 + F_{w34}k_{w12}k_{w23}k_{w4x4}k_{x42}$
$A_{(EOSx)}1 = F_{w34}k_{w12}k_{w23}k_{w4x4}k_{x21}X1$

Part B: Rapidly Interconvertible Nondissociative Mechanism (Branched-Chain Mechanism)[b]

$W2_n = Y2_n = k_{w4x4}W1$ $X2_n = k_{x4w4}X1$
$W3_n = Y3_n = k_{w4x4}$ $X3_n = k_{x4w4}$
$W5_n = k_{w21}k_{x21}[k_{w4x5}W1(k_{x46}X1 + k_{x42}) + k_{x4w4}(k_{w46H}W1 + k_{w42})X1]$
$Y5_n = k_{w21}k_{x21}[(k_{x46}X1 + k_{x42})k_{w4x4}W1 + k_{x4w4}(k_{w46D}W1 + k_{w42})X1]$

[a] Boldfaced terms contain the isotope-sensitive rate constant, $k_{w46}$. [b] All other terms are the same as those defined in part A.

In the present study, the various equations have been derived and condensed with the aid of Macsyma (Symbolics, Inc., East Burlington, MA).

## DERIVATION OF EQUATIONS

*Nondissociative Mechanisms. (A) General Mechanism.* As shown in Figure 3, the intermediate forms present in a competitive experiment, and therefore in the denominator of eq 1, include

$$(\text{denominator})_{H,D} = (E) + (ES_w)_H + (EO_2S_w)_H + (EOS_w)_H + (EP_w)_H + (ES_x)_H + (EO_2S_x)_H + (EOS_x)_H + (EP_x)_H + (ES_w)_D + (EO_2S_w)_D + (EOS_w)_D + (EP_w)_D + (ES_x)_D + (EO_2S_x)_D + (EOS_x)_D + (EP_x)_D$$

The solutions for the steady-state concentrations of these intermediates are shown in Table 1. Table 2A gives the appropriate mechanism-dependent definitions for the general mechanism that should be substituted into the equations in Table 1.

In noncompetitive experiments (Figure 2), the denominator for the rate equations for the nondeuterated substrate will be

$$(\text{denominator})_H = (E) + (ES_w)_H + (EO_2S_w)_H + (EOS_w)_H + (EP_w)_H + (ES_x)_H + (EO_2S_x)_H + (EOS_x)_H + (EP_x)_H$$

and for the deuterated substrate will be

$$(\text{denominator})_D = (E) + (ES_w)_D + (EO_2S_w)_D + (EOS_w)_D + (EP_w)_D + (ES_x)_D + (EO_2S_x)_D + (EOS_x)_D + (EP_x)_D$$

The equations for each of the nondeuterated forms in the noncompetitive experiment may be derived from the equations

Table 3: Nondissociative Mechanisms: Direction of Isotope Effects for Various Parameters[a]

| row no. | ratio or parameter | general (A) | rapidly interconvertible (B) |
|---|---|---|---|
| | Competitive Experiments[b] | | |
| | plots: | linear | linear |
| 1 | $\frac{(v_{pw})_H/(v_{px})_H}{(v_{pw})_D/(v_{px})_D}$ | normal[B,c] | intrinsic[F,f] |
| 2 | $(v_{pw})_H/(v_{pw})_D$ | normal[C,c] | normal[G,c] |
| 3 | $(v_{px})_H/(v_{px})_D$ | <1.0 to 1.0[C,f] | inverse[H,d] |
| 4 | $(v_{pw+px})_H/(v_{pw+px})_D$ | normal[E,h] | normal[I,h] |
| | Noncompetitive Experiments | | |
| | plots: | linear | linear |
| 5 | $\frac{(v_{pw})_H/(v_{px})_H}{(x_{pw})_D/(v_{px})_D}$ | normal[B,c] | intrinsic[F,f] |
| 6 | $\frac{(V_{max,pw}/K_m)_H}{(V_{max,pw}/K_m)_D}$ | normal[C,c] | normal[G,c] |
| 7 | $\frac{(V_{max,px}/K_m)_H}{(V_{max,px}/K_m)_D}$ | <1.0 to 1.0[D,g] | inverse[H,d] |
| 8 | $\frac{(V_{max,(pw+px)}/K_m)_H}{(V_{max,(pw+px)}/K_m)_D}$ | normal[E,h] | normal[I,h] |
| 9 | $(V_{max,pw})_H/(V_{max,pw})_D$ | normal[c] | normal[c] |
| 10 | $(V_{max,px})_H/(V_{maxpx})_D$ | <1.0 to >1.0[e,i] | <1.0 to >1.0[e,k] |
| 11 | $\frac{(V_{max,(pw+px)})_H}{(V_{max,(pw+px)})_D}$ | <1.0 to >1.0[e,j] | <1.0 to >1.0[e,l] |

[a] In a normal observed isotope effect the value of (parameter)$_H$/(parameter)$_D$ is greater than 1.0. In an inverse isotope effect the value of (parameter)$_H$/(parameter)$_D$ is less than 1.0. An intrinsic isotope effect for the (parameter)$_H$/(parameter)$_D$ equals $k_{46H}/k_{46D}$. Superscript capital letters identify corresponding identical equations. [b] $S_H = S_D$. [c] Case 2. [d] Case 3. [e] Case 4. [f] Case 5. [g] Case 4. Subtraction of the denominator from the numerator reveals that the denominator will always be larger when $k_{w4x4}$ and $k_{x4w4}$ are significant. Thus, the isotope effect will always be either 1.0 or inverse. [h] Case 4. Subtraction of the denominator from the numerator, however, gives an equation which will always be positive, and therefore, the isotope effect will always be normal when either ($EOS_w$) or ($EOS_x$) is reduced to (ES) and water. The isotope effect will be 1.0 when neither of these intermediates is reduced. [i] Case 4. Subtraction of the equation for the $(V_{max,px})_D$ from that for $(V_{max,px})_H$ reveals that if virtually all of the enzyme existed as ($EP_w$), the isotope effect would be inverse, whereas if it existed as ($EOS_w$), the isotope effect would be normal. If virtually all of the enzyme existed as ($ES_w$) and/or ($EO_2S_w$), the direction of the isotope effect depends on whether ($EOS_w$) is reduced to ($ES_w$) and water; if it is not, the isotope effect will be inverse, but if it is, the isotope effect could be normal. If virtually all of the enzyme existed as ($ES_x$) and/or ($EO_2S_x$), the isotope effect would be inverse. But if virtually all of the enzyme existed as either ($EOS_x$) or ($EP_x$), the isotope effect would approach 1.0. [j] Case 4. Subtraction of the equation for the $(V_{max,(pw+px)})_D$ from that for $(V_{max,(pw+px)})_H$ reveals that if the predominant form is ($EP_w$), the isotope effect will be inverse, but if the other intermediate forms predominate, the isotope effect will be normal. ($ES_w$), ($EO_2S$), ($ES_x$), and ($EO_2S$), however, will contribute to a normal isotope effect only when either $k_{w42}$ or $k_{x42}$ is significant; otherwise, they will contribute only to the masking term. [k] Case 4. Subtraction of the equation for the deuterated substrate from the equation for the nondeuterated form reveals, however, that the isotope effect will be normal only when ($EOS_w$) is the predominant form. Moreover, ($EOS_x$) and ($EP_x$) will contribute only to the masking term. [l] Case 4. Subtraction of the equation for the deuterated form from that for the nondeuterated form shows that the isotope effect would be inverse only when ($EP_w$) is the predominant form. If the other forms predominate, the isotope effect would be normal, but ($ES_w$), ($EO_2S$), ($ES_x$), and ($EO_2S_x$) will contribute to a normal isotope effect only when either $k_{w42}$ or $k_{x42}$ is significant.

Table 4: General Dissociative Mechanisms, Competitive Experiment: Steady-State Solutions for the Concentrations of the Intermediate Forms[a,b]

$(E)_{H,D} = k_{w21}\{[S_D(\mathbf{W2_d})k_{w46D} + S_H(\mathbf{Y2_d})k_{w46H}](W1)A_{(E)}1)\ [S_D(\mathbf{W2_d}) + S_H(\mathbf{Y2_d})]k_{w42}(A_{(E)}1) + (S_H + S_D)(\mathbf{W2_d})(\mathbf{Y2_d})(A_{(E)}2)\}$

$(EO)_{H,D} = k_{w21}\{[S_D(\mathbf{W2_d}) + S_H(\mathbf{Y2_d})](A_{(EO)}1) + (S_D + S_H)(\mathbf{W2_d})(\mathbf{Y2_d})(A_{(EO)}2)\}$

$(ES_w)_H = k_{w21}S_H\{S_D(A_{(Esw)}4)(k_{w46H}k_{w46D}W1 + k_{w45}k_{w46D}W1 + k_{w42}k_{w46H}) + (\mathbf{Y2_d})(S_H + S_D)[(A_{(Esw)}2)(\mathbf{W3_d}) + A_{(ESw)}3] + [S_Hk_{w46H} + k_{w42}(S_H + S_D)](\mathbf{Y2_d})(A_{(ESw)}1)\}$

$(ES_w)_D = k_{w21}S_D\{S_H(A_{(ESw)}4)(k_{w46H}k_{w46D}W1 + k_{w45}k_{w46H}W1 + k_{w42}k_{w46D}) + (\mathbf{W2_d})(S_H + S_D)[(A_{(ESw)}2)(\mathbf{Y3_d}) + A_{(ESw)}3] + [S_Dk_{w46D} + k_{w42}(S_H + S_D)](\mathbf{W2_d})(A_{(ESw)}1)\}$

$(ES_x)_H = S_Hk_{w21}[(A_{(ESx)}1)(W1)(S_Hk_{w46H}\mathbf{Y2_d} + S_Dk_{w46D}\mathbf{W2_d}) + (\mathbf{W2_d})(\mathbf{Y2_d})(S_H + S_D)(A_{(ESx)}2] + [(A_{(ESx)}1)k_{w42} + A_{(ESx)}3](S_H\mathbf{Y2_d} + S_D\mathbf{W2_d})$

$(ES_x)_D = S_Dk_{w21}[(A_{(ESx)}1)(W1)(S_Hk_{w46H}\mathbf{Y2_d} + S_Dk_{w46D}\mathbf{W2_d}) + (\mathbf{W2_d})(\mathbf{Y2_d})(S_H + S_D)A_{(ESx)}2] + [(A_{(ESx)}1)k_{w42} + A_{(ESx)}3](S_H\mathbf{Y2_d} + S_D\mathbf{W2_d})$

$(EO_2S_w)_H = (ES_w)_H[k_{w23}/(k_{w32} + k_{w34})]$

$(EO_2S_x)_H = (ES_x)_H[k_{x23}/(k_{x32} + k_{x34})]$

$(EO_2S_w)_D = (ES_w)_D[k_{w23}/(k_{w32} + k_{w34})]$

$(EO_2S_x)_D = (ES_x)_D[k_{x23}/(k_{x32} + k_{x34})]$

$(EOS_w)_H = S_H(\mathbf{Y2_d})k_{w21}(A_{(EOSw)}1)(S_H + S_D)$

$(EOS_w)_D = S_D(\mathbf{W2_d})k_{w21}(A_{(EOSw)}1)(S_H + S_D)$

$(EOS_x)_H = S_Hk_{w21}[(A_{(EOSx)}1)(S_Hk_{w46H}\mathbf{Y2_d} + S_Dk_{w46D}\mathbf{W2_d}) + (A_{(EOSx)}2)(S_H\mathbf{Y2_d} + S_D\mathbf{W2_d}) + (A_{(EOSx)}3)(S_H + S_D)(\mathbf{W2_d})(\mathbf{Y2_d})]$

$(EOS_x)_D = S_Dk_{w21}[(A_{(EOSx)}1)(S_Hk_{w46H}\mathbf{Y2_d} + S_Dk_{w46D}\mathbf{W2_d}) + (A_{(EOSx)}2)(S_H\mathbf{Y2_d} + S_D\mathbf{W2_d}) + (A_{(EOSx)}3)(S_H + S_D)(\mathbf{W2_d})(\mathbf{Y2_d})]$

$(EP_w)_H = (EOS_w)_H(k_{w46H}/k_{w61})$

$(EP_x)_H = (EOS_x)_H(k_{x46}/k_{x61})$

$(EP_w)_D = (EOS_w)_D(k_{w46D}/k_{w61})$

$(EP_x)_D = (EOS_x)_D(k_{x46}/k_{x61})$

[a] All equations have been divided by $(k_{w32} + k_{w34})$, $(k_{x32} + k_{x64})$, $k_{w61}$, $k_{x61}$, and $X2_d$. [b] Boldfaced terms contain the isotope-sensitive rate constant, $k_{w46}$.

for the analogous forms in the competitive experiment (Table 1) by setting the $S_D$ to 0, and dividing the equation by $Y5_n$. Similarly, the equations for each of the forms of the enzyme with the deuterated form may be derived from the equations for the corresponding forms in the competitive experiment by setting the $S_H$ to 0 and dividing them by $W5_n$.

*(B) Rapidly Interconvertible Nondissociative Mechanism (Branched Mechanism).* In this mechanism the rates at which ($EOS_w$) and ($EOS_x$) are interconvertible are much greater than the rates at which they are reduced to ($ES_w$) and ($ES_x$) or converted to ($EP_w$) and ($EP_x$), i.e., $k_{w4x4} >> (k_{w46} + k_{w42})$ and $k_{x4w4} >> (k_{x46} + k_{x42})$. The equations for this model will be the same as those shown in Table 1 for the general mechanism, but the definitions of the terms are those shown in Table 2B.

*(C) Properties of the Nondissociative Mechanisms.* Substitution of the steady-state concentrations of the various intermediates into eq 1 reveals that the rate equations for nondissociative model may be rearranged to a Michaelis–Menten form. Lineweaver–Burk plots, as well as other graphs based on rearrangements of the Michaelis–Menten equation, will be linear (Table 3). Substitution of the equations shown in Table 1 into eqs 2n, 3n, and 5–8, as appropriate, leads to several conclusions (Table 3). The equations for the ratio of the ratios of the metabolites, $(P_w/P_x)_H/(P_w/P_x)_D$, formed in the competitive and noncompetitive experiments are identical (cf. Table 3, rows 1 with 5). The isotope effect on the $(P_w/P_x)_H/(P_w/P_x)_D$ will be normal; in the rapidly interconvertible (branched-chain) mechanism the value will be the intrinsic isotope effect. Moreover, the equations for the ratio of the metabolites formed in the competitive experiment are identical to the corresponding equations for the $(V_{max}/K_m)_H/(V_{max}/K_m)_D$ in the noncompetitive experiments (cf. Table 3, rows 2 with 6, 3 with 7, and 4 with 8). The $(V_{max}/K_m)_H/(V_{max}/K_m)_D$ will be normal for the $P_w$. It may also be normal for the total metabolism of the substrate, $P_w + P_x$, but only when ($EOS_w$) or ($EOS_x$) is reduced to (ES) and water. The $(V_{max}/K_m)_H/(V_{max}/K_m)_D$ for $P_x$ will be inverse, but will approach 1.0 when $k_{w4x4}$ and $k_{x4w4}$ are small. By contrast, the isotope effects on $V_{max}$ will be normal for $P_w$, but may be either normal or inverse for $P_x$ or for the sum, $P_w + P_x$.

*Dissociative Mechanisms. (A) General Mechanisms.* In the competitive experiment (Figure 4), the following inter-

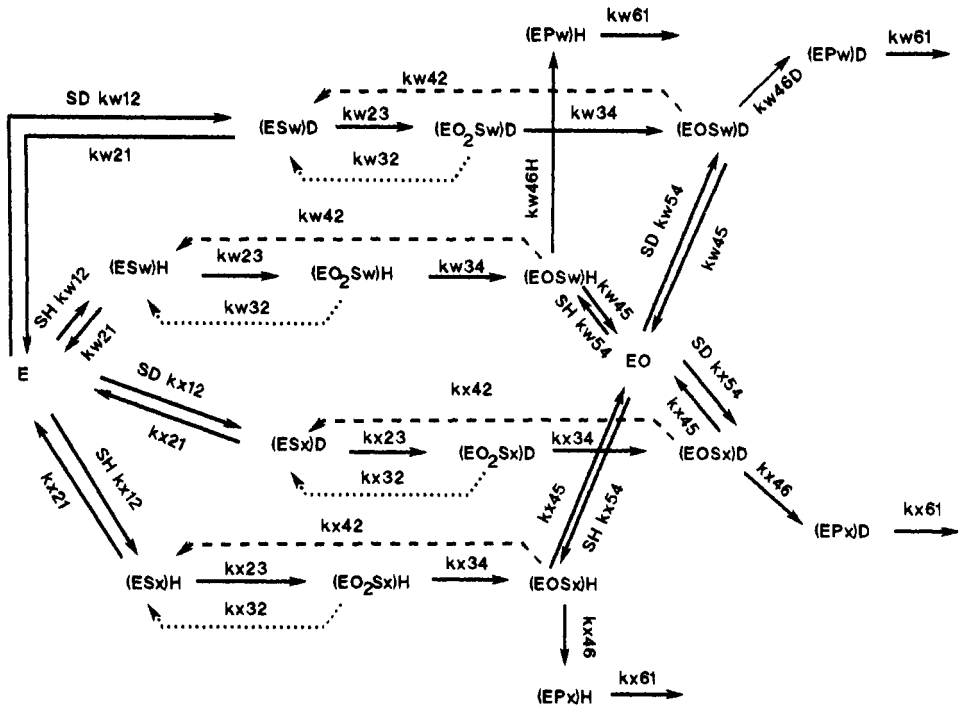


FIGURE 4: The dissociative mechanisms in competitive experiments. The rate constants shown in the figure have been used in the derivation of the equations in Tables 4–6.

Table 5: Dissociative Mechanism: Definitions[a]

Part A: General Dissociative Mechanism

| | |
|---|---|
| $F_{w34} = k_{w34}/(k_{w32} + k_{w34})$ | $F_{x34} = k_{x34}/(k_{x32} + k_{x34})$ |
| $W1 = (F_{w34}k_{w23}/k_{w21}) + 1$ | $X1 = (F_{x34}k_{x23}/k_{x21}) + 1$ |
| $W12 = k_{w23}F_{w34}k_{x21}k_{w12}$ | $X12 = k_{w21}k_{x23}F_{x34}k_{x12}$ |
| $\mathbf{W2_d} = (k_{w45} + \mathbf{k_{w46H}})W1 + k_{w42}$ | $X2_d = (k_{x45} + k_{x46})X1 + k_{x42}$ |
| $\mathbf{W3_d} = k_{w45} + \mathbf{k_{w46H}} + k_{w42}$ | $X3_d = k_{x45} + k_{x46} + k_{x42}$ |
| $\mathbf{Y2_d} = (\mathbf{k_{w46D}} + k_{w45})(W1) + k_{w42}$ | |
| $\mathbf{Y3_d} = \mathbf{k_{w46D}} + k_{w45} + k_{w42}$ | |
| $A_{(E)}1 = k_{w21}k_{x21}k_{w54}$ | $A_{(ESx)}1 = k_{w21}k_{x12}(X3_d)k_{w54}$ |
| $A_{(E)}2 = k_{w21}k_{x21}k_{w54}(k_{x46}X1 + k_{x42})$ | $A_{(ESx)}2 = k_{w21}k_{x12}(k_{x46} + k_{x42})k_{x54}$ |
| $A_{(EO)}1 = k_{x21}k_{w12}k_{w23}F_{w34}k_{w45}X2_d$ | $A_{(ESx)}3 = k_{w12}k_{w23}F_{w34}k_{w45}k_{x42}k_{x54}$ |
| $A_{(EO)}2 = k_{w21}k_{x12}k_{x23}F_{x34}k_{x45}$ | $A_{(EOSx)}1 = k_{w54}(W1)(X12)$ |
| $A_{(ESw)}1 = k_{x21}k_{w12}(X2_d)k_{w54}$ | $A_{(EOSx)}2 = k_{w42}k_{w54}(X12) + k_{w45}k_{x54}(W12)(X1)$ |
| $A_{(ESw)}2 = k_{x21}k_{w12}(k_{x46}X1 + k_{x42})k_{w54}$ | $A_{(EOSx)}3 = k_{x54}(X12)$ |
| $A_{(ESw)}3 = k_{w12}k_{w23}F_{w34}k_{x45}k_{w42}k_{w54}$ | |
| $A_{(ESw)}4 = k_{x21}k_{w12}k_{w54}X2_d$ | |
| $A_{(EOSw)}1 = k_{x45}k_{w54}(W1)(X12) + k_{x54}(W12)(k_{x46}X1 + k_{x42}) + k_{w54}(W12)(X2)$ | |

Part B: Rapidly Equilibrated Dissociative Model[b]

| | |
|---|---|
| $W2_d = Y2_d = k_{w45}W1$ | $X2_d = k_{x46}X1$ |
| $W3_d = Y3_d = k_{w45}$ | $X3_d = k_{x45}$ |

[a] Boldfaced terms contain the isotope-sensitive rate constant, $k_{w46}$. [b] All other definitions are the same as those shown in part A.

mediate forms will be present in the denominator of eq 1:

$$\begin{aligned}(\text{denominator})_{H,D} = {} & (E)_{H,D} + (EO)_{H,D} + (ES_w)_H + \\ & (EO_2S_w)_H + (EOS_w)_H + (EP_w)_H + (ES_x)_H + (EO_2S_x)_H + \\ & (EOS_x)_H + (EP_x)_H + (ES_w)_D + (EO_2S_w)_D + (EOS_w)_D + \\ & (EP_w)_D + (ES_x)_D + (EO_2S_x)_D + (EOS_x)_D + (EP_x)_D\end{aligned}$$

The equations for the steady-state concentrations of these intermediate forms are given in Table 4, and the definitions of the general mechanism-dependent terms are shown in Table 5A.

Appropriate substitution of these equations into eq 1 for either $(P_w)_H$ or $(P_x)_H$ leads to the following general form:

$$v = \frac{(AS_H + BS_D)S_H}{(C + DS_D + ES_H)S_H + (F + GS_D + HS_H)S_D}$$

This equation cannot be rearranged to a simple Michaelis–Menten equation, and therefore, Lineweaver–Burk and other plots based on rearrangements of the Michaelis–Menten equation will be curved (Table 6).

In noncompetitive experiments (Figure 2), the denominator of eq 1 for the nondeuterated substrate will be

$$\begin{aligned}(\text{denominator})_H = {} & (E)_H + (EO)_H + (ES_w)_H + \\ & (EO_2S_w)_H + (EOS_w)_H + (EP_w)_H + (ES_x)_H + \\ & (EO_2S_x)_H + (EOS_x)_H + (EP_x)_H\end{aligned}$$

whereas that for the deuterated substrate will be

$$\begin{aligned}(\text{denominator})_D = {} & (E)_D + (EO)_D + (ES_w)_D + \\ & (EO_2S_w)_D + (EOS_w)_D + (EP_w)_D + (ES_x)_D + \\ & (EO_2S_x)_D + (EOS_x)_D + (EP_x)_D\end{aligned}$$

The equations for the forms of the enzymes for the noncompetitive experiment with the nondeuterated substrate may be derived from the equations for the corresponding forms in the competitive experiment, shown in Table 4, by setting $S_D$ to 0 and dividing the resulting equations by $S_H$. Similarly, the equations for the forms of the enzyme for the noncompetitive experiment with the deuterated form may be derived from the equations for the corresponding forms in the competitive experiment by setting the concentration of $S_H$ to 0, and dividing the resulting equations by $S_D$.

Appropriate substitution of these equations into eq 1 leads to equations that can be rearranged to Michaelis–Menten forms and therefore can give linear Lineweaver–Burk plots. It should be pointed out, however, that the validity of these considerations is based on the assumption that (EO) is not reduced to (E) and water; if it were, the equation would comprise quadratic equations in both the numerator and the

Table 6: Dissociative Mechanisms: Direction of Isotope Effects of Various Parameters[a]

| row no. | ratio or parameter | general | rapid equilibrium |
|---|---|---|---|
| | Competitive Experiments[b] | | |
| | plots: | nonlinear | nonlinear |
| 1 | $\frac{(v_{pw})_H/(v_{px})_H}{(v_{pw})_D/(v_{px})_D}$ | normal[B,c] | intrinsic[C,f] |
| 2 | $(v_{pw})_H/(v_{pw})_D$ | normal[B,c] | intrinsic[C,f] |
| 3 | $(v_{px})_H/(v_{px})_D$ | 1.0[g] | 1.0[g] |
| 4 | $(v_{w+x})_H/(v_{w+x})_D$ | normal[h] | normal[h] |
| | Noncompetitive Experiments | | |
| | plots: | linear[i] | linear[i] |
| 5 | $\frac{(v_{pw})_H/(v_{px})_H}{(v_{pw})_D/(v_{px})_D}$ | normal[e] | normal[e] |
| 6 | $\frac{(V_{max,pw}/K_m)_H}{(V_{max,pw}/K_m)_D}$ | normal[e] | normal[e] |
| 7 | $\frac{(V_{max,px}/K_m)_H}{(V_{max,px}/K_m)_D}$ | <1.0 to >1.0[j] | <1.0 to >1.0[j] |
| 8 | $\frac{(V_{max,(pw+px)}/K_m)_H}{(V_{max,(Pw+px)}/K_m)_D}$ | normal[k] | normal[k] |
| 9 | $(V_{max,pw})_H/(V_{max,pw})_D$ | normal[e] | normal[e] |
| 10 | $(V_{max,px})_H/(V_{max,px})_D$ | <1.0 to >1.0[j] | <1.0 to >1.0[j] |
| 11 | $\frac{(V_{max,(pw+px)})_H}{(V_{max,(pw+px)})_D}$ | <1.0 to >1.0[m] | <1.0 to >1.0[m] |

[a] In a normal observed isotope effect the value of $(\text{parameter})_H/(\text{parameter})_D$ is greater than 1.0. An intrinsic isotope effect for $(\text{parameter})_H/(\text{parameter})_D$ equals $k_{46H}/k_{46D}$. Superscript capital letters identify corresponding identical equations. [b] $S_H = S_D$. [c] Case 2. [d] Case 3. [e] Case 4. [f] Case 5. [g] Case 1. [h] Case 4. Subtraction of the rate of metabolism of the deuterated substrate from that of the noneduterated substrate reveals that the isotope effect would be normal. [i] If (EO) is reduced to water, the plots will be nonlinear. [j] Case 4. Subtractions of the equation for the deuterated substrate from that for the nondeuterated substrate reveals that the direction of the isotope effect will depend on the direction of the following equation: $(V_{max,px}/K_m)_H - (V_{max,px}/K_m)_D = (C)\{(k_{x54}/k_{x12})[k_{x21}/(k_{x23}F_{x34}) + 1] - 1\}(k_{w46D} - k_{w46H})$, where $C$ is a set of terms containing isotope-insensitive rate constants. Values >0.0 will be normal, and values <0.0 will be inverse. Inspection reveals that the direction of the isotope effect is governed largely on the ratios of $k_{x54}/k_{x12}$ and $k_{x21}/k_{x23}$ and the values of $k_{w46D}$ and $k_{w46H}$. Since $k_{w46D} < k_{w46H}$, the isotope effect would be inverse if $k_{x54} = k_{x12}$ and would be normal if $k_{x54}$ were zero. It would also be inverse if $(k_{x54}/k_{x12})[k_{x21}/(k_{x23}F_{x34})]$ were >1.0 even when $k_{x54} < k_{x12}$; in most cases this is likely, because the introduction of the first electron, represented by $k_{x23}$, is nearly always slow compared to the dissociation of the $(ES_x)$ complex to (E) and substrate, represented by $k_{x21}$; the intermediates in the formation of estrogens from androgens by aromatase, however, would be exceptions. [k] Case 4. Subtraction of the equation for $V_{max}/K_m$ for the total metabolism of the deuterated substrate reveals that the isotope effect will always be normal. If (E) ≫ (EO) and both $(EOS_w)$ and $(EOS_x)$ were not reduced to water, however, the isotope effect would approach 1.0. [l] Subtraction of the equation for the $V_{max}$ for $P_x$ formed from the deuterated substrate from that formed from the nondeuterated substrate reveals that $(ES_x)$, $(EO_2S_x)$, and $(EP_w)$ terms will contribute to an inverse isotope effect. $(EOS_w)$ terms will contribute to a normal isotope effect. $(EOS_x)$ and $(EP_x)$ terms will not affect the direction of the isotope effect but will contribute to the masking term. However, the $(ES_w)$ and $(EO_2S_w)$ terms may contribute to either a normal or an inverse isotope effect, but will be inverse if $k_{w42}$ were zero. [m] Case 4. Subtraction of the equation for the $V_{max}$ for the total metabolism of the deuterated substrate from that of the nondeuterated substrate reveals that the $(ES_w)$, $(EO_2S_w)$, $(EOS_w)$, $(ES_x)$, $(EO_2S_x)$, $(EOS_x)$, and $(EP_x)$ terms will contribute to a normal isotope effect, but $(EP_w)$ will contribute to an inverse isotope effect.

denominator (derivation not shown), and nonlinear Lineweaver–Burk plots would occur.

*(B) Rapid Equilibrium Mechanism.* In this mechanism, the rate constants for the dissociation of $(EOS_w)$ and $(EOS_x)$ are assumed to be very much larger than the rate constants for their conversion to $(EP_w)$ and $(EP_x)$ and their reduction to $(ES_w)$ and $(ES_x)$, i.e., $k_{w45} >> (k_{w46} + k_{w42})$ and $k_{x45} >> (k_{x46} + k_{x42})$. These modifications of the definition of the mechanism-dependent terms, shown in Table 5B, are then substituted into the equations for the steady-state concentrations of the various intermediates shown in Table 4.

Table 7: Parallel Pathway Mechanism, Competitive Experiment: Steady-State Solutions for the Intermediate Forms of the Enzyme[a,b]

$$(E) = (\mathbf{W5_{pp}})(\mathbf{Y5_{pp}})$$
$$(ES_w)_H = S_H(\mathbf{Y5_{pp}})k_{w12}k_{x21}(\boldsymbol{k_{w46H}} + k_{w42})X2_{pp}$$
$$(EO_2S_w)_H = (ES_w)_H k_{w23}/(k_{w32} + k_{w34})$$
$$(EOS_w)_H = S_H(\mathbf{Y5_{pp}})A_{(EOSw)}1$$
$$(EP_2)_H = (EOS_w)_H \boldsymbol{k_{w46H}}/k_{w61}$$
$$(ES_x)_H = S_H(\mathbf{Y5_{pp}})k_{w21}k_{x12}(\boldsymbol{k_{w46H}}W1 \;\; k_{w42})X3_{pp}$$
$$(EO_2S_x)_H = (ES_x)_H k_{w23}/(k_{w32} + k_{w34})$$
$$(EOS_x)_H = S_H(\mathbf{Y5_{pp}})F_{x34}k_{w21}k_{x12}k_{x23}\mathbf{W2_{pp}}$$
$$(EP_x)_H = (EOS_x)_H k_{x46}/k_{x61}$$
$$(ES_w)_D = S_D(\mathbf{W5_{pp}})k_{w12}k_{x21}(\boldsymbol{k_{w46D}} + k_{w42})X2_{pp}$$
$$(EO_2S_w)_D = (ES_w)_D k_{w23}/(k_{w32} + k_{w34})$$
$$(EOS_w)_D = S_D(\mathbf{W5_{pp}})A_{(EOSw)}1$$
$$(EP_w)_D = (EOS_w)_D \boldsymbol{k_{w46D}}/k_{w61}$$
$$(ES_x)_D = S_D(\mathbf{W5_{pp}})k_{w21}k_{x12}(\boldsymbol{k_{w46D}}W1 + k_{w42})X3_{pp}$$
$$(EO_2S_x)_D = (ES_x)_D k_{x23}/(k_{x32} + k_{x34})$$
$$(EOS_x)_D = S_D(\mathbf{W5_{pp}})F_{x34}k_{w21}k_{x12}k_{x23}\mathbf{Y2_{pp}}$$
$$(EP_x)_D = (EOS_x)_D k_{x46}/k_{x61}$$

[a] All of the equations have been divided by $(k_{w32} + k_{w34})$, $k_{w61}$, $(k_{x32} + k_{x34})$, and $k_{x61}$. [b] Boldfaced terms contain the isotope-sensitive rate constant, $k_{w46}$.

*(C) Properties of the Dissociative Mechanisms.* Appropriate substitutions of the equations into eqs 2d–4d and 5–8 lead to several conclusions (Table 6). In competitive experiments, the ratio of the ratios of the metabolites, $(P_w/P_x)_H/(P_w/P_x)_D$, will be normal and will approach the intrinsic isotope effect if the system approaches the rapid equilibrium mechanism (Table 6, row 1). Moreover, the equation for the ratio, $(P_w/P_x)_H/(P_w/P_x)_D$, will be identical to that for the ratio, $(P_w)_H/(P_w)_D$ (cf. Table 6, rows 1 and 2). This follows because $(P_x)_H/(P_x)_D$ will be 1.0 (Table 6, row 3). Appropriate substitutions into eqs 2d–3d also reveal that in noncompetitive experiments the isotope effect on the ratio of the ratios of the metabolites, $(P_w/P_x)_H/(P_w/P_x)_D$, and on the $(V_{max}/K_m)_H/(V_{max}/K_m)_D$ for $P_w$ and for the sum, $P_w + P_x$, will be normal (Table 6, rows 5, 6, and 8). But the equations for the isotope effects on these parameters will not be identical to those for the corresponding ratio of metabolites obtained in the competitive experiment (cf. Table 6, rows 1 with 5, 2 with 6, and 4 with 8). Moreover, the $(V_{max}/K_m)_H/(V_{max}/K_m)_D$ for $P_x$ may be either inverse or normal (Table 6, row 7), depending on the relative values of the rate constants in the nondeuterium abstraction pathway (Table 6, footnote *j*). The isotope effects on the $V_{max}$ for $P_w$ will be normal (Table 6, row 9), but the isotope effects on $P_x$ or the sum, $P_w + P_x$, may be either normal or inverse (Table 6, rows 10 and 11).

*Parallel Pathway Mechanism.* In this mechanism, the $(EOS_w)$ and the $(EOS_x)$ complexes are so rigid that there is neither interconversion between them nor dissociation to (EO) and the substrate. The mechanism may thus be viewed as a special case of either the nondissociative model, in which $k_{w4x4}$ and $k_{x4w4}$ are zero, or the dissociative mechanism, in which both $k_{w54}$ and $k_{x54}$ are zero. This mechanism thus has characteristics of both general mechanisms. Because (EO) is not present, the intermediate forms of the enzyme will be the same as those for the nondissociative mechanism, but the equations which govern their concentrations will be different.

Substitution of the mechanism-dependent definitions of the terms shown in Table 8 into the equations for the steady-state concentrations of the various intermediates (Table 7) leads to the conclusions shown in Table 9. Substitution into eq 1

Table 8: Parallel Pathway Mechanism: Definitions[a]

| | |
|---|---|
| $F_{w34} = k_{w34}/(k_{w32} + k_{w34})$ | $F_{x34} = k_{x34}/(k_{x32} + k_{x34})$ |
| $W1 = (F_{w34}k_{w23}/k_{w21}) + 1$ | $X1 = (F_{x34}k_{x23}/k_{x21}) + 1$ |
| $\mathbf{W2_{pp}} = \mathbf{k_{w46H}}W1 + k_{w42}$ | $X2_{pp} = k_{x46}X1 + k_{x42}$ |
| $\mathbf{W3_{pp}} = \mathbf{k_{w46H}} + k_{w42}$ | $X3_{pp} = k_{x46} + k_{x42}$ |
| $\mathbf{Y2_{pp}} = \mathbf{k_{w46D}}W1 + k_{w42}$ | |
| $\mathbf{Y3_{pp}} = \mathbf{k_{w46D}} + k_{w42}$ | |
| $\mathbf{W5_{pp}} = k_{w21}k_{x21}(\mathbf{W2_{pp}})(k_{x46}X1 + k_{x42})$ | |
| $\mathbf{Y5_{pp}} = k_{w21}k_{x21}(k_{x46}X1 + k_{x42})\mathbf{Y2_{pp}}$ | |
| $A_{(E)Sw)}1 = F_{w34}k_{w12}k_{w23}k_{x21}X2_{pp}$ | |

[a] Boldfaced terms contain the isotope-sensitive rate constant, $k_{w46}$.

Table 9: Parallel Pathway Mechanism: Direction of Isotope Effects for Various Parameters[a]

| row no. | ratio or parameter | parallel pathway (B) |
|---|---|---|
| | Competitive Experiments[b] | |
| | plots: | linear |
| 1 | $\frac{(v_{pw})_H/(v_{px})_H}{(v_{pw})_D/(v_{px})_D}$ | normal[B,g] |
| 2 | $(v_{pw})_H/(v_{pw})_D$ | normal[B,g] |
| 3 | $(v_{px})_H/(v_{px})_D$ | 1.0[C,h] |
| 4 | $(v_{w+x})_H/(v_{w+x})_D$ | normal[D,g] |
| | Noncompetitive Experiments | |
| | plots: | linear |
| 5 | $[(v_{pw})_H/(v_{px})_H]/[(v_{pw})_D/(v_{px})_D]$ | normal[B,g] |
| 6 | $(V_{max,pw}/K_m)_H/(V_{max,pw}/K_m)_D$ | normal[B,g] |
| 7 | $(V_{max,px}/K_m)_H/(V_{max,px}/K_m)_D$ | 1.0[C,h] |
| 8 | $(V_{max,(pw+px)}/K_m)_H/(V_{max,(pw+px)}/K_m)_D$ | normal[D,g] |
| 9 | $(V_{max,pw})_H/(V_{max,pw})_D$ | normal[c] |
| 10 | $(V_{max,px})_H/(V_{max,px})_D$ | <1.0 to >1.0[e,i] |
| 11 | $(V_{max,(pw+px)})_H/(V_{max,(pw+px)})_D$ | <1.0 to >1.0[e,j] |

[a] In a normal observed isotope effect the value of (parameter)$_H$/(parameter)$_D$ is greater than 1.0. Superscript capital letters identify corresponding identical equations. [b] $S_H = S_D$. [c] Case 2. [d] Case 3. [e] Case 4. [f] Case 5. [g] Case 2. The isotope effect will be normal, but only when $k_{w42}$ is significant. [h] Case 1. [i] Subtraction of the equation for the deuterated substrate from that for the nondeuterated substrate reveals, however, that the isotope effect would be inverse, only if virtually all of the enzyme existed as ($EP_w$) and $k_{w42}$ were significant. If nearly all of its existed as ($EOS_w$), the isotope effect would be normal. But ($ES_w$) and ($EO_2S_w$) would contribute to normal isotope effect only if $k_{w42}$ is significant. Otherwise, they would contribute to the masking term. If virtually all of the enzyme existed as ($ES_x$), ($EO_2S_x$), ($EOS_x$), or ($EP_x$), the isotope effect would be 1.0. [j] Case 4. Subtraction of the equation for the deuterated form from that for the nondeuterated form shows that the isotope effect will be inverse only when ($EP_w$) is the predominant form and $k_{w42}$ is significant. If the other intermediate forms are predominant, the isotope effect will be normal, but ($ES_w$), ($EO_2S$), ($ES_x$), ($EO_2S_x$), and ($EP_x$) will contribute to a normal isotope effect only when $k_{w42}$ is significant.

reveals that the rate equations for both the competitive and the noncompetitive experiment may be rearranged to a Michaelis–Menten form; Lineweaver–Burk and similar plots thus will be linear (Table 9). There will be no isotope effect on either the $(v_{px})_H/(v_{px})_D$ in the competitive experiment (Table 9, row 3) or the $(V_{max,px}/K_m)_H/(V_{max,px}/K_m)_D$ in the noncompetitive experiment (Table 9, row 7); i.e., both will be 1.0. Moreover, the equation for the isotope effects for $(v_{pw})_H/(v_{pw})_D$ in the competitive experiment (Table 9, row 2) will be identical to the equation for the $(V_{max,pw}/K_m)_H/(V_{max,pw}/K_m)_D$ in the noncompetitive experiment (Table 9, row 6) and will be >1.0, but only when $k_{w42}$ is significant (Table 9, footnote *g*). Consequently, the equation for the isotope effects on the ratio of metabolites, $(v_{px})/(v_{pw})$ in the competitive experiment is identical to the equation for the $(V_{max,pw}/K_m)/(V_{max,px}/K_m)$ in the noncompetitive experiment and will be >1.0 when $k_{w42}$ is significant (Table 9, footnote *g*; cf. Table 9, row 1 with row 5, and row 4 with row 8).

The $(V_{max})_H/(V_{max})_D$ for $P_w$ will be normal (Table 9, row 5), but the $(V_{max})_H/(V_{max})_D$ for $P_x$ and for the sum, $P_w + P_x$, may be either normal or inverse (Table 9, rows 10 and 11).

## DISCUSSION

The principal objective of the present study was to provide insights into various kinds of preabstraction kinetic mechanisms. Inspection of the various equations for the different mechanisms reveals that the magnitude of the rate constant for the abstraction of a deuterium from a deuterated substrate, $k_{w46}$, relative to the magnitudes of the other rate constants which emanate from the immediate precursor, ($EOS_w$), is key to the different mechanisms. If $k_{w46}$ is at least 2 orders of magnitude greater than $k_{w42}$, $k_{w4x4}$, and $k_{w45}$, the value of $(V_{max}/K_m)_H/(V_{max}/K_m)_D$ in noncompetitive experiments and the ratio of $(v_{pw})_H/(v_{pw})_D$ in competitive experiments for the metabolite formed by the deuterium abstraction pathway will approach 1.0. In this case, the parallel pathway mechanism would be applicable (Table 9, footnote *g*). The finding that the $(V_{max}/K_m)_H/(V_{max}/K_m)_D$ for the formation of the metabolite by the deuterium abstraction pathway is greater than 1.0 thus implies that the perferryl oxygen species in the ($EOS_w$) complex is rather stable, because the values of $k_{w42}$, $k_{w4x4}$, or $k_{w45}$ must be of the same order of magnitude or greater than the $k_{w46}$ constant. Which of these rate constants approaches or exceeds the $k_{w46}$ rate constant determines the preabstraction kinetic mechanism.

Inspection of Tables 3, 6 and 9 reveals that comparisons of the isotope effects on a metabolite formed by a non-deuterium abstraction pathway provide sufficient information to differentiate between the mechanisms. If both $(V_{max,px}/K_m)_H/(V_{max,px}/K_m)_D$ in noncompetitive experiments and $(v_{px})_H/(v_{px})_D$ in competitive experiments for the metabolite formed by the non-deuterium abstraction pathway, $P_x$, approach 1.0, the parallel pathway would be applicable (Table 9). In both kinds of experiments, a normal isotope effect on the metabolite formed by the deuterium abstraction pathway, $P_w$, would be caused by a significant reduction of ($EOS_w$) to ($ES_w$) and water; i.e., $k_{w42}$ would approach or exceed $k_{w46}$. Theoretically, if $k_{w42} >> k_{w46}$, the observed isotope effect on the metabolite formed by the deuterium abstraction pathway could approach the intrinsic isotope effect, but the system would be extremely "leaky" and the rate of formation of the metabolite would be small.

If both $(V_{max,px}/K_m)_H/(V_{max,px}/K_m)_D$ in noncompetitive experiments and $(v_{px})_H/(v_{px})_D$ in competitive experiments for the metabolite formed by the nondeuterium abstraction pathway were <1.0, a nondissociative mechanism would be applicable (Table 3). In such cases, the finding that the $(V_{max}/K_m)_H/(V_{max}/K_m)_D$ for the metabolite formed by the deuterium abstraction pathway, $P_w$, was >1.0 would indicate that $k_{w4x4}$ approached or exceeded $k_{w46}$. The reduction of ($EOS_w$) and ($EOS_x$) to their corresponding (ES) complexes and water could also contribute to the unmasking of the isotope effect on the formation of $P_w$, but the extent to which they do so depends on a complex interaction of the various rate constants. A finding that $(V_{max}/K_m)_H/(V_{max}/K_m)_D$ for the sum of the metabolites, $P_w$ and $P_x$, is virtually 1.0 would indicate that there was very little reduction of either ($EOS_w$) or ($EOS_x$) to water and the corresponding (ES) complexes (Table 3, footnote *h*).

When the perferryl oxygens in the ($EOS_w$) and ($EOS_x$) complexes are very long-lived relative to the rates of reorientation of the substrate within the active site, i.e., $k_{w4x4}$ and $k_{x4w4} >> k_{w46}$ and $k_{x46}$, the nondissociative mechanisms

approach the branched-pathway mechanisms described by many other investigators [e.g., Korzekwa et al. (1989b)]. In such cases, the ratio of $(v_{pw}/v_{px})_H/(v_{pw}/v_{px})_D$ obtained in either the noncompetitive experiment or the competitive experiment will approach $k_{w46H}/k_{w46D}$, the intrinsic isotope effect (Table 3, column B, rows 1 and 5).

If $(V_{max,px}/K_m)_H/(V_{max,px}/K_m)_D$ in noncompetitive experiments is not 1.0, but $(v_{px})_H/(v_{px})_D$ in competitive experiments is 1.0, the dissociative mechanisms are applicable (Table 6, row 7). In such cases, the perferryl oxygen in the $(EOS_w)$ complex must be long-lived relative to the rates of dissociation and reassociation of the complex; i.e., $k_{w45}$ and $Sk_{w54}$ must approach or exceed the values of $k_{w46}$. Moreover, for the sake of simplicity, we have assumed that (EO) is not reduced to water, but reacts solely with the substrates to form the various (EOS) complexes. At low concentrations of the substrate, therefore, the enzyme may exist in its activated form, (EO), as well as in its nonactivated form, (E). Since there can be an isotope effect on the steady-state concentrations of both (E) and (EO) (Table 4), the $(V_{max}/K_m)_H/(V_{max}/K_m)_D$ for the metabolite, $P_w$, formed by the deuterium abstraction pathway may be greater than 1.0 even when $(EOS_w)$ is not reduced to $(ES_w)$ and water; i.e., $k_{w42}$ becomes negligible. For the same reason the value of $(V_{max,px}/K_m)_H/(V_{max,px}/K_m)_D$ in noncompetitive experiments may also be greater than 1.0 when the formation of $(EOS_x)$ from (EO) and the substrate is negligible; i.e., $k_{x54}$ approaches zero compared with $k_{x12}$ (Table 6, footnote *j*). However, when $k_{x54}/k_{x12} > 1.0$ the value of $(V_{max,px}/K_m)_H/(V_{max,px}/K_m)_D$ will be less than 1.0. The dissociative mechanism, however, will always give a normal isotope effect on the $(V_{max}/K_m)_H/(V_{max}/K_m)_D$ for the total metabolism of the substrate when neither $(EOS_w)$ nor $(EOS_x)$ is reduced to (ES) and water (Table 6, footnotes *h* and *k*).

When the rate constants for the dissociation of $(EOS_w)$ and $(EOS_x)$ complexes are much larger than the rate constants for the abstraction of the deuterium and hydrogen, i.e., $k_{w45}$ and $k_{x45} >> k_{w46}$ and $k_{x46}$, the system approaches the rapid equilibrium version of the dissociative mechanism. Under these conditions the ratio of the ratio $(v_{pw})_H/(v_{px})_H$ to the ratio $(v_{pw})_D/(v_{px})_D$ will approach $k_{w46H}/k_{w46D}$, the intrinsic isotope effect, in competitive experiments but not in noncompetitive experiments. In noncompetitive experiments the value will be >1.0 but less than the intrinsic isotope effect.

The possibility that cytochrome P450 enzymes may act through the dissociative mechanism raises the possibility of a kind of substrate interaction that can be significant even at low concentrations of the substrates. For example, suppose that an "inhibitor" were able to combine with the P450 and to form (EOI), but is not metabolized. Instead, it dissociates to (EO) and (I), and the (EO) is converted to (EOS). The total rate of metabolism of the substrate thus depends not only on the (E) → $(EO_2S)$ → (EOS) → (EP) pathway but also on the (E) → $(EO_2I)$ → (EOI) → (EO) → (EOS) → (EP) pathway. In this way the "inhibitor" might stimulate the metabolism of the substrate at low concentrations. On the other hand, if (EOI) is more rapidly reduced to water than is (EOS), then low concentrations of the "inhibitor" might inhibit the metabolism of low concentrations of the substrate by increasing the formation of water.

It is theoretically possible to detect a dissociative mechanism by showing that increases in the rate of metabolism with increases in substrate concentration in competitive experiments do not fit the Michaelis–Menten equation. But detection of the difference would require extraordinarily precise data. Indeed, most computer programs for curve fitting of nonlinear systems would fail to detect the lack of conformity to a Michaelis–Menten equation.

Isotope effects on $V_{max}$ for the formation of metabolites by deuterium and non-deuterium abstraction pathways are of no diagnostic value in differentiating between the preabstraction mechanisms. All three mechanisms predict that $(V_{max})_H/(V_{max})_D$ for a metabolite formed by a deuterium abstraction pathway will be greater than 1.0, and all three mechanisms predict that the $(V_{max})_H/(V_{max})_D$ for a metabolite formed by a non-deuterium abstraction pathway may range from <1.0 to >1.0. Nevertheless, studies of the isotope effects are useful for elucidating other aspects of the mechanisms of the enzyme. Some of the effects may be visualized intuitively from the realization that, at any given substrate concentration, the concentration of $(EOS_w)_D$ tends to be greater than that of $(EOS_w)_H$. The observed isotope effect at very high concentrations of the substrate therefore depends on the extent to which $(EOS_w)_H$ represents the total concentration of the enzyme. If $(EOS_w)_H$ represents most of the enzyme, then $(EOS_w)_D$ cannot represent a much greater proportion of the enzyme and $(V_{max})_H/(V_{max})_D$ may approach the intrinsic isotope effect. But if $(EOS_w)_H$ represents a minor fraction of the enzyme at very high concentrations of the substrate, then $(EOS_w)_D$ may achieve greater concentrations at the expense of the concentrations of the other intermediate forms of the enzyme, and $(V_{max})_H/(V_{max})_D$ may be only slightly greater than 1.0. The isotope effect on the $V_{max}$ of a metabolite formed by a non-deuterium abstraction pathway is more difficult to visualize, because the tendency for the concentration of $(EOS_w)_D$ to be greater than that of $(EOS_w)_H$ may result in a tendency for the concentration of $(EOS_x)_D$ to be either greater than or less than that of $(EOS_x)_H$. The footnotes to Tables 3, 6, and 9 provide some insights into the contribution of various intermediate forms of the enzyme to the direction of the isotope effects.

The solutions for the steady-state equations shown in Tables 1, 4, and 7 may be used to obtain the equations for other parameters, such as the apparent $K_m$ values (see eqs 3n and 3d), when appropriate. In addition, the general mechanisms may be modified to include those in which hydrogen peroxide or superoxide anion are not formed by setting $k_{w32}$ and $k_{x32}$ to zero and simplifying the resulting equations. The equations may also provide the basis for describing systems in which the radical formed by the abstraction of a hydrogen may lead to several metabolites. For example, the steady-state concentration of the radical $(EOSrad_w)$ formed by the deuterium abstraction pathway may be represented by $(EOS_w)k_{w47}/(k_{w78} + k_{w79})$, in which $k_{w47}$ is the rate constant for the abstraction of the hydrogen and $k_{w78}$ and $k_{w79}$ are the rate constants for the formation of two metabolites formed by a postabstraction mechanism. The steady-state concentration of the $(EP_{w9})$, the complex between the enzyme and one of these "postabstraction metabolites", may be represented by

$$(EP_{w9}) = (EOS_w)k_{w47}k_{w79}/[(k_{w78} + k_{w79})k_{w91}], \text{ etc.}$$

Substitution of these modifications into eq 1 may then be used to elucidate the effects, if any, on the isotope effects for such mechanisms. The equations thus should be useful as the basis for making many other comparisons.

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