

AUG 22 1973  
50133

A Heuristic Model of the Oxygenation  
of Hydrocarbons by Cytochrome P-450

My  
student  
at Stanford

  
Larry Hjelmeland  

---

Since 1959, a considerable amount of research effort has been directed towards the development of a non-enzymatic model of the monooxygenases. These enzymes are also known as the mixed function oxygenases and their general function is appropriately described by the following equation.



RH is an organic substrate and DH<sub>2</sub> is a reduced electron donor which serves as an essential cofactor for this reaction.

The monooxygenases are especially important to the field of pharmacology since the major component of the drug metabolizing enzymes of the human liver, cytochrome P-450, is a member of this class. Thus although the non-enzymatic model systems were devised to study the chemical activation of molecular oxygen, they also serve the purpose of demonstrating how organic substrates might be metabolized by the human liver. This paper will explore the possibility of using a heuristic computer program to serve as a model of enzyme P-450.

Before exploring the actual operation of the program, though, it is essential to discuss some of the assumptions which are made

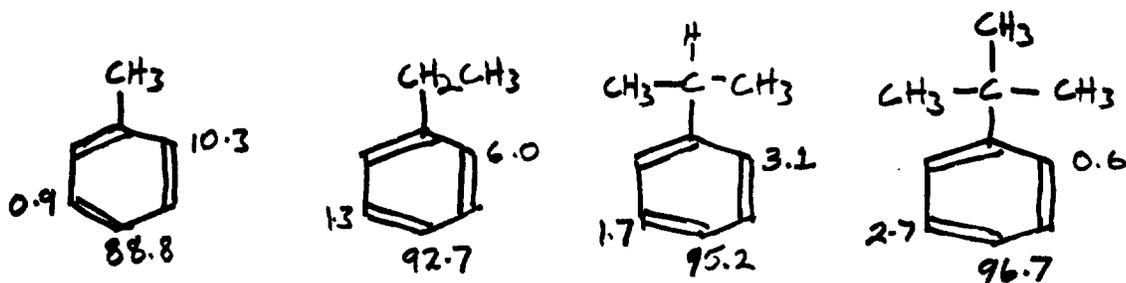
in using this method and also to show that these assumptions are justified at least in part by the information which is currently available on enzyme P-450. The first section of this paper will therefore be a short discussion of the factors which affect chemical reactivity and how they are represented in non-chemical systems. The second section will relate these factors of reactivity to the function of the enzyme. Section III will describe the heuristic model and the fourth section will evaluate the performance of the model on a simple class of compounds.

## I. Factors Affecting Reactivity

The factors which affect enzymatic catalysis are thought to be more limited than those which affect chemical reactivity in general. Most simple discussions of reactivity are centered on steric qualification of the substrate with respect to the active site with the additional factors of electrostatic interaction and Van der Waals forces also receiving some consideration. A more general perspective of reactivity will be given here, though, since cytochrome P-450 does not follow our usual ideas of enzyme substrate interaction and in addition it is of some value to be comprehensive in an attempt to show which factors may be modeled in a heuristic program such as the one proposed later in this paper. This general treatment roughly follows the same section in (11).

### Steric and Strain Effects

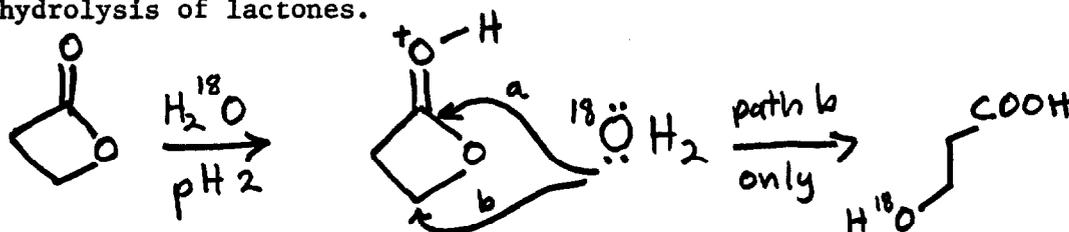
In the reaction of  $\text{PhCO}^+ \text{SbF}_6^-$  with a series of alkylbenzenes, the effect of steric hinderance in the transition state may be seen in the distribution of isomers.



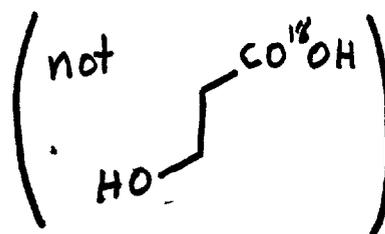
Isomer distribution in the Friedel-Crafts benzoylation of alkylbenzenes

The amount of orthosubstitution clearly decreases with increasing size of the alkyl substituent. This is directly due to steric interference. Steric interaction is usually sited as the major factor involved in the lock and key fit required for the high specificity of most enzymes.

The relief of angle-strain may also direct the course of a reaction. When a bond is strained out of its normal geometry, an increase in the ground state energy of the molecule is also seen. Relief of this strain energy may direct a reaction such as the hydrolysis of lactones.



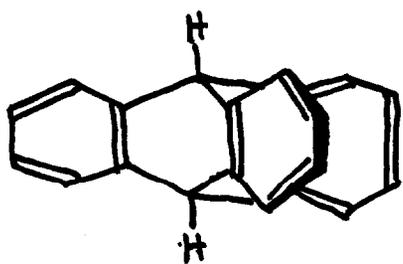
Lactone hydrolysis  
by O-Alkyl cleavage



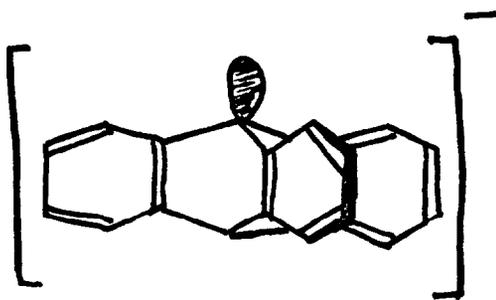
A general pathway for lactone hydrolysis is via the cleavage of the O-Acyl bond after nucleophilic attack at the carbonyl carbon. In the example shown above, though, the relief of angle strain in the transition state directs the reaction towards o-alkyl cleavage, as is conclusively demonstrated by the distribution of  $^{18}\text{O}$  in the product.

Stereoelectronic Effects

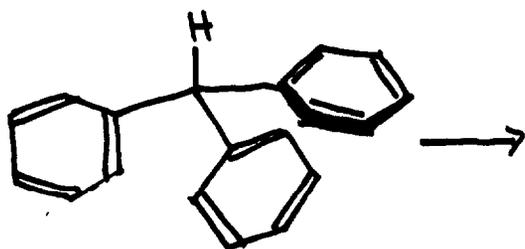
The degree of overlap between two orbitals is of crucial importance in determining the energy of activation for a reaction which involves either bond breaking or bond making between those orbitals. This degree of overlap depends not only on the distance between the two, but also on the angular disposition of the bonding lobes. An important example is the deprotonation of triptycene and triphenyl methane by strong bases.



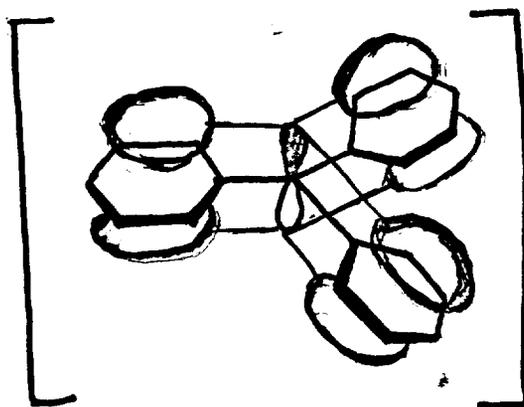
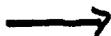
Triptycene



Triptycenylyde anion



Triphenylmethane



Triphenylmethylyde anion

In the former, no delocalization of charge to the phenyl groups in the transition state is possible since the  $\pi$  electron clouds are orthogonal to the lone pair electrons of the carbanion. This situation achieves minimum overlap between the lone pair and  $\pi$  clouds.

The triphenylmethylyde anion is considerably stabilized, though, since maximum overlap is achieved due to the parallel disposition of the bonding lobes. The deprotonation of triphenylmethane proceeds about 1/2 million times faster than triptycene accordingly.

### Electronic Effects

Substituents in organic molecules can markedly affect chemical reactivity due to electronic effects. These electronic effects are transmitted across the molecules by polar and conjugative mechanisms. The well known change in acidity due to addition of electronegative substituent is demonstrated by the  $pK$  of the following carboxylic acids in water at 25° c.

Values of  $pK$  for carboxylic acids

<u>Acid</u>	<u><math>pK</math></u>
$CH_3COOH$	4.80
$ClCH_2COOH$	2.86
$Cl_2CHCOOH$	1.30
$Cl_3CCOOH$	0.65

The polar effect consists of two separate components. The first is a polarization of bonds between the reaction center and the substituent which is normally termed an inductive effect. The second is called the field effect and involves the direct electrostatic interaction of the substituent and reaction center through space. Experiments to clarify the relative strengths of these components in the overall substituent effect have proved difficult to interpret, but it is important to note that most authors agree that the field effect is more often the most significant.

The other mechanism for the transmission of electronic effects is by the conjugative effect or delocalization. This is achieved by the overlap of a p-orbital at the reaction center with some  $\pi$ -electron system which may then overlap with p-electrons of the substituents. The increased acidity of toluene with respect to methane is due to a conjugative effect on the benzyl anion. This lowers the energy difference between the charged and neutral species and hence increases the acidity.

### Analysis of Reactivity in Model Systems

In order to analyze the factors that affect reactivity in model systems, it is first essential to discuss the space which is used to build the model. For example -- if an analysis of a molecule by the molecular orbital method is being done then the space we are dealing with is geometric. There is a measure of distance between points in this space and all arguments which pertain to the geometry of a molecule are therefore applicable. Unfortunately, it is often difficult or even impossible to construct models and perform an analysis even with empirical molecular orbital methods.

A simpler space in which molecules may be represented is a topological space. This space has no measure of distance or geometry, but only of connectedness. Given an atom in a molecule, for example, it is only possible to ascertain what atoms it is connected or bonded to. Without a measure of distance, it is not possible to say how far away these atoms are. This greatly simplifies the mechanics of the representation. Molecules become labelled graphs and are subject to manipulation by fairly simple computer programs, as opposed to those which deal with geometric representations (14). This simplicity is bought at the price of a tremendous restriction of any analysis of chemical reactivity. Steric and strain effects are clearly related to a notion of geometry and distance as are obviously all stereoelectronic effects. This means we may not incorporate these ideas into any program which deals with a topological representation. In a similar fashion, the field component of the substituent effect is also disallowed. Conjugative effects, on the other hand, are possible to represent, but will not always give satisfactory results due to their

obvious relation to stereoelectronic effects. Any usefulness of conjugative mechanisms will depend on a fortuitous situation involving free rotation about a bond connecting the p-orbital of the reaction center involved and the  $\pi$ -electron system.

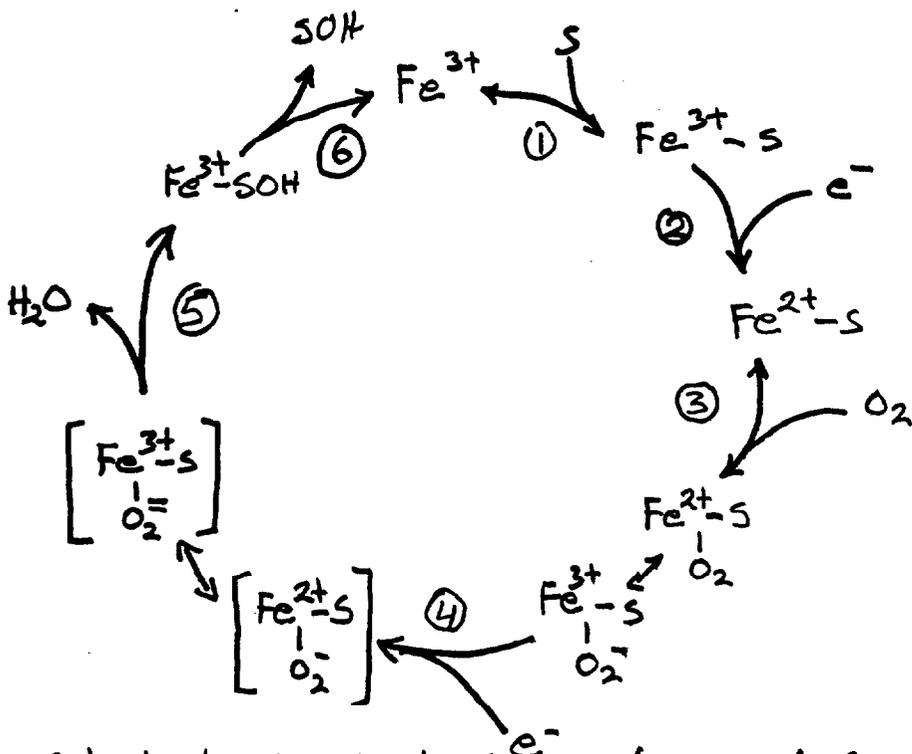
If for example, the  $\pi$ -system is locked in a position orthogonal to the reaction center, then no conjugative effect will be seen. This is precisely the type of question which is indeterminable in a topological space, since no notion of orthogonality exists without geometry.

The only effect which may be completely represented with no notion of geometry is the inductive component of the field effect. It is extremely important to realize this in dealing with the topological representation of molecules.

## II. The Mechanism of P-450 Oxygenations

With this brief discussion of reactivity in mind, I would now like to explore the mechanism of P-450 oxygenations and relate that mechanism to the factors discussed so far.

Perhaps one of the most remarkable facts to emerge about this enzyme is its surprising lack of molecular specificity. Where as most enzymes are specific for only a handful of molecules, P-450 appears to be capable of binding and introducing an atom of oxygen into almost any lipophilic substrate (5). The details of the exact sequence of events at P-450 are now commonly accepted to correspond to the following sequence (3).



1. Substrate binds to ferric form of P-450
2. Ferric substrate complex reduced to ferrous form.
3. Ferrous substrate complex binds molecular oxygen
4. Oxygenated ferrous complex reduced
5. One atom of oxygen inserted into substrate, the other is released as  $\text{H}_2\text{O}$
6. Hydroxylated substrate released, regenerating ferric form of P-450.

In the rate limiting step of this sequence, the second electron is added to further reduce the P-450/substrate/ $\text{O}_2$  complex. Model systems such as 2-Mercaptobenzoic Acid/ $\text{Fe}(\text{II})/\text{O}_2$  have been studied in order to learn more about the species of oxygen which is incorporated into the molecule (1). From these studies and similar experiments with liver microsomes, the active form of oxygen has been deduced. Since the typical reactions of this complex are carbon-hydrogen bond insertion and electrophilic addition to double bonds, it was reasoned that the active oxygen was a neutral oxygen atom or oxene, a species isoelectronic to carbenes which show exactly the same reaction products. Experimental evidence supports this conclusion (2).

Oxygenation proceeds with the retention of a deuterium label, which would be expected for the insertion reaction but not for a mechanism involving displacement by a hydroxyl ion. In addition, this reaction shows an isotope effect when hydrogen is replaced by deuterium in those C-H bonds involved. This indicates that the rate limiting step of the overall reaction involves C-H bond breaking and again supports the insertion mechanism.

It is now appropriate to discuss those factors which might affect the reactivity of various substrates with respect to the oxene electrophilic mechanism. Steric effects seem to play a small role as mentioned earlier. That fact that such a variety of molecules may be bound to P-450 does not exclude the possibility of steric effects, in fact experimental evidence does support some steric interaction, particularly for larger molecules (2). On the other hand, conjugation seems to be very important. Benzylic and allylic positions are commonly activated towards oxygenation by P-450 (2). Inductive effects are also present. Carbon-hydrogen bonds which are adjacent to heteroatoms are often oxygenated. This effect is commonly seen in the dealkylation of ethers and thioethers and the dealkylation of secondary and tertiary amines (2). In a series of studies on the simple alkanes, it was also determined that the reactivity of carbon-hydrogen bonds in these compounds followed the order of bond weakness, that is:  $3^\circ > 2^\circ > 1^\circ$  (6,7,8,9).

### III. The Heuristic Model

The ~~brief~~ <sup>brief</sup> survey of the factors affecting reactivity of substrates

for cytochrome P-450 given in the previous section reveals an interesting trend. All the important factors (ignoring steric interactions for large molecules) are electronic in nature and are precisely those which admit to being represented in a topological rather than geometrical space. To test this possibility, I have constructed a simple set of heuristics to be incorporated into a model which would select the best sites for P-450 oxygenations within any candidate molecule. The heuristic is simple: choose those C-H bonds, in order, which are weakest as the best sites. First year organic chemistry textbooks provide relative strengths for the following groups:

Strengths of C-H bonds in hydrocarbons

Benzylic < 3° < 2° < 1°  
 Allylic

The actual computer program to be used is the Dendral Predictor (14). This is essentially a graph manipulation program based on the situation action rule. The program accepts a candidate molecule and a list of situation - action rules. The rules are applied to the candidate in the following fashion:

1. Search the candidate for any subgraph which might match the situation given in the first rule.
2. If such a match is found, perform the action indicated in the second part of the rule. An example will illustrate this process. Suppose the rule is composed of the following situation and action.



molecules.

### Alkanes

2,2 dimethylpropane, 2 - methylbutane, isobutane, n - pentane, n - butane, and n- heptane, the observed results are in accordance with the programs prediction except that the primary alcohols of n - pentane, n - butane, and isobutane were not detected (6,7,8,9).

This confirms the order of reactivity for  $3^{\circ}$  <sup>vs  $2^{\circ}$</sup>  vs  $1^{\circ}$  carbon hydrogen bonds.

### Alicyclic Rings

Methylcyclohexane is metabolized in agreement with the program. The tertiary bond is favored as a site of attack (9). Trans and cis decalin are oxygenated at the 2 position, in disagreement with the program, which predicts attack at the 9 and 10 positions.

### Benzylic Positions

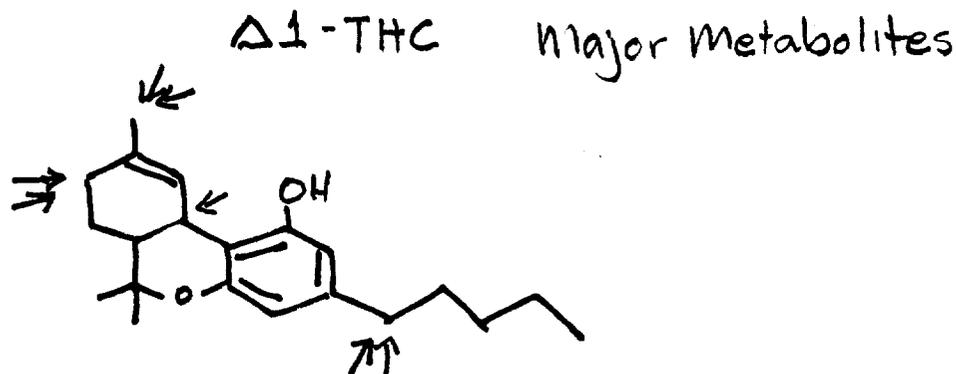
Ethylbenzene, propylbenzene, butylbenzene and p - xylene are all attacked at the benzylic position as would be expected (2).

### Other Results

While experiments with hydrocarbons are difficult to find, many hydrocarbon like molecules which contain one or two functional groups have been experimented with. The data here suggests, for example, that steric effects play an important part for larger molecules. Thus most steroids are attacked only at axial groups (2B, 6B, ~~7B~~ 11B, and 16B) (2). This appears to be a general rule for larger hydrocarbon

like alicyclic ring compounds.

The metabolism of  $\Delta^1$ -THC demonstrates the validity of this method for large molecules (12, 13).



→ Indicates sites predicted

→ Indicates sites actually found

Of the twelve positions available for attack by P-450, 3 of the 4 sites selected by the model as being most important are found to be the metabolites produced in greatest quantity. The next observed metabolites are hydroxylations on the side chain. Again, the failure of the program in this case is most likely due to some steric effect.

## V. Conclusion

This paper is intended only to provide an introductory survey of the heuristic technique for predicting the metabolites of P-450 reactions. Further research on the exact mechanism of monooxygenations might allow the development of better and more sophisticated heuristics and a consequent improvement of performance. The inherent limitations of the representation will always cause problems such as the avoidance

of steric interactions. Possibilities for further study include the expansion of the heuristics to include functional molecules and more primitive methods of estimating relative bond strengths.

#### REFERENCES

1. Ullrich V., Staudinger H.: Model Systems in Studies of the Chemistry and the Enzymatic Activation of Oxygen. In: Handbuch der Experimentellen Pharmakologie, XXVIII/2, Springer Verlag. Berlin-Heidelberg-New York (1971).
2. Daly, J.: Enzymatic Oxidation at Carbon. In: Handbuch der Experimentellen Pharmakologie, XXVIII/2, Springer Verlag, Berlin-Heidelberg-New York (1971).
3. Estabrook, R.W., et. al.: Studies on the Molecular Function of Cytochrome P-450 during Drug Metabolism. Drug Met. and Disp. 1, 98 (1972).
4. Ullrich, V.: Enzymatic Hydroxylations with Molecular Oxygen. Angew. Chem. Internat. Edit. 11, 701 (1972).
5. Frommer U., Ullrich V.: Model Hydroxylation Reactions. Z. Naturforsch. 26b, 322 (1971).
6. Frommer U., et. al.: Hydroxylation of Aliphatic Compounds by Liver Microsomes. Hoppe-Seyler's Z. Physiol. Chem. 351, 903 (1970).
7. Frommer U., et. al.: Hydroxylation of Aliphatic Compounds by Liver Microsomes. Hoppe-Seyler's Z. Physiol. Chem. 351, 913 (1970).
8. Frommer U., et. al.: The Monooxygenation of N-Heptane by Rat Liver Microsomes. Biochim. et Biophys. Acta 280, 487 (1972).
9. Frommer U., et. al.: Hydroxylation of Aliphatic Compounds by Liver Microsomes. Archiv Für Pharm. 266, 328 (1970).
10. Ullrich, V.: Oxygen Activation by the Iron (II) -2- Mercaptobenzoic Acid Complex. A Model for Microsomal Mixed Function Oxygenases. Z. Naturforsch. 24b, 699 (1969).

11. Alder, R.W., et.al. Mechanism in Organic Chemistry. Wiley-Interscience, London-New York-Sydney-Toronto, 1971.
12. Augrell, S., et.al.: Metabolic Fate of Tetrahydrocannabinol. In: Cannabis and its Derivatives. Oxford University Press, London 1972.
13. Burnxtein, S., et.al.: The Urinary Metabolites of D1 THC. In: Cannabis and its Derivatives. Oxford University Press, London 1972.
14. Mass Spectrum Prediction In Dendral. (Dendral Group documentation.)