MECHANISTIC STUDIES OF A MODEL REACTION FOR ENZYMATIC HYDROXYLATION

Thesis by

David Michael Mog

In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1970

(Submitted March 9, 1970)

To the dedicated educators in my life.

"Science in our age has social, economic, and political functions, and however remote one's own work is from technical application it is a link in the chain of actions and decisions which determine the fate of the human race."

— Max Born

"Three passions, simple but overwhelmingly strong, have governed my life: the longing for love, the search for knowledge, and unbearable pity for the suffering of mankind."

— Bertrand Russell

ACKNOWLEDGMENTS

I wish to thank Professor John Richards for ideas, guidance, patience, understanding, and moral support, the Caltech community for a stimulating intellectual environment; the Woodrow Wilson Foundation, the National Institutes of Health, the Paint Research Foundation, the California Institute of Technology, and my wife for financial support; Eileen McKoy for superb secretarial assistance; and most importantly my wife, Gloria, for love, comfort, and a reason for being.

ABSTRACT

A study has been made of the reaction mechanism of a model system for enzymatic hydroxylation. The results of a kinetic study of the hydroxylation of 2-hydroxyazobenzene derivatives by cupric ion and hydrogen peroxide are presented. An investigation of kinetic orders indicates that hydroxylation proceeds by way of a coordinated intermediate complex consisting of cupric ion and the mono anions of 2-hydroxyazobenzene and hydrogen peroxide. Studies with deuterated substrate showed the absence of a primary kinetic isotope effect and no evidence of an NIH shift. The effect of substituents on the formation of intermediate complexes and the overall rate of hydroxylation was studied quantitatively in aqueous solution. The combined results indicate that the hydroxylation step is only slightly influenced by ring substitution. The substituent effect is interpreted in terms of reaction by a radical path or a concerted mechanism in which the formation of ionic intermediates is avoided. The reaction mechanism is discussed as a model for enzymatic hydroxylation.

TABLE OF CONTENTS

Chapter 1. INTRODUCTION

1.1 Historical Background

The oxygen we breathe every minute of our lives is structurally one of the simplest metabolites used by living systems. Functionally and mechanistically, however, oxygen is probably the most complex metabolite known. Nitrogen metabolism is, in comparison, quite simple (1).

The importance of oxygen as the terminal electron acceptor in the electron transport system is generally well known. Compounds are oxidized through the electron transport system by an enzymatic dehydrogenation reaction. The electrons released are transported by a series of coupled redox reactions to molecular oxygen which is reduced to water (or hydrogen peroxide). The energy released in the process is stored as chemical energy in the phosphate ester bond of adenosine triphosphate. The life processes of all higher animals are sustained by the energy channeled in this way through the electron transport system (2).

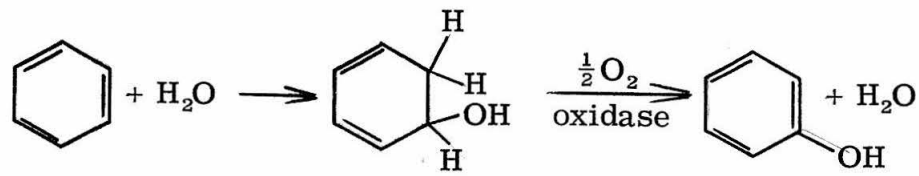
Early in this century, H. Wieland postulated that all biological oxidations proceed by a series of dehydrogenations in which, for example, an aldehyde is oxidized to an acid by dehydrogenation to ketene followed by addition of water. The opposing view was held by Otto Warburg, who felt that oxygen was activated and transferred directly to substrate. The bulk of the early evidence supported the former view to such an extent that in 1932 Wieland was able to state:

"Limiting ourselves to the chief energy-supplying foods, we have carbohydrates, amino acids, the higher fatty acids and glycerol. There is no known example among them of [the production of] an unsaturated compound in the case of which it is necessary to assume direct addition of oxygen, that is, additive oxidation" (3).

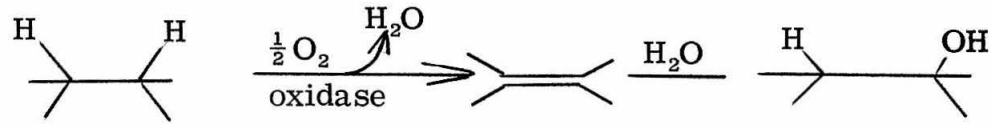
Wieland's statement went unchallenged for more than 20 years, and thus during the 1930's and 1940's, in order to be consistent with the Wieland postulate, biological hydroxylation mechanisms were written in terms of hydration-dehyrogenation sequences, Scheme I (4).

That oxygen might have a larger biological role than Wieland allowed and that it might function directly in biosynthesis, was suggested as early as 1867 when Pasteur observed that yeast cells capable of living under strictly anaerobic conditions degenerated if not given periodic exposure to oxygen (5). Unambiguous evidence that oxygen participates directly in biosynthesis did not come until the atomic age and the ready availability of oxygen-18.

In 1955, two research groups independently studied the products of enzymatic hydroxylation in the presence of oxygen-18 (6). Contrary to the requirements of Wieland's postulate, oxygen was incorporated from the atmosphere, but not from water; Scheme II (6). Warburg's oxygen transfer enzymes had been found.

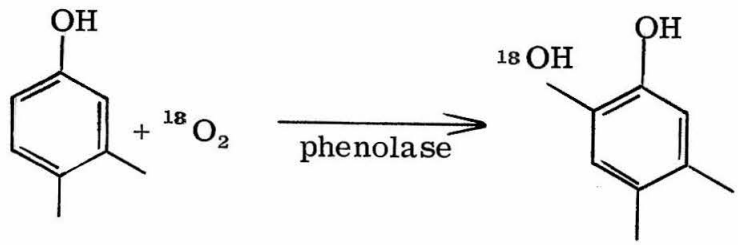
(a) 

(1)

(b) 

(2)

Scheme I



(3)

Scheme II

1.2 Classification of Oxygen Transfer Enzymes

Oxygen-18 studies during the past fifteen years have demonstrated the existence of a large variety of enzymes which catalyze the fixation of molecular oxygen. Enzymes of this type, which are generally widespread in plants, animals, and microorganisms, have come to be known as oxygenases (5,7). Two subclasses of oxygenases are known, and the distinctions between them are illustrated in equations 4-6 where S is substrate and AH_2 is a hydrogen donor:

Monooxygenases

Internal: $$SH_2 + O_2 \longrightarrow SO + H_2O \qquad (4)$$

External: $$S + O_2 + AH_2 \longrightarrow SO + H_2O + A \qquad (5)$$

Dioxygenases

$$S + O_2 \longrightarrow SO_2 \qquad (6)$$

The monooxygenases are often referred to as mixed-function oxidases or hydroxylases (8). The term hydroxylase is widely used because it is often more descriptive.

In the middle ground between oxidases (equation 1) and oxygenases (equations 4-6) is a class of enzymes called peroxidases (equation 7) (9).

$$ROOH + AH_2 \xrightarrow{\text{peroxidase}} ROH + H_2O + A \qquad (7)$$

The peroxidases are included at this point because there is good evidence of a peroxidase-oxygenase duality. There exist studies, for example, which indicate that under certain conditions peroxidases can

function as oxygenases (10), while recent reports have shown that reactions previously attributed to some oxygenases may in fact be due to peroxidases (11).

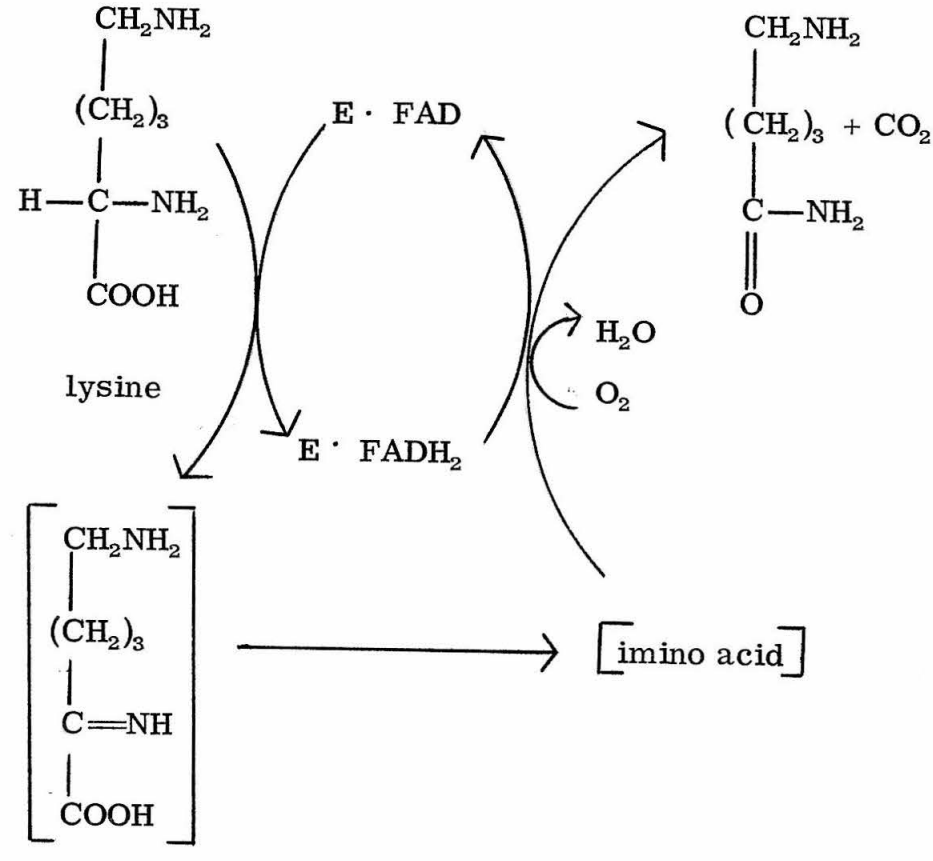
1.3 Biological Background

1.3.1 Oxygenases

The oxygenases participate in a large number of biosynthetic and degradative pathways. They are capable of oxidizing inert systems, for example saturated or aromatic carbon atoms, as well as unsaturated carbon, nitrogen, and sulfur. Examples of oxygenase reactions are given below to demonstrate the range of function of these enzymes and the types of oxygenated compounds they produce.

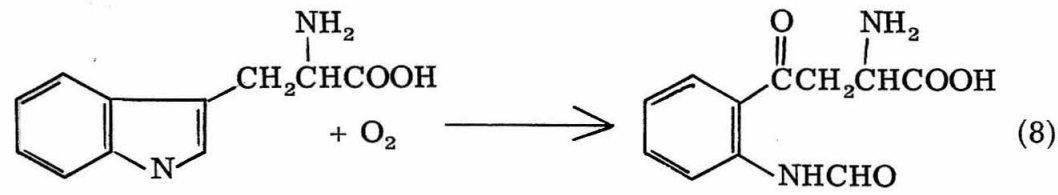
1.3.1.1 Amino Acid Biosynthesis: Phenylalanine and proline are hydroxylated to tyrosine (12) and 4-hydroxyproline (13) respectively by monooxygenases. Proline hydroxylase has been the subject of intensive study in recent years in part because hydroxyproline is an important constituent of collagen. These studies have shown that the substrate for proline hydroxylase is not proline itself, but proline residues in a polypeptide precursor, protocollagen (14).

1.3.1.2 Amino Acid Metabolism: The amino acids tyrosine, tryptophan, lysine, and cysteine are subject to reactions involving molecular oxygen. Lysine monooxygenase is an example of an internal monooxygenase (Scheme III). The decarboxylation which accompanies lysine oxidation is a common adjunct to monooxygenase reactions which are also known to bring about dealkylation and deamination (5).


CH2NH2
(CH2)3
H—C—NH2
COOH
lysine
E · FAD
E · FADH2
H2O
O2
CH2NH2
(CH2)3 + CO2
C—NH2
O
CH2NH2
(CH2)3
C=NH
COOH
imino acid
imino acid

Scheme III

Several mono- and dioxygenases participate in the metabolism of tryptophan whose degradation products are involved in various aspects of brain chemistry. A typical dioxygenase is tryptophan 2,3-dioxygenase which breaks down the aromatic indole ring (equation 8) (15).

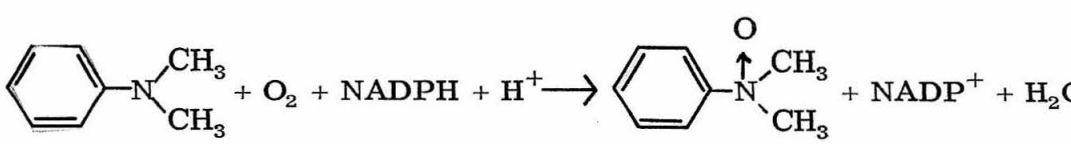


Tryptophan — Formylkynurenine (8)

1.3.1.3 Sulfur and Nitrogen Oxide Formation: The sulfur atom of the amino acid cysteine is converted by a dioxygenase to cysteine sulfinic acid (equation 9) (16).

$$\text{cys-SH} + O_2 \longrightarrow \text{cys-SOOH} \qquad (9)$$

Oxygenases are also known which convert amines to nitro compounds (17) and N-oxides (equation 10) (18).



(10)

1.3.1.4 Fatty Acid Metabolism and Peroxide Intermediates: The major route of fatty-acid metabolism in mitochondria is the β-oxidation pathway (19) which involves a dehydrogenation-hydration sequence similar to that in Scheme Ib. In recent years, the existence of an alternate route has been demonstrated in mammalian liver. It is called the ω-oxidation scheme, and it requires an NADPH dependent monooxygenase (20). The two pathways differ in the nature of the metabolites produced. More important, perhaps, are the differences from the standpoint of energetics; because while the normal β-oxidation scheme provides a source of energy for the cell, the route involving the monooxygenase step consumes energy, as do all oxygenase reactions. It has been suggested that for this reason the oxygenases may play a regulating role in energy distribution (21).

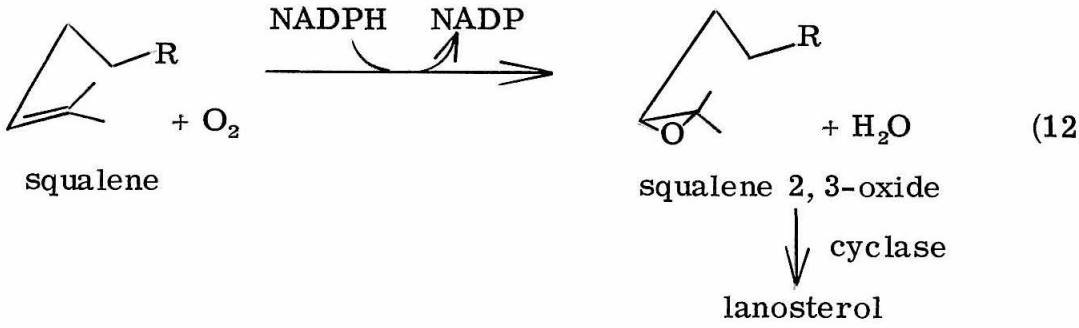
Oxygenases generally lead only to stable products in which the oxygen-oxygen bond has been broken. Exceptions are the enzymatic systems which result in isolable hydroperoxides (22,23). The best examples are the long-chain fatty acid hydroperoxides which are produced when unsaturated fatty acids are incubated with soybean lipoxygenase (equation 11) (23).

$$CH_3-(CH_2)_4-CH=CH-R + O_2 \xrightarrow{\text{lipoxygenase}} CH_3-(CH_2)_4-\underset{\displaystyle OOH}{\overset{|}{C}}=CH-R \qquad (11)$$

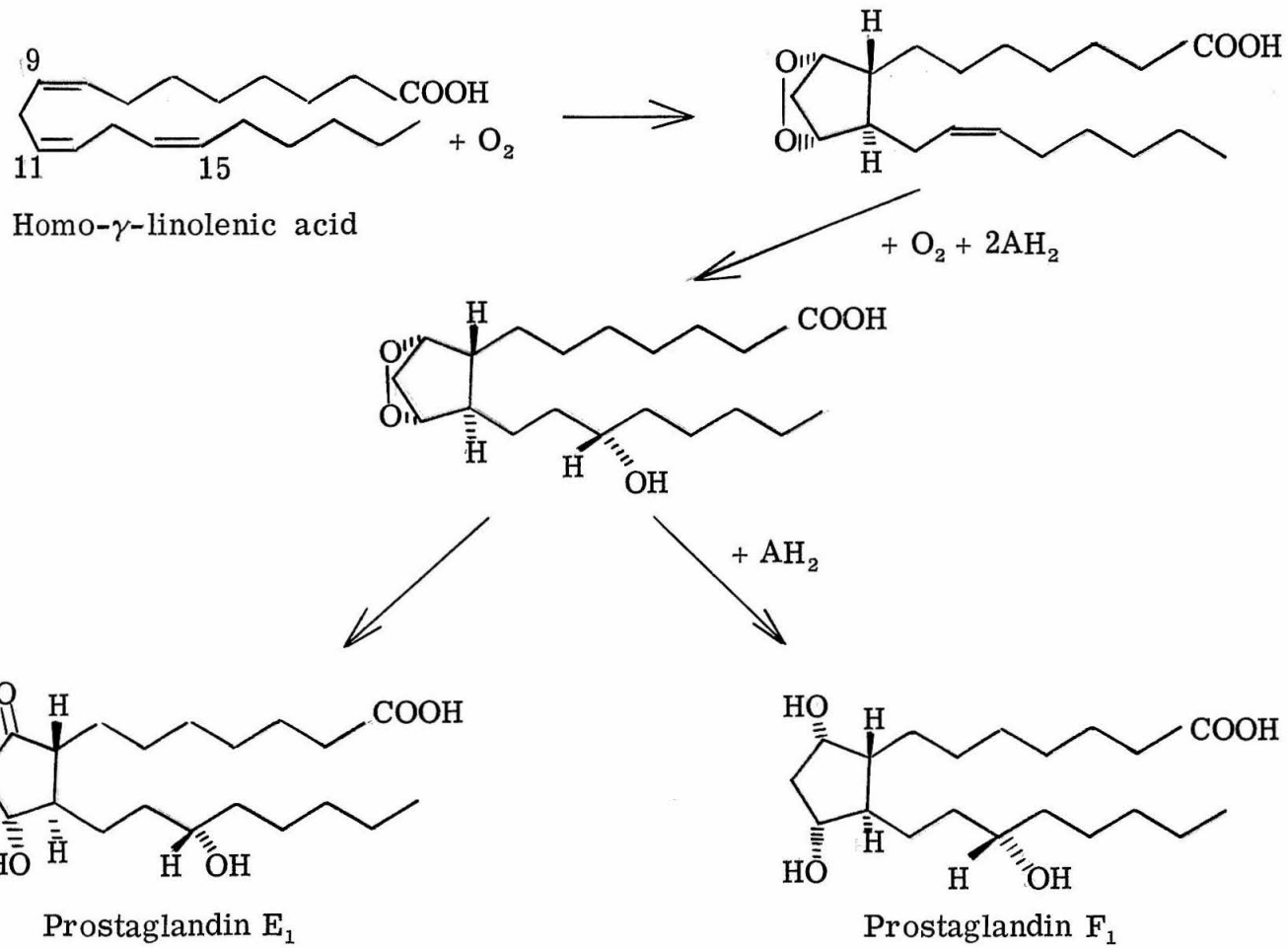
The biological significance of this reaction in vitro is unknown (5).

There are a number of oxygenase systems in which cyclic peroxides have been postulated but never isolated. One example in fatty acid metabolism is the conversion of homo-γ-linolenic acid to the prostaglandin hormones by sheep vesicular gland homogenates. Both a dioxygenase and a monoxygenase are known to be involved, and furthermore it has been shown that the oxygen atoms introduced at positions 9 and 10 are derived from the same molecule of oxygen. The latter observation leads to speculation that a cyclic peroxide is involved in prostaglandin formation (Scheme IV) (5).

1.3.1.5 Steroids: The oxygenases play a major role in steroid biosynthesis. The first enzyme of the series is a monooxygenase which converts the C-30 hydrocarbon squalene to squalene 2,3-oxide (25). The squalene oxide is then converted to the steroid lanosterol by a separate enzyme (equation 12) (26).



Other microsomal and mitochondrial oxygenases are involved in the degradation and transformation of various steroids including cholesterol (5,27).



Prostaglandin E_1

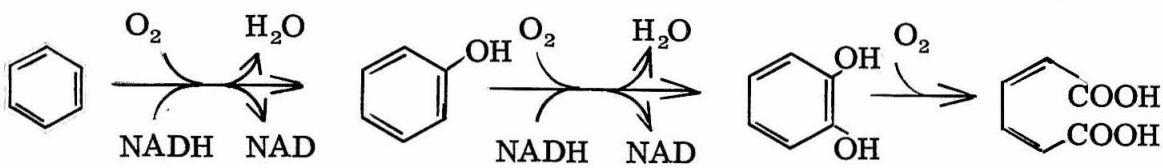
Prostaglandin F_1

Scheme IV

1.3.1.6 Drugs - Detoxification Mechanisms: The rational use of drugs and food additives depends on a knowledge of what occurs when a drug (or environmental pollutant) is ingested. The breakdown and elimination (or utilization) of such foreign substances is called detoxification--a process which nearly always begins with an oxygenase reaction (28). At least some of these oxygenases are inducible; that is, the cell can be stimulated to produce enzyme (29). For these reasons, the oxygenases which participate in detoxification processes have received considerable attention from pharmacologists, toxicologists, and geneticists.

Many drugs and a host of carcinogens contain aromatic rings which can be metabolized via hydroxylation and cleavage by oxygenases. A simple example is the metabolism of benzene by microorganisms as shown in equation 13 (30).

(13)



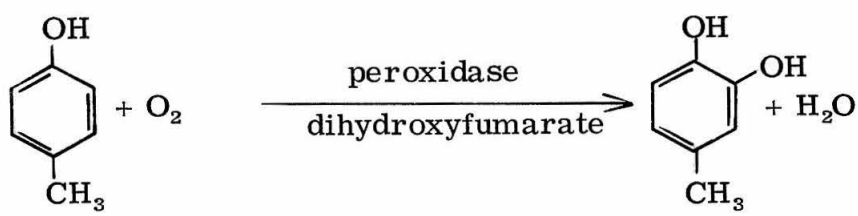
In this example, a hydrophobic, biologically inert compound has been converted by oxygenase reactions to a water soluble compound which can easily be further metabolized to normal biochemical intermediates. Transformations of this kind are a general characteristic of many oxygenase reactions (5).

1.3.2 Peroxidases

Peroxidases are hemoproteins which can be isolated from plant and animal sources (9). In many ways, they are very similar to oxidases in that the oxygen atoms they use as substrate are electron acceptors (see equation 7). In spite of all that has been learned about the peroxidases from *in vitro* experiments, their biological function remains obscure. A good example is catalase, a peroxidase which prefers hydrogen peroxide as both substrate and hydrogen donor, i.e., R = H and A = O_2 in equation 7. It was believed at one time that the sole function of catalase was to protect cells from damage by excess peroxide generated by various redox processes (31). But calculations have revealed that the body contains far more catalase than it should need simply for protection from hydrogen peroxide (31).

Experiments have been done (see 1.2) which indicate that, in addition to their "normal" function (equation 7), the peroxidases are capable of exhibiting oxygenase activity. The relationship of such systems to the oxygenases themselves is of interest.

An example of oxygenase activity exhibited by a peroxidase is the conversion of *p*-cresol to 4-methyl catechol by horseradish peroxidase in the presence of dihydroxyfumarate (equation 14) (10).



(14)

The situation above is reversed in the case of tryptophan oxygenase (see 1.3.1.2), which has been shown to utilize hydrogen peroxide and to exhibit general behavior characteristic of peroxidases (Scheme V) (32,33). The suggested intermediate (our structure) has never been isolated (32).

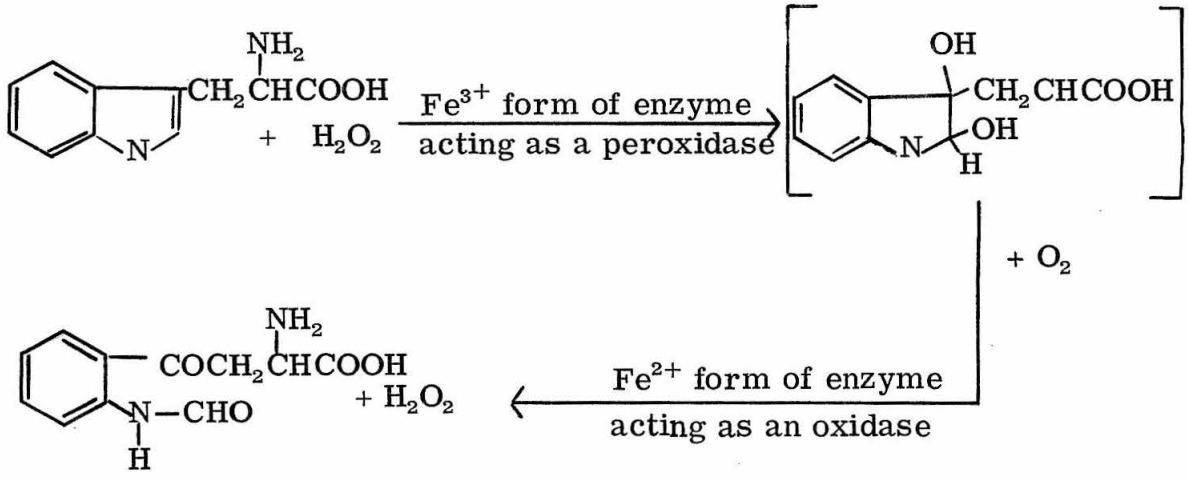
The two examples above indicate that at the present time there is no clear distinction between oxygenases and peroxidases from the standpoint of function and behavior in vitro.

1.4 Oxygen and Its Activation

1.4.1 Oxygen and Transition Metals

The preceding examples give an indication of the versatility of the enzymes which fix oxygen in biological systems. The one thing these enzymes have in common is their ability to use oxygen or peroxide as substrate. In order to understand the possible role of the enzyme, it is helpful to know something about oxygen itself.

Experience in basic chemistry indicates that the oxygen molecule is a fairly inert species with respect to organic compounds. This has been attributed to the fact that oxygen is a triplet in its ground state, so that reduction by two electrons to a singlet species such as hydrogen peroxide is a spin-forbidden process (34). Therefore, the most common reaction of unactivated oxygen is its combination with organic free radicals (35). The unstable peroxy radical thus formed is reduced in a second one-electron step by a second free radical. Although such one-electron, radical, reactions involving oxygen have been postulated in biological systems (usually in the absence of metal ions)


NH2
CH2CHCOOH
+ H2O2
Fe3+ form of enzyme
acting as a peroxidase
OH
CH2CHCOOH
OH
H
+ O2
NH2
COCH2CHCOOH
N—CHO
H
+ H2O2
Fe2+ form of enzyme
acting as an oxidase

Scheme V

(36), there is good evidence that a great many metalloenzymes utilize oxygen in two-electron steps with no detectable radical intermediates (34).

A question of interest from a mechanistic point of view is, how do the enzymes activate the oxygen molecule to react with non-radical substrates? Since most of the oxygenases have appeared to require the presence of a transition metal ion, usually iron or copper (7), the answer has generally been approached from the perspective of transition metal-oxygen chemistry. Transition metal ions can act as catalysts for oxygen activation for two principal reasons: First, transition metal ions often have unpaired electrons, and are therefore similar to organic free radicals. Such ions can readily complex with oxygen thereby changing the overall spin state which permits the oxygen molecule to react further (34). A second important factor is the ability of the transition metal ion to undergo changes of oxidation state, or to act indirectly as an agent for electron transfer from various reducing agents to oxygen (37). These properties of transition metal ions are often used to rationalize the mechanisms proposed for enzymatic and model oxygenases (see 1.5 and 1.6).

1.4.2 Hydrogen Peroxide and Transition Metal Ions

Although the very stable oxygen-oxygen bond in O_2 is considerably weakened by two-electron reduction to the formal O_2^{-2} state (38) (that found in hydrogen peroxide), hydrogen peroxide itself is found to be a stable molecule in the absence of catalysts in neutral or acidic aqueous solution (39). We find that the very same properties which make

transition metal ions good catalysts for oxygen activation, also make them good catalysts for the activation or decomposition of peroxides (40,41). It is not surprising then that nearly all of the peroxidases have been found to contain at least one atom of iron (9).

1.4.3 The Question of Metal Participation in the Oxygenases

As a result of considerations like those above, it was once believed that all oxygenases and peroxidases would be found to contain metal ions. That an oxygenase might be fully active in the absence of metal ions has been demonstrated only recently with highly purified, crystalline oxygenases (5). Oddly enough, all four crystalline monooxygenases prepared thus far have been found to lack metal ion participation. It is also of note that the four recently isolated crystalline dioxygenases have been shown to contain at least one iron atom (5).

In the past the question of metal ion participation has been resolved by studies of activation by added metal ions, or inhibition by organic chelating agents. In at least one case, the previously observed oxygenase inhibition by a chelating agent is now attributed to a hydrophobic interaction between active protein and the chelating agent itself (42).

1.4.4 Oxygen Activation in the Absence of Metal Ions and the Question of Singlet Oxygen

The generality of the results showing no metal ion participation in four crystalline monooxygenases (see 1.4.3) remains to be seen. It should be clear, however, that more attention must be paid to the

question of possible oxygen activation in the absence of metal ions. Very little work has been done in this area, but one study has been published in which the oxygen atom is proposed to complex with a flavin free radical (36). A second possibility, which has been explored on theoretical grounds, is that the enzyme activates substrate which is then capable of reacting directly with ground-state oxygen (43).

For clarity, it will be necessary to confine the discussion below to the question of oxygen activation by metalloenzymes. In addition, the case of singlet oxygen is ignored. Although it is believed to be the active state of oxygen in photo-oxygenations (44) and in certain heterolytic decompositions of hydrogen peroxide (45), the existence of singlet oxygen in biological systems has been limited to its postulated participation in two dioxygenase reaction mechanisms (22,46). Since this work deals primarily with monooxygenase mechanisms, we feel the exclusion of singlet oxygen is justifiable.

1.5 Background on Enzyme Mechanisms

In light of the information given in the previous two sections, we are now faced with the question of what is known about oxygenase mechanisms.

1.5.1 Oxygenases

1.5.1.1 Some Reasons for Lack of Progress: When we began this study several years ago, the phrase "primitive and incomplete" had been used to describe the copper enzymes involved with oxygen metabolism (47). A similar phrase would have been appropriate for

the oxygenases in general. One need only be aware of the problem of metal participation (see 1.4.3) to understand that the enzyme systems are still poorly understood. This is true in spite of the numerous studies dealing with oxygenases which have been reported in recent years. Most of the results are of a descriptive nature, and, to date, very few quantitative studies have been published.

The prevailing situation is due to a number of factors not the least of which has been the unavailability of sufficient quantities of purified enzyme. Isolation and purification of oxygenases from higher organisms are particularly difficult because the enzymes are tightly bound to microsomal or mitochondrial particles. Recent successes in obtaining reasonable quantities of crystalline enzymes from inducible, bacterial sources is encouraging (see 1.4.3).

A second problem stems from the fact that the oxygenases require labile cofactors. Identification of the cofactors is a major task in itself, and their presence makes isolation, purification, and activation of the enzymes more complex. From a mechanistic point of view it would be helpful to know something about the environment of participating metal ions, but unlike the peroxidases (see 1.5.2) most oxygenases are non-heme proteins and, consequently, the nature of metal atom binding to the enzyme is almost completely unknown.

A third problem which makes the elucidation of oxygenase mechanisms difficult is one which is inherent to redox reactions in general; namely, the difficulty of determining oxidation states and the sequence in which oxidation-reduction steps occur when various

interacting species are present. From a chemist's point of view, even factors as elementary as reaction stoichiometry and stable product identification are complicated because the first formed products of oxygenase reactions are usually metabolized further. Good kinetic studies have been limited, because such circumstances have made it difficult to develop satisfactory enzyme assays. Although limited mechanistic information has been derived from an application of classical methods of enzymology to the oxygenases, the increasing use of fast reaction techniques and tools like electron spin resonance spectroscopy with highly purified enzyme preparations has future promise (48).

1.5.1.2 Oxygenase Mechanisms: It is probably fair to say that, at the enzymatic level, the mode of action of the oxygenases is obscure, and that this is especially true at the level of oxygen activation and its incorporation into substrate (49,50). At the present time there are basically three lines of evidence, from enzyme studies, from which one can speculate about the oxygenation step: (1) Product studies and oxygen labelling experiments, (2) deuterium isotope studies of hydroxylation at saturated carbon (51), and (3) discovery of the "NIH shift" (52,53).

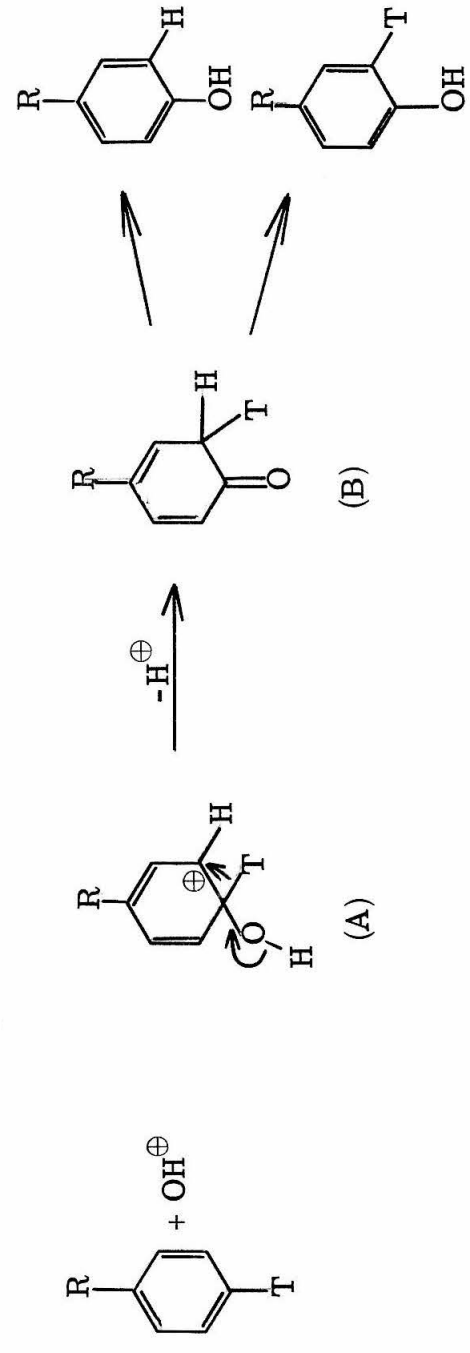
The first and second pieces of evidence are useful in that they put boundary conditions on the possible mechanisms one might consider, but they say little about oxygen activation or the nature of the oxygenating species. The NIH shift, on the other hand, involves an apparent 1,2-shift of substituents at the point of hydroxylation on aromatic rings,

and represents an unusual deviation from standard aromatic substitution processes. If its existence continues to be substantiated, the NIH shift should prove to be a useful mechanistic probe.

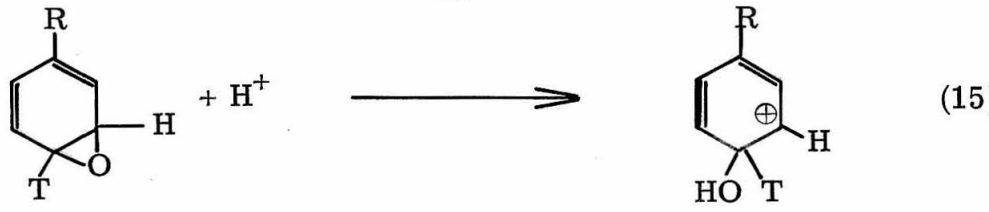
To date, all but one (54) of the reported cases of the NIH shift have come from the National Institutes of Health laboratories of Udenfriend, Witkop, Daly, and Guroff, who have evidence that the shift occurs with hydroxylases for phenylalanine, tryptophan, tyrosine, and 4-hydroxyphenyl pyruvate, and the nonspecific hydroxylases of rabbit liver microsomes (55). They have also shown that the shift occurs in vivo as well as in vitro (53).

On the basis of the observed shift in studies of enzymatic and model systems (56), the following mechanism has been proposed (Scheme VI) (53,57).

A concerted mechanism has been ruled out by these authors (58), and they seem to favor a hydroxyl cation type attack on the aromatic ring which implies heterolytic oxygen-oxygen bond breaking by the enzyme. More recently they have pointed out, however, that there is no evidence to rule out the intermediate formation of a benzene epoxide, which has been proposed as an intermediate in aromatic hydroxylation (4). Since protonation of an epoxide could lead (equation 15) to the same intermediate (A) as that proposed in Scheme VI, it seems clear that the case for $OH^{\oplus}$ attack from NIH shift studies is weak and that at the present time there is no conclusive evidence from which to draw conclusions concerning the mechanism of the hydroxylation step.



Scheme VI



(15)

1.5.2 Peroxidases

1.5.2.1 Enzyme Preparations and Structure: As a class, the peroxidases (catalase is included as a special case) do not have all of the drawbacks which were discussed in connection with the oxygenases (see 1.5.1.1). Pure enzyme preparations have long been available. Horseradish peroxidase for example was obtained in crystalline form more than 25 years ago (59), and it has recently been reported that the amino acid sequence of bovine liver catalase is near completion (60). Secondly, classical peroxidases are all iron hemoproteins, which means that the nature of the metal complex is better understood than is the case with the oxygenases. Finally, since peroxidases use as substrate a partially reduced form of oxygen, the complex problem of oxidation states, inherent with the oxygenases, is simplified. Thus, as demonstrated below, in vitro peroxidase mechanisms are better understood than oxygenase mechanisms, although the biological significance of the former remain obscure (61).

1.5.2.2 Catalase Mechanisms: The overall picture of the mechanism of action of catalase has changed only slightly from that suggested 20 years ago by Chance on the basis of kinetic studies (62). The mechanism shown in equations 16 and 17 was published in a recent paper on beef liver catalase (63):

$$Fe^{3+}\text{—OH} + H_2O_2 \rightleftharpoons Fe^{3+}\text{—O—O—H} + H_2O \quad (16)$$

$$Fe^{3+}\text{—O—O—H} + H_2O_2 \longrightarrow Fe^{3+}\text{—O—H} + O_2 + H_2O \quad (17)$$

It has been suggested that decomposition occurs as shown in Scheme VII. This mechanism is consistent with the observed absence of free radicals in the decomposition of hydrogen peroxide by peroxidase (64) and with the results of a number of recent model studies (see 1.6.2.3). Note that the oxygen-oxygen bond is cleaved in a concerted 2-electron step.

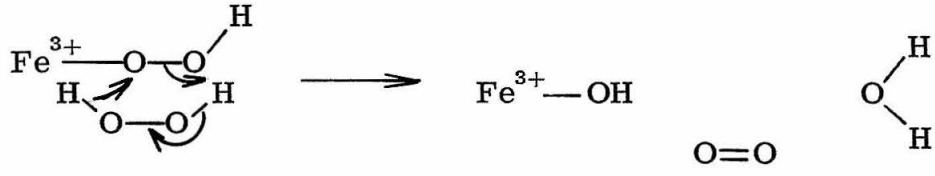
1.5.2.3 Peroxidase Mechanism: Unlike the catalase system, peroxidase activity seems to be associated with the formation of free radicals via 1-electron reduction (Scheme VIII) (65,66). The exact nature of Compound II is a source of current debate (67). It is generally presumed to be a species of oxygenated iron with an unknown distribution of electrons and protons.

The free radicals generated in the peroxidase reaction may subsequently disproportionate (equation 18) (65),

$$2AH\cdot \rightleftharpoons A + AH_2 \quad (18)$$

or dimerize (equation 19).

$$2AH\cdot \longrightarrow HA - AH \quad (19)$$



Scheme VII

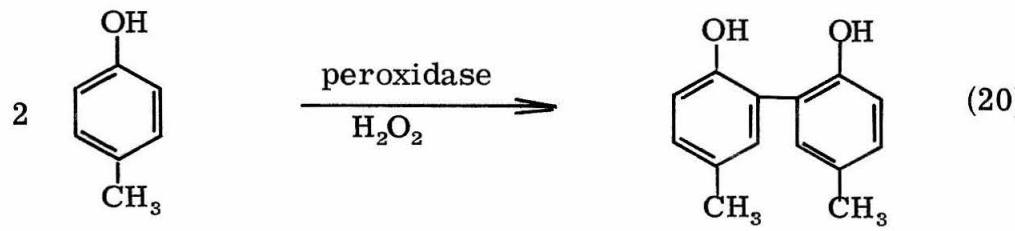
$$Fe^{3+} + ROOH \rightleftharpoons Fe^{3+} \cdot ROOH$$

$$Fe^{3+} \cdot ROOH + AH_2 \longrightarrow \text{Compound II} + AH\cdot + ROH$$

$$\text{Compound II} + AH_2 \longrightarrow Fe^{3+} + AH\cdot$$

Scheme VIII

Dimerization is very common with phenols; thus p-cresol in the presence of hydrogen peroxide and horseradish peroxidase gives 2,2′-dihydroxy-5,5′-dimethyl biphenyl (equation 20) (68):



(20)

Note that hydroxylation has never been found to occur under the above conditions (69). Hydroxylation with peroxidase (see equation 14) requires oxygen and dihydroxy fumaric acid. The oxygen/dihydroxy fumarate/peroxidase system is believed to involve free radicals (70). It has been reported, however, that no free radicals have been detected during substrate hydroxylation by the same system (69). The reasons for this difference are not clear, and the nature of possible hydroxylating species is presently being debated.

It would be possible to go on at some length discussing the pros and cons of various proposals for the monooxygenase activity of peroxidase, but inevitably one is forced to admit that there are too many unknown factors in the enzymatic system. It then becomes necessary to turn to relatively simple nonenzymatic systems.

1.6 Model Studies

We have discussed above (see 1.5.1.1) some of the factors which have contributed to the relatively slow development toward an understanding of oxygenase mechanisms. One other factor which has not

been mentioned is a function, not of the enzymes, but of their non-enzymatic model systems. A survey of the chemical literature pertaining to oxidations and oxygen fixation reveals two things: First, the limited number of known reactions which might serve as oxygenase models, and second, the limited degree of understanding of the mechanistic details of these reactions. At this point some of the systems which have been suggested as models for oxygenase or peroxidase action are briefly reviewed. The discussion will be limited to systems involving metal ions, all of which happen to be models for aromatic hydroxylases or peroxidases.

1.6.1 Systems Utilizing Molecular Oxygen

1.6.1.1 Udenfriend's and Related Systems: Udenfriend's system, which consists of ferrous iron, EDTA, ascorbic acid, and molecular oxygen (71), is the oldest, most widely known and best studied model for the monooxygenases. A variation on the basic system, which has received some attention recently, employs tetrahydropteridine (THP) in place of ascorbic acid (72). It has been argued that the mechanism of action is the same in either case, with THP or ascorbic acid acting as a two-electron reducing agent (72) (see below).

Oddly enough, studies of Udenfriend's system were begun a year prior to Mason's and Hayashi's experiments demonstrating the existence of the oxygenases. After 15 years of study, however, the only points of agreement concerning the mechanism of Udenfriend's system are that the iron atom complexes with oxygen, and that the complex is reduced by a one or a two-electron transfer from ascorbate (or THP)

to produce the oxygen-transfer species. During the past five years no less than four distinct mechanisms have been written for the process of oxygen activation and transfer to substrate. The main features of each are summarized below:

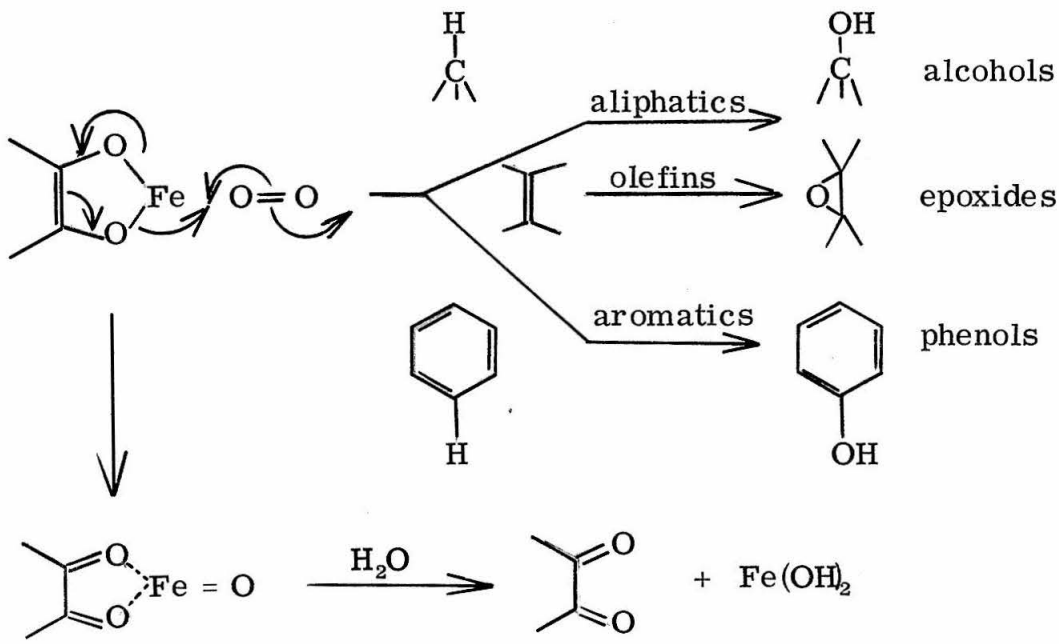
(1) Oxygen atom transfer from an $Fe^{2+} \cdot O_2$ complex to substrate (73,74) (Scheme IX) (73). It has been suggested that these oxygen insertion reactions be called "oxene" reactions because of their similarity to reactions of carbene and nitrene analogs (75).

(2) Oxygen atom transfer from an iron (IV)-oxygen complex (76) (Scheme X). The participation of ferryl iron, Fe^{IV}, has been invoked in a number of other studies (see 1.6.2.1).

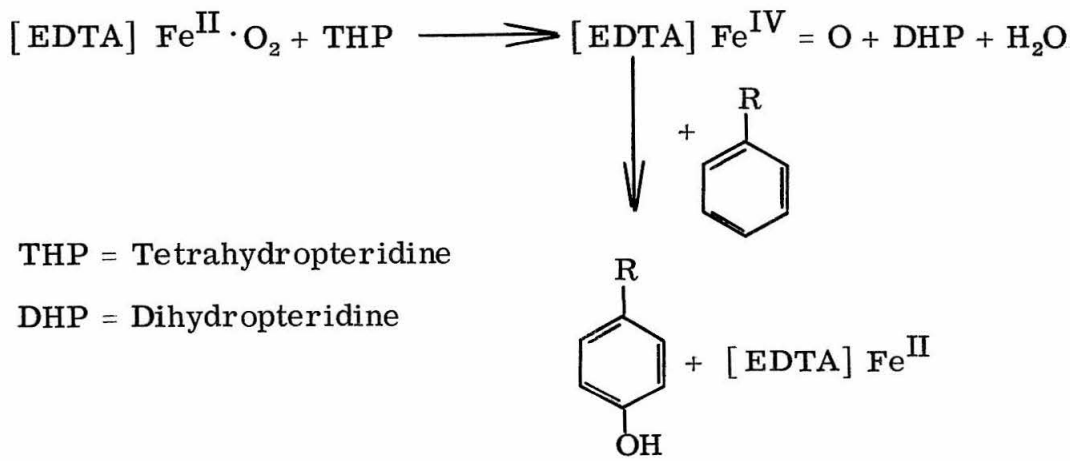
(3) Transfer of superoxide-radical ($O_2^{\ominus}\cdot$) to substrate (Scheme XI) (77). The $Fe^{2+}O$ is presumably reduced to $Fe^{2+} \cdot H_2O$ by ascorbic acid.

(4) Transfer of hydroxyl radical to substrate (78,79) (Scheme XII) (78).

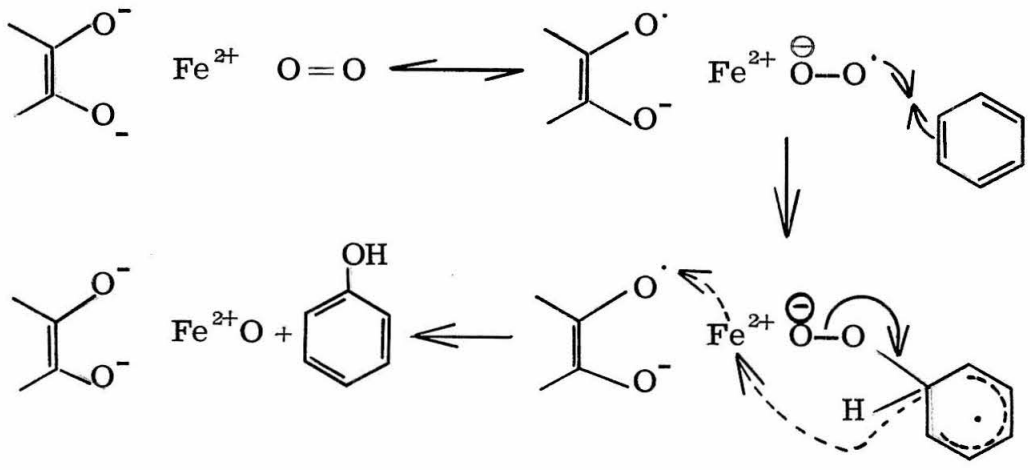
In each of the above cases the proposed mechanism is based on studies of reaction stoichiometry and reaction products.



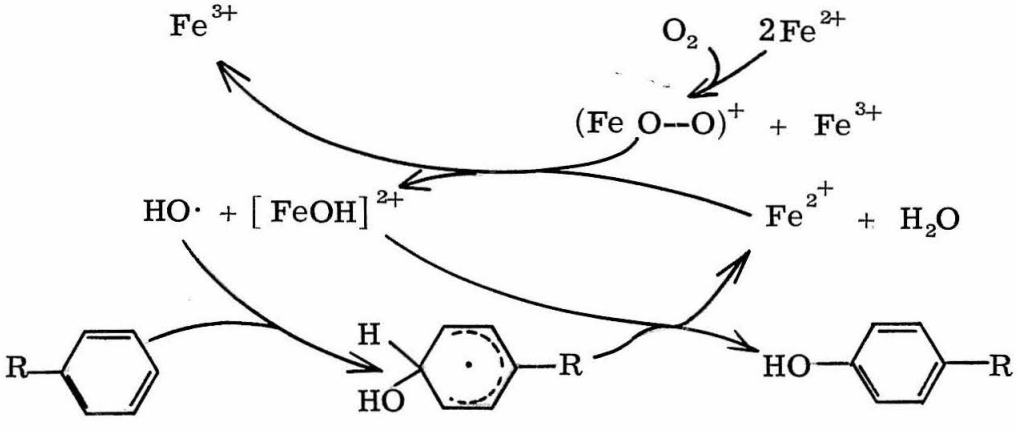
Scheme IX



Scheme X



Scheme XI



Scheme XII

1.6.1.2 Autoxidation of Metal Ions: Hydroxylation reactions have been observed during the autoxidation of various metal ions. In general these systems have received less attention than the Udenfriend system. At least three oxygen-transfer species have been postulated.

(1) An oxygen atom (oxene) from a system containing $Sn^{2+}/HPO_4^{2-}/O_2$ (80).

(2) An $[FeO_2]^{2+}$ species in the autoxidation of Fe^{2+} (81).

(3) The perhydroxyl radical (HOO·) in the autoxidation of Ti^{3+} (82).

The nature of postulated hydroxylating agents is based on product studies and comparison with better known systems such as Fenton's reagent (see 1.6.2.1). The highly speculative nature of the proposed mechanisms is clear in view of the fact that the details of autoxidation processes for ferrous and cuprous ions in the absence of substrate have only recently been elucidated (83,84).

1.6.2 Systems Employing Hydrogen Peroxide

1.6.2.1 Fenton's Reagent: Fenton's reagent, Fe^{2+}/H_2O_2, is the best studied system known to bring about aromatic hydroxylation. The results of many years of study were summarized in a recent paper by Shiga and Isomoto who said, "Despite considerable effort to clarify the nature of the reactive species involved in Fenton's reagent since the 1930's, no definite conclusion has yet been reached" (85).

Hydroxyl Radical: Over the years, the hydroxyl radical has been the favored hydroxylating species (77,86) generated by Fenton's reagent. Recent electron spin resonance studies have been published in support of hydroxyl radical (85,87). A postulated mechanism for hydroxyl radical production by Fenton's reagent is given in Scheme XIII (77). There is a fair amount of evidence to show that the hydroxyl radical, produced by Fenton's reagent, or by the pulse radiolysis of water, is capable of converting aromatic compounds to phenols by way of cyclohexadienyl radicals (see 2.5.1.2).

Ferryl Iron ($Fe^{IV}O)^{2+}$: A recently published study of Fenton's reagent suggests that ferryl iron ($Fe^{IV}O)^{2+}$ exists in place of hydroxyl radical in all but the most acid media (i.e., $Fe^{IV}O^{2+}$ exists if pH>2) (88). Based on rate studies employing electron spin resonance spectroscopy, the mechanism in Scheme XIV has been proposed.

The author suggests that the FeO^{2+} species forms in the absence of complexing agents, otherwise, aspects of the mechanism are quite similar to proposed mechanisms of interaction of peroxide with transition metal complexes (see 1.6.2.2 and 1.6.2.3).

1.6.2.2 Hamilton's System: Hamilton's system (89), catechol/Fe^{3+}/H_2O_2, is a relatively recent discovery, and has not received as much attention as the models discussed above. Two possible hydroxylating species have been suggested on the basis of product studies and the kinetics of peroxide disappearance.

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + HO\cdot$$

$$Fe^{2+} + HO\cdot \longrightarrow Fe^{3+} + OH^-$$

$$H_2O_2 + HO\cdot \longrightarrow H_2O + HO_2\cdot$$

$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + HO_2\cdot + H^+$$

$$Fe^{3+} + HO_2\cdot \longrightarrow Fe^{2+} + O_2 + H^+$$

Scheme XIII

$$Fe^{2+} + H_2O \rightleftharpoons Fe\cdot OH^+ + H^+$$

$$FeOH^+ + H_2O_2 \longrightarrow FeO^{2+} + H_2O + OH^-$$

$$FeO^{2+} + H_2O_2 \longrightarrow HO_2\cdot + OH^- + Fe^{3+}$$

$$FeO^{2+} + \text{scavenger} \longrightarrow \text{products}$$

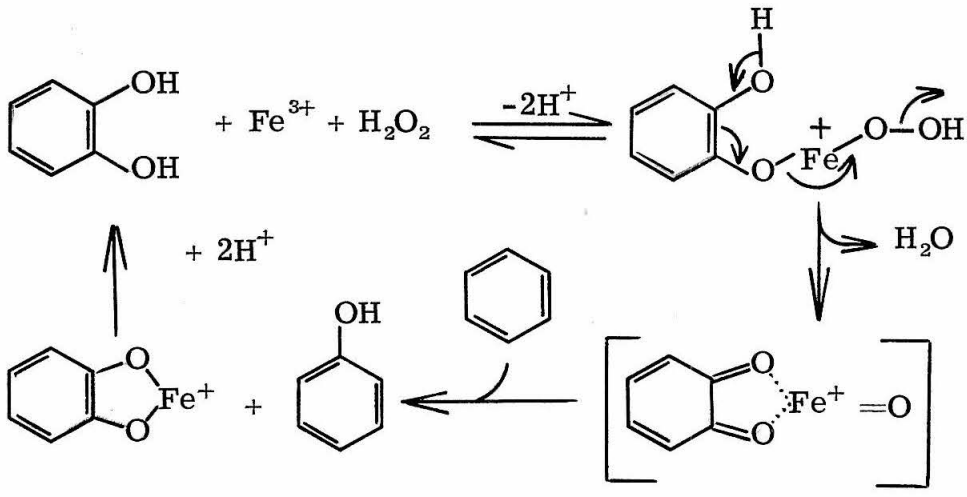
Scheme XIV

(1) Oxygen atom transfer: The mechanism favored by Hamilton consists of oxygen atom transfer from a catechol-iron-oxygen complex (Scheme XV) (90). Note that the oxygen activation process takes place in two-electron steps, which excludes the formation of free hydroxyl radicals.

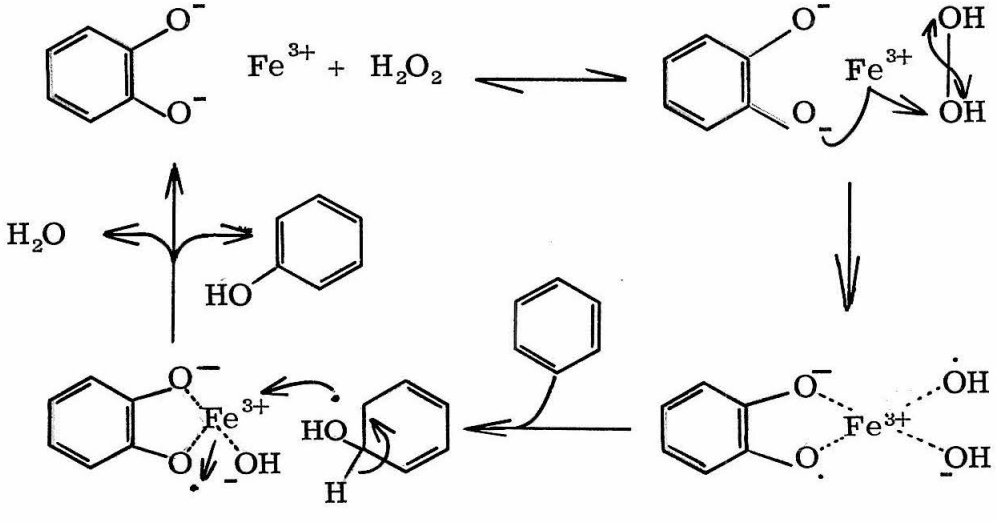
(2) Hydroxyl radical transfer: An alternate mechanism employing one-electron steps and hydroxyl radical formation has been proposed by Norman and Smith (Scheme XVI) (77).

In addition to the hydroxylase action of the above system, Hamilton has suggested that the system behaves as a peroxidase in that it is capable of converting alcohols to ketones (91). He has proposed that the same catechol-iron-oxygen complex shown in Scheme XV is responsible for peroxidase activity. In the section which follows, two systems are discussed which show only peroxidase-like activity.

1.6.2.3 Cupric Ion Complexes: It was pointed out recently that, "no significant quantitative studies on catalytic reactions of copper complexes exist from which reaction mechanisms can be deduced." The existing situation has improved during the last year as the result of several kinetic studies in buffered aqueous solution. The two systems noted below represent the first published studies of peroxide decomposition in the presence of complexes of known concentration.



Scheme XV



Scheme XVI

Cupric Ion Histamine System (92): The overall rate expression for oxygen evolution in the pH region 6-9 for a system consisting of cupric ion, histamine (Hm), and hydrogen peroxide was found to obey equation 21:

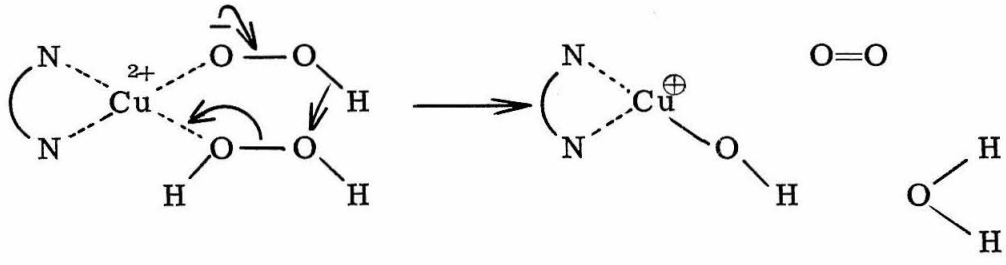
$$\frac{dO_2}{dt} = k_1'[CuHm^{2+}][HOO^-] + k_2'[CuHm(HOO^-)^+][HOO^-] \qquad (21)$$

The results indicate that only the 1:1 complex of cupric ion and histamine is active in the decomposition process which the authors suggest occurs by a combination of radical and ionic steps.

Cupric Ion 2,2′-Bipyridyl System (93,94): Erlenmeyer, Sigel, and co-workers have studied peroxide decomposition in the presence of cupric ion and 2,2′-bipyridyl (bipy). They have reported that the rate of peroxide decomposition is consistent with equation 22 in the pH region 5.5-7.5:

$$\frac{-d[H_2O_2]}{dt} = \frac{k[Cu\ bipy^{2+}][H_2O_2]^2}{[H^+]} \qquad (22)$$

As with the cupric ion-histamine system, the above result indicates that the 1:1 complex of cupric ion and bipyridyl is the only one of several possible complexes which is actually involved in the decomposition process. The kinetic expression implies that one molecule of hydrogen peroxide and one peroxide anion participate in the transition state for decomposition. The mechanism shown in Scheme XVII has been suggested by the authors.



Scheme XVII

A study of systems involving hydrogen donors other than hydrogen peroxide indicates that very similar decomposition mechanisms may be operative (94). These systems have been suggested as catalase and peroxidase models (compare with proposed catalase mechanism, Scheme VII).

1.6.3 Summary

In the foregoing discussion, an attempt has been made to describe the nature of the most recent studies pertaining to those systems which have been seriously considered as models for oxygenases and peroxidases. In summary, the following observations are made:

(1) The model systems which employ molecular oxygen are the most complex and consequently the least understood. On the basis of available information, it is not possible to choose between the diverse mechanisms which have been proposed.

(2) The nature of Fenton's reagent is still not entirely clear; but even if the hydroxyl free radical hypothesis is accepted, one must conclude that Fenton's reagent is an inadequate model, as truly free hydroxyl radicals are unlikely hydroxylating agents in substrate-specific enzymatic systems.

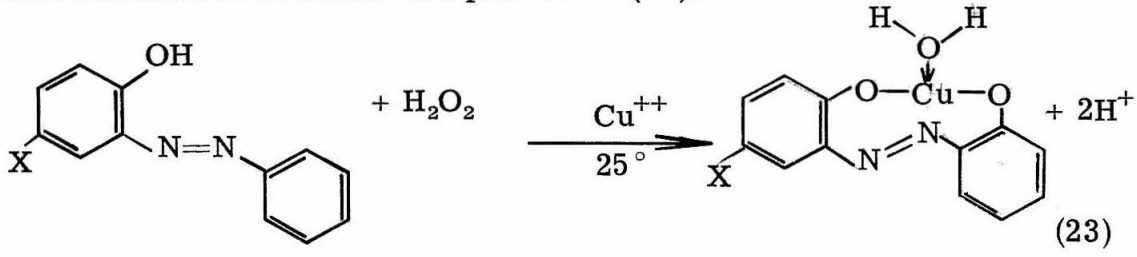
(3) Although Hamilton's system appears to have many interesting features, especially as a potential model for peroxidase-oxygenase function, it has not yet received enough attention to allow one to draw any firm conclusions regarding the mechanisms of oxygen activation or

hydroxylation.

(4) The systems involving cupric ion complexes and hydrogen peroxide may be valuable models for peroxidase-oxidase functions, but to date there is no evidence that they participate in oxygenase-type reactions, and in this sense they must be regarded as incomplete models.

1.7 The 2-Hydroxyazobenzene, Cupric Ion, Hydrogen Peroxide System

In view of growing interest in the oxygenases and of the relative lack of unambiguous information concerning their modes of action, we were interested in studying some mechanistic aspects of the hydroxylation reaction illustrated in equation 23 (95):



(23)

The 2,2′-dihydroxyazobenzenes are important mordant dyes. For this reason, the reaction has received some attention from dye chemists, but with almost no consideration from a mechanistic point of view. The only known facts about the above reaction beyond the present study are:

(1) The hydroxylation occurs exclusively in the 2′ -position (95).

(2) That the reaction appears to be specific for cupric ion and the 2-hydroxyazobenzenes.

(3) That the reaction is inhibited by 2′ -substituents which are capable of coordination with cupric ion, i.e., 2′ -methoxy or 2′ -carboxy (96).

(4) That upon substitution in the 5-position, higher product yields are observed with electron-withdrawing substituent ($x = NO_2$) than with electron-donating substituents ($x = OCH_3$) (97).

The results of a number of new studies of the above reaction are presented below from which we draw conclusions regarding its mechanism. The significance of these results in terms of related biological and chemical systems is discussed.

Chapter 2. RESULTS AND DISCUSSION

2.1 Synthesis

2.1.1 Derivatives of 2-Hydroxyazobenzene

Hydroxyazobenzenes are most easily prepared by the diazo-coupling reaction of amines with phenol derivatives. Phenol itself couples with aniline diazonium salts to give 99% 4-hydroxyazobenzene (98). High yields of 2-hydroxyazobenzenes can be obtained if 4-substituted phenols are used. Most of our syntheses were done with phenol substituted in the 4-position by methyl or sodium sulfonate. In addition the parent compound, 2-hydroxyazobenzene (IV), was synthesized by a more involved procedure (see 3.2.4).

2.1.1.1 2-Hydroxyazobenzenes: Table 1 summarizes the 2-hydroxyazobenzenes used in a study of hydroxylation in non-aqueous solvents. All of the derivatives listed in Table 1 are known compounds, and from the melting point data we conclude that all are reasonably pure materials. The possible exception is the nitro derivative (III) which received only one recrystallization; however, we do not feel that this factor seriously affects the results obtained with this derivative.

2.1.1.2 2-Hydroxyazobenzene-5-Sodium Sulfonates: The data in Table 2 summarize the derivatives of 2-hydroxyazobenzene-5-sodium sulfonate used in a study of hydroxylation in aqueous solution.

Table 1. Derivatives of 2-Hydroxyazobenzene

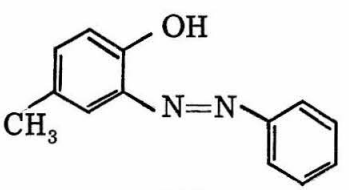
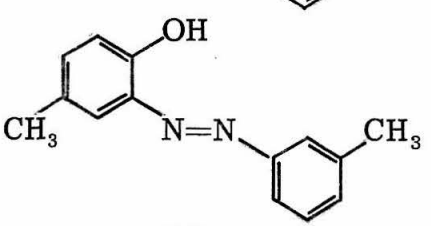
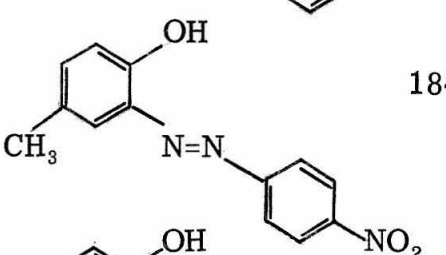
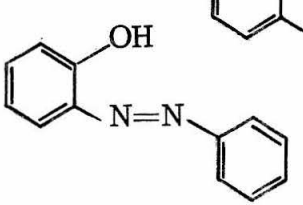
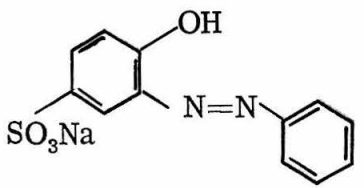
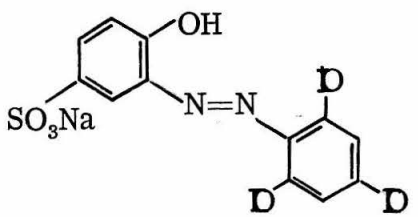
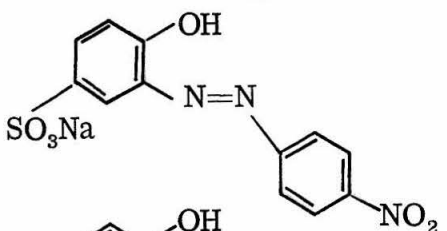
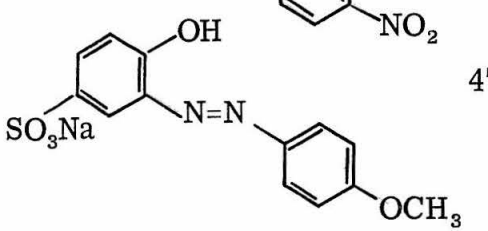
Compound No.	Structure	MP	Lit MP	Extinction Coefficient in Glacial Acetic Acid	λ in mμ
I		107-108.5°	108° (99)	0.78×10^{4} M^{-1}	385
				0.04	490
II		99-101°	98-100° (100)	0.85	390
				0.05	495
III		184-190°	191° (101)	0.87	400
				0.10	520
IV		81-82°	82-83 (102)	0.92	375
				0.02	485

Table 2. Derivatives of 2-Hydroxazobenzene-5-Sodium Sulfonate

Compound No.	Structure	Calculated			Found			% Purity	Extinction Coefficient[b] at 500 mμ
		C	H	C/N	C	H	C/N		
V		48.0	3.0	5.13	48.3	2.8	5.09	94	$0.17 \times 10^4\ M^{-1}$ (0.57)[a]
VI									
VII		41.7	2.3	3.42	40.8	2.4	3.39	96	0.61 (0.92)[a]
VIII		47.4	3.33		46.4	3.53		90	0.15 (1.00)[a]

[a]Numbers in brackets refer to extinction coefficient of 1 : 2 copper complex. [b]At pH 7.3 in 0.26 M sodium perchlorate.

The poor quality of the analytical data in Table 2 results from the fact that sodium sulfonates are generally difficult to purify. All of the compounds used in quantitative studies (V-VIII) were crystallized at least three times from ethanol-water and dried for two hours at 120° before use. From literature reports (103, 104) on the preparation of similar compounds, we expect that after simple recrystallization and drying, inorganic salts and water of hydration might still be present as major impurities. Although compound VII, for example, undergoes a 10 percent weight loss on drying, the presence of a few remaining weight percent water even after drying is apparent from the low titration analysis (96%), and the low carbon and high hydrogen microanalyses. Note that for VII the experimental C/N ratio (3.39) is in fairly good agreement with the calculated value (3.42). This is to be expected in the absence of impurities which contain carbon or nitrogen. The presence of such impurities, though not expected to interfere directly with our experiments, made it necessary to compute concentration values from analytical data.

The nature of the 2′, 4′, 6′-trideuterio derivative, VI, was established by comparing its visible spectrum with that of compound V. The two compounds had identical spectra in aqueous solution in the region from 350 mμ to 700 mμ in the presence and in the absence of cupric ion. In comparative studies (see 2.5) we, therefore, assumed that both compounds were of the same purity. The deuterium substitution pattern is discussed in section 2.5.2.1.

2.1.2 Derivatives of 2, 2′-Dihydroxyazobenzene

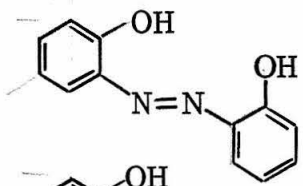
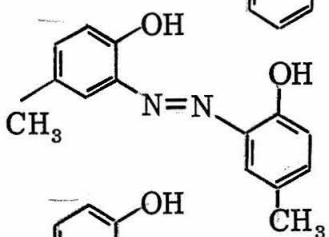
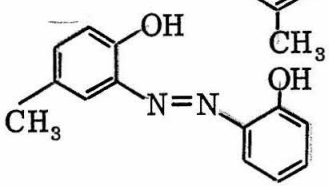
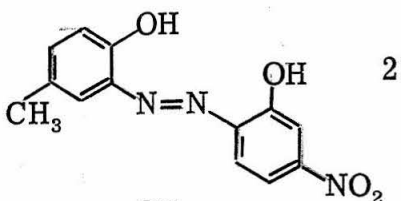
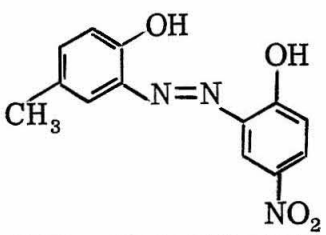
The 2, 2′-dihydroxyazobenzenes cannot be prepared by the straightforward approach used to prepare the 2-hydroxyazobenzenes, because the diazo-coupling reaction fails if 2-aminophenols are used (96). The hydroxylation reaction (equation 23) with cupric ion and hydrogen peroxide offers the only direct route to 2, 2′-dihydroxyazobenzene derivatives. Unfortunately, the method has not been used for the preparation of pure, metal-free compounds. Therefore, the compounds listed in Table 3 were synthesized by alternate routes.

2.1.2.1 2, 2′-Dihydroxyazobenzenes: Table 3 summarizes the 2, 2′-dihydroxyazobenzenes which were prepared for the study of hydroxylation in glacial acetic acid. The parent compound, X, was prepared in 14% yield from o-aminophenol by a coupling method which has been described for the preparation of symmetrical 2, 2′-dihydroxyazobenzenes (105).

As the unsymmetrical derivatives XII to XIV could not be synthesized by the coupling procedure, a reasonable alternative path to these previously unreported compounds seemed to be demethylation of 2′-methoxy-2-hydroxyazobenzenes, which could be prepared by diazocoupling o-anisidines with 4-substituted phenols.

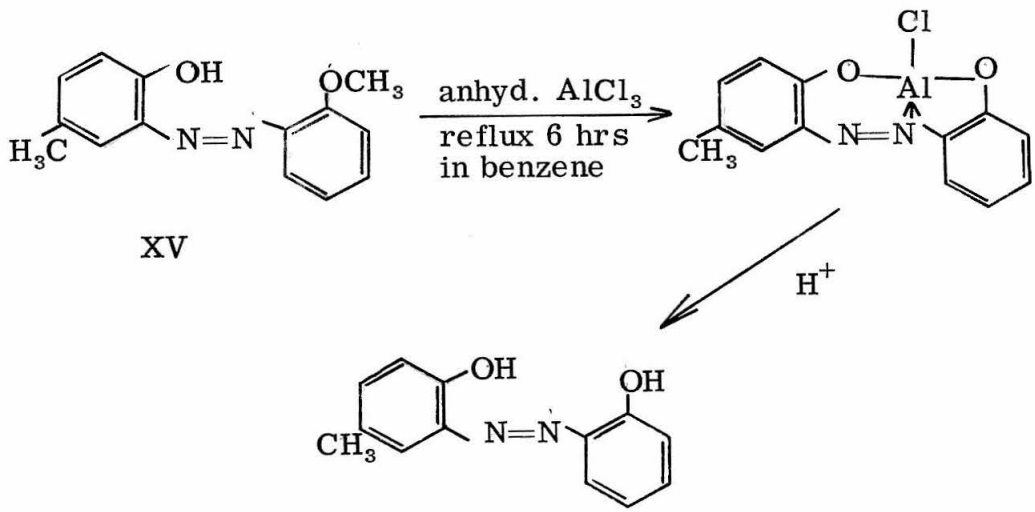
Attempts to demethylate 2′-methoxy-5-methyl-2-hydroxyazobenzene (XV) with such demethylating agents as hydrobromic acid (106), hydriodic acid (107), or sodium borohydride and iodine (108), were unsuccessful. The infrared spectrum of the compound isolated after treatment with sodium borohydride in the presence of iodine, for instance, matched that of unaltered starting material. Treatment of

Table 3. Derivatives of 2, 2′ -Dihydroxyazobenzene

Compound No.	Structure	M.P.	Lit. M.P.	Calc. C	Calc. H	Found C	Found H	Extinction Coefficient Copper Complex in HOAc	$\lambda_{m\mu}$
X		174-175°	172-172.7° (105)					0.49×10^4 M^{-1}	375
								1.49×10^4 M^{-1}	485
XI		217-219°	219-220° (105)					0.38	390
								1.48	495
XII		164-166°		68.5	5.28	68.0	5.30	0.45	385
								1.45	490
XIII		215-223°		57.2	4.03	57.1	4.06	0.44	400
								1.74	520
XIV		248-253°		57.2	4.03	57.2	4.05		

XV with either hydrobromic or hydriodic acids gave dark products which were most likely mixtures of compounds in which the azo group had been reduced as infrared spectra of the isolated materials showed new bands in the N—H stretch region around 3500 cm^{-1}.

Compounds XI, XII, and XIII were successfully prepared by a demethylation procedure utilizing anhydrous aluminum chloride, equation 24. Since the aluminum complexes isolated from the reaction



XV

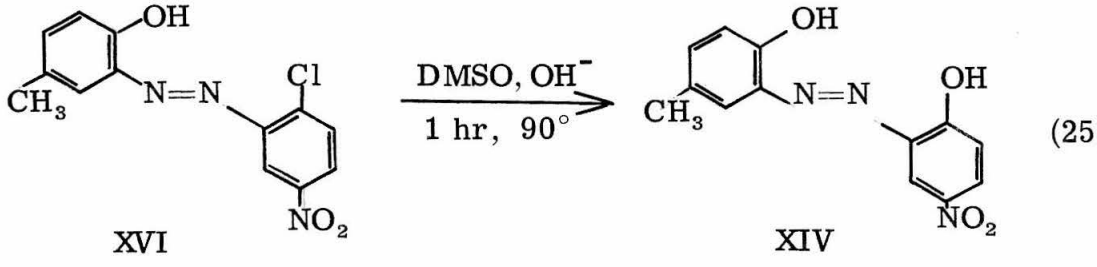
XII

(24)

mixtures were insoluble in strong acid, the metal-free compounds could not be recovered, as is often done, by heating with strong acid. The aluminum complexes were, however, soluble in alkali; and yields of metal-free 2, 2′-dihydroxyazobenzenes as high as 60% were obtained by precipitating the free ligands through the rapid acidification of solutions of the complexes in concentrated sodium hydroxide. (If acid was added slowly, the complex precipitated however.)

The identity of the compounds prepared by aluminum chloride demethylation is supported by agreement between the melting points of XI and the same compound prepared by a different method, and by the microanalyses of previously unreported compounds XII and XIII.

We conclude this section by mentioning the successful preparation of 5′-nitro-2′-hydroxy derivative, XIV, which was recovered in 20 percent yield after heating the 2′-chloro derivative, XVI, at 90° with sodium hydroxide in dimethylsulfoxide for one hour (equation 25).



XVI XIV (25)

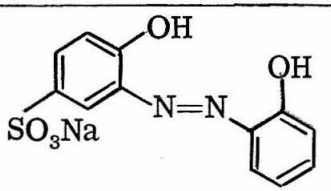
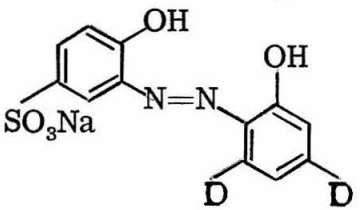
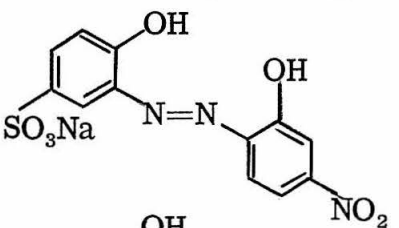
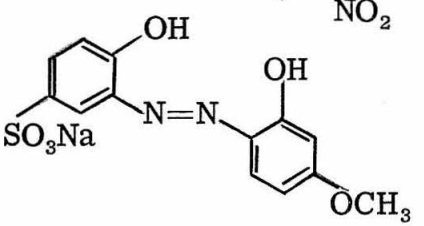
This reaction represents a new approach to the preparation of 2, 2′-dihydroxyazobenzene derivatives by nucleophilic substitution. Previously reported nucleophilic substitution methods (96) have employed cupric ion in aqueous ammonium hydroxide. These methods have the disadvantage of giving a 2, 2′-dihydroxyazobenzene chelate from which the metal-free compound must be recovered, whereas reaction 25 gives the desired product directly. The key factor in reaction 25 is the solvent, dimethyl sulfoxide (DMSO), which has been shown to be an excellent solvent for nucleophilic substitution reactions (109). We have not investigated the utility of reaction 25 with other substituents.

2.1.2.2 2, 2′ -Dihydroxyazobenzene-5-Sodium Sulfonates: Table 4 summarizes the 2, 2′ -dihydroxyazobenzene-5-sodium sulfonates used in a study of hydroxylation in aqueous solution. Attempts to prepare these compounds by demethylation with aluminum chloride (see equation 24) were complicated by the insolubility of the sodium sulfonates in benzene and also by their solubility in water. In several attempts to prepare compound XVII by the demethylation of IX, only a few milligrams of product were isolated. An attempt to prepare XIX by nucleophilic substitution in DMSO (see equation 25) also failed.

In view of these difficulties, we decided to employ the hydroxylation reaction with cupric ion and hydrogen peroxide (equation 23). This procedure (see 3.2.7 for details) gave, after the removal of copper ion, a mixture of 2, 2′-dihydroxyazobenzene-5-sodium sulfonate and starting material, i.e., 2-hydroxyazobenzene-5-sodium sulfonate. In experiments involving deuterio derivative, XVIII, the components of the mixture were separated by preparative thin-layer chromatography (see 3.5). There was no reason to separate the 2, 2′-dihydroxy derivatives XVII, XIX, or XX from their 2-hydroxy precursors, however, as this was found to be unnecessary because the desired extinction coefficients could be determined directly from the mixtures (see 2.6.2.2).

The weight percents of the 2, 2′ -dihydroxyazobenzene-5-sodium sulfonates were determined in the presence of their 2-hydroxy analogs by titration with cupric ion at pH3 (see 3.5 for details). At this pH the dihydroxy derivatives, but not the monohydroxy compounds, are

Table 4. Derivatives of 2, 2′ -Dihydroxyazobenzene-5-Sodium Sulfonate

Compound No.	Stucture	Percent in Mixture by Weight	Extinction Coefficient of Copper Complex 500 mμ [b]
XVII		73	$1.19 \times 10^4\ M^{-1}$
XVIII		80[a]	1.44
XIX		41	1.97
XX		94	

[a] Estimated from NMR . [b] Measured at pH 3

capable of forming stable complexes with cupric ion. Titration end points were determined from plots of absorbance near λ_{max} for the complex (corrected for dilution by titrant) versus milliliters of added cupric ion. A sample plot is given in Figure 1. From the end point, normality of cupric ion, and weight of sample, the weight percent of 2,2′-dihydroxyazobenzene in the mixture was determined. The results are given in Table 4.

2.2 Qualitative Observations of the Hydroxylation Reaction

Summarized in the introduction (section 1.7) are the results of previous studies of the hydroxylation of 2-hydroxyazobenzenes with hydrogen peroxide in the presence of cupric ion. Because the published reports generally lacked detailed information about the reaction, it was necessary to begin this study with a number of qualitative experiments. The results presented in this section were important in that they helped to determine the direction of the work which led to the more definitive experiments which are discussed in subsequent sections.

In brief summary, the results reported in this section demonstrate that:

(1) Formation of a complex between cupric ion and 2-hydroxyazobenzene is not favored in glacial acetic acid.

(2) Hydroxylation in acetic acid proceeds slowly with an interfering side reaction which destroys the initial product.

(3) Hydroxylation is relatively rapid in solvents (i.e., dimethylsulfoxide, methanol, water) where copper complex formation

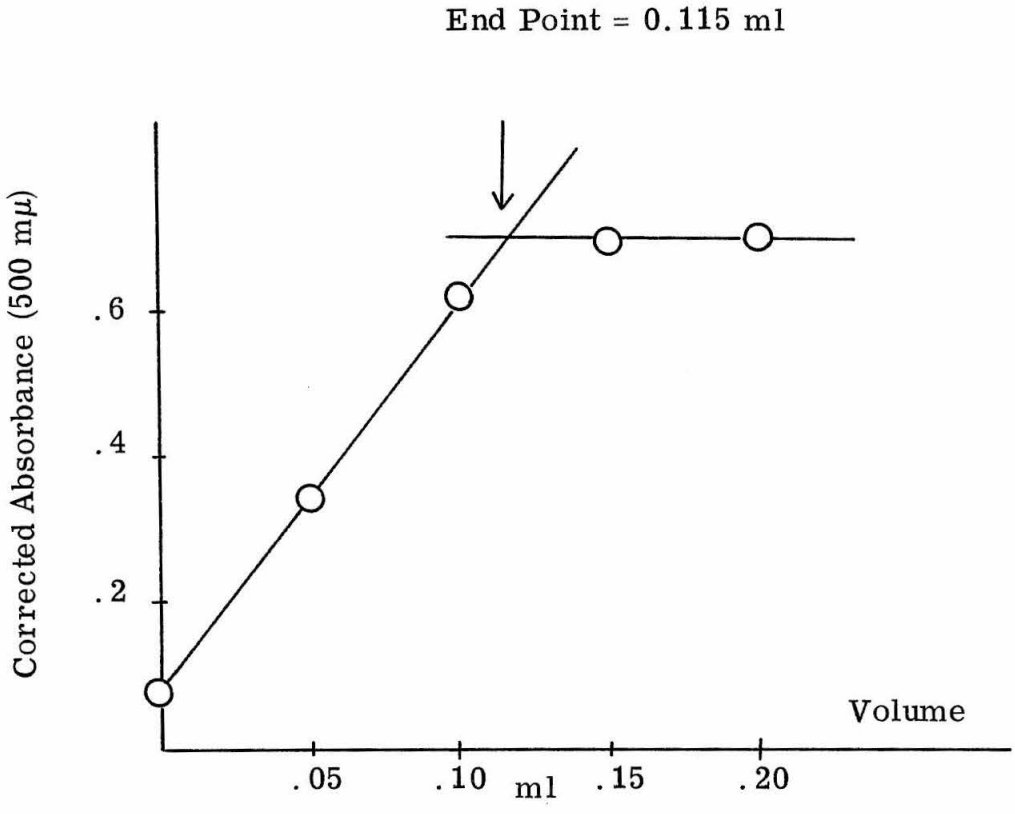


Figure 1. Titration of 0.100 mg of compound XIX with 10^{-3} M cupric nitrate solution. The absorbance values are corrected for dilution by titrant.

is favored.

(4) Whereas increased ligand concentrations accelerate reaction rates in acetic acid, hydroxylation in dimethylsulfoxide/methanol is inhibited by excess ligand.

(5) The hydroxylation reaction appears to exhibit a small substituent effect in acetic acid and in dimethylsulfoxide.

2.2.1 Hydroxylation in Glacial Acetic Acid

2.2.1.1 The Reaction: Glacial acetic acid was the solvent of choice in the most recently published study (97) of the hydroxylation reaction because it is both inert to peroxide and a good solvent for each of the components of the reaction. In glacial acetic acid at 37°, and at concentrations of copper and 2-hydroxyazobenzene near 10^{-2} M, a reaction was observed, i.e., a color change occurred, upon addition of a ten-fold excess of hydrogen peroxide. Progress of the reaction was followed by diluting aliquots of the reaction mixture 100-fold with solvent. Aliquot dilution effectively stopped the reaction and permitted spectral analysis of the mixture. The formation of a copper complex of 2, 2′-dihydroxyazobenzene was detected by the appearance of an absorption band at 485 mμ which corresponds to the absorption maximum of the authentic material.

2.2.1.2 Question of Complex Formation: Although it was possible to observe the formation of a copper complex of 2, 2′-dihydroxyazobenzene in acetic acid, no evidence was found for the existence of a copper complex of starting material. Thus the visible spectrum of 5-methyl-2-hydroxyazobenzene in glacial acetic acid

remained unchanged by the addition of an equimolar amount of cupric ion. This was contrary to expectations, since previous workers had mentioned a 1:1 copper complex under these conditions (97). In addition it had been tacitly assumed that a copper complex was somehow involved in the hydroxylation step.

Additional evidence which suggests the absence of any significant quantity of copper complex with 2-hydroxyazobenzene derivatives in glacial acetic acid was obtained in the following way: The cupric bis (2-benzeneazo-4-methylphenolate) complex (XXI) was prepared in methanol (3.2.10). Such complexes are well known, and the X-ray crystal structure of one is given in Figure 2 (110). A benzene solution of the isolated complex (XXI) showed a λ max at 470 mμ. The addition of a drop of glacial acetic acid to the spectral cell caused a shift to 375 mμ, which is characteristic of the uncomplexed ligand. Since it is unreasonable to assume that such a large shift is merely due to solvent effect on the complex, we conclude that the copper complex is unstable with respect to its components in glacial acetic acid, and that the 1:1 complex implied in previous work does not exist in significant amount.

Although the presence of a 1:1 complex could not be detected spectrally, the results of kinetic studies in acetic acid (2.4.1), which are consistent with results obtained from studies in aqueous solution (2.4.2), indicate that a 1:1 complex participates directly in the hydroxylation step. We conclude (2.4.1) that a very small amount of 1:1 complex exists in acetic acid in rapid equilibrium with its

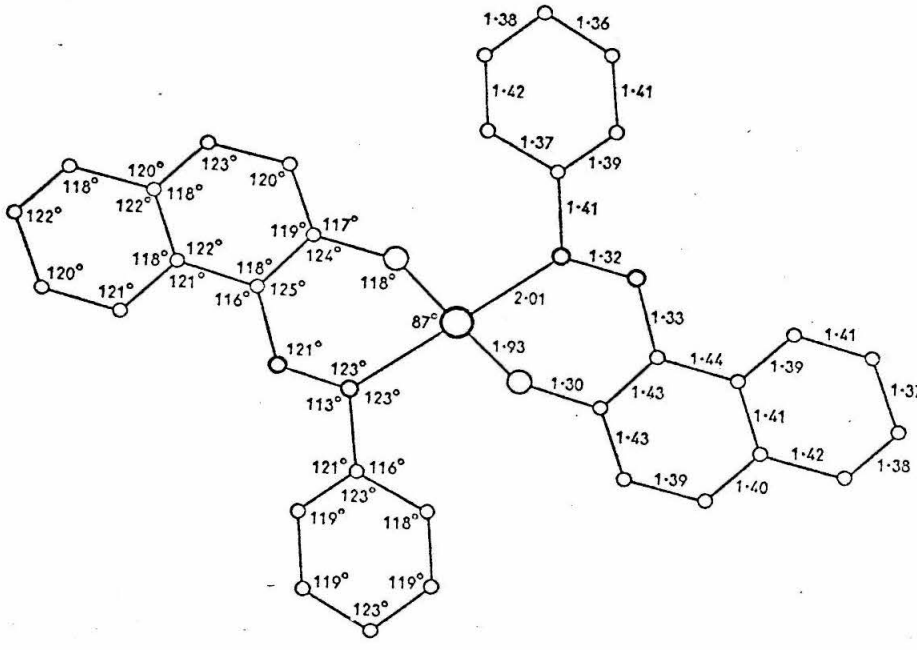


Figure 2. Cu(II)bis (benzene-azo-β-naphthol): bond lengths (in A) and bond angles (110).

components, and that this complex reacts with added peroxide to form the hydroxylated product.

2.2.1.3 Product Complex Destruction: The 2,2′-dihydroxyazobenzene derivatives are much better ligands than their 2-hydroxy analogs. Although their copper complexes were stable in glacial acetic acid, it was found that the complexes were slowly destroyed under the reaction conditions by excess hydrogen peroxide. Thus when 0.1 ml of 30% hydrogen peroxide was added to 5 ml of a glacial acetic acid solution 1×10^{-4} M in copper complex of X, the yellow solution became clear on standing overnight. The disappearance of color is interpreted as an indication that the azo group is being destroyed by the peroxide. Further evidence of a deterioration of product complex was obtained when quantitative calculations showed a decrease in total concentration of azo compounds under reaction conditions (2.4.1.2). The existence of this interfering side reaction was taken into account during the studies of kinetic behavior in acetic acid.

2.2.1.4 Substituent Effect: One of the goals of this work was to study the kinetic effect of substituents on hydroxylation. In qualitative experiments comparing the hydroxylation of 5-methyl-2-hydroxyazobenzene (I) with the 4′-nitro derivative (III), a reaction was observed in both cases on about the same time scale. The existence of a small substituent effect was verified in later quantitative work (see 2.6.1), however, an adequate interpretation of the effect could not be made without a better understanding of the role of complexation. We turned, therefore, to studies in solvents where stable complexes

of copper with 2-hydroxyazobenzenes were formed.

2.2.2 Methanol

Methanol is a solvent in which copper complexes of 2-hydroxyazobenzene are known to be stable, and so a methanol solution of cupric bis (2-benzenazo-4-methyl phenolate), XXI, 2×10^{-4} M, was prepared. [Note that this solution is two orders of magnitude more dilute than the acetic acid systems studied above (2.2.1)]. The effect of hydrogen peroxide on the visible spectrum of the solution at room temperature was observed on quenching the reaction by the addition of an equal volume of glacial acetic acid. Addition of a 100-fold excess of hydrogen peroxide resulted in a very fast reaction as indicated by the fact that in less than 15 seconds the absorbance reading at λ_{max} for product (485 mμ) was 90% of the maximum absorbance attained after several minutes. This result indicates a rather large, qualitative rate enhancement over the reaction in acetic acid where component concentrations are 100-fold greater. The difference in rates as a function of solvent is consistent with the notion that complexation is important in the hydroxylation step, and that the quantity of complex present in acetic acid is small.

When an attempt to study the effect of substituents in methanol was rendered impossible by the insolubility of the copper complex of the 4′-nitro derivative (III), a search for a better solvent became necessary.

2.2.3 Dimethylsulfoxide (DMSO)

In order to study the kinetic effect of substituents and complexation on the hydroxylation reaction, a solvent was required in which the nitro derivative of the complex was both soluble and stable. Furthermore, the solvent had to be miscible with water and relatively inert to hydrogen peroxide. A number of different solvents were tested including dimethylformamide, acetonitrile, benzonitrile, and tetrahydrofuran, but only DMSO was able to satisfy the necessary requirements. In this section are summarized the results of studies in pure DMSO and mixtures of DMSO with water or methanol.

2.2.3.1 Pure DMSO: The visible spectra in DMSO of 5-methyl-2-hydroxyazobenzene (L), an equimolar mixture of cupric ion and L, and a mixture of cupric ion and 5-methyl-2, 2′-dihydroxyazobenzene (D) are shown in Figure 3. The shift in λ_{max} from 380 to 485 mμ on the addition of cupric ion is characteristic of complexation. When a 400-fold excess of hydrogen peroxide (30% diluted 1:10 with DMSO) was added to the 1×10^{-4} M solution of cupric ion and ligand, a product was observed to form slowly which had a λ_{max} at 535 mμ, which is identical to that of the expected product complex (CuD). Under the same conditions the nitro derivative (III) was found to behave similarly, although it reacted more slowly.

In the absence of other reagents, we could show that hydrogen peroxide-DMSO mixtures were stable, but it was found that the addition of 10^{-4} M cupric acetate resulted in a reaction which produced an unknown product exhibiting a λ_{max} at 380 mμ. This

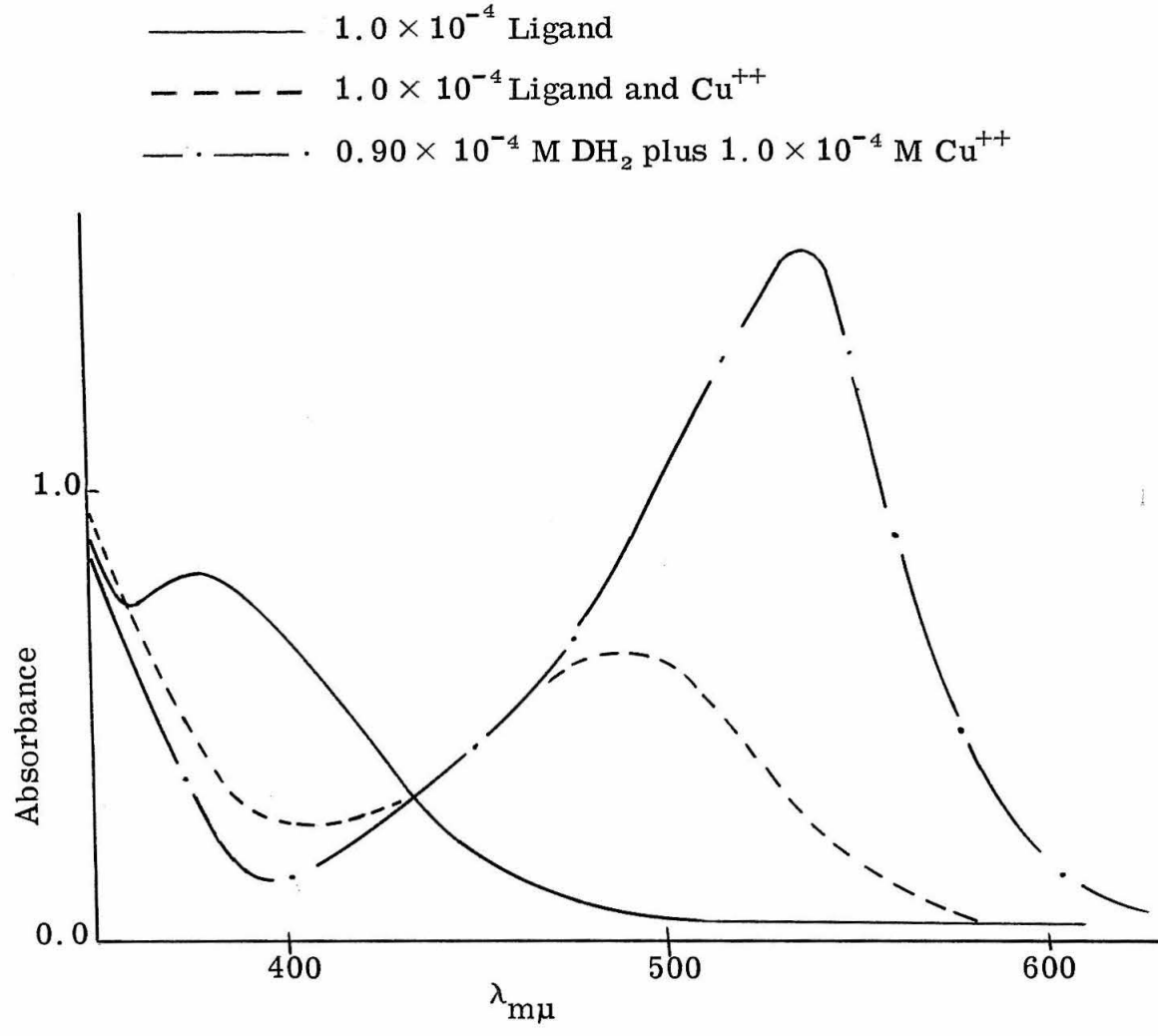


Figure 3. Visible spectra of solutions in DMSO. Ligand = 5-methyl-2-hydroxyazobenzene (I). DH_2 = 5-methyl-2, 2′-dihydroxyazobenzene (XII).

relatively slow side reaction was not observed in systems where the cupric ion was complexed. For this reason reaction mixtures with an excess of cupric ion were avoided.

2.2.3.2 Dimethylsulfoxide/Water (3:1) : In a solvent system of DMSO-water in a 3:1 ratio, uncomplexed ligand was found to exist in equilibrium with its copper complex. The equilibrium is indicated by the effect on the visible spectrum of changes in the copper/ligand ratio. As expected, increasing the copper concentration shifted the equilibrium in favor of complex formation as shown by the increase in absorbance at 485 mμ, Figure 4.

The addition of hydrogen peroxide to a solution containing 2×10^{-4} M cupric ion and ligand gave a rapid reaction. As in the case with methanol, however, the substituent effect could not be studied in this solvent because the 4′-nitro complex was too insoluble.

2.2.3.3 Dimethylsulfoxide/Methanol: The spectrum in DMSO/methanol (3:1) of a solution 1×10^{-4} M in both cupric ion and ligand is very similar to the same system in DMSO alone (Figure 3). The absence of a peak at 380 mμ indicates that, as in DMSO, most of the ligand is complexed. But what of the copper ion? At a copper:ligand ratio of 1:1 it is conceivable that all of the ligand is bound in a 2:1 complex with half of the copper remaining in the free state. An effort was made to shift the equilibrium in favor of a 2:1 complex by adding a large excess of ligand. The results of increasing the copper:ligand ration from 1:10 to 1:40 are given in Table 5. The results, which show a decrease in rate with increasing ligand, and hence complexation,

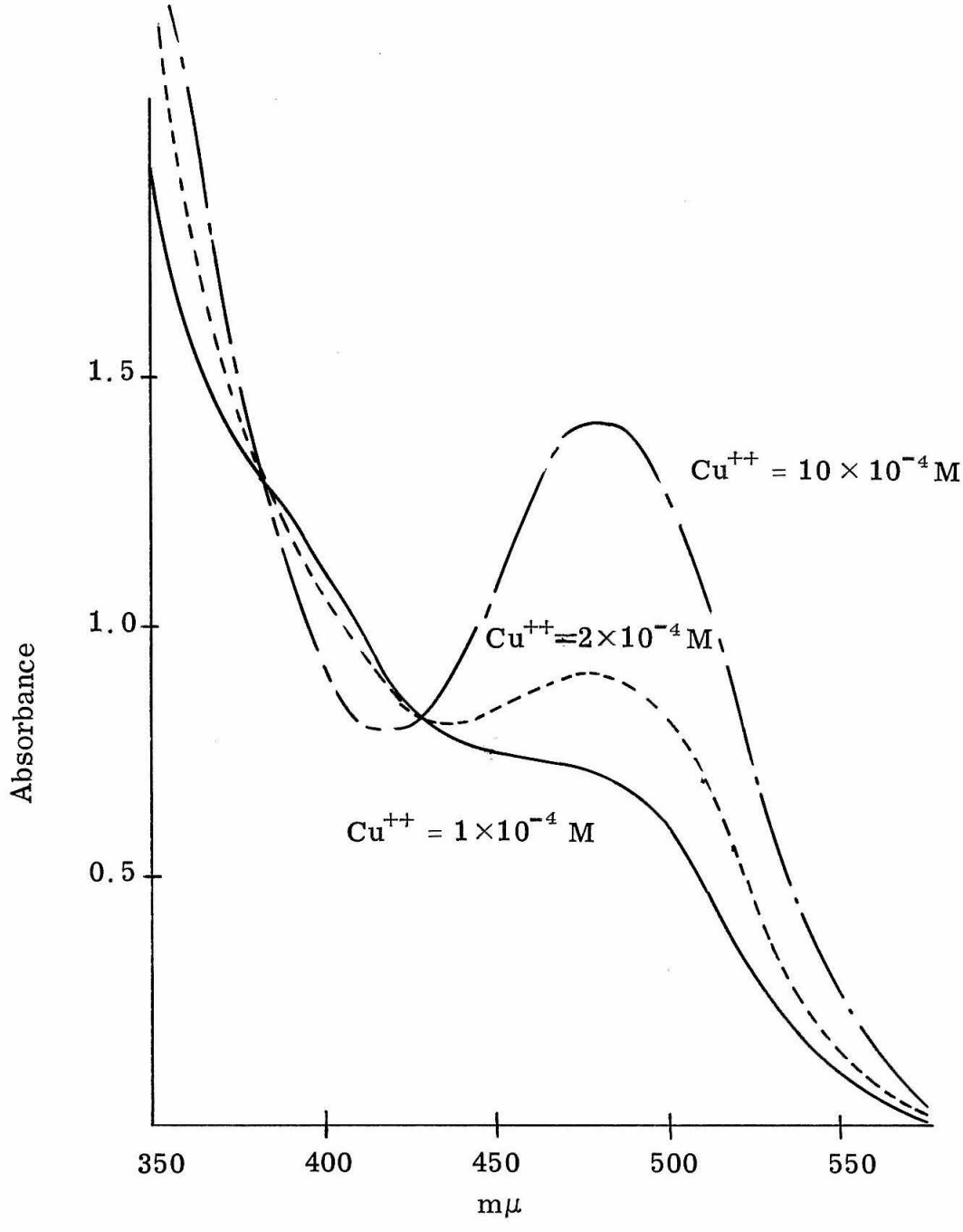


Figure 4. Visible spectra in DMSO/water (3:1) of 5-methyl-2-hydroxyazobenzene, 2×10^{-4} M and $[Cu^{++}] = 1 \times 10^{-4}$ M, ———, 2×10^{-4} M, -----, 10×10^{-4} M, ——— · ———.

Table 5[c]. Kinetic Effect of Excess Ligand[a]

Sample No.	Total Ligand	Initial Slope $\left(\frac{dA}{dt}\right)_o^b$
1	1.0×10^{-3} M	19×10^{-3} min^{-1}
2	1.2	15
3	1.6	5.8
4	2.0	3.2
5	4.0	0.7

[a]Ligand = 5-methyl-2-hydroxyazobenzene. [b]Initial slope is proportional to initial rate; λ = 535 mμ. [c]Total copper concentration = 1.0×10^{-4} M.

were unexpected in view of the assumed importance of complexation (see 2.2.2); but it is quite understandable if one assumes that a series of complexes exist in rapid, multiple equilibrium (see 2.3.1, equations 26-28), and that only the 1:1 complex participates in the hydroxylation reaction. Under circumstances where most of the ligand is complexed, increasing ligand concentration shifts the equilibrium (equation 29) away from 1:1 complex in favor of the kinetically inactive 2:1 form. The results of kinetic studies in support of this proposition are presented in section 2.4, but first it will be necessary to study the complex formation quantitatively.

2.3 Complex Formation

2.3.1 General Considerations

The 2-hydroxyazobenzenes are known to from chelate complexes with transition metal ions (111). The X-ray crystal structure of an isolated copper chelate of phenylazo-2-napthol was presented in section 2.2.1.2. The term complexation was employed in the previous section to describe an interaction which could be detected by changes in visible spectra. A more quantitative consideration of the solution behavior of 2-hydroxyazobenzene-cupric ion systems is now presented.

In solution a system consisting of a monoacidic ligand, LH, and cupric ion can be described in terms of equations 26-29:

$$L^- + H^+ \underset{}{\overset{K_A^*}{\rightleftharpoons}} LH \qquad (26)$$

$$Cu^{++} + L^- \underset{}{\overset{K_1}{\rightleftharpoons}} CuL^+ \qquad (27)$$

$$CuL^+ + L^- \underset{}{\overset{K_2}{\rightleftharpoons}} CuL_2 \qquad (28)$$

$$CuL_2 + L^- \underset{}{\overset{K_3}{\rightleftharpoons}} CuL_3^- \text{ etc.} \qquad (29)$$

*Note that K_A as defined here is equal to $1/K_a$ where K_a is the normally defined acidity constant.

In general only the first three equations need to be considered, as cupric ion forms higher order complexes, i.e., CuL_3^-, with difficulty (112). In the discussion which follows questions of ion solvation, polynuclear complexes, and of complexation with hydroxide ion are

ignored. With these simplifications the system can be described in terms of a proton association constant K_A, and step-wise complex formation constants K_1 and K_2.

An examination of equilibria 26-28 reveals two important considerations: First, complexation should be proton dependent; and second, the solution should contain three states of copper ion whose relative concentrations depend on the values of K_A, K_1, K_2, and the initial concentrations of ligand and cupric ion. The situation becomes clearer if, for example, equation 28 is rewritten as equilibrium expression 30:

$$K_2 = \frac{[CuL_2]}{[CuL^+][L^-]} \quad \text{or} \quad K_2[L^-] = \frac{[CuL_2]}{[CuL^+]} \tag{30}$$

Clearly the predominant complex in solution is expected to be CuL_2 if $K_2[L^-] \gg 1$, whereas CuL^+ should be the major species when $K_2[L^-] \ll 1$.

2.3.2 Complex Formation in Mixed Solvents

Under the conditions of hydroxylation in DMSO-methanol (see 2.2.3.3), what is the nature of the copper complex (or complexes) in solution?

2.3.2.1 Isosbestic Point: In an effort to determine whether one or more complex species exist under reaction conditions, a number of DMSO-methanol (7:3) solutions were prepared at constant ligand, but varying copper ion, concentrations. Since the absorbance due to copper ion is negligible at the concentrations used in the

experiment (except in the last case, i.e. curve 5, where the absorbance due to excess copper ion was blanked) the spectrum obtained will be a composite of the spectra of the free and complex forms of the ligand. Under these conditions the existence of a single copper complex would be demonstrated by the presence of an isosbestic point in the series of spectra (113). The results are shown in Figure 5. Solutions with less than a five-fold excess of cupric ion (curves 1-4) have near isosbestic points at 360 and 435 mμ. This can be taken as evidence for a single complex predominating, but the pronounced deviation observed at higher excess copper ion (curve 5) indicates that significant quantities of a second complex may be present under certain circumstances.

2.3.2.2 Job's Method: What is the predominant species of complex in DMSO-methanol at low copper concentrations? In an effort to answer this question, Job's method (see Appendix I) of continuous variations (114) was applied to the DMSO-methanol system. In its simplest form, Job's method is applicable to systems in which only a single complex is formed. To apply the method, plots are made of the change in some measurable quantity (in this case absorbance) versus mole fraction of ligand in a series of solutions in which ligand plus metal ion is equal to a constant. The formation of a 1:1 complex has been shown to correspond to a maximum at 0.5 on a Job plot; and maxima at 0.67 and 0.75 are expected for 1:2 and 1:3 complexes respectively. A Job plot of the cupric ion-2-hydroxyazobenzene system in DMSO-methanol is given in Figure 6. The existence of an ill-defined maximum between 0.50 and 0.67 confirms the

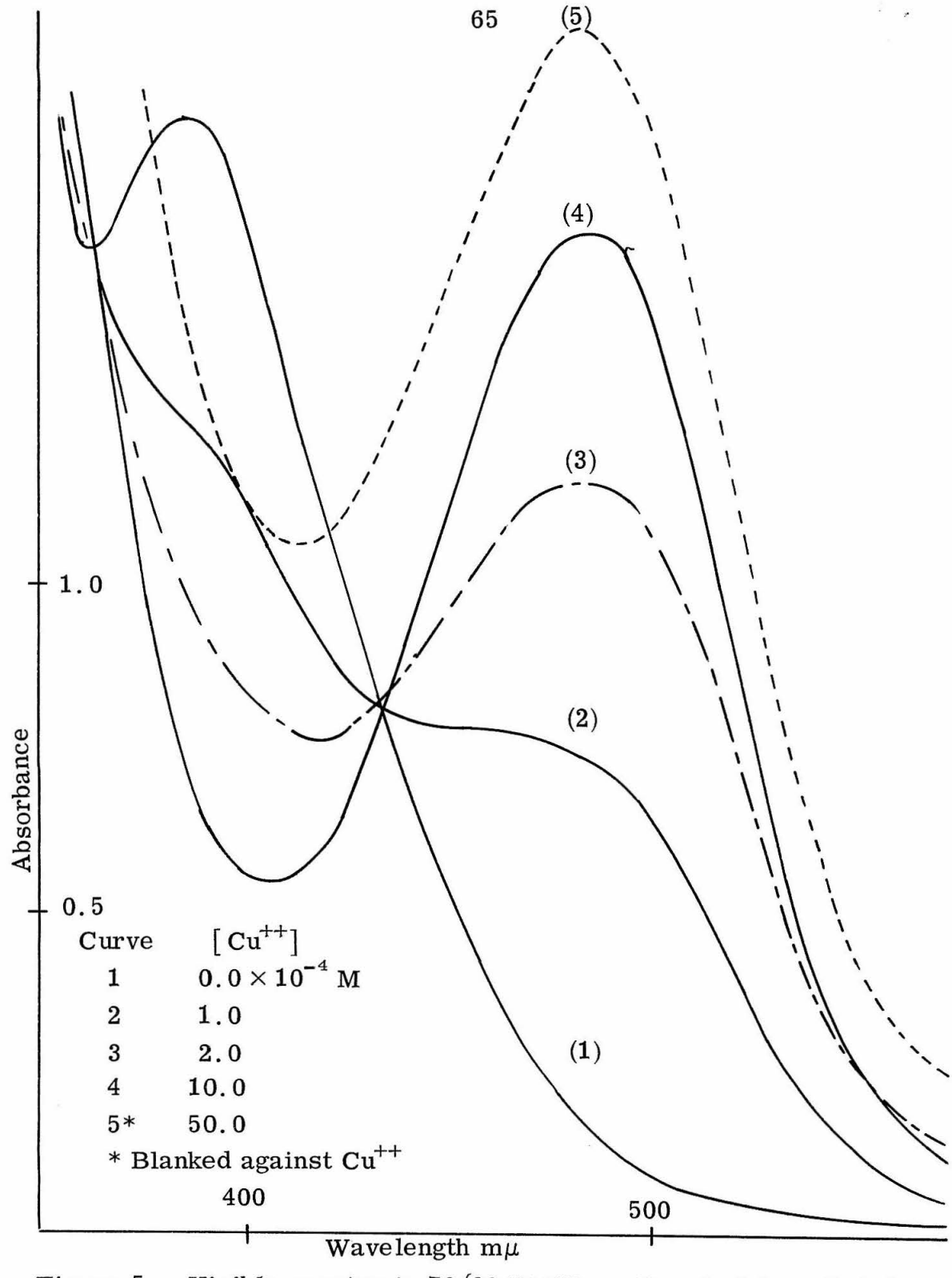


Figure 5. Visible spectra in 70/30 DMSO-methanol of 5-methyl-2-hydroxyazobenzene at 2×10^{-4} M with varying copper acetate.

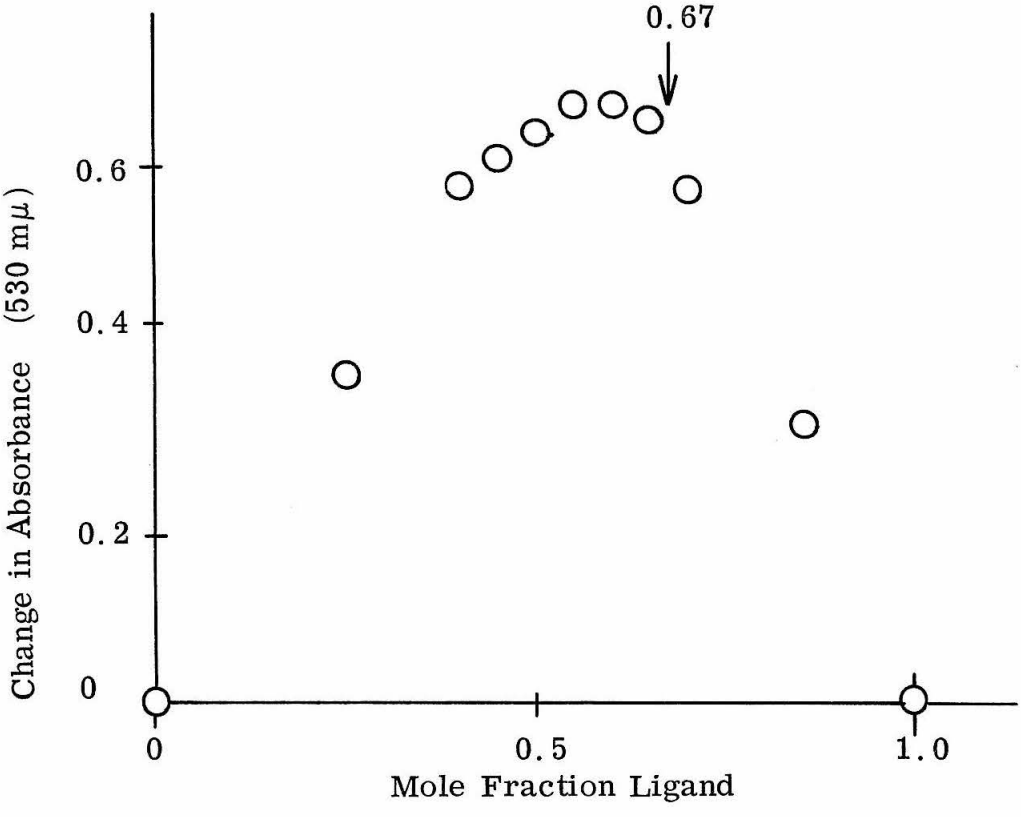


Figure 6. Job Plot. Change in absorbance at 530 mμ vs. mole fraction 2-hydroxyazobenzene. $[L] + [Cu^{++}] = 4.0 \times 10^{-4}$ M in DMSO-methanol (7:3).

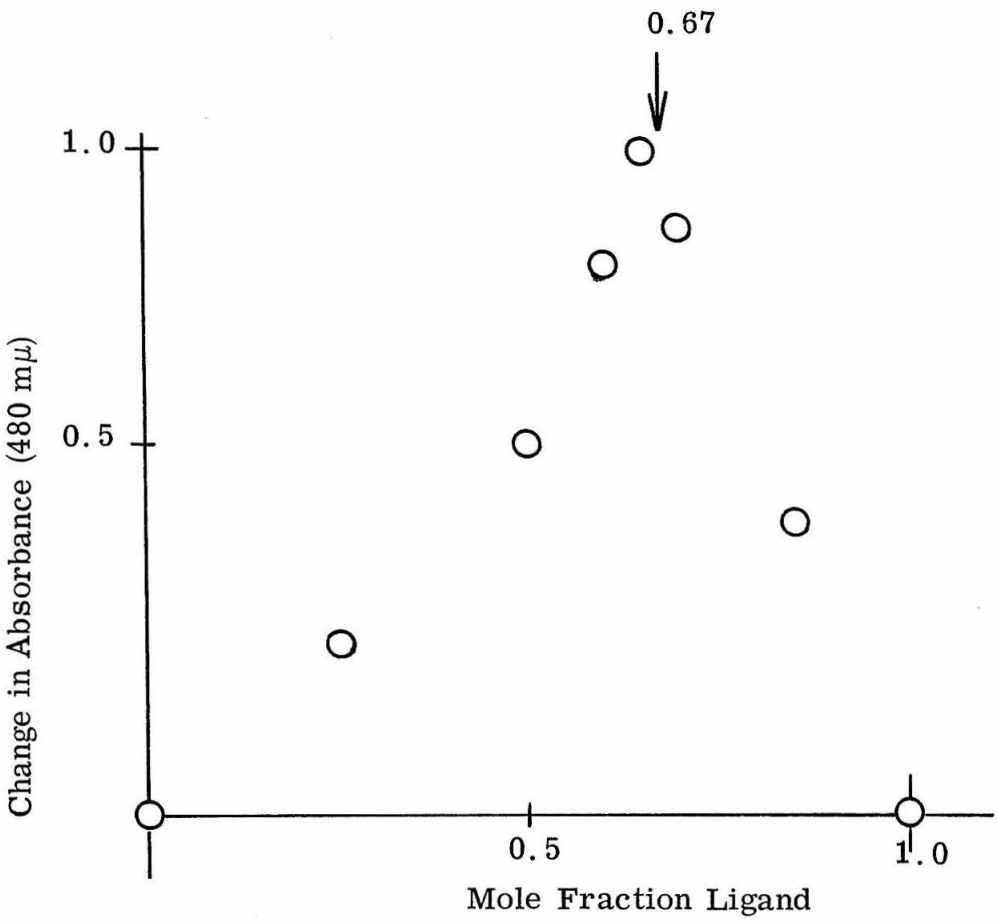


Figure 7. Job Plot. Change in absorbance at 480 mμ vs. mole fraction of 2-hydroxyazobenzene.
$[L] + [Cu^{++}] = 3.6 \times 10^{-4}$ M in DMSO- methanol-pH 7.35 borate buffer (.2 M), (7:3:1).

suspicion that more than one complex is formed and indicates that the solutions may contain a mixture of 1:1 and 2:1 complexes (115), however, the system may be somewhat complicated by the absence of pH control. The effect of pH was investigated by repeating the above experiment in the presence of buffer. A Job plot of the DMSO-methanol system with 1 ml of pH 7.35 borate buffer (0.2 M) added to each 10 ml sample is given in Figure 7.

The sharp maximum near 0.67 in Figure 7 is understandable in view of the prediction (equation 30) that the ratio of CuL_2 to CuL^+ will be increased with increasing ligand anion (L^-) concentration. We suggest that in both the buffered and the unbuffered systems in DMSO-methanol the predominant complex at low copper concentrations is CuL_2, but that increasingly significant amounts of 1:1 complex are formed as the ratio of copper ion to ligand increases. In order to know exactly how the concentrations of complex species vary with initial ligand and copper concentration, a determination of K_A, K_1, and K_2 is required.

2.3.3 Complexation in Aqueous Solution. Formation Constants.

2.3.3.1 pH Titration Method: A general method for studying proton dependent equilibria of the type described in equations 26-28 has been discussed by Irving and Rossotti (116). The pH titration method makes use of a series of "pH" measurements relative to each other so that the method can be used to study complexation in mixed solvents where activity coefficients and electrode calibration factors are unknown. Although a few experiments were conducted in DMSO-

water and in aqueous dioxane, the fact that kinetic measurements at controlled pH would ultimately be desired caused us to shift our attention to aqueous solution.

The titration data are recorded in Appendix II. A typical experiment is illustrated by the plot in Figure 8. The three curves shown correspond to the titration of (A) perchloric acid, (B) perchloric acid plus ligand, and (C) perchloric acid plus ligand and cupric nitrate. In each case the quantity of perchloric acid was held constant, as was the amount of ligand in B and C. All of the titrations were conducted under a stream of nitrogen at 30.0° in 0.265 M sodium perchlorate. The raw titration data are reduced in terms of volume differentials, ΔV and $\Delta V'$ (see Figure 8), as a function of pH. Qualitatively, a strong interaction between copper ion and ligand will result in larger values of $\Delta V'$ at low pH. The specific way in which these quantities are related to formation constants K_1 and K_2 is described in the following section.

2.3.3.2 Data Analysis: The development which follows has been adapted from the treatment of Irving and Rossotti (116).

The formation constant, K_n, for complex ML_n between metal ion M, and ligand, L, is defined as[a]

$$K_n = \frac{[ML_n]}{[ML_{n-1}][L]} \qquad (31)$$

---

[a]Ionic charges are not shown.

Figure 8. pH vs. ml added NaOH. Titration at 30.0° in 0.265 M $NaClO_4$ with 0.0980 N NaOH. □ $HClO_4$; O $HClO_4$ and 2-hydroxyazobenzene-5-sodium sulfonate (V) at 4.9×10^{-4} M; ▽ $HClO_4$, V at 4.9×10^{-4} M, and $Cu(NO_3)_2$ at 1.00×10^{-4} M.

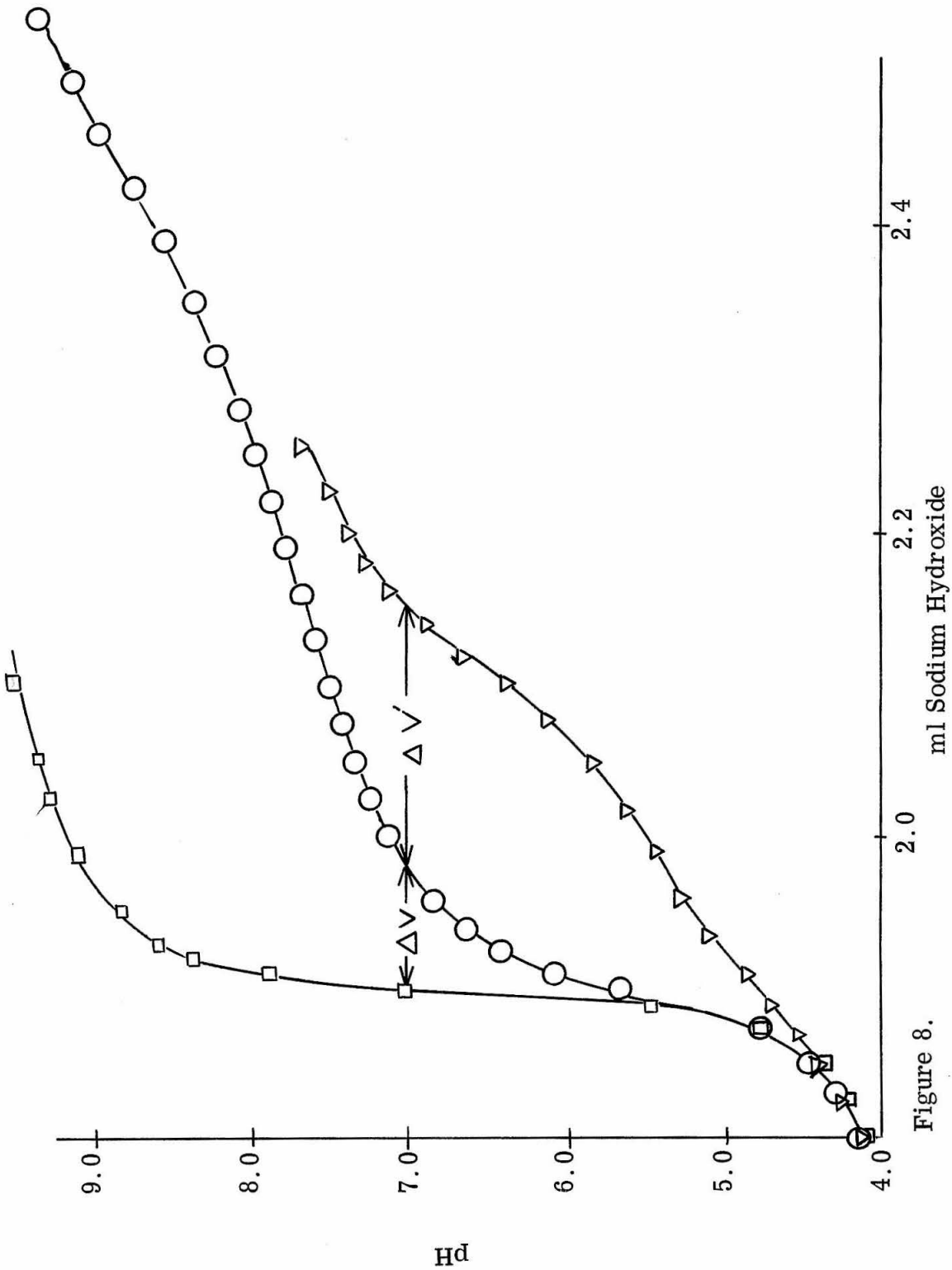


Figure 8.

For a simple mono-acidic ligand, LH, the proton association constant, K_A, is defined as

$$K_A = \frac{[LH]}{[L][H]} \tag{32}$$

Two ratios which are useful in analysis of multiple equilibria are defined in equations 33 and 34.

$$\bar{n}_A = \frac{\text{concentration of protons bound to ligand}}{\text{concentration of ligand not bound to metal}} = \frac{[LH]}{[L]+[LH]} \tag{33}$$

$$\bar{n} = \frac{\text{total concentration of ligand bound to metal}}{\text{total concentration of metal}} = \frac{\sum_{i=o}^{n} i[ML_i]}{\sum_{i=o}^{n} ML_i} \tag{34}$$

Let us consider the titration of solution at constant ionic strength by sodium hydroxide. Let

T_L = total concentration of mono-acidic ligand (LH)

T_M = total concentration of metal ion

E = total concentration of strong mineral acid (i.e., perchloric)

Na = total concentration of sodium ion (from added sodium hydroxide)

OH = concentration of hydroxide ion from hydrolysis of water (i.e., $H_2O \rightleftharpoons H^+ + OH^-$)

V^o = initial volume of solution to be titrated

N = normality of sodium hydroxide titrant

Consider first the titration of mineral acid itself. The free hydrogen ion concentration $[H]'$ can be expressed as

$$[H]' = E' + OH' - Na' \tag{35}$$

For a system containing ligand in addition to mineral acid, the concentration of hydrogen ion $[H]''$ becomes

$$[H]'' = E'' + OH'' - Na'' + T_L'' + \bar{n}_A'' T_L'' \tag{36}$$

where the last two terms are simply the concentration of ionized ligand, since

$$T_L - \bar{n}_A T_L = T_L \left(1 - \frac{[LH]}{[L] + [LH]}\right) = T_L \cdot \frac{[L]}{[L] + [LH]} \tag{37}$$

$$= [L]$$

because $T_L = [L] + [LH]$ in systems which do not contain metal ion.

If the system to be considered contains a metal ion in addition to ligand and mineral acid, the hydrogen ion concentration $[H]'''$ is found to be

$$[H]''' = E''' + OH''' - Na''' + T_L''' - \bar{n}_A''' T_L''' + \bar{n}''' \bar{n}_A''' T_M''' \tag{38}$$

where the last three terms represent the total concentration of protons released from the ligand, i.e., T_L - LH, because

$$T_L - \bar{n}_A T_L + \bar{n}\bar{n}_A T_M = T_L - \bar{n}_A (T_L - \bar{n}T_M)$$

$$= T_L - \frac{[LH]}{(T_L - \bar{n}T_M)} \cdot (T_L - \bar{n}T_M)$$

$$= T_L - [LH]$$

The substitution of $[LH]/T_L - \bar{n}T_M)$ for $\bar{n}_A$ follows directly from the definitions of $\bar{n}_A$ and $\bar{n}$.

Now consider the three systems at constant pH and constant ionic strength where $[H]' = [H]'' = [H]'''$, $OH' = OH'' = OH'''$, and $\bar{n}_A'' = \bar{n}_A'''$.

Subtracting 35 from 36 and rearranging terms gives

$$\bar{n}_A'' = \left[(E''-E') - (Na''-Na') + T_L''\right] / T_L'' \qquad (39)$$

If we let V', V'', and V''' be volumes of sodium hydroxide added to each system, then under conditions where $V^0 \gg V'$, V'', V''', we may neglect slight differences in volume during the titrations so that $E' \cong E'' \cong E'''$ and $V^0 + V' \cong V^0 + V'' \cong V^0 + V''' \cong V^0$. Equation 40 now simplifies to

$$\bar{n}_A'' = (Na' - Na'' + T_L^0)/T_L^0 \qquad (40)$$

and since

$$Na' = \frac{V'N}{V^0} \text{ and } Na'' = \frac{V''N}{V^0}$$

equation 40 becomes

$$n_A'' = \frac{(V' - V'')N}{V^0 T_L^0} + 1 \qquad (41)$$

In a similar way, subtracting 36 from 38 gives 42 if the same simplifying assumptions are made.

$$\bar{n} = \frac{Na''' - Na''}{\bar{n}_A''' T_M^0} \cong \frac{(V''' - V'')N}{\bar{n}_A T_M^0 V^0} \qquad (42)$$

If we define volume differentials ΔV and $\Delta V'$ (see Figure 8) as

$$\Delta V = V'' - V'$$

$$\Delta V' = V''' - V''$$

then equation 41 becomes

$$\bar{n}_A = 1 - \frac{\Delta V N}{T_L^0 V^0} \qquad (43)$$

and equation 42 becomes

$$\bar{n} = \frac{\Delta V' N}{\bar{n}_A T_M^0 V^0} \qquad (44)$$

Perhaps the easiest way to understand equation 44 is to consider Figure 8 in the following way: Imagine a solution of ligand and mineral acid which has been titrated to pH 7.0. The state of this

solution corresponds to a particular point on curve B. Now imagine adding copper ion to the solution with simultaneous addition of base to maintain pH 7.0. Since the pH remains constant, the ratio $[LH]/[L]$ is constant. The point of intersection of curve C with pH 7.0 will be attained when T_M reaches 1.00×10^{-4} M. We can imagine that the added copper complexes with ligand by two paths

$$Cu^{++} + LH \rightleftharpoons CuL^{+} + H^{+}$$

$$Cu^{++} + L^{-} \rightleftharpoons CuL^{+}$$

The fraction of ligand bound by the first path is reflected in $\Delta V'$, the quantity of base required to compensate for the released protons. Since the ratio of ligand to its anion remains constant, the fraction of ligand bound by the second path must be proportional to $\Delta V'[L]/[LH]$. The sum of these two processes will give the total amount of ligand bound to metal, i.e., $\bar{n} T_M$. Thus

$$\bar{n} T_M = (\Delta V' + \Delta V'[L]/[LH])(N/V^0) \qquad (45)$$

$$= \frac{\Delta V' N}{V^0} \cdot \frac{[LH] + [L]}{[LH]}$$

Equation 45 rearranges to 44 on substitution for the value of $\bar{n}_A$ as defined in equation 33.

From pH titration data it is now possible to compute $\bar{n}_A$ and $\bar{n}$ as functions of measured pH. One additional quantity which will be needed to calculate formation constants is $[L]$, the concentration of

free, ionized ligand in solution. From $\bar{n}$ and the hydrogen ion concentration we can write that

$$K_A = \frac{T_L^o - [L] - \bar{n}T_M^o}{[L][H]} \tag{46}$$

Equation 46 follows from 32 since

$$LH = T_L^o - L - \bar{n}T_m^o \tag{47}$$

Solving equation 46 for L gives 48.

$$L = \frac{T_L^o - \bar{n}T_M^o}{1 + K_A[H]} \tag{48}$$

The relationship between $\bar{n}$, L, and formation constants K_1 and K_2 is found in the classic formulation of Bjerrum who showed (see Appendix III-A) that equation 49 is true for a system of mononuclear complexes (117).

$$\sum_{n=0}^{N} (\bar{n} - n)\,\beta_n[L]^n = 0 \tag{49}$$

where $\beta_n = K_1K_2K_3 \cdots K_n$ and $\beta_0 = K_0 = 1$

If we limit ourselves to the case where N = 2, equation 49 becomes

$$\bar{n} + (\bar{n} - 1)K_1[L] + (\bar{n} - 2)K_1K_2[L]^2 = 0 \tag{50}$$

By dividing equation 50 by $(n - 1)[L]$ and rearranging terms,

equation 51 is obtained.

$$\frac{\bar{n}}{(\bar{n} - 1)[L]} = \frac{(2-\bar{n})[L]}{\bar{n} - 1} K_1K_2 - K_1 \qquad (51)$$

Equation 51 has the form y = mx + b which is the equation of a straight line of slope m and intercept b. The formation constants can in principle be determined by plotting values of

$$y = \frac{\bar{n}}{(\bar{n} - 1)[L]} \qquad (52)$$

versus

$$x = \frac{(2 - \bar{n})[L]}{(\bar{n} - 1)} \qquad (53)$$

Such a plot will have a slope of K_1K_2 and a y-intercept of $-K_1$.

2.3.3.3 Results of Studies with Derivatives of 2-Hydroxyazobenzene-5-Sodium Sulfonate: The formation constants K_1 and K_2 were determined for three derivatives of 2-hydroxyazobenzene-5-sodium sulfonate from pH titration data using equation 51. The results of least squares analysis of the data are given in Table 6.

A sample of the least squares fit to the data for run 33c is included in Figure 9 to give an idea of the type of data obtained from the calculations. Inspection of equation 51 reveals that small errors in $\bar{n}$ can result in large errors in x or y for $\bar{n}$ values near 0, 1, and 2. For this reason only values of $0.2 < \bar{n} < 0.8$ and $1.2 < \bar{n} < 1.8$ were used in the calculation of K_1 and K_2.

Table 6.[a] Formation Constants for Derivatives of 2-Hydroxyazobenzene-5-Sodium Sulfonate

Run	Compound	4′-Substituent	$K_1 \times 10^{-5}$	$K_2 \times 10^{-5}$	Ligand pK_a
33c	V	-H	2.3	7.2	
33d	V	-H	2.8	7.2	7.70
ave			2.5	7.2	
100	VII	$-NO_2$	3.8	4.4	
104	VII	$-NO_2$	4.1	2.3	7.10
ave			4.0	3.4	
96	VIII	$-OCH_3$	3.2	22	
97	VIII	$-OCH_3$	2.2	14	7.75
ave			2.7	18	

[a]At 30.0° in 0.265 M sodium perchlorate.

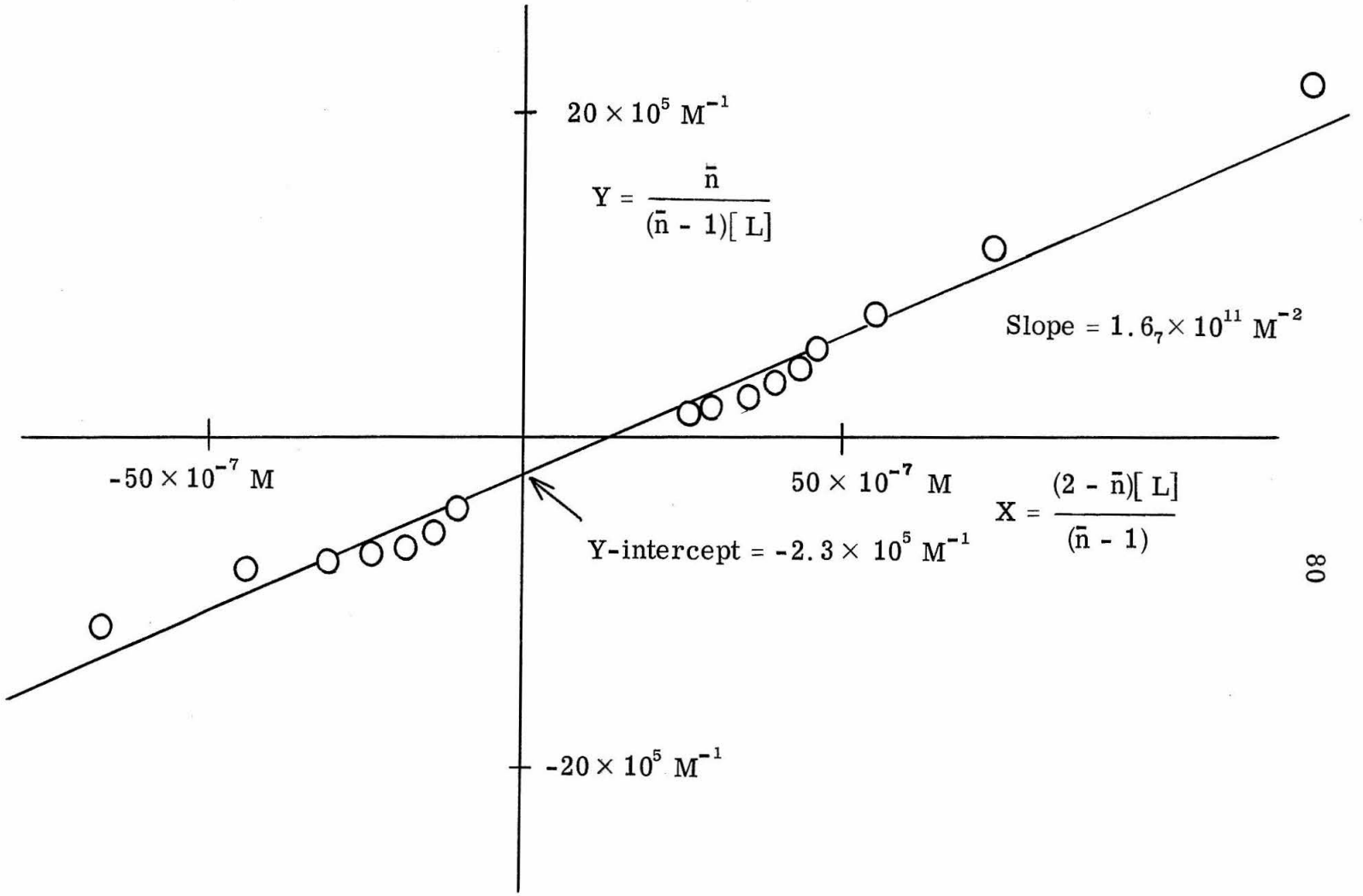


Figure 9. Plot of $Y = \bar{n}/(\bar{n} - 1)[L]$ vs. $X = (2 - \bar{n})[L]/(\bar{n} - 1)$ for run 33c.

2.3.3.4 Correction for Hydrolysis: The formation constants, K_1 and K_2, reported in Table 6 were calculated on the (often valid) assumption that hydrolysis (equations 54 and 55) is negligible.

$$Cu(H_2O)_x^{++} \rightleftharpoons^{K^H_{Cu}} CuOH(H_2O)_{x-1}^{+} + H^+ \qquad (54)$$

$$CuL(H_2O)_x^{n+} \rightleftharpoons^{K^H_{CuL}} CuLOH(H_2O)_{x-1}^{(n-1)+} + H^+ \qquad (55)$$

Since our pH titrations were conducted in aqueous solution at low ligand concentrations, we felt that it was important to test this assumption. The extent to which hydrolysis occurred under experimental conditions was estimated by combining expressions for equilibria 54 and 55 with the definition of formation constants, K_1 and K_2 (equation 31). The combined equations give the ratios 56 and 57.

$$\frac{K_1}{K^H_{Cu}} = \frac{[CuL]}{[CuOH][L][H]} \qquad (56)$$

$$\frac{K_2}{K^H_{CuL}} = \frac{[CuL_2]}{[CuLOH][L][H]} \qquad (57)$$

The ratios CuL/CuOH and CuL_2/CuLOH were determined from equations 56 and 57. The necessary K_1 and K_2 values were taken from Table 6, and ligand concentrations at various pH's were available from workup of the pH titration data. A value for the hydrolysis constant, $pK^H_{Cu} = 6.8$, has been reported (118) under conditions of

temperature and ionic strength similar to those used in our experiments. An estimate of the hydrolysis constant for the complex (pK^{H}_{CuL}) can be made by comparison with values reported for the monoanionic ligands histidine ($pK^{H}_{CuL} = 7.4$) (119) and glycine amide ($pK^{H}_{CuL} = 8.1$) (120). For the purposes of discussion, we shall assume that pK^{H}_{CuL} for the 2-hydroxyazobenzene monoanion is 7.5.

Table 7 summarizes the results of applying equations 56 and 57 to the data of runs 33c, 104, and 97. The low CuL/CuOH ratios in Table 7 (especially for runs 104 and 97, where ligand concentrations were low due to limited solubility) indicate that a significant fraction of the free copper ion is hydrolyzed under experimental conditions; but there is no way, short of refining the experimental data to include hydrolysis terms, to predict the error incurred in the K_1 and K_2 values in Table 6, which were calculated assuming hydrolysis was negligible. What follows is an attempt to refine the experimental data by viewing the cupric ion hydrolysis equilibrium (equation 54) as a perturbation on the equations used to determine the constants reported in Table 6.

In accord with the derivation in 2.3.3.2, we begin by correcting equation 38 for hydrolysis to give

$$[H]''' = E''' + OH''' - Na''' + T_L''' - \bar{n}_A T_L''' + \bar{n}''' \bar{n}_A''' T_M''' + CuOH \quad (58)$$

where all of the original definitions and assumptions hold, and the last term (CuOH) represents the concentration of protons released by hydrolysis (equation 54). When the mathematical steps are repeated,

Table 7. Approximate Degree of Hydrolysis During pH Titrations[a]

Run	pH 5.0		pH 6.0		pH 7.0	
	CuL/CuOH	CuL_2/CuLOH	CuL/CuOH	CuL_2/CuLOH	CuL/CuOH	CuL_2/CuLOH
33c	13	200	10	140	-	-
104	9	25	6	16	2	6
97	2.5	80	2.2	55	0.4	13

[a]Ratios calculated assuming $pK^{H}_{Cu} = 6.8$ and $pH^{H}_{CuL} = 7.5$.

equation 44 is replaced by 59.

$$\bar{n} + \frac{CuOH}{T_M} \cdot \frac{1}{\bar{n}_A} = \frac{\Delta V' N}{\bar{n}_A T_M^0 V^0} \tag{59}$$

Equation 59 is further simplified by choosing to limit data analysis to pH values such that $\bar{n}_A > 0.95$, in which case

$$\boxed{\bar{n}' = \bar{n} + \frac{CuOH}{T_M} \cong \frac{\Delta V' N}{\bar{n}_A T_M V^0}} \tag{60}$$

where

$$\bar{n}' = \frac{CuOH + CuL + 2CuL_2}{T_M}$$

A relationship between $\bar{n}'$ and formation constants K_1 and K_2 was derived by working backward through the proof of Bjerrum's equation in Appendix III-A. The result is equation 61.

$$\bar{n}' + (\bar{n}' - 1)(K_1 L) + (\bar{n}' - 2)(K_1 K_2 L^2) - \Delta(\bar{n}' - 1)(L) = 0 \tag{61}$$

where

$$\Delta = \frac{(K_{Cu}^H/H)(1 - K_1 K_2 L^2)}{(\bar{n}' - 1)(L)(1 + K_1 L + K_1 K_2 L^2 + K_{Cu}^H/H)}$$

A proof of equation 61 is given in Appendix III-B.

A more useful form of equation 61 is obtained by dividing through by $(\bar{n} - 1)(L)$ to give

$$Y' - \Delta = K_1K_2X' - K_1 \qquad (62)$$

where

$$Y' = \frac{\bar{n}'}{(\bar{n}' - 1)(L)} \quad \text{and} \quad X' = \frac{(2 - \bar{n}')(L)}{(\bar{n}' - 1)}$$

Equation 62 is a perturbed form of equation 51. New values of K_1 and K_2 can be determined by solving equation 62 by an iterative procedure (see Appendix IV for details). Pertinent data are included in Appendix V.

The results of recalculating the formation constants with correction terms for copper ion hydrolysis are presented in Table 8. Figure 10 shows the least squares fit to the corrected and the uncorrected data for run 97. (Note that run 97 showed the greatest change in K_1 and K_2)

Table 8.[a, b, c] Corrected Formation Constants for Derivatives of 2-Hydroxyazobenzene-5-Sodium Sulfonate

Run	Compound	4′-Substituent	$K_1 \times 10^{-5}$	$K_2 \times 10^{-5}$	pK_a
33c	V	H	2.3 (2.3)	7.2 (7.2)	
33d	V	H	2.8 (2.8)	7.2 (7.2)	7.7
ave			2.5	7.2	
100	VII	$-NO_2$	3.1 (3.8)	5.3 (4.4)	
104	VII	$-NO_2$	3.6 (4.1)	2.4 (2.3)	7.1
ave			3.4	3.8	
96	VIII	$-OCH_3$	3.5 (3.2)	16 (22)	
97	VIII	$-OCH_3$	3.3 (2.2)	6 (14)	7.75
ave			3.4	11	

[a]At 30.0° in 0.265 M sodium perchlorate. [b]pK_{Cu}^{H} assumed to be 6.8 (118). [c]Figures in parentheses ( ) are uncorrected values, Table 6.

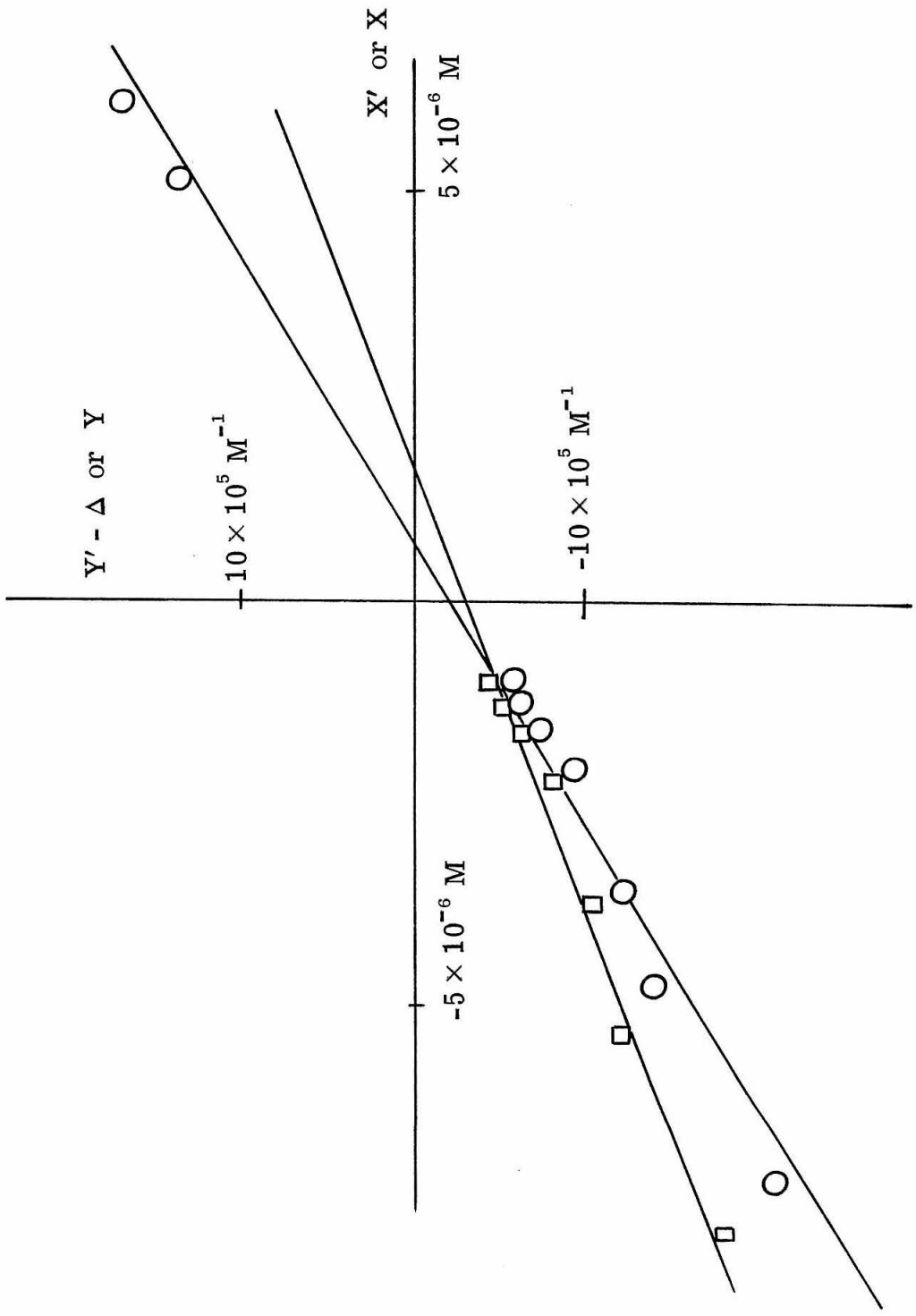


Figure 10. Plot of Y' - Δ vs. X' for run 97, □.
Plot of Y vs. X for run 97, O.

2.3.3.5 Discussion: The relative corrections to formation constants K_1 and K_2 for runs 33c, 104, and 97 (compare values in Table 8) are in the order which might have been expected based on CuL/CuOH ratios given in Table 7, i.e., 97 > 104 > 33c. The negligible correction to run 33c, even though CuL/CuOH ≅ 10, gives us some confidence that since CuL_2/CuLOH ratios are considerably larger, the constants reported in Table 8 would be essentially unchanged even if an additional correction term for CuLOH formation had been included.

To the best of our knowledge only one other determination of formation constants of copper complexes of 2-hydroxyazobenzenes has been reported. The results of Kido's studies with 5-methyl-2-hydroxyazobenzene derivatives in 75% dioxane at 30° are presented in Table 9 (121).

Table 9.[a] Formation Constants for Derivatives of 5-Methyl-2-Hydroxyazobenzene in 75% Dioxane-Water at 30°

	Copper		Nickel		
4′-Substituent	log K_1	log K_2	log K_1	log K_2	pK_a
H	10.75	10.21	8.34	7.90	14.01
NO_2			7.63	6.93	12.91
OCH_3	10.81	10.35	8.48	7.99	14.21

[a]H. Kido, Sci. Repts. Saitama Univ., 2A, 157 (1957).

If the results in Table 8 are compared with those in Table 9, one finds that the trends in values of pK_a and K_2 with substituent are in good agreement. Secondly, it is observed that in both systems the values of K_1 and K_2 appear to differ by less than an order of magnitude. Although the proximity of K_1 to K_2 complicates the experimental determination of formation constants, its occurrence is not unusual. More unusual and different from Kido's results is the fact that for all three derivatives the experimental average of K_2 is greater than K_1. Although such reversals of normal order (i.e., $K_1 > K_2 > K_3$ etc.) have been reported for the silver (I) -ammonia complexes (122), iron (II) -9, 10-phenanthroline (122), and copper (II) -2, 9-dimethyl-phenanthroline systems (123), no cases have yet been reported with ionic ligands. Irving has shown (124) that plots of $\bar{n}/L$ versus L for normal systems (i.e., $K_1 > K_2$) show a monotonic decrease in $\bar{n}/L$ with increasing L, but that "abnormal" systems show an initial increase and exhibit at least one maximum. A check of the data (Appendix V) shows the expected abnormal behavior of $\bar{n}/L$ vs. L, which demonstrates that the recorded data are consistent with the unusual case of $K_2 > K_1$. Although possible explanations of this behavior have been discussed (123, 124), a consideration of them is beyond the scope of this work and the extent of our data.

In concluding this section, we note first that calculations of complex concentrations from K_1 and K_2 values at pH 7.3 in aqueous solution give results (see 2.4.2.1) which are consistent with the conclusion, drawn from studies in mixed solvents, that CuL_2 is the

predominant complex species in solutions with an excess of ligand. Finally, from the standpoint of the mechanistic conclusions which we wish to draw from results based on calculations involving values of K_1 and K_2, we conclude that the accuracy with which the constants have been determined is adequate. The results in section 2.4, for example, are only slightly affected by including the hydrolysis correction, and, more importantly, the conclusions remain unchanged. The conclusions, which are based on the kinetic effect of substituents in aqueous solution (2.6.2), rely more heavily on the accuracy of K_1 and K_2 than do those of any other section; but even those conclusions are based on considerations which allow a greater degree of error than is likely to exist in the results recorded in Table 8.

2.4 Kinetics of Hydroxylation

The results of kinetic studies to determine reaction orders for the hydroxylation of 2-hydroxyazobenzenes are presented in this section. The experiments conducted in acetic acid and buffered aqueous solution indicate that, in both solvents, hydroxylation is first-order with respect to ligand, copper ion, and hydrogen peroxide. The mechanistic implications of these results are discussed. Additional kinetic studies are found in sections 2.5 (isotope effects) and 2.6 (substituent effects).

2.4.1 Kinetic Studies in Glacial Acetic Acid

The results of some early kinetic experiments in glacial acetic acid are presented here for comparison with later studies in buffered aqueous solution, which proved to be a more flexible solvent for

kinetic work.

2.4.1.1 Determination of Species Concentrations:

Qualitative aspects of the hydroxylation reaction in glacial acetic acid have been discussed (see 2.2.1). The gross features of the reaction in acetic acid are expressed by equation 63

$$LH + Cu^{++} + H_2O_2 \longrightarrow P + H_2O + 2H^+ \qquad (63)$$

where LH is 2-hydroxyazobenzene and P is a 1:1 complex (CuD) of 2, 2′-dihydroxyazobenzene (DH_2) and cupric ion. Quantitative information about the concentrations of ligand and product as a function of time was obtained from the visible spectra of diluted aliquots of the reaction mixture. Since neither cupric ion nor hydrogen peroxide absorb significantly in the visible region at the concentrations used, the total absorbance, A_λ, at any wavelength was assumed to be

$$A_\lambda = A_L + A_p \qquad (64)$$

where A_L is the absorbance of ligand alone, and A_p is the absorbance due to product. Rewriting equation 64 in terms of concentrations and extinction coefficients (ϵ) gives equations 65 and 66 at wavelengths λ_1 and λ_2 respectively.

$$A_{\lambda_1} = \epsilon_{1L}[LH] + \epsilon_{1p}[P] \qquad (65)$$

$$A_{\lambda_2} = \epsilon_{2L}[LH] + \epsilon_{2p}[P] \qquad (66)$$

Equations 65 and 66 are solved for [P] and [LH] to give equations 67 and 68.

$$[P] = \frac{\frac{\epsilon_{1L}}{\epsilon_{2L}} A_{\lambda_2} - A_{\lambda_1}}{\frac{\epsilon_{2p}\epsilon_{1L}}{\epsilon_{2L}} - \epsilon_{1p}} \qquad (67)$$

$$[LH] = \frac{A_{\lambda_1} - \epsilon_{1p}[P]}{\epsilon_{1L}} \qquad (68)$$

Values of [LH] and [P] were calculated from A_{λ_1} and A_{λ_2} measurements as a function of time. The extinction coefficients used in equations 67 and 68 are listed in Tables 1 and 3.

2.4.1.2 Instability of Product Complex and of Hydrogen Peroxide under Reaction Conditions: The results in Table 10 indicate that the total concentration of product plus ligand, [LH + P], decreases with time and that the product concentration goes through a maximum. This result is consistent with qualitative experiments in acetic acid (2.2.1.3) which showed that the copper complex of 2, 2′-dihydroxy-azobenzene was converted to colorless by-products in the presence of hydrogen peroxide. In order to demonstrate that product decomposition was fast enough to account for the observed decrease in total ligand, [LH + P], a kinetic experiment was conducted in the presence of product, $[CuD]_o$, added at the start of the reaction. If the decrease in [LH + P] were not due to a secondary reaction of product with peroxide to form colorless compounds (p′), i.e., if path 1 in equation

Table 10.[a] Hydroxylation of 2-Hydroxyazobenzene in Glacial Acetic Acid at 37° (R-5)

Elapsed Time	A_{375}	A_{485}	[LH]	[P]	[LH + P]
0.0 min			1.14×10^{-2} M	0.00×10^{-2} M	1.14×10^{-2} M
2.0	0.99	0.19	1.00	0.11	1.11
5.0	0.89	0.40	0.82	0.25	1.07
8.0	0.83	0.56	0.69	0.36	1.05
12.5	0.76	0.70	0.57	0.46	1.03
23.0	0.69	0.79	0.48	0.52	1.00
32.0	0.61	0.88	0.35	0.58	0.93
64.0	0.50	0.78	0.26	0.52	0.78
117	0.39	0.49	0.25	0.32	0.57
218	0.20	0.29	0.13	0.19	0.32
302	0.15	0.13	0.12	0.08	0.20
463	0.11	0.08	0.10	0.05	0.15

[a] $[Cu^{++}]_0 = 2.19\times10^{-2}$ M, $[LH]_0 = 1.14\times10^{-2}$ M, $[H_2O_2] = 0.8$ M.

69 is not significant, then a plot of $[LH + P] - [CuD]_o$ vs. time should be identical to a plot of $[LH + P]$ for the same experiment run in the absence of product added initially ($[CuD]_o = 0$).

$$LH + Cu^{++} + H_2O_2 \xrightarrow{k_1} CuD \xrightarrow{(1)} p' \qquad (69)$$
$$\xrightarrow{(2)} p'$$

The results for comparable runs, R-3 and R-9, are given in Figure 11. The lower curve shows the decrease in $[LH]$ with time for both runs and is presented to demonstrate the similarity of the two runs. The uppermost plots of $[L + P] - [CuD]_o$ vs. time for R-3 and R-9 do not fall along the same curve, but the points for R-9 drop off more rapidly, especially at the beginning of the reaction when $[CuD]_o$ is a maximum in R-9. This is exactly the behavior expected of a system in which the first reaction product undergoes a secondary reaction. We take these results to mean that most, if not all, of the change in $[LH + P]$ results from the simultaneous destruction of CuD under reaction conditions (i.e., reaction by path 1, equation 69).

Included in Figure 11 is a plot of

$$\frac{[H_2O_2]}{[H_2O_2]_o}$$

vs. time under the conditions of R-3. Since R-3 had an eightyfold excess of peroxide, and since peroxide was found to be stable in the absence of copper ion, we conclude that copper catalyzed decomposition of peroxide occurs in addition to hydroxylation. A proposed

Figure 11. Concentration versus time curves at 37° in glacial acetic acid. O, R-3, $[LH]_0 = 1.14 \times 10^{-2}$ M, $[Cu^{++}]_0 = 1.14 \times 10^{-2}$ M, $[CuD]_0 = 0$, $[H_2O_2]_0 = 80 \times 10^{-2}$ M. Δ, R-9, $[LH]_0 = 1.14 \times 10^{-2}$ M, $[Cu^{++}]_0 = 1.14 \times 10^{-2}$ M, $[CuD]_0 = 0.37 \times 10^{-2}$ M, $[H_2O_2]_0 = 80 \times 10^{-2}$ M. □, fraction of peroxide titre vs. time for a solution identical to R-3.

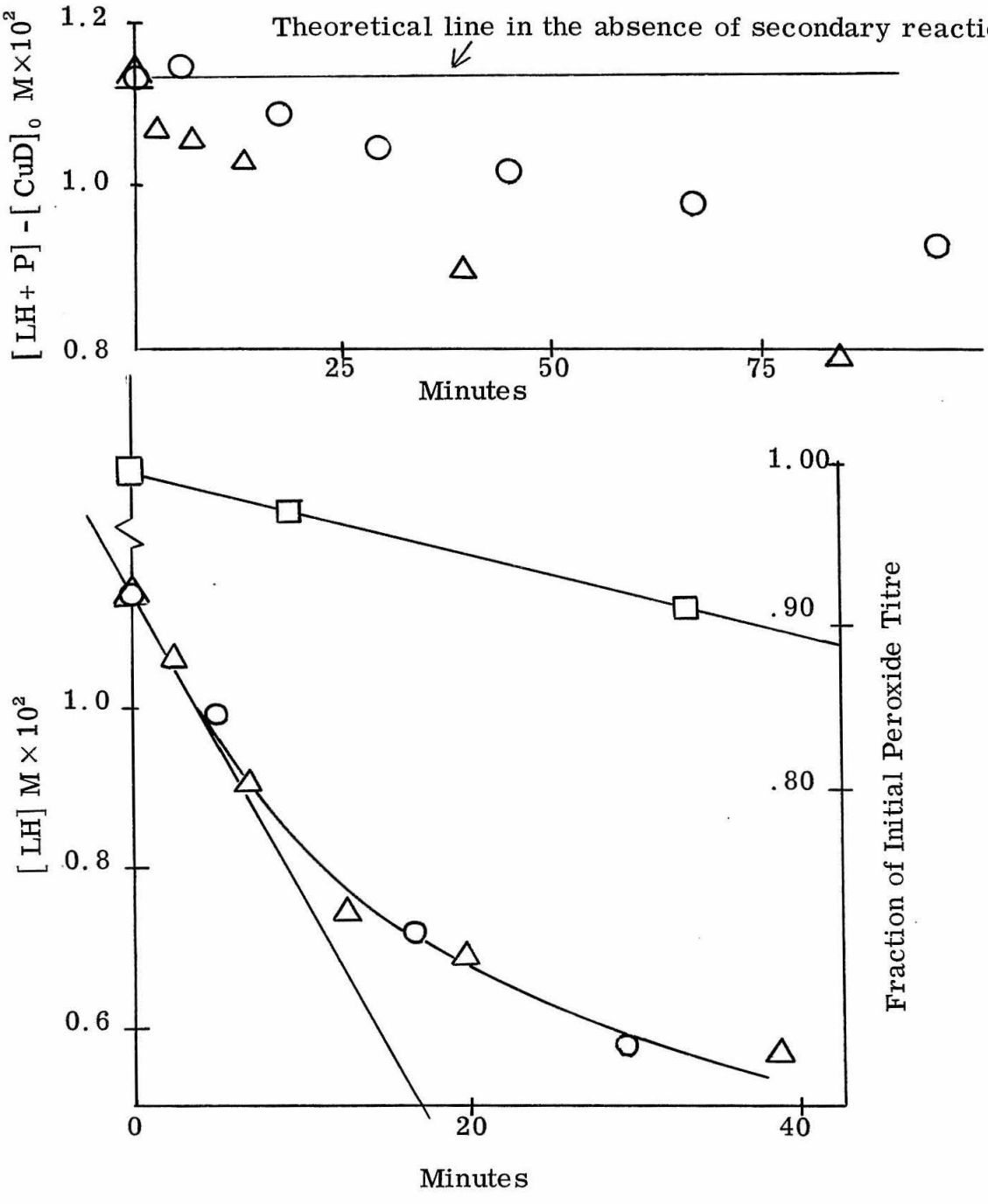

Theoretical line in the absence of secondary reactions
[LH + P] - [CuD]$_0$ M×10^2
1.2
1.0
0.8
25
50
75
Minutes
1.00
.90
.80
[LH] M × 10^2
1.0
0.8
0.6
0
20
40
Minutes
Fraction of Initial Peroxide Titre

Figure 11

mechanism which ties peroxide decomposition together with product destruction is given in Appendix VII.

We chose not to make detailed kinetic studies in acetic acid because of the complications of product and peroxide decomposition. Initial rates, which are not affected by either process, were measured, however, to determine approximate reaction orders with respect to the three constituents of the reaction. The results of these experiments are presented in the next section.

2.4.1.3 Reaction Orders in Acetic Acid: The kinetics of hydroxylation in glacial acetic acid were studied assuming the validity of equations 68 and 69 (i.e., that any secondary reaction products are effectively colorless) and that reaction of ligand by path 2, equation 69, was negligible. With these assumptions the initial rate of disappearance of ligand, $(-\frac{dLH}{dt})_0$ is correlated with reactant concentrations by equation 70

$$(-\frac{dLH}{dt})_0 = k_1[Cu^{++}]_0^a\ [LH]_0^b\ [H_2O_2]_0^c \qquad (70)$$

where k_1 is the rate constant for hydroxylation.

Initial rates were determined graphically from the slope of tangents drawn at t = 0 to plots of [LH] vs. time, an example of which is found in Figure 11.

The results of a series of experiments in glacial acetic acid with varying initial amounts of 2-hydroxyazobenzene, cupric ion, and hydrogen peroxide are given in Table 11.

Table 11. Initial Rates of Disappearance of Ligand in Glacial Acetic Acid at 37° at Varying Concentrations of 2-Hydroxyazobenzene, Cupric Acetate, and Peroxide

Run	$[LH]_0$	$[Cu^{++}]_0$	$[H_2O_2]_0$	$(-\frac{dLH}{dt})_0$
R-2	1.19×10^{-2} M	1.19×10^{-2} M	42×10^{-2} M	2.2×10^{-4} M/min
R-3	1.14	1.14	80	3.5
R-4	0.57	0.57	80	0.75
R-5	1.14	2.19	80	7.1
R-6	1.14	0.57	80	1.8
R-9[a]	1.14	1.14	80	3.5

[a]In the presence of 0.37×10^{-2} M copper 2, 2′-dihydroxyazobenzene complex.

The reaction order with respect to copper ion, a, was determined from the initial rates of runs R-3, R-5, and R-6, for which equation 70 simplifies to

$$\left(-\frac{dLH}{dt}\right)_0 = k_1'' [Cu^{++}]_0^{\ a} \qquad (71)$$

where $k_1'' = k_1[LH]_0[H_2O_2]_0$. Taking the log of both sides of

equation 71 gives

$$\log \left(-\frac{dLH}{dt}\right)_0 = \log k_r'' + a \log [Cu^{++}]_0 \qquad (72)$$

The value of $\underset{\sim}{a}$ was estimated to be 1.04 from the slope of a straight line through a plot of $\log \left(-\frac{dLH}{dt}\right)_0$ vs. $\log [Cu^{++}]_0$. By the same procedure, $\underset{\sim}{b}$ was estimated to be 1.2 from runs 4 and 6, and runs 2 and 3 gave results consistent with a value of 0.9 for $\underset{\sim}{c}$. Allowing for experimental error and assuming integral reaction orders, we conclude that the hydroxylation of 2-hydroxyazobenzene with cupric ion and hydrogen peroxide proceeds according to equation 73.

$$\left(-\frac{dLH}{dt}\right) = k_1[Cu^{++}][LH][H_2O_2] \qquad (73)$$

2.4.1.4 Discussion: Two dissimilar reaction mechanisms, which are consistent with equation 73, are given in Schemes XVIII and XIX.

A fundamental difference between Schemes XVIII and XIX is the role of chelate formation between copper ion and LH. We have already noted from studies in methanol and DMSO, that a dramatic rate enhancement occurs when complex formation is favored. Quantitative evidence in support of a mechanism based on Scheme XIX is found in studies of hydroxylation in buffered aqueous solution (2.4.2) which indicates that hydroxylation is first order with respect to a 1:1 complex of copper with ligand.

$$Cu^{++} + H_2O_2 \underset{k_1}{\overset{k_1}{\rightleftharpoons}} [O] + Cu^{++} + H_2O$$

$$[O] + CH_3COOH \underset{k_2}{\overset{k_2}{\rightleftharpoons}} CH_3CO_3H$$

$$[O] + LH \xrightarrow[k_3]{\text{slow}} DH_2 \xrightarrow[Cu^{++}]{\text{fast}} CuD + 2H^+$$

Scheme XVIII

$$Cu^{++} + LH \rightleftharpoons CuL^+ + H^+$$

$$CuL^+ + H_2O_2 \xrightarrow{\text{slow}} CuD + H^+ + H_2O$$

Scheme XIX

2.4.2 Kinetics Studies in Aqueous Solution.

Buffered aqueous solution was a better solvent for studying hydroxylation than acetic acid for the following reasons:

(1) The hydrogen ion concentration, which is involved in the multiple equilibrium expressions, could be controlled, varied, and measured.

(2) The concentrations of possible reacting species could be determined.

(3) The complicating secondary reactions observed in acetic acid were apparently absent.

(4) The reaction could be studied directly in a spectrophotometric cell.

The results presented here correlate well with the experiments conducted in acetic acid, which showed that hydroxylation is first-order with respect to all three reactants. The major additional result of these studies is a better understanding of the role of hydrogen ion concentration in the reaction. We conclude from these experiments that the results are consistent with a reaction intermediate composed of a 1:1:1 complex of cupric ion and the monoanions of 2-hydroxyazobenzenes and hydrogen peroxide.

2.4.2.1 Calculation of Initial Concentrations: Hydroxylation reactions were conducted in aqueous solution at 30° and 0.26 M ionic strength so that the formation constants reported in Table 8 could be used to calculate initial concentrations of the various copper species. The buffers used were especially selected from a series of buffering

agents which do not complex with copper ion (124) so that complexation of copper with buffer could be ignored. From equilibrium expressions 31, 32, and 54, and the definitions of T_L and T_M, we can write that

$$T_M = (1 + \frac{K_{Cu}^H}{H} + K_1L + K_1K_2L^2)(Cu) \qquad (74)$$

and that

$$T_L = (K_AH + 1)L + (K_1L + 2K_1K_2L^2)(Cu) \qquad (75)$$

An iterative method (see Appendix VI) was used to solve equations 74 and 75 for $[L^-]_0$ and $[Cu^{++}]_0$ from which the values of $[CuOH^+]_0$, $[CuL^+]_0$, and $[CuL_2]_0$ were determined for given values of T_L, T_M, and pH. The results of calculations for a number of runs are found in Table 12. It should be noted that the existence of CuLOH complexes was not considered in computing the concentrations in Table 12, because the value of pK_{CuL}^H (see equation 55) is not known. The results of a calculation of the values for 29A (the worst case), using a conservative estimate of the hydrolysis constant, are given in brackets in Table 12. The resulting differences are small, and we conclude that a negligible error is introduced by ignoring CuLOH. We have also left out of our calculations polynuclear complexes like $Cu_2(OH)_2$. A calculation based on the free copper ion concentrations in Table 12 and a literature value (126) for the formation constant of $Cu_2(OH)_2$ indicates that $[Cu_2(OH)_2] < 10^{-11}$ M, so such species are safely ignored.

Inspection of Table 12 reveals that at pH 7.3 $>$94% of the copper ion is complexed as CuL_2. At lower pH's and higher copper concentrations, as in 39-C′, the $[CuL]/[CuL_2]$ ratio increases. These results are in accord with the conclusions about the predominance of CuL_2 which were drawn from studies in mixed solvents (section 2.3.2).

2.4.2.2 Determination of Relative Initial Rates: In contrast to acetic acid where reactant concentrations of 10^{-2} M were required to obtain reaction half-lives of several minutes, the reaction in aqueous solution at $5 < pH < 8$ was rapid enough to give similar half-lives at reactant concentrations of 10^{-4} M. At these concentrations, the hydroxylation reaction could be observed directly in the spectrophotometer, and continuous plots of absorbance vs. time were obtained.

Tangent lines drawn to spectrophotometric curves at t = 0 were used to determine $(\frac{dA}{dt})_0$ values for each experiment, where A is observed absorbance. The relationship between $(\frac{dA}{dt})_0$ and initial rates $(\frac{dCuD}{dt})$ is derived below:

Under conditions of pH and ligand concentration where most of the copper is in the form of CuL_2, the hydroxylation reaction can be considered to occur in accordance with equation 75-1.

$$CuL_2 \xrightarrow[H_2O_2 \quad H_2O]{} CuD + LH \qquad (75\text{-}1)$$

Table 12.[a,b,c] Concentration and Initial Rate Data for the Hydroxylation of 2-Hydroxyazobenzene-5-Sodium Sulfonate at pH 7.3 and 5.9

	Run	T_M	T_L	$[Cu^{++}]_0$	$[CuOH^+]_0$	$[CuL^+]_0$	$[CuL_2]_0$	$\left(\frac{dA}{dt}\right)_0$[e]
pH 7.3	29-A	1.93×10^{-5} M	1.44×10^{-4} M	0.110×10^{-6}	0.35×10^{-6}	0.82×10^{-6}	18.0×10^{-6}	7.2×10^{-3} sec^{-1}
	(29-A)[d]			(0.105)	(0.33)	(0.81)	(17.5)	
	29-B	1.93	2.16	0.040	0.13	0.50	18.6	4.6
	29-C	1.93	2.88	0.020	0.065	0.37	18.8	3.4
	29-D	1.93	3.61	0.012	0.039	0.28	18.9	2.6
	29-E	1.93	4.32	0.008	0.027	0.23	19.0	1.8
pH 5.9	38-C′	3.86	2.63	9.7	0.12	8.15	19.6	2.6

[a]At 30° in 0.1 M buffer of 0.26 M ionic strength. [b]Constants used to determine concentrations: $K_1 = 2.5\times10^5$, $K_1K_2 = 1.8\times10^{11}$, $K_A = 5.01\times10^7$, $K_H^{Cu} = 1.59\times10^{-7}$ (118). [c]$[H_2O_2]$ held constant at $\sim3\times10^{-2}$ M. [d]Corrected values including CuLOH and assuming $pK_{CuL}^{H} = 7.5$. Calculated $[CuLOH] = 0.53\times10^{-6}$ M. [e]Absorbance at 500 mμ.

The absorbance of the solution, at constant wavelength, is expressed by equation 75-2.

$$A = \epsilon_X[CuL_2] + \epsilon_D[CuD] + \epsilon_L[LH + LH_0] \qquad (75\text{-}2)$$

where LH_0 is the constant, excess ligand concentration, and ϵ_X, ϵ_D, and ϵ_L are the extinction coefficients[a] of CuL_2, CuD, and LH. A time derivative of equation 75-2 gives 76.

$$\frac{dA}{dt} = \epsilon_X \frac{d[CuL_2]}{dt} + \epsilon_D \frac{d[CuD]}{dt} + \epsilon_L \frac{d[LH]}{dt} \qquad (76)$$

If the absence of any competing side reactions is assumed, we can state that $[CuD] = [LH]$, and that $[CuL_2] = [CuL_2]_0 - [CuD]$. Equation 77 is obtained by rewriting 76 in terms of $[CuD]$.

$$\frac{dA}{dt} = -\epsilon_X \frac{d[CuD]}{dt} + (\epsilon_D + \epsilon_L) \frac{d[CuD]}{dt} \qquad (77)$$

so that

$$\frac{d[CuD]}{dt} = \frac{dA}{dt} \cdot \frac{1}{\epsilon_D + \epsilon_L - \epsilon_X} \qquad (78)$$

---

[a] ϵ_L is an apprent extinction coefficient which is pH dependent because LH and L^- have different actual extinction coefficients.

2.4.2.3 Reaction Orders: Summarized in Table 12 are values of $(\frac{dA}{dt})_0$ for a series of experiments at various concentrations of total ligand, T_L. The decrease in rate with increasing ligand concentration shown in Table 12 was also observed during hydroxylation in DMSO/methanol (Table 5). The decrease in rate is accompanied by a similar decrease in $[CuL^+]_0$. If a rate law of the form

$$\frac{d[CuD]}{dt} = k[CuL^+]^a \tag{79}$$

is assumed, the value of a can be determined (see 2.4.1.3) from the slope of a line drawn through a plot of $\log (\frac{dA}{dt})_0$ vs. $\log[CuL^+]_0$. A least squares fit to the data for runs 29A-29E, Table 12, gave a line of slope 1.08. If run 29-E, which deviates badly from the line is excluded, a value for the slope of 0.96 is obtained. If integral reaction orders are assumed, we conclude that the reaction is first order with respect to $[CuL^+]$.

The reaction order with respect to hydrogen peroxide was determined in a similar fashion from the results for a series of kinetic runs in which only peroxide concentration was varied. The kinetic effect of varying initial peroxide concentration is illustrated in Table 13.

Least-squares analysis of $\log[H_2O_2]_0$ vs. $\log (\frac{dA}{dt})_0$ gives a value of 0.92 for the slope if data for runs 112-A to 112-E are included. If we exclude the slowest run (112-A), a value for the slope of 1.06 is obtained. These results imply that hydroxylation is

also first order with respect to peroxide.

Table 13.[a,b] Initial Rates as a Function of Peroxide Concentration for the Hydroxylation of 2-Hydroxyazobenzene-5-Sodium Sulfonate

Run	Relative Initial Peroxide Concentration	$(\frac{dA}{dt})_o$ [c]
112-A	1.0	2.8×10^{-3} sec^{-1}
112-B	2.0	5.3
112-C	3.0	7.2
112-D	4.0	9.2
112-E	6.0	14.8

[a]At 25°, pH 7.3 in 0.1 M buffer at 0.26 M ionic strength. [b]$[H_2O_2]_o \cong 1.7 \times 10^{-2}$ M for 112-A. $T_M = 2 \times 10^{-5}$ M and $T_L = 1.9 \times 10^{-4}$ M for all runs. [c]Absorbance at 500 mμ.

2.4.2.4 Effect of pH: Based on the results in section 2.4.2.3, we can write that

$$\frac{d[CuD]}{dt} = k_r [CuL^+][H_2O_2] \qquad (80)$$

A change in pH will result in a change in $[CuL^+]$ in accord with equilibria discussed in 2.3. The peroxide equilibrium (equation 81) (127) is also pH dependent.

$$\frac{[H^+][HOO^-]}{[H_2O_2]} = 10^{-12}$$

Since our kinetic experiments are run at constant pH the concentration of peroxide is proportional to the concentration of its anion so that equation 80 could also be written as

$$\frac{d[CuD]}{dt} = k_r[CuL^+][HOO^-] \qquad (81)$$

A distinction between these two possibilities was made by comparing values of $(\frac{dA}{dt})_0$ for runs 29-C and 38-C′ (Table 12) in light of the changing concentrations of complex and peroxide anion. The comparison is made in Table 14.

Table 14. pH Effect on Apparent Hydroxylation Rates

Run	pH	$(\frac{dA}{dt})_0/[CuL^+]_0$	$[H_2O_2]_0$	$[HOO^-]_0$	$(\frac{dA}{dt})_0/[CuL^+]_0[HOO^-]_0$
38-C′	5.9	3.2×10^2 sec^{-1} M^{-1}	3.0×10^{-2} M	2.3×10^{-8} M	1.4×10^{10} sec^{-1} M^{-2}
29-C	7.3	93	3.0	60	1.5

One should recognize the potential error introduced by making a direct comparison between $(dA/dt)_0$ values at pH 7.3 and pH 5.9. The error, which results from changes in $1/(\epsilon_D + \epsilon_L - \epsilon_X)$ with pH, equation 78, is not expected to be large so that the value of $(\frac{dA}{dt})_0 / [CuL^+]_0 [HOO^-]_0$ should be nearly proportional to the rate constant. We conclude from these results that equation 81 more accurately describes hydroxylation in aqueous solution than equation 80 because $(\frac{dA}{dt})_0 / [CuL^+]_0 [HOO^-]_0$ is approximately constant at pH 7.3 and 5.9, whereas $(\frac{dA}{dt})_0 / [CuL^+]_0 [H_2O_2]_0$ differs by a factor of 30. This factor is too large to attribute to changes in extinction coefficients or inaccurate formation constants. One other possibility, of course, is that a fundamental change in reaction mechanism occurs from pH 7.3 to 5.9; but the reaction similarities in making more drastic solvent changes, from acetic acid to water for example, make this possibility very unlikely.

2.4.2.5 Discussion: Before any mechanistic conclusions are drawn from the rate law for hydroxylation (equation 81), two equally valid ways of writing the rate expression should be mentioned. First, since by definition

$$K_1 = \frac{[CuL^+]}{[Cu^{++}][L^-]} \quad ,$$

substitution for $[CuL^+]$ in equation 81 gives

$$\frac{d[CuD]}{dt} = k_r' [Cu^{++}][L^-][HOO^-] \qquad (82)$$

where $k_r' = k_r K_1$. Note that except for the proton dependence, which was not considered in acetic acid, equation 82 is equivalent to the rate law for hydroxylation in acetic acid (equation 73). Secondly, CuL^+ is in equilibrium with CuLOH, and hydrogen peroxide is in equilibrium with its anion. If

$$K^{H}_{CuL} = \frac{[CuLOH][H^+]}{[CuL^+]} \quad \text{and} \quad K^{H}_{H_2O_2} = \frac{[HOO^-][H^+]}{[H_2O_2]}$$

are combined, equation 83 is obtained.

$$\frac{K^{H}_{CuL}}{K^{H}_{H_2O_2}} = \frac{[CuLOH][H_2O_2]}{[CuL^+][HOO^-]} \tag{83}$$

Substitution for $[CuL^+][HOO^-]$ in equation 81 gives rate expression 84.

$$\frac{d[CuD]}{dt} = k_r'' [CuLOH][H_2O_2] \tag{84}$$

where $k_r'' = k_r \cdot K^{H}_{H_2O_2} / K^{H}_{CuL}$.

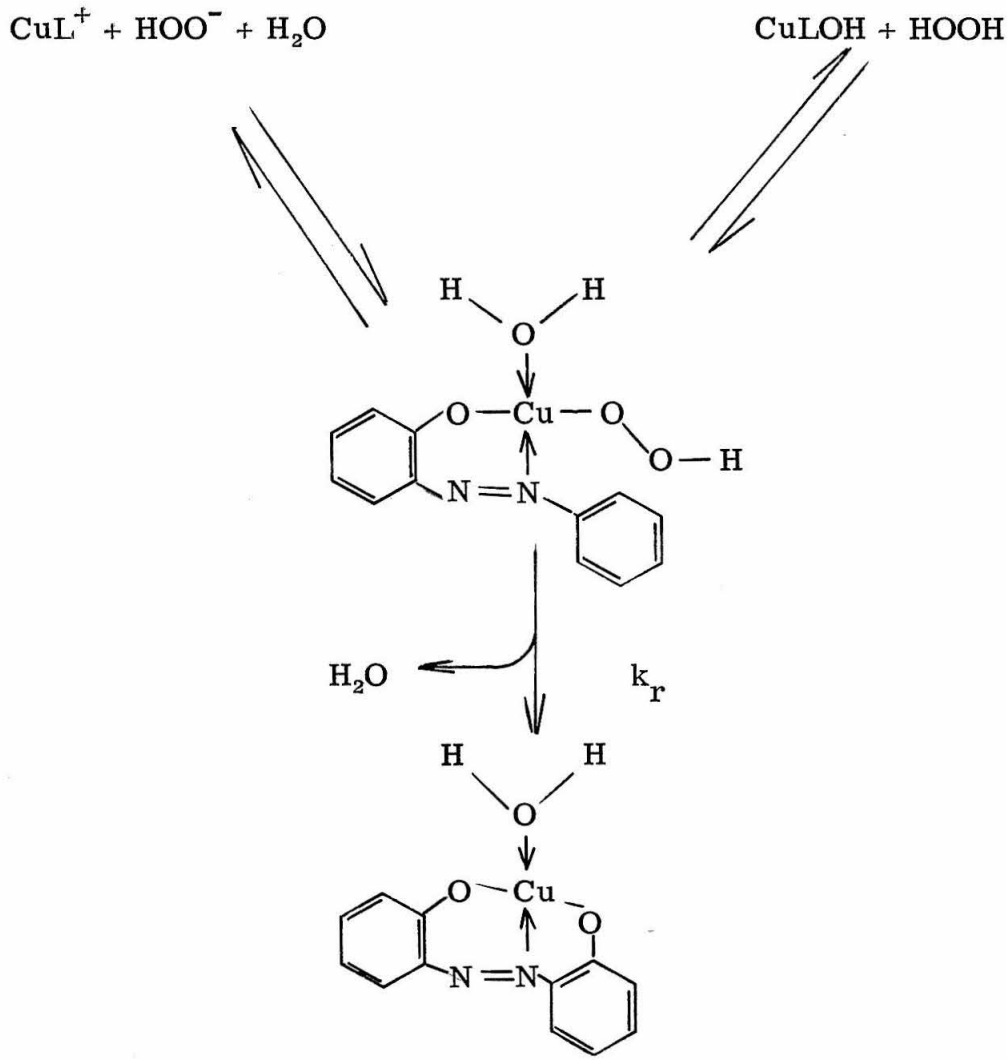
Let us first consider the implications of expression 82. Although the observed increase in hydroxylation rate in solvents which favor complex formation has been taken as evidence for the participation of copper complexes in the rate determining step, equation 82 reveals that appearances can be deceiving. On the basis of kinetic results alone, the hydroxylation reaction could also be explained by assuming

that solvents which favor complex formation also favor proton dissociation, and that the observed rate enhancement results from an increase in ligand and peroxide anion concentrations rather than an increase in complex formation. Scheme XVIII could, for example, be rewritten in terms of HOO^- and L^-. The resulting expressions will give equation 82 by applying the steady-state approximation to [O] and assuming that $k_2 \gg k_{-1}[Cu^{++}] + k_3[L^-] - k_{-2}[CH_3CO_3H]$.

Rate expressions 81 and 84, on the other hand, imply the participation of a 1:1 complex (CuL^+) in the rate determining step. Equations 81 and 84 look different from each other; but they are equivalent from a mechanistic point of view, if we assume that hydroxylation proceeds through a coordinated intermediate of the type shown in Scheme XX.

The difference between Scheme XX and multi-step mechanisms similar to Scheme XVIII might be described as the difference between coordinated hydroxlation and "free" hydroxylation. The coordinated mechanism is the conceptually more appealing mechanism, especially if an enzyme model system is sought. There are also a number of good reasons for favoring the coordinated mechanism:

(1) Specificity. The simplest argument for hydroxylation via a coordinated intermediate is specificity. In the introduction (1.7) it was noted that hydroxylation occurs specifically at the 2′ position. The coordinated intermediate tends to orient the attacking oxygen for preferential substitution at the 2′-position. A "free" hydroxylating (oxidizing) species would be expected to favor the less sterically

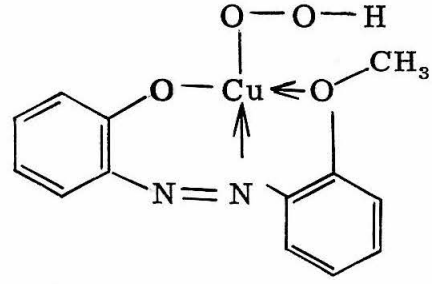


Scheme XX

hindered 3′ and 4′ or 3 and 4 positions. In fact, the azo nitrogens might be preferred by a "free" oxidizing agent. This is apparently true of hydrogen peroxide at 70°; for when hydrogen peroxide is decomposed thermally in the presence of 2-hydroxyazobenzenes in glacial acetic acid, the products are 2-hydroxyazoxybenzenes (128).

We noted in the introduction that the hydroxylation reaction was also specific for 2-hydroxy-(as opposed to 3 or 4-hydroxy) azobenzenes. There is nothing inherent in Scheme XVIII to account for this requirement.

(2) Inhibition by 2′-methoxy substitution. A second observation, mentioned in the introduction, was the fact that hydroxylation is inhibited by 2′-substituents which are capable of coordination with copper ion. We have also observed such inhibition. When 2′-methoxy-2-hydroxyazobenzene-5-sodium sulfonate (IX) was subjected to our usual reaction conditions in aqueous solution at pH 7.3, no reaction was observed. The inhibition is most easily explained by assuming that the 2′-methoxy derivative forms a complex of the type shown below, where the peroxide bond is isolated from an available



carbon atom and peroxide decomposition may occur without hydroxylation.

(3) Studies of copper chelates in decomposition of hydrogen peroxide. Finally, we note that recent studies of peroxide decomposition by copper chelates (92, 93, 94, 129) have shown that the complexes, rather than free copper ion, are the most catalytically active species in solution. Furthermore, the complexes which exhibit catalytic activity always have at least one site in the coordination sphere of the copper ion which is not occupied by ligand--a condition which permits the peroxide moiety to have access to the copper ion. Some examples of studies of peroxide decomposition by copper complexes are listed in the introduction (1.6.2.3).

In our system the CuL^+ ion, but not the CuL_2 complex, satisfies the requirements for peroxide decomposition; and so our observation that CuL^+ is the active species in hydroxylation. which accompanies peroxide decomposition, is in agreement with studies on decomposition alone.

Our conclusion, based on observed reaction orders and the considerations of specificity, inhibition, and analogy to peroxide decomposition, is that cleavage of the peroxide bond and ring hydroxylation occur by way of a 1:1:1 complex of cupric ion and the monoanions of 2-hydroxyazobenzene and hydrogen peroxide.[b] The experiments discussed in the following two sections provide some information which is useful in elucidating the mechanism of peroxide bond cleavage and of the substitution process itself.

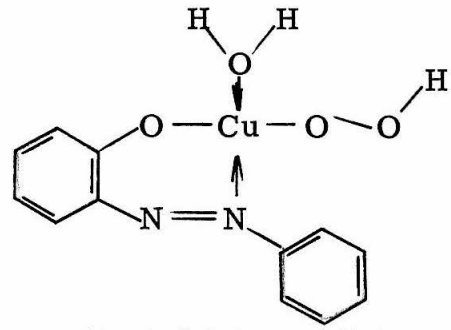
---

[b]The participation of L^- and HOO^- has been demonstrated only for the reaction in aqueous solution, but the degree of protonation of the complex in acetic acid remains unknown.

2.5 Studies Employing 2′, 4′, 6′-Trideuterio-2-Hydroxybenzene-5-Sodium Sulfonate

2.5.1 Kinetic Isotope Effect

We have concluded (in section 2.4.2.5) from initial rate studies and from several non-kinetic criteria that hydroxylation of 2-hydroxyazobenzene by hydrogen peroxide in the presence of cupric ion proceeds by way of a coordinated intermediate (XXII). We will now be concerned



coordinated intermediate

XXII

with possible mechanisms by which intermediate XXII can be converted to a copper complex of 2, 2′-dihydroxyazobenzene (CuD). A number of detailed mechanisms can be conceived; but for purposes of discussion four general possibilities are considered:

(1) Mechanisms in which cleavage of the peroxide bond is the rate determining step.

(2) Mechanisms in which ring substitution is rate determining.

(3) Mechanisms in which the slow step of the reaction involves C—H bond breakage at the point of ring substitution.

(4) Concerted mechanisms which involve various combinations of all three.

A study of the kinetic effect of deuterium substitution at the 2′-carbon offers a means of determining the relative importance of mechanisms in category three. The results of some studies which indicate that carbon-hydrogen bond breaking is not important in the transition state are given below.

2.5.1.1 Results of Studies to Determine $\frac{k_H}{k_D}$ for 2′-Hydroxylation: The 2′,4′,6′-trideuterio derivative (VI-Table 2) of 2-hydroxyazobenzene-5-sodium sulfonate (V) was prepared as described in section 3.2.5. We have shown (see 2.1.1.2) that dried samples of V and VI appear to be identical except for replacement of the 2′, 4′, and 6′ hydrogens by deuterium.[c] The results of five (H-D) pairs of kinetic runs in which initial rates of absorbance change were graphically determined for comparable reaction mixtures are presented in Table 15.

The approximate nature of our method of estimating initial rates is reflected in the deviation between individual results (note especially 65B and 65B′). Despite the inaccuracies of the method, the results in Table 15 clearly indicate that there is no large difference in the rates of hydroxylation of compounds V and VI, i.e., $0.8 < \frac{k_H}{k_D} < 1.2$. As a further test of this result the change of absorbance ($\Delta A = A - A_0$, where A_0 = initial absorbance) was measured at three time intervals for four (H-D) pairs of kinetic runs.

---

[c] For the NMR spectrum of this compound and a discussion of isotope purity, see 2.5.2.1.

Table 15.[a] Kinetic Effect of Deuterium Substitution on the Relative Rates of Hydroxylation of Compounds V and VI

Run	Initial Concentrations		Initial Slopes $(\frac{dA}{dt})_0$ at 500 mμ		$\frac{k_H}{k_D}$
	Ligand $[V]_o = [VI]_o$	$[Cu^{++}]_o$	Compound V	Compound VI	
62	3.7×10^{-4} M	9.5×10^{-5} M	1.35 min^{-1}	1.19 min^{-1}	1.13
65A	3.1	1.9	0.096	0.090	1.06
65B	2.6	1.9	0.077	0.076	1.01
65B′	2.6	1.9	0.065	0.085	0.77
65D	3.1	3.8	0.230	0.235	1.02
ave					1.00 ± 0.05

[a]All runs are in 0.1 M buffer at pH 7.3, 0.26 M ionic strength at room temperature with $[H_2O_2]_0 = 3 \times 10^{-2}$ M.

If there were no real kinetic differences between compounds V and VI, then one would expect the absorbance changes for comparable runs to be the same, i.e., $\Delta A_H/\Delta A_D = 1.0$, where ΔA_H refers to V and ΔA_D refers to VI. The results of this test are given in Table 16. These data confirm the conclusion, drawn from Table 15, that there is no observable difference in the rate of hydroxylation as a result of deuterium substitution at the 2′-carbon.

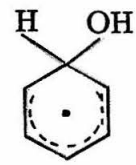
2.5.1.2 Discussion: The results in Tables 15 and 16 are consistent with the absence of a primary deuterium isotope effect, i.e., $\frac{k_H}{k_D} < 2$, in the hydroxylation reaction. Since the data are not good enough to detect small secondary effects, i.e., $0.8 < \frac{k_H}{k_D} < 1.2$, the discussion is limited to an interpretation of primary effects.

The absence of a primary deuterium isotope effect has been reported for a large number of electrophilic aromatic substitution reactions (129). These results have been taken as evidence for a two step substitution mechanism and the intermediacy of a σ-complex. Many free radical aromatic substitution processes also do not exhibit a primary isotope effect, although the interpretations in these cases are less clear cut (130). In the field of free radical aromatic oxygenations, both the isopropoxycarboxy radical (131) and the hydroxyl radical (132) show no primary deuterium isotope effect in their reaction with aromatic nuclei. These results have been used as evidence for direct radical attack on the aromatic nucleus, and for the formation of cyclohexadienyl radical intermediates in the rate determining step.

Table 16. Absorbance Changes at Three Time Intervals during the Hydroxylation of Compounds V and VI under Comparable Conditions[a]

Run	Initial Concentrations [V] = [VI]	Initial Concentrations [Cu^{++}]	$\frac{\Delta A_H}{\Delta A_D}\Big)_{1\ min}$	$\frac{\Delta A_H}{\Delta A_D}\Big)_{2\ min}$	$\frac{\Delta A_H}{\Delta A_D}\Big)_{3\ min}$	ave
93-A	1.4×10^{-4} M	1.9×10^{-5} M	$\frac{0.161}{0.138} = 1.17$	$\frac{0.200}{0.178} = 1.12$	$\frac{0.220}{0.198} = 1.11$	1.13
93A′	1.4×10^{-4} M	1.9	$\frac{0.111}{0.098} = 1.13$	$\frac{0.144}{0.143} = 1.00$	$\frac{0.170}{0.162} = 1.06$	1.06
93A″	1.4×10^{-4} M	5.8	$\frac{0.595}{0.598} = 0.99$	$\frac{0.651}{0.671} = 0.97$	$\frac{0.685}{0.710} = 0.97$	0.98
93B	2.3×10^{-4} M	1.9	$\frac{0.076}{0.078} = 0.98$	$\frac{0.117}{0.117} = 1.00$	$\frac{0.140}{0.143} = 0.98$	0.99
ave						1.04 ± 0.04

[a]All runs are in 0.1 M buffer at pH 7.3, 0.26 M ionic strength at room temperature with $[H_2O_2]_0 = 3 \times 10^{-2}$ M.



cyclohexadienyl radical

Direct evidence from electron spin resonance studies for the existence of such intermediates has been reported recently (85, 133).

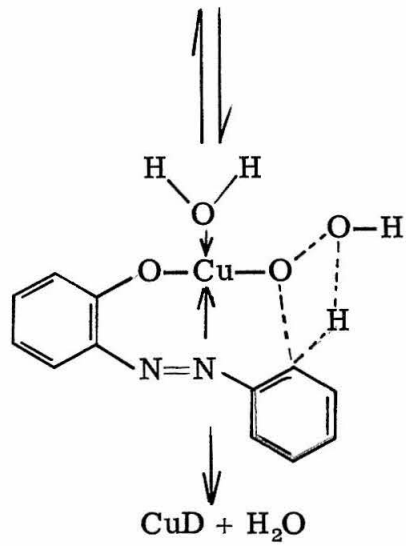
Finally, in biological systems, it has been found that 4-^{3}H-phenylalanine undergoes hydroxylation in the 4 position by rat liver phenylalanine hydroxylase without evidence of a primary isotope effect (58).

In all of the cases cited above, the absence of a primary hydrogen isotope effect was considered to be evidence for the irreversible addition of a radical or an ion to the aromatic ring in the rate determining step. The intermediate σ-complex thus formed is assumed to convert to the observed product in a fast step.

The absence of a primary isotope effect in our system is, therefore, consistent with results for a variety of aromatic substitution processes. We conclude from this study that type three mechanisms, for example, those where proton removal or hydrogen atom abstraction are rate determining, can be eliminated. The results also rule out concerted mechanisms, like that shown in Scheme XXI, where the carbon-hydrogen bond is broken during the hydroxylation step.

Additional information about the reaction mechanism is found in section 2.6, which deals with studies of the substituent effect.

[Intermediate XXII]

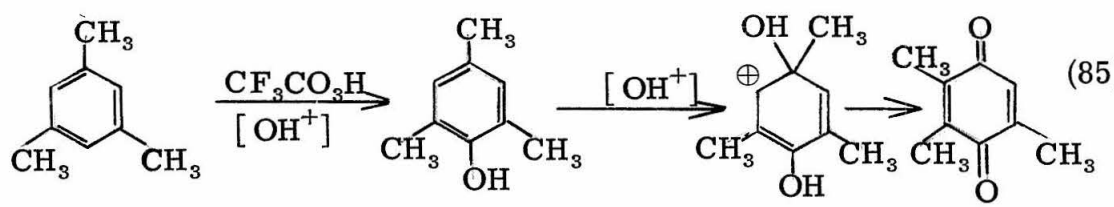


Scheme XXI

2.5.2 The NIH Shift

The NIH shift, which has been described briefly (see 1.5.1.2), is of interest in a study of models for enzymatic hydroxlations because it represents a major difference between hydroxylation reactions and other electrophilic substitution processes. The existence of a 1-2 shift of substituent from the carbon which is undergoing hydroxylation has been reported to occur with a number of aromatic hydroxylases (53, 54, 55), including an aromatic steriod hydroxylase (134).

The existence of the NIH shift is apparently not limited to enzymatic hydroxylation. A 1-2 methyl migration was postulated in 1959 by Chambers, Goggin, and Musgrave (135) to explain the results obtained in the dihydroxylation of mesytilene with trifluoroperacetic acid (equation 85).



(85)

This result is of interest in that, of a number of model systems for enzymatic hydroxylation studied by the NIH group, only trifluoroperacetic acid was observed to give any significant amount of NIH shift (Table 17).

The results in Table 17 have been explained in terms of a difference in the nature of the model hydroxylating agents; for while the various iron-peroxide (or oxygen) systems are believed to

Table 17.[a] Percent Tritium Retention in the Conversion of 4-Tritioacetanilide to 4-Hydroxyacetanilide

Hydroxylating System	% Tritium Retention
Enzymatic	50
CF_3CO_3H	9.6
Fe(II), H_2O_2, EDTA (1.6.2.1)[b]	1.9
Fe(II), O_2, ascorbate, EDTA (1.6.1.1)[b]	1.0
Fe(III), H_2O_2, catechol (1.6.2.2)[b]	1.0

[a]From D. Jerina, J. Daly, W. Landis, B. Witkop, and S. Udenfriend, J. Am. Chem. Soc., 89, 3347 (1967). [b]Numbers in brackets refer to section describing these model systems.

hydroxylate by radical or atom transfer mechanisms (see 1.6.2), trifluroperacetic acid is generally thought to be a source of hydroxyl cation (OH^+) (136) and might be expected to produce a cyclohexadienyl cation intermediate.

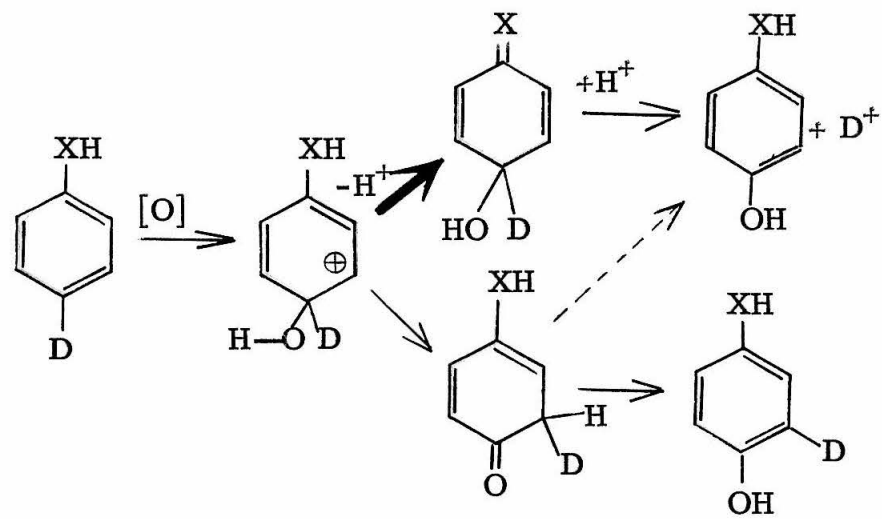
The possibility that cationoid intermediate formation occurs in enzymatic hydroxylation and plays a role in the NIH shift has been explored by Daly, Jerina, and Witkop. In an extensive study of the

NIH shift as a function of ring substituent (53), they found that the degree of deuterium retention was highly substituent dependent. The results were interpreted in terms of the ability of the substituent to stabilize the intermediate cation by loss of a proton (Scheme XXII).

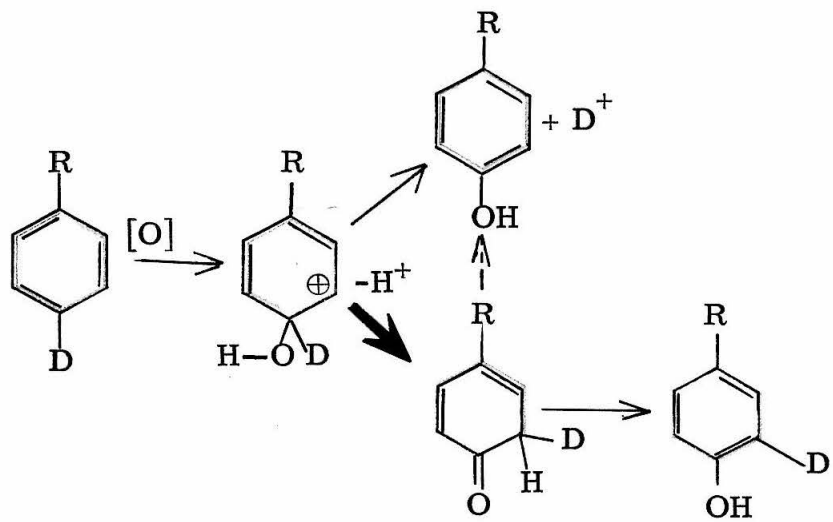
The mechanism illustrated in Scheme XXII can be used to account for the fact that substituents in Class I gave 4-hydroxy derivatives with between zero and 30 percent deuterium retention, while retention values of between 40 and 65 percent were observed with substituents in Class II.

In view of the unique place which the NIH shift occupies in aromatic hydroxylation mechanisms, we attempted to determine the extent to which it occurs in our system. The results presented below indicate that the NIH shift does not occur to any great extent, i.e., $<20\%$, in the hydroxylation of 2-hydroxyazobenzenes.

2.5.2.1 Results: The nuclear magnetic resonance spectrum of a sample of 2′,4′,6′-trideuterio-2-hydroxyazobenzene-5-sodium sulfonate (VI) is given in Figure 12. The peak assignments are based upon our experience with the NMR spectra of a number of derivatives of 2-hydroxyazobenzene-5-sodium sulfonate, all of which have a similar 3, 4, 6 pattern, and the expected coupling constants. The peak areas are in about the expected 1:1:2:1 ratio, however, there appears to be some extra peak area beneath the peaks labelled 3′, 5′, and 4. This is undoubtedly due to the presence of some isotopically impure material produced by hydrogen exchange under the conditions of the diazocoupling reaction. The presence of some exchanged material



Class I: X = O, NH, $NCOCF_3$, $NCOCH_3$



Class II: R = OCH_3, CH_3, NO_2, Cl

Scheme XXII

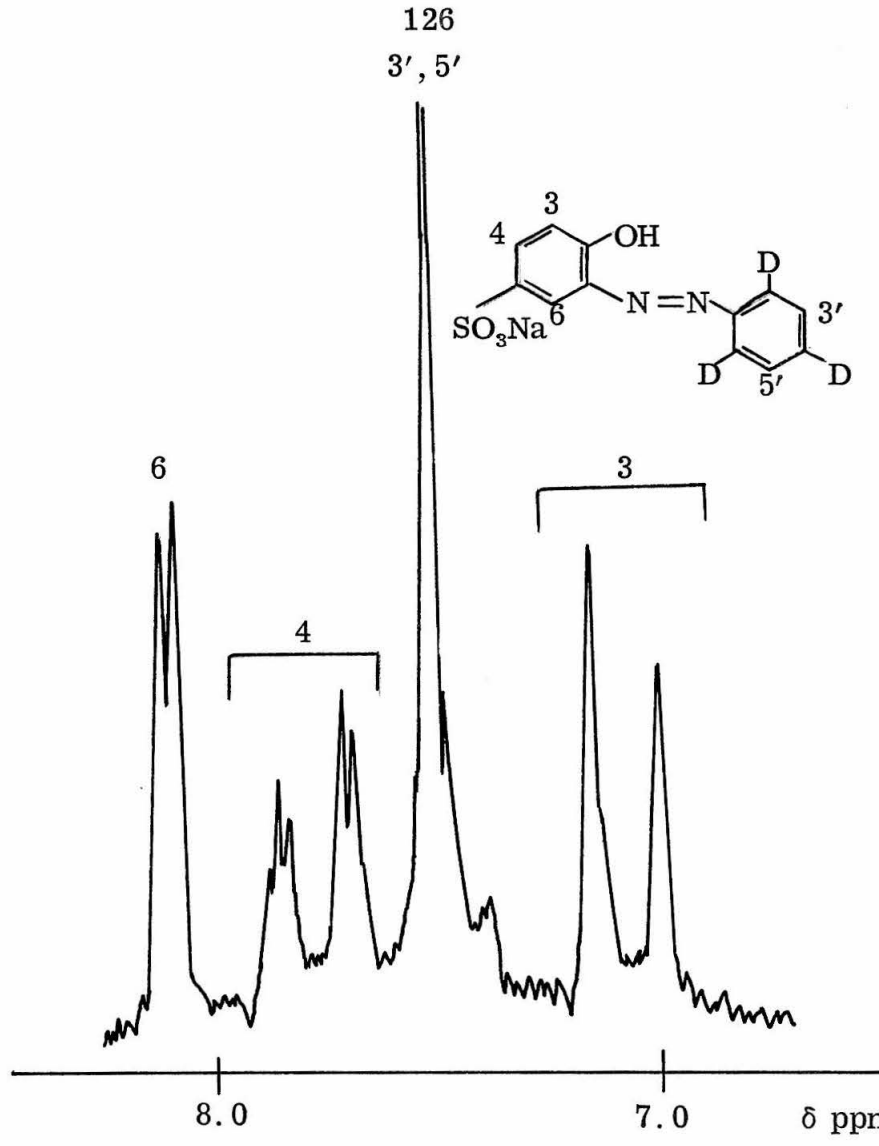
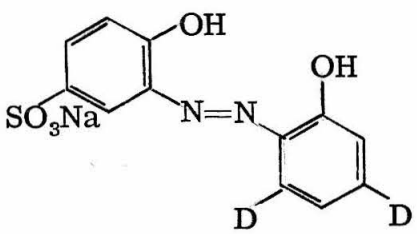


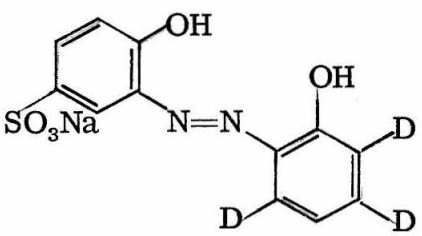
Figure 12. Nuclear magnetic resonance spectrum of 2′, 4′, 6′- trideuterio-2-hydroxyazobenzene-5-sodium sulfonate (VI) in D_6·DMSO at 60 Mcps.

makes the detection of small amounts of NIH shift impossible by NMR, but does not effect the conclusion in section 2.5.1 regarding the absence of a primary kinetic isotope effect.

The two possible products of the hydroxylation of VI are the normal isomer (XVIII) and the NIH shifted product (XXIII). Compound XVIII has five aromatic protons and XXIIIhas only four, so it should be



XVIII



XXIII

possible to find evidence for XXIIIby measuring the number of aromatic protons in the product.

Trideuterio derivative VI was hydroxylated on a preparative scale (see 3.2.7 for details) at pH 7 in the presence of cupric ion and hydrogen peroxide at room temperature. Under these mild conditions, deuterated phenols and catechols have been shown to be stable to exchange of deuterium ortho to the hydroxyl group (137). One half hour after the peroxide was added the reaction mixture was passed through a cation exchange column in order to remove the copper. The possibility of deuterium exchange in the acidic medium was minimized by keeping the solutions cold, and by neutralizing the eluant as it came off the column by the simultaneous addition of sodium hydroxide. The NMR spectrum of the crystallized, metal-free product obtained is

given in Figure 13.

Just as might have been expected, the introduction of the phenolic group at the 2′-position appears to have caused an upfield shift of the 3′, 5′ protons so that they nearly coincide with proton 3. If the assumption is made that the spectrum in Figure 13 is that of a mixture of compounds XVIII and XXIII, then the integral over A and B (i.e., protons 4 and 6) should represent two protons, while the integral over C should be the sum over the rest of the molecule (i.e., protons 3, 3′, and 5′, where some of the 3′ hydrogen may be replaced by deuterium).

The peak area ratio, A:B:C, was found from the spectrum in Figure 13 to be 1:1.4:2.7. This ratio fits neither of the two products under consideration, since XVIII is expected to give 1:1:3 and XXIII should be 1:1:2. The reason for the high value for group B is clear from Figure 14, which gives the nuclear magnetic resonance spectrum of the product isolated in a second experiment. The sharp peak at ~ 7.5 ppm corresponds to that of the 3′, 5′ singlet for the starting compound (VI). Inspection of Figure 13 reveals a shoulder at 7.5 ppm, and we conclude that the material recovered from the preparative hydroxylation reactions is actually a mixture of product (XVIII and/or XXIII) and starting material (VI). This conclusion was supported by subsequent titration analyses (Table 4) and by preparative thin layer chromatography (section 3.5).

The peak area ratio for the spectrum in Figure 13 can be corrected for the assumed presence of starting material to give 1:1:3.1 for the sum of the products (which are about 80% of the

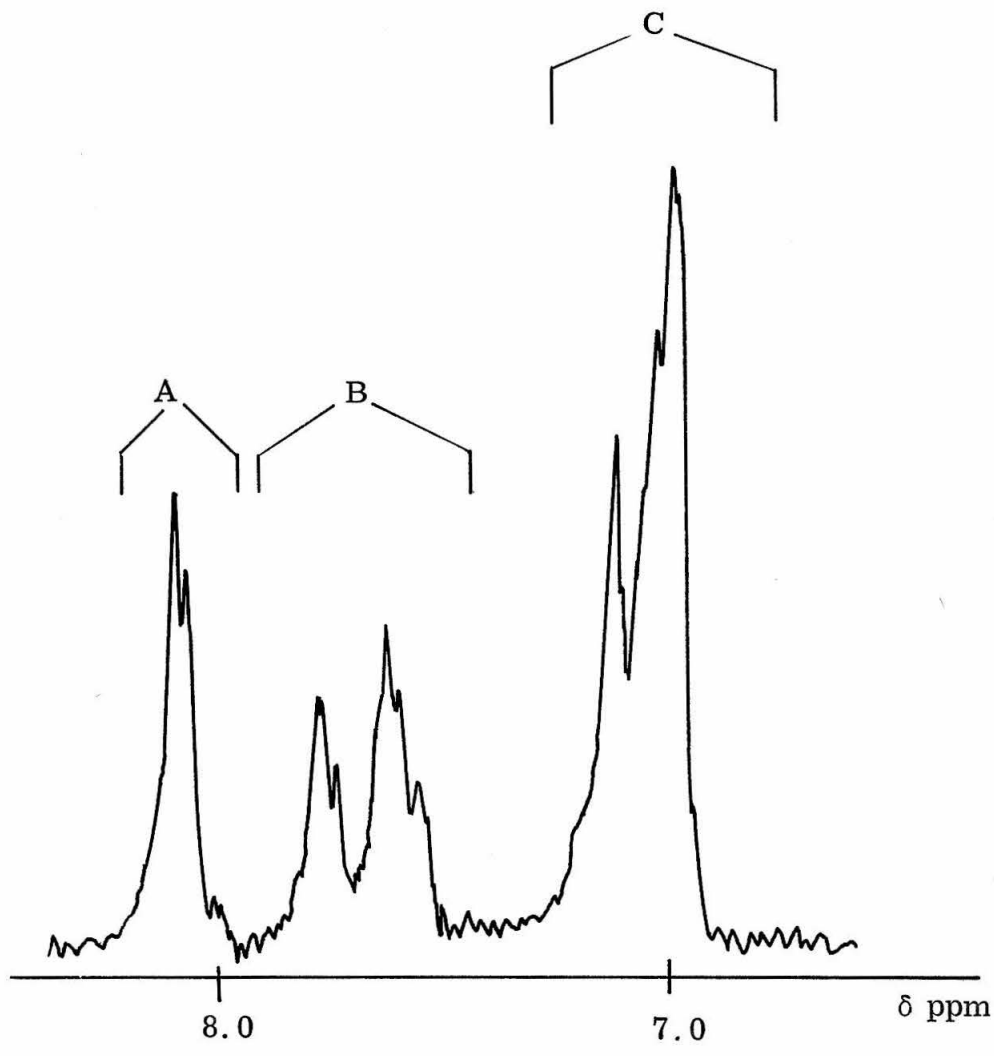


Figure 13. Nuclear magnetic resonance spectrum of the product obtained on hydroxylation of VI the first time. Run in D_6-DMSO at 60 Mcps.

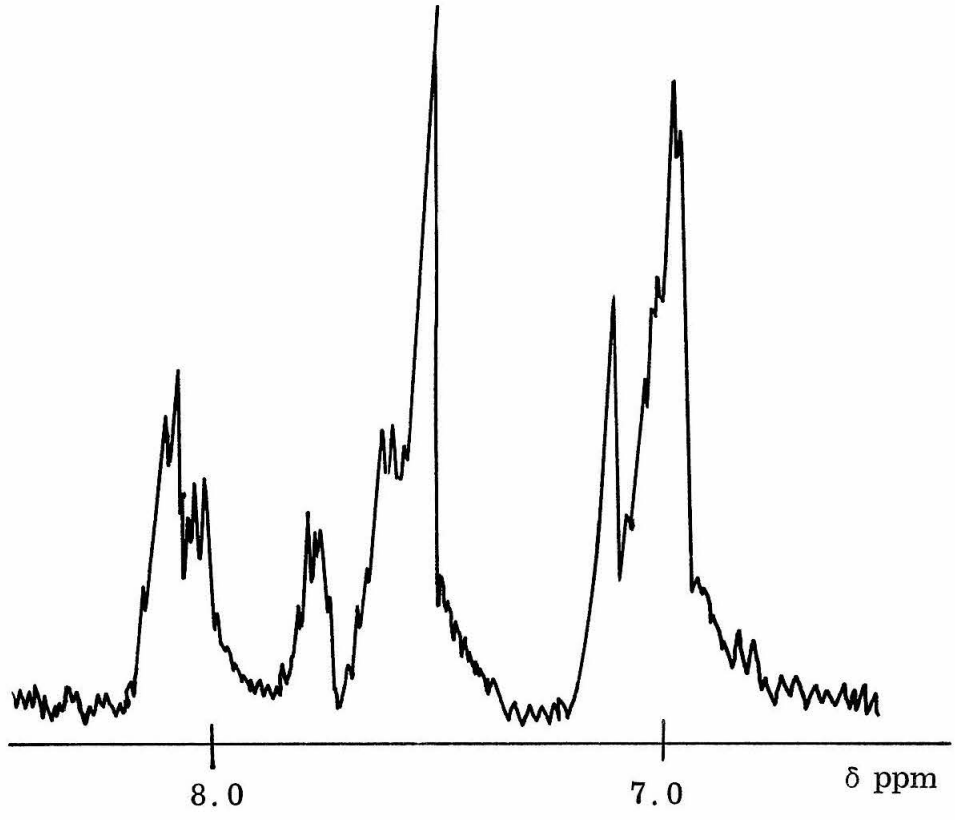


Figure 14. Nuclear magnetic resonance spectrum of the product obtained on hydroxylation of VI the second time. Run in D_6-DMSO at 60 Mcps

mixture). The corrected ratio is consistent with five protons in the product and, therefore, no NIH shift.

2.5.2.2 Discussion: The NMR results presented above indicate the absence of any substantial deuterium migration of the type reported to occur in enzymatic hydroxylation reactions. The analysis of the data is somewhat complicated, however, by incomplete conversion to dihydroxyazobenzene and the noted lack of isotopic purity in the starting material. To these two known sources of error must be added the problem of isotopic exchange of hydrogen ortho to the hydroxyl group, for if the NIH shift does occur, the deuterium which undergoes migration becomes situated ortho to the newly formed hydroxyl group. Although hydrogen ortho to hydroxyl is stable to exchange at room temperature and pH 7 (137), it can be readily replaced in basic solution (138) or less easily in concentrated acid solution at reflux (139).

The point at which exchange was most likely to occur in our systems was during the cation exchange procedure where the product was subjected to dilute acid in the cold for several minutes. We believe that the possibility of extensive exchange at this point was minimal since the half-life for deuterium-hydrogen exchange at the ortho position of p-cresol is calculated to be 2.2 hours at 25° in 6N hydrochloric acid (139).

In view of the real and potential sources of error in these experiments, we hesitate to exclude the possibility of some NIH shift occurring (i.e., $< 20\%$), however, we feel confident that it is not a

major factor in 2-hydroxyazobenzene hydroxylation. The absence of an NIH shift is consistent with the conclusion, drawn from substituent effects, that the 2-hydroxyazobenzene hydroxylation process is not strongly electrophilic and, therefore, that cationoid intermediates of the type postulated as intermediates in the NIH shift are unlikely to occur. Results of substituent effect studies are presented in the following section.

2.6 Substituent Effects in the Hydroxylation of 2-Hydroxyazobenzenes

The results of experiments to determine the effect of ring substituents on the rate of 2-hydroxyazobenzene hydroxylation by hydrogen peroxide in the presence of cupric ion are presented in this section. Hydroxylation rate data gathered from early studies of the substituent effect in glacial acetic acid proved to be ambiguous in light of the unknown effect of substituents on the equilibrium constants for formation of a coordinated intermediate similar to XXII. In aqueous solution, however, some of the equilibrium constants could be determined, so that it was possible to account for several pre-equilibrium factors in the overall rate expression and thereby obtain a better estimate of the effect of substituents on the rate of the hydroxylation step itself.

The results of substituent effect studies in aqueous solution indicate that the hydroxylation step is not strongly affected by the introduction of substituents in the 4′-position, e.g., that $k_H/k_{NO_2} \cong 20$. These results imply that hydroxylation is rate determining, and that it is not brought about by attack on the aromatic ring of a strongly electrophilic species like $OH^{\oplus}$. Possible mechanistic implications of this effect are discussed in terms of proposed coordinated intermediate, XXII. We conclude that the kinetic data are consistent with a radical like mechanism or a concerted two-electron process in which peroxide bond cleavage is assisted by formation of a carbon-oxygen bond at the 2′-carbon.

2.6.1 Results of a Study of Substituent Effects on the Rate of Hydroxylation in Glacial Acetic Acid

Initial rates of hydroxylation for four derivatives of 2-hydroxyazobenzene in glacial acetic acid at 37° were determined by the procedure described in section 2.4.1.3. Since all of the reactions were carried out at the same initial concentration of peroxide, pseudo-second order rate constants could be determined from initial rates and the concentrations of cupric ion and ligand by means of equation 86, which follows directly from equation 73.

$$k_1[H_2O_2]_o = (\frac{dLH}{dt})_o / [Cu^{++}]_o [LH]_o \qquad (86)$$

Values of $k_1[H_2O_2]_o$ as a function of substituent are reported in Table 18.

The results in Table 18 confirm the qualitative observation (section 2.2.1.4) that hydroxylation in acetic acid is only slightly substituent dependent. The absence of a large rate effect (from which mechanistic conclusions might have been drawn) prompted the initial study of hydroxylation in aqueous solution (2.4.2). The results of a study of substituent effects on the rate of hydroxylation in aqueous solution, where the effect of substituent groups on formation constants could be accounted for, are presented below.

2.6.2 Effect of Substituents on the Rate of Hydroxylation in Buffered Aqueous Solutions

If we begin with the assumption that hydroxylation proceeds by way of a coordinated intermediate similar to that illustrated in

Table 18. Pseudo-Second Order Rate Constants for Hydroxylation of Substituted 2-Hydroxyazobenzenes in Glacial Acetic Acid at 37°.[a]

Compound No.	Substituents	$[Cu^{++}]_0 = [LH]_0$ $M \times 10^2$	$(\frac{dLH}{dt})_0$ $M\,min^{-1} \times 10^4$	$k_1[H_2O_2]_0$ $M^{-1}\,min^{-1}$
I	5-methyl	1.82	8.3	2.5
II	3′,5-dimethyl	1.82	8.3	2.5
III	4′-nitro-5-methyl	0.73	0.80	1.5
IV[b]	none	-	-	2.7

[a]In all runs $[H_2O_2]_0 = 0.8$ M. [b]Average result from data reported in Table 11 for runs 3, 4, 5, and 6.

Scheme XX, then it can be seen that in buffered solution (at constant initial concentrations of cupric ion, ligand, and hydrogen peroxide) the observed reaction rate should be influenced by changes in ligand substituents. This must be true, even if the hydroxylation rate itself were independent of substituent, because there are known substituent effects on the formation constants and pKa's (Table 8) which govern the effective concentration of the 1:1 complex (CuL^+). In contrast to acetic acid, where formation constants are not known, the concentration of CuL^+ can be calculated in aqueous solution as a function of initial concentrations. If we further assume that the

equilibrium constant, K_i, for the formation of the intermediate which contains the peroxide ligand, XXII, (see equation 87), is only slightly substituent dependent (see discussion, 2.6.3.1), then we are in a position to study the effect of substituents on the rate determining step for the conversion of XXII to product ($CuD \cdot H_2O$).

$$CuL^+ + HOO^- \underset{}{\overset{K_i}{\rightleftharpoons}} CuLOOH \xrightarrow[k_{rds}]{rds} CuD \cdot H_2O \qquad (87)$$

The results of experiments to determine relative values of k_{rds} as a function of substituents are presented below.

2.6.2.1 General Method: An expression for the observed rate constant (k_r) for hydroxylation in terms of initial rates of change of absorbance $(\frac{dA}{dt})_0$ and initial concentrations of CuL^+ and hydrogen peroxide is obtained by combining equations 78 and 80 to give

$$k_r = \left(\frac{dA}{dt}\right)_0 \cdot \frac{1}{\epsilon_D + \epsilon_L - \epsilon_X} \cdot \frac{1}{[CuL^+]_0[H_2O_2]_0} \qquad (88)$$

If all experiments are conducted at constant initial peroxide concentration, equation 88 simplifies to

$$k_{r'} = \left(\frac{dA}{dt}\right)_0 \cdot \frac{1}{\epsilon_D + \epsilon_L - \epsilon_X} \cdot \frac{1}{[CuL^+]_0} \qquad (89)$$

where $k_{r'} = k_r[H_2O_2]_0$.

Methods have been discussed for determining values of $(\frac{dA}{dt})_0$ (see 2.4.2.2) and $[CuL^+]_0$ (see 2.4.2.1). We will now discuss how values of $\epsilon_D + \epsilon_L - \epsilon_X$ were determined for each substituted

2-hydroxyazobenzene-5-sodium sulfonate.

2.6.2.2 Determination of Extinction Coefficients, ϵ_D, ϵ_L, ϵ_X:

(1) Extinction coefficient of reaction product, ϵ_D. It has been noted (in section 2.1.2.2) that the available 2,2′-dihydroxyazobenzene-5-sodium sulfonates exist as mixtures with the corresponding 2-hydroxy derivatives. However, since the 2,2′-dihydroxy compounds have much larger formation constants (140) than the 2-hydroxy derivatives, it was possible to selectively titrate the dihydroxy compound with standardized cupric ion in aqueous solution at pH3. A titration curve showing absorbance at 500 mμ versus volume of added cupric ion in the titration of nitro derivative XIX was given in Figure 1. The total concentration of cupric ion and the absorbance at the end point were used to determine ϵ_D.

$$\epsilon_D = \frac{A^*}{[Cu^{++}]_{EP}} \tag{90}$$

The values of ϵ_D, which were determined in this way, are reported in Table 4. In the determination of initial rates (2.6.2.3) it is assumed that ϵ_D is not affected by changing pH from 3 to 7.3. Since the ionization state of the complex is unchanged in this pH range, we feel the assumption is valid.

---

*In each case the absorbance at the end point was corrected for a small amount (i.e., $< 6\%$) of absorbance due to uncomplexed 2-hydroxyazobenzene present in the mixture.

(2) Extinction coefficient of ligand at pH 7.3, ϵ_L. We have pointed out (2.4.2.2) that the extinction coefficient of the ligand in the visible region is pH dependent. Values of ϵ_L at 500 mμ for each ligand under the conditions of the hydroxylation reaction were determined by measuring the absorbance of a solution of known total ligand concentration in the reaction medium at pH 7.3 in the absence of cupric ion. The results at 500 mμ are reported in Table 2.

(3) Extinction coefficient of CuL_2 at pH 7.3, ϵ_X. The determination of ϵ_X is somewhat complicated by equilibria 26-28, but calculations based on known formation constants show (see Table 12 above and Table 19 below) that at pH 7.3 and in the presence of excess ligand, $> 95\%$ of the copper ion in solution exists as CuL_2. If the equilibria governing the effect of adding cupric ion to a solution of ligand at pH 7.3 are considered, then we can say that during the early stages of the titration complexation occurs (within 5 per cent) according to equation 91.

$$Cu^{++} + 2LH \underset{\text{buffer}}{\rightleftharpoons} CuL_2 \qquad (91)$$

During the titration, however, the total copper concentration increases relative to ligand, and the fraction of copper ion complexed as CuL^+ or $CuOH^+$ can be shown to increase as the equivalence point for CuL_2 is approached.

The result of this equilibrium effect during the titration of compound VIII by cupric ion is shown in Figure 15. Instead of reaching a sharp plateau, as was true for the titration of the dihydroxy

derivatives (Figure 1), the titration curve gradually decreases in slope as the equivalence point is approached. This difficulty did not permit us to determine ϵ_X exactly, however, an approximate value for ϵ_X was determined in the following way from the slope of the straight line portion of Figure 15.

The total absorbance of the solution can be expresses as equation 92

$$A = \epsilon_X[CuL_2] + \epsilon_L[LH] \tag{92}$$

if we assume that CuL_2 and LH are the only absorbing species in solution. If we further assume that all of the copper is complexed as CuL_2, then $[CuL_2] = [Cu^{++}]$ and $[LH] = [LH_0] - 2[Cu^{++}]$. Substituting these expressions into 92 and taking the derivative with respect to copper gives an expression for the extinction coefficients as a function of slope

$$\text{slope} = \frac{\Delta A}{\Delta[Cu^{++}]} = \epsilon_X - 2\epsilon_L \tag{93}$$

where ϵ_L is a known constant (Table 2).

The extinction coefficients determined from the slopes of titration curves at 500 mμ and pH 7.3 according to equation 93 are recorded in Table 2. It should be pointed out that the extinction coefficients determined in this approximate fashion are probably more suitable for use in equation 89 than the exact values would be, since the derivation of 89 involves an approximation (equations 75-1 and 75-2)

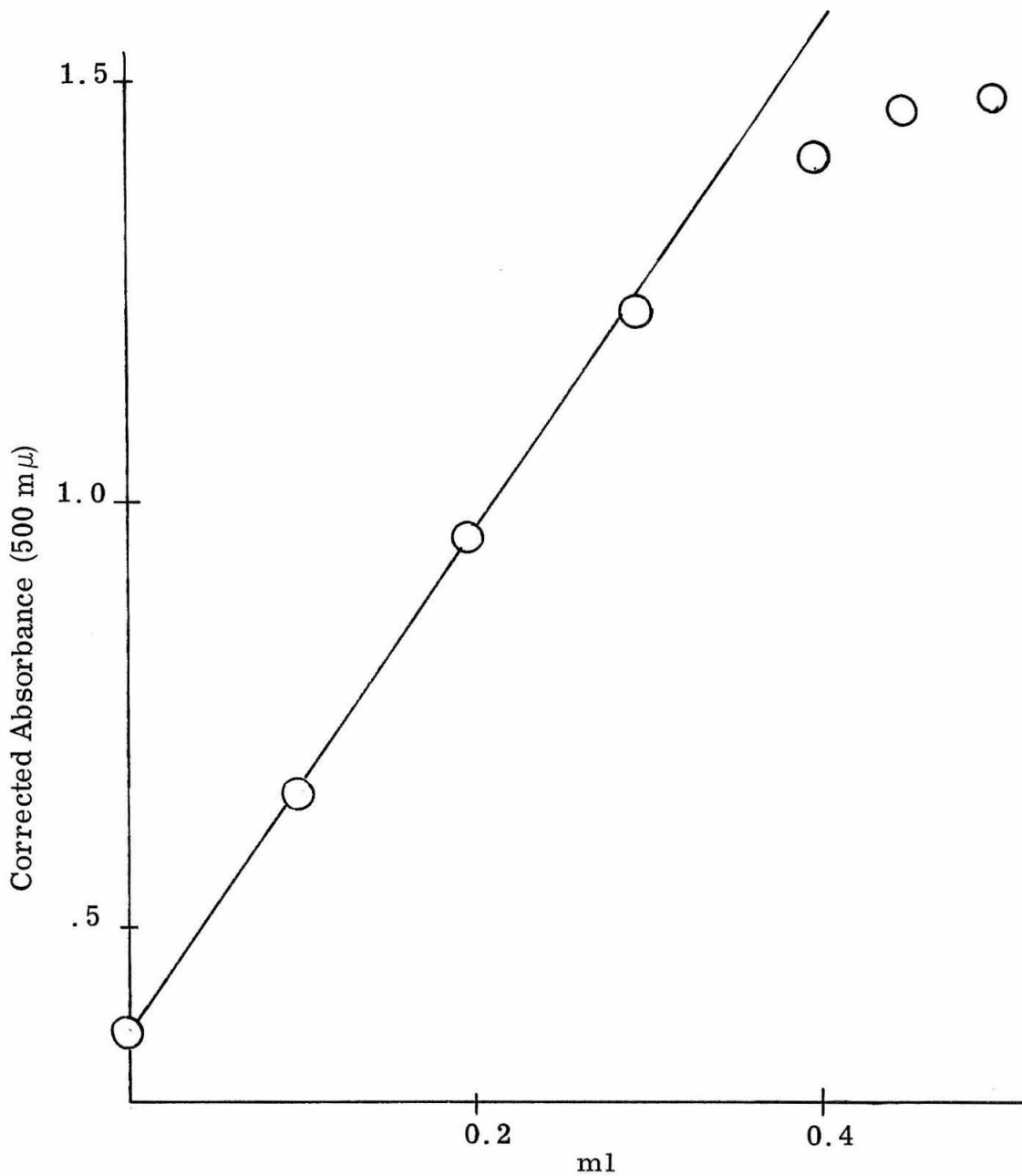


Figure 15. Titration of 2.50 ml of 2.72×10^{-4} M VIII with 10^{-3} M cupric nitrate. Absorbance is corrected for dilution with titrant.

whose error is partially cancelled by the error in the assumption implicit in equation 91, i.e. that all of the copper is complexed as CuL_2.

2.6.2.3 Calculation of Hydroxylation Rates: Summarized in Table 19 are the data used to calculate the relative rates of hydroxylation for three derivatives of 2-hydroxyazobenzene-5-sodium sulfonate in buffered aqueous solution at pH 7.3. In view of the many assumptions and experimental constants which have gone into the determination of the relative rate values reported in Table 19, we must ask whether the small rate difference between compound V and methoxy derivative VIII is significant. For the answer, we need only consider the values for the formation constants reported in Table 8 and the values shown in brackets in Table 19. The results given in brackets were calculated assuming a 20% increase in the value of K_2 over the reported average. This change is well within the limits of experimental error, yet it reverses the apparent order of reaction rates between compounds V and VIII. We must conclude, therefore, that for all intents and purposes $k_H \cong k_{OCH_3}$. The rate differences between these two derivatives and nitro-derivative VII, on the other hand, can not be attributed to experimental error, and must be regarded as real, i.e., that k_H and k_{NO_2} differ by at least an order of magnitude.

With these considerations in mind, we now discuss the mechanistic implications of these results.

Table 19. The Effect of Substituents on Hydroxylation Rates in Aqueous Solution[a]

Compound No. 4′-Substituent	V Hydrogen	VII Nitro	VIII Methoxy
T_M, $M \times 10^5$	1.93	1.93	1.93
T_L, $M \times 10^4$	2.31	2.30	2.62
$[CuL^+]_0$, $M \times 10^7$	4.6	4.2	2.9 (2.3)[c]
$[CuL_2]_0$, $M \times 10^5$	1.85	1.89	1.90 (1.90)[c]
$(\epsilon_D + \epsilon_L - \epsilon_X)$,[b] $M^{-1} \times 10^{-4}$	0.8	1.1	1.1
$(\frac{dA}{dt})_0^b$ $sec^{-1} \times 10^3$	3.5	0.17	2.6
	3.0	0.19	2.2
$k_1[H_2O_2]_0$ M^{-1} $sec^{-1} \times 10$	8.8	0.38	7.4 (9.3)[c]
Relative rates $(\frac{kx}{k_{NO_2}})$	23	1	20 (24)[c]

[a]At 30° in 0.26 M ionic strength and pH 7.3, $[H_2O_2]_0 \cong 3 \times 10^{-2}$ M in all runs. [b]At 500 mμ. [c]Numbers in brackets refer to values calculated assuming K_2 for compound VIII was 13×10^5 instead of average value given in Table 8, i.e., 11×10^5.

2.6.3 Discussion

2.6.3.1 Discussion of Results in Aqueous Solution:

2.6.3.1.1 Formation of the Coordinated Intermediates:

The question of the equilibrium for formation of the coordinated intermediate, CuLOOH, was introduced in equation 87. Since the rate determining step for hydroxylation is first order in CuLOOH, we can write that

$$\frac{d[CuD]}{dt} = k_{rds}[CuLOOH] \qquad (94)$$

If it is further assumed that equation 95 represents the equilibrium constant for the formation of the ternary intermediate (XXII)

$$K_i = \frac{[CuLOOH]}{[CuL^+][HOO^-]} \qquad (95)$$

then, by substitution for $[CuLOOH]$, 94 becomes

$$\frac{d[CuD]}{dt} = k_{rds} \cdot K_i[CuL^+][HOO^-] \qquad (96)$$

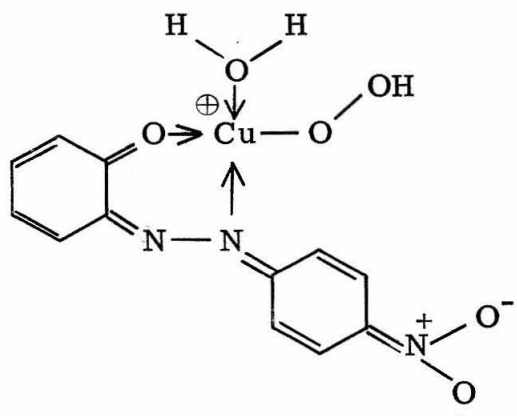
Clearly, the relative rates reported in Table 19 are not strictly ratios of k_{rds} values, but ratios of $k_{rds} \cdot K_i$, i.e.,

$$\frac{(k_{rds})_H}{(k_{rds})_{NO_2}} \neq 23$$

$$\frac{(k_{rds})_H}{(k_{rds})_{NO_2}} = 23 \frac{(K_i)_{NO_2}}{(K_i)_H} \quad .$$

We estimate that $1 < (K_i)_{NO_2} / (K_i)_H < 10$ for the following reasons:

(1) The ratio is likely to be greater than one because the Cu—OOH bond in XXII is probably stabilized by the nitro group as a consequence of decreased electron density at the copper ion which is brought about by electron withdrawal and the contribution of resonance structures like XXIV.



XXIV

That this effect is probably less than a factor of 10 in size is supported by the observation that the $C\overset{\oplus}{u}OH$ complex (118) is only about an order of magnitude more stable than LCuOH complexes (119), where L is a monoanion. (Note that in $C\overset{\oplus}{u}OH$ and LCuOH the copper atom differs by a full positive charge, whereas the contribution of resonance structure XXIV to the complex is expected to be much less than 100%).

In a similar fashion, it can be argued that $(K_i)_{OCH_3} < (K_i)_H$ due to electron donating contributions of the methoxy group. The similarity in the pKa's of V and VIII (Table 8), however, would argue that the effect of K_i is much smaller than the effect caused by the nitro group.

We conclude from this that in aqueous solution the relative values of k_{rds} are about 1:1:0.04 for $H:OCH_3:NO_2$.

2.6.3.1.2 Limiting the Mechanistic Possibilities: In the introduction to section 2.5.1, four general classes of mechanisms were discussed. The k_H/k_D results eliminated the possibility that the C—H bond breaks in the rate determining step. The conclusions drawn in the preceding section permit us to further eliminate the possibility of peroxide bond cleavage as the rate determining step. We believe this to be true because the nitro group would be expected to accelerate, rather than retard, peroxide bond cleavage. In a series of para substituted peroxybenzoic acids, for example, the relative decomposition rates were found to be 0.5:1:4 for $OCH_3:H:NO_2$ (141).

We are left with two mechanistic possibilities: (1) A concerted mechanism and (2) a mechanism wherein an oxidizing species is produced which attacks the aromatic ring in a slow step.

Reactions of the second type are known with hydroxylating agents, and the effect of substituents on their reaction rates has been determined. Two reagents of interest are trifluoroperacetic acid and Fenton's reagent.

Mechanisms Involving Ionic Intermediates. Trifluoroperacetic acid was discussed in connection with the NIH shift (2.5.2). Hydroxylation with this reagent is often interpreted in terms of electrophilic attack by OH^+ (135,136). The rate of hydroxylation of anisole relative to benzene was reported to be 530 with trifluoroperacetic acid (142), and the product was found to be substituted exclusively in the ortho and para positions. A rate and orientation effect of this kind for the methoxy group is characteristic of electrophilic aromatic substitution, and the effect is indicative of the formation of cationoid intermediates (143). By comparison, the rate effect of substituents in the hydroxylation of 2-hydroxyazobenzenes (Table 19) is much too small to be accounted for in terms of the ionic intermediates generally postulated for electrophilic substitution reactions. The absence of cationic intermediates in the 2-hydroxyazobenzene system would also be consistent with the observed absence of an NIH shift (section 2.5.2).

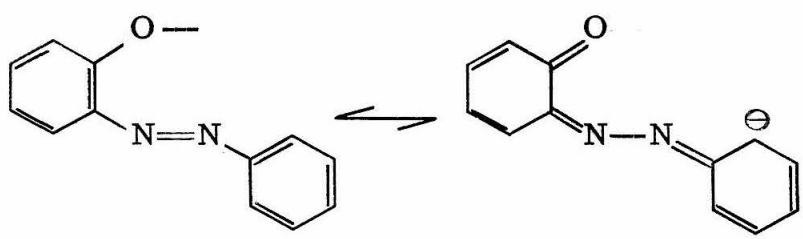
Radical Mechanisms. The substituent effect on hydroxylation rates by Fenton's reagent (see 1.6.2.1) is much more like that observed in our system. Norman and Radda (144) showed that the relative rates for hydroxylation by Fenton's reagent were 6:1:0.1 for anisole, benzene, and nitrobenzene respectively, and it was concluded that the hydroxyl radical generated by Fenton's reagent was a weak electrophile. The result with Fenton's reagent has been confirmed for the less ambiguous case of hydroxylation by hydroxyl radical in the γ-irradiation of aqueous solutions (145). (Note that in the

2-hydroxyazobenzene system the methoxy group should be deactivating toward electrophilic attack since the meta position is the one which undergoes hydroxylation).

Two radical mechanistic paths which are expected to be consistent with the kinetic results and the effect of substituents are presented in Scheme XXIII.

Concerted Mechanisms. One way to avoid radicals or two electron mechanisms involving ionic intermediates is to consider the possibility that the coordinated intermediate (XXII) is converted to product by a concerted mechanism.

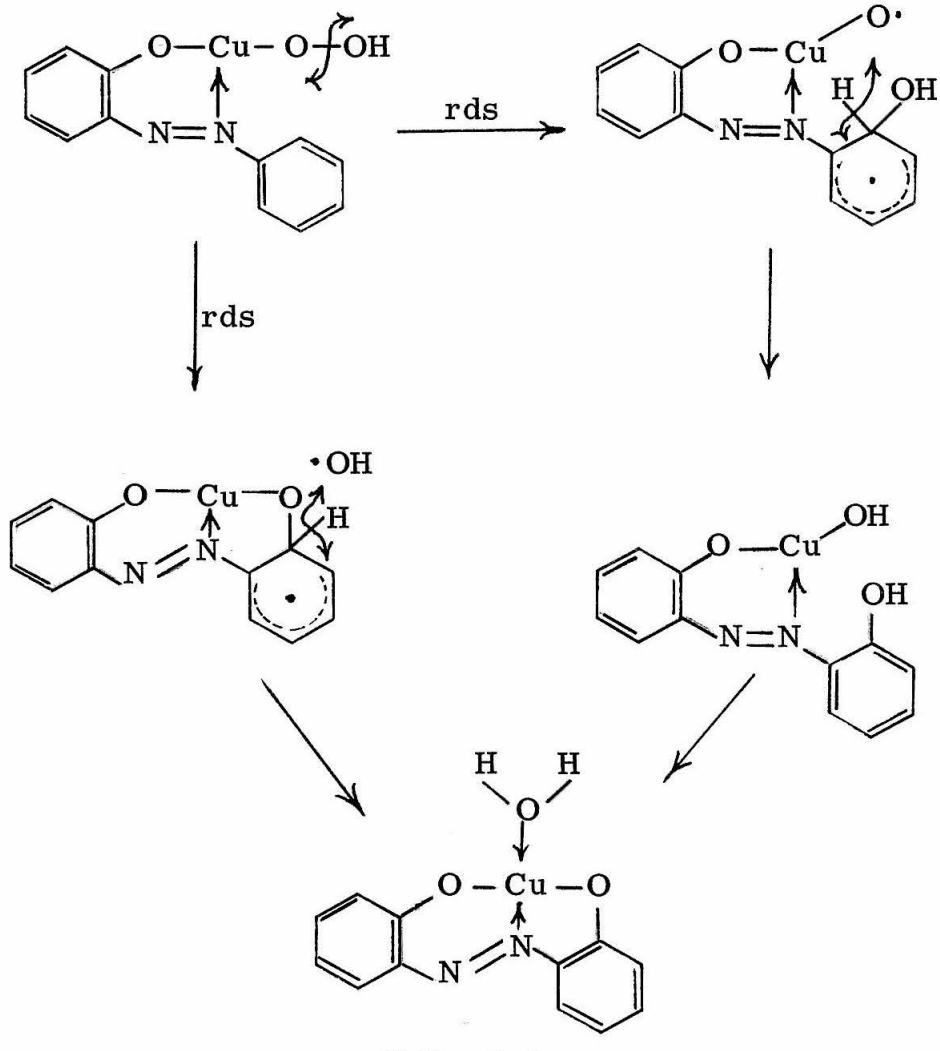
Consider first two resonance structures for 2-hydroxyphenolate ion.



XXV

Secondly, consider the fact that there is a body of evidence which indicates that many peroxide decompositions occur by way of nonradical mechanisms in which the peroxide bond is attacked by a nucleophile (146).

Two concerted reaction mechanisms in which the 2-hydroxyazo-



$CuD \cdot H_2O$

Scheme XXIII

benzene complex can be viewed as a nucleophile are presented in Schemes XXIV and XXV. We favor Scheme XXV over Scheme XXIV for three reasons:

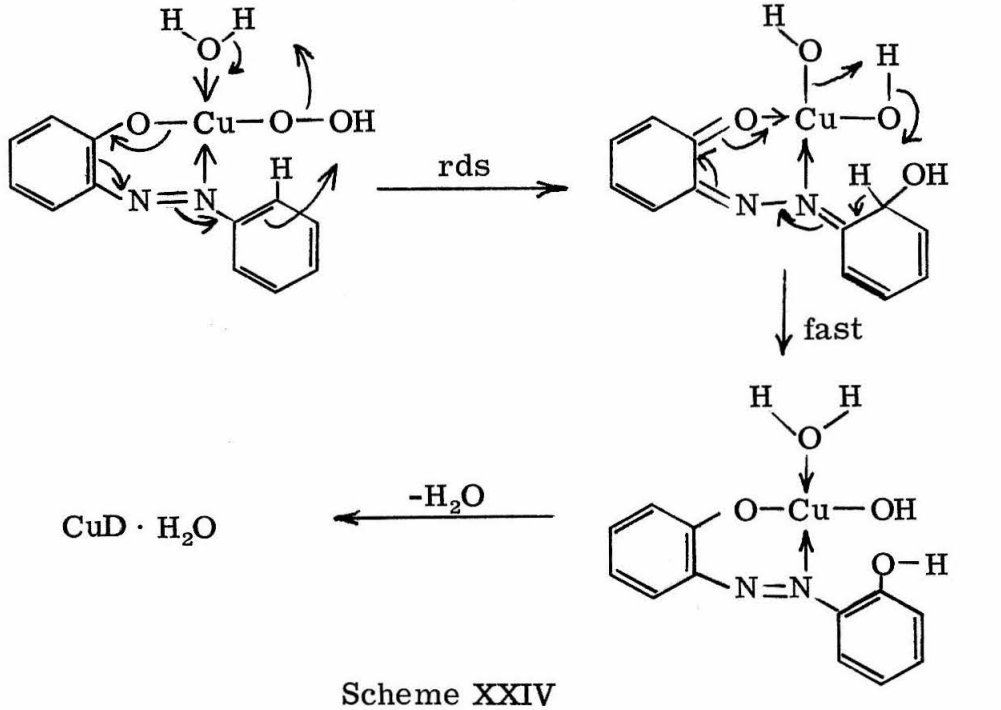
(1) The atoms in the CuLOOH complex are in a more favorable geometry for the rearrangement via Scheme XXV than by XXIV.

(2) The oxygen attached to copper is the more electrophilic oxygen in the peroxide bond.

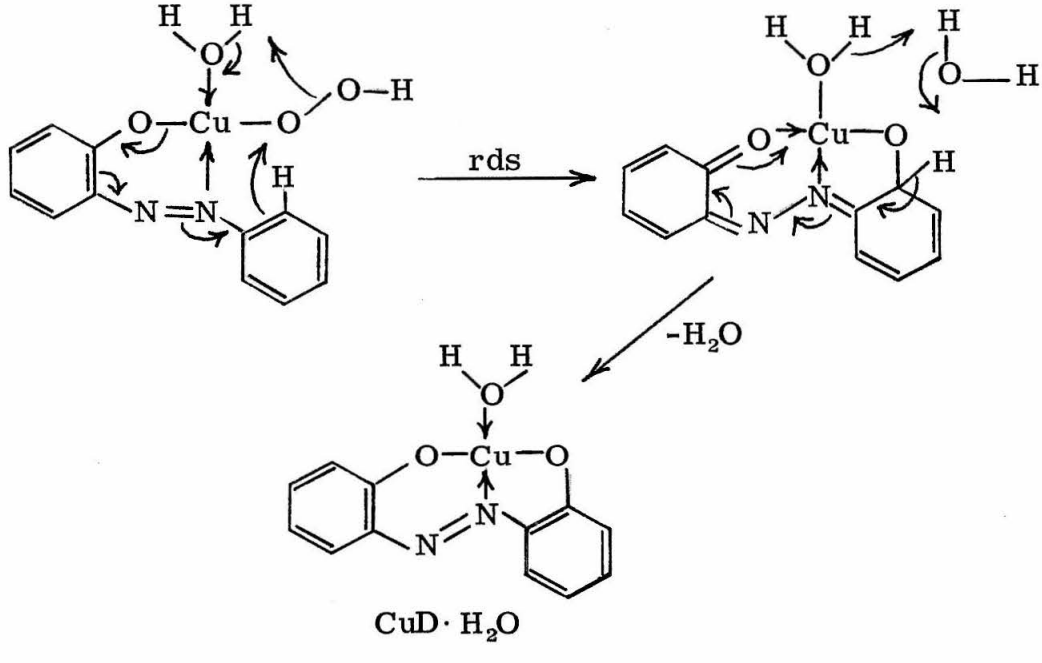
(3) Scheme XXV gives the product directly.

Either concerted mechanism is consistent with the observed reaction orders (section 2.4.2) and with the absence of a deuterium isotope effect (section 2.5.1). We feel that a concerted mechanism is also reasonable in terms of the observed substituent effect for the reasons discussed below.

In classical electrophilic substitution (i.e., substitution by a positively charged [polarized] reagent), the electronic effect of a substituent on reaction rates is generally explained in terms of its effect on the electron density at the carbon which undergoes substitution, and its ability to stabilize a positively charged intermediate. In the case of the concerted reaction mechanism, the transition state for the reaction would presumably be similar to that shown in Figure 16. Since there is no charge to stabilize in the intermediate, the greatest effect of the substituent would be inductive, i.e., the most important factor would be the stability of the nascent carbon-oxygen bond. An estimate of the inductive effect is found in the acidity



Scheme XXIV



Scheme XXV

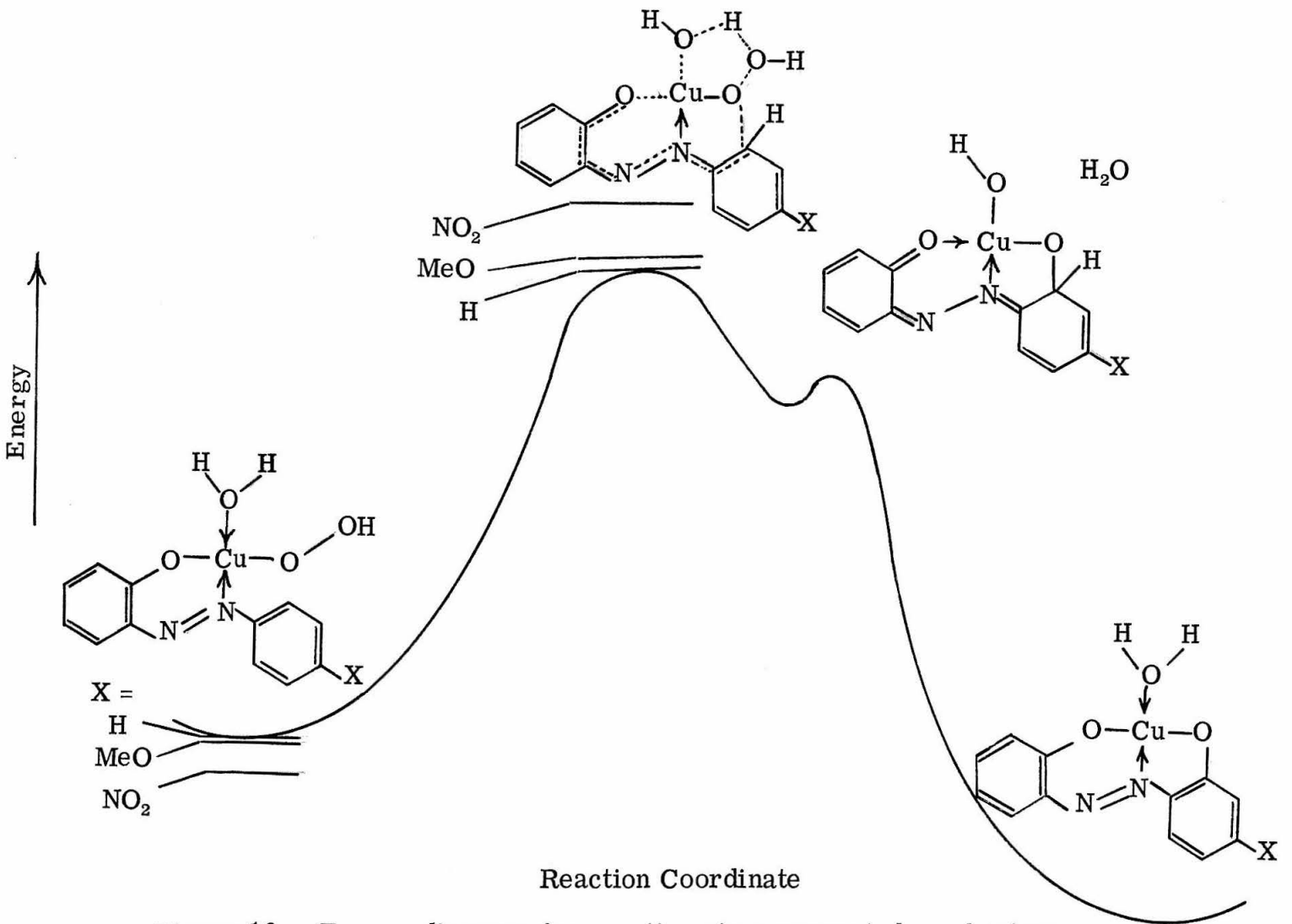
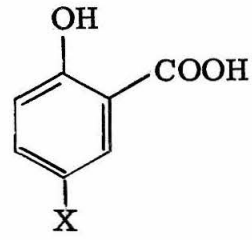


Figure 16. Energy diagram for reaction via a concerted mechanism.

constants for the 5-substituted salicyclic acids (147).



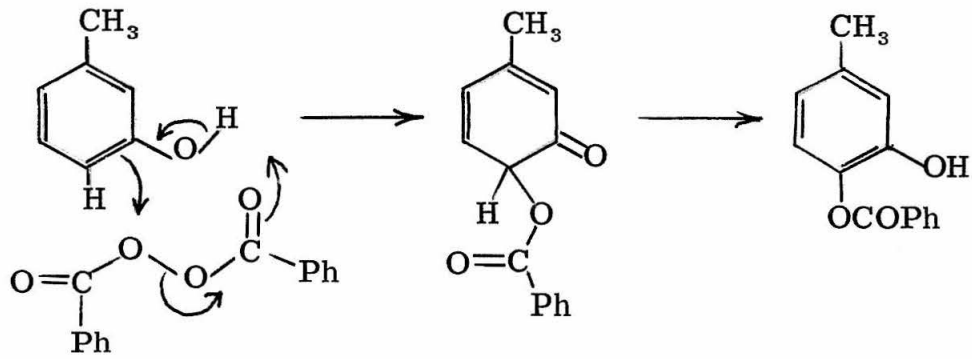
X	dissociation constant
H	1.05×10^{-3}
OCH_3	1.15
NO_2	4.8

If we assume that these values are roughly related to the inductive effect at the meta carbon atom and, therefore, to the carbon-oxygen bond energy, then clearly the energy in the transition state would be expected to fall in the order $H \cong OCH_3 < NO_2$, and the reaction rates would be expected to fall in the reverse order, i.e. $H \cong OCH_3 > NO_2$.

An additional factor which might be expected to further increase the relative activation energy for hydroxylation of the nitro derivative would be a difference in the ground state energies of the CuLOOH complexes. A lower value for the nitrogroup is rationalized in terms of the extra resonance energy, from extended conjugation in the nitro derivative, which would be lost to a certain extent in the proposed transition state. Obviously results with additional substituents would provide a useful means of testing the proposed mechanism.

2.6.3.1.3 Summary of Conclusions: From a study of the substituent effect in aqueous solution, we conclude that the rate determining step for conversion of the CuLOOH complex (XXII) to the copper complex of 2, 2′-dihydroxyazobenzene is not simply cleavage of the peroxide bond, nor does the mechanism involve the formation of a cationic intermediate in the transition state. The available data

are consistent with either a radical mechanism or a transition state in which cleavage of the peroxide bond is accompanied by formation of an oxygen-carbon bond and where any developing charge is effectively cancelled by resonance interaction within the ligand-copper complex. Although our data do not distinguish between the radical and concerted paths, we tend to favor the concerted mechanism (Scheme XXV) in view of the apparent absence of radicals in peroxide decomposition by copper chelates (93, 94) and peroxidase (64), and in the conversion of phenol to catechol by hydrogen peroxide in acetic acid (148). In addition, precedence for the concerted path exists by analogy with the mechanism proposed for the reaction of m-cresol with benzoyl peroxide (Scheme XXVI) (149).



Scheme XXVI

2.6.3.2 Discussion of Substituent Effects in Acetic Acid:

In view of the mechanistic conclusions drawn from studies of hydroxylation in aqueous solution, we return briefly to the results of the substituent effect in acetic acid. The most obvious difference between the kinetic results in water and those in acetic acid is the relatively rapid rate for the 4′-nitroderivative, i.e., $k_H/k_{NO_2} = 1.6$ (Table 18). This result can be explained if one assumes that the overriding factor governing the reaction rates in acetic acid is the concentration of ligand anion, $[L^-]$. In this regard the 4′-nitro derivative would have the largest pK_a in acetic acid, and thus the greatest concentration of intermediate XXII. One can imagine that the increased concentration of intermediate is balanced by the decrease in rate expected on the basis of the considerations in section 2.6.3.1.2.

If this assumption were true, then substituents in the 5-position, which are not connected to the 2′-position by resonance, and which are too far removed to have much inductive effect, should affect the rate in the same order as they affect ligand dissociation constants. If compounds I and IV in Table 18 are compared, it can be seen that the hydroxylation rate is decreased slightly by the introduction of a methyl group in the 5-position. Additional support for the hypothesis that $[L^-]$ is important comes from a literature report regarding product yields in the hydroxylation of five substituted 2-hydroxyazobenzenes. These results, which were mentioned in the introduction (see 1.7 number 4), are presented in Table 20 (97).

Table 20. Yields of 2, 2′ -Dihydroxyazobenzenes in the Hydroxylation of 5-Substituted-2-Hydroxyazobenzenes with Cupric Ion and Hydrogen Peroxide in Glacial Acetic Acid at 30°.

5-Substituent	Yield of Product
$-OCH_3$	0
$-CH_3$	49
$-H$	55
$-Cl$	65
$-NO_2$	68

The yields reported are in exactly the order which one would predict on the basis of the acidity of the 2-hydroxy group.

In conclusion we feel that the reaction mechanism in acetic acid is probably similar to the mechanism in aqueous solution and that the apparent differences in substituent effects are due to differences in the concentration of the intermediate complex (CuLOOH).

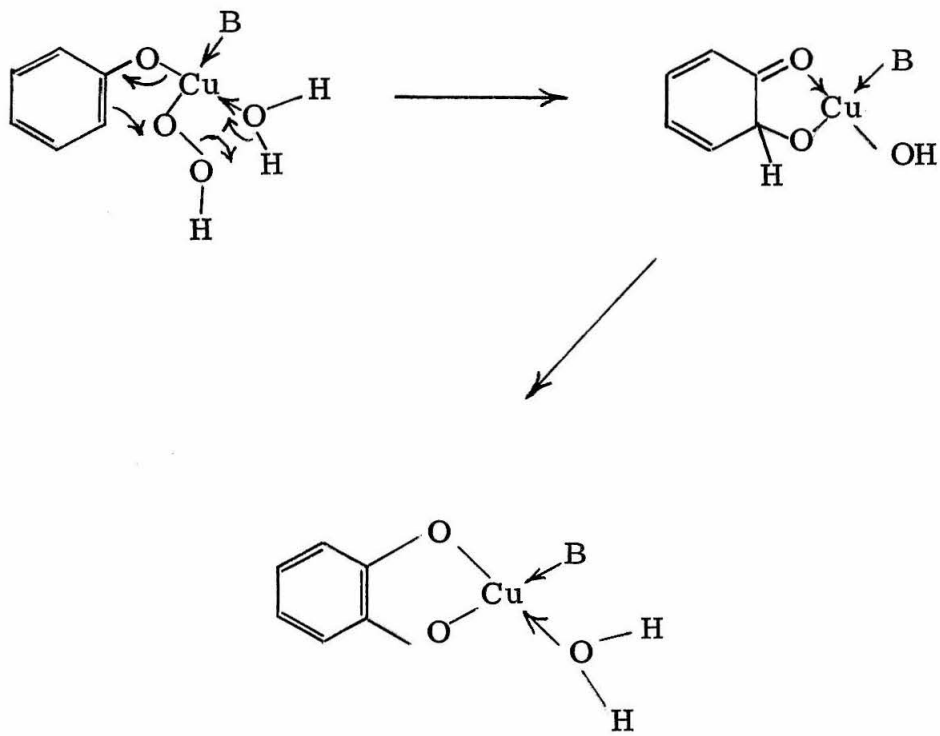
2.7 The Hydroxylation of 2-Hydroxyazobenzene by Cupric Ion and Hydrogen Peroxide as a Model for Enzymatic Hydroxylation

In the final section of this chapter, the proposed mechanisms for the hydroxylation of 2-hydroxyazobenzene by cupric ion and hydrogen peroxide are discussed in relation to previously proposed model hydroxylases and with respect to selected enzymatic reactions.

A number of the nonenzymatic systems which have been proposed as models for hydroxylase and/or peroxidase function were discussed in the introduction (see section 1.6). The present study is unique in that it is the only known hydroxylation system in which the details of complex formation have been examined. As a result this is the first reported hydroxylase model system whose mechanism has been investigated by direct kinetic studies of reaction orders and substituent effects. The 2-hydroxyazobenzene system is also unique in that none of the hydroxylase models previously discussed appear to orient or activate the substrate molecule with respect to the hydroxylating agent in the way that is implied in Scheme XXV.

In view of the orientation effect, the results of this study do not bear directly on any of the model hydroxylases discussed in section 1.6, although they may shed some light on the reported ortho hydroxylation of phenols by cupric-amine complexes (150). The mechanism of the latter reaction was originally described in terms of a complex of cupric ion, phenol, and hydrogen peroxide which was said to be converted to ortho-dihydroxy phenol by nucleophilic attack on the aromatic

ring (150). By analogy with the mechanism proposed in Scheme XXV, we suggest that the mechanism of phenol hydroxylation by cupric ion-amine complexes may occur instead by a mechanism similar to that shown in Scheme XXVII.

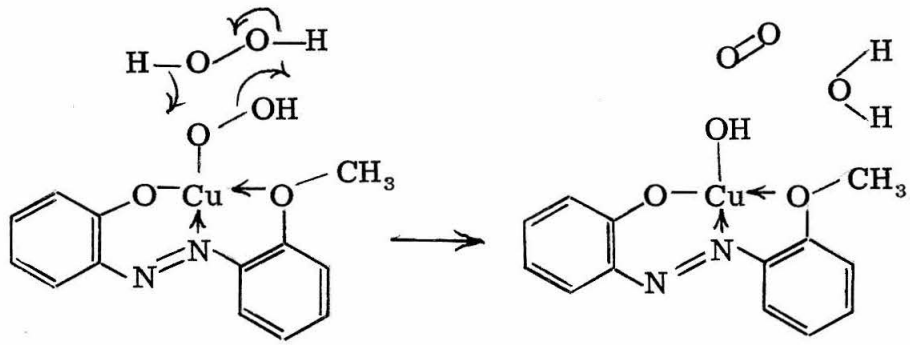


Scheme XXVII

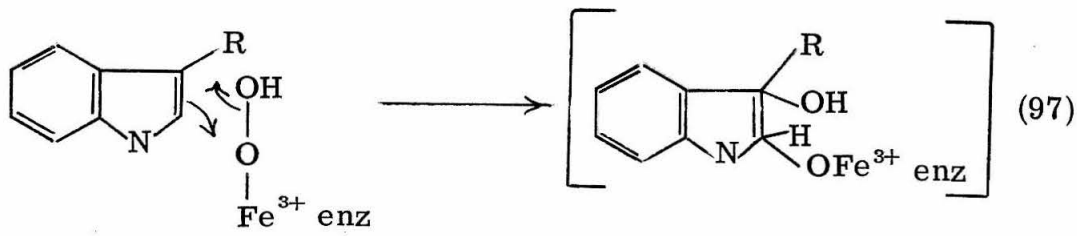
A mechanism involving electrophilic attack, but different from Scheme XXVII was proposed several years ago by Ingraham (151).

In many ways the results of our study of the 2-hydroxyazobenzene system correlate well with the studies of catalase and peroxidase models which were discussed in section 1.6.2.3. A comparison of Schemes VII and XXV shows that the only formal difference between the catalase and hydroxylase mechanisms is the nature and orientation of the substrate. We suggest that when the normal substrate in the 2-hydroxyazobenzene system is altered in such a way as to inhibit hydroxylation, that then the complex is capable of destroying peroxide by a catalase-type mechanism (without concomitant ring hydroxylation). An example is the introduction of a 2′-methoxy group; and although the 2′-methoxy derivative showed no evidence of hydroxylation, it is very likely that the complex participated in peroxide decomposition, perhaps as outlined in Scheme XXVIII. It is also possible that a certain amount of catalase-type activity occurs via the CuLOOH complex even when hydroxylation is not inhibited. Our experiments, however, were not designed to test this possibility although we have noted (in section 2.4.1.2) that the rate of peroxide decomposition exceeds the rate of hydroxylation in acetic acid.

The implied similarity between peroxidase and hydroxylase function in the model reactions may be indicative of a basic similarity at the enzymatic level. We have already noted the observed hydroxylase activity of peroxidase (equation 14, section 1.3.2) and the reported peroxidase activity of tryptophan oxygenase (Scheme V, 1.3.2). Based on schemes VII and XXV, one can imagine the possibility that the first step in Scheme V occurs according to equation 97.



Scheme XXVIII



(97)

Finally, we are led to speculate on the possibility that *in vivo* (as opposed to *in vitro* where free radicals have sometimes been shown to occur) many of the metalloenzymes which participate in monooxygenase, dioxygenase, peroxidase, and catalase function bring about the metabolism of oxygen by the same basic mechanism. We have given examples of mechanisms in which metal-peroxide complexes function as hydroxylases (Scheme XXV) and catalases (Scheme VII). Sigel *et al.* (94) have attempted to show that in the presence of hydrogen donors, the complexes which show catalase activity are capable of peroxidase function; and to complete the picture equation 97 represents a portion of a scheme in which a metal-peroxide complex could conceivably be involved in a dioxygenase reaction. Common features in all four systems are the formation of a metal-peroxide complex, and a concerted, heterolytic cleavage of the metal-peroxide bond with loss of $OH^{\ominus}$ and either the formation of an oxygen-carbon bond with the π electrons of substrate, or the abstraction of hydride ion by electron deficient oxygen.

In conclusion we note that the list of enzymes involved in oxygen metabolism grows continually, but that much work remains to be done at the mechanistic level in both enzymatic and model systems before the mystery of the catalytic function of this important class of enzymes is understood. The problem is as fascinating as it is complex, and for that reason the solution will come, with time and the patient probing of those who have a passion for understanding the intimate details of life at the molecular level.

Chapter 3. EXPERIMENTAL

3.1 General--Reagents and Instrumentation

Commercially available reagents used in this study were: 2-chloro-5-nitroaniline, 2-methoxy-5-methylaniline, and 2-methoxy-4-nitroaniline from Aldrich Chemical; aluminum chloride (anhydrous), ammonium chloride, benzene (thiophene free), cupric acetate monohydrate, cupric nitrate trihydrate, hydrogen peroxide (30%), methanol (spectral grade), perchloric acid (60%), potassium iodide, sodium carbonate, sodium nitrite, sodium sulfate (anhydrous), and sodium thiosulfate from Allied Chemical; dimethylsulfoxide, and potassium acid phthalate (primary standard grade) from J. T. Baker; N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid, and 2-(N-morpholino)ethane sulfonic acid from Calbiochem; D_6-DMSO from Chemi Standards Inc; deuterium oxide from Columbia Organic Chemicals; glacial acetic acid from Du Pont; o-aminophenol (practical), o-anisidine, p-anisidine, and nitrobenzene from Eastman Kodak; peracetic acid from FMC; sodium perchlorate (anhydrous) from G. F. Smith; aniline, copper sulfate pentahydrate, potassium dichromate (primary standard grade), sodium

dichromate, and zinc dust from Mallinkrodt; and aniline hydrochloride, p-cresol (practical grade), hydroxylamine hydrochloride, p-nitroaniline, 1-phenol-4-sodium sulfonate, sodium methoxide, and m-toluidine from Matheson, Coleman, and Bell.

All reported melting points were determined with a Fisher-Johns melting block, and are uncorrected. Sample weights were determined using a Mettler single pan balance Ultraviolet and visible spectra were obtained with a Cary Model 11 recording spectrophotometer. A Perkin-Elmer Model 237 was used to measure infrared spectra; and nuclear magnetic resonance spectra were obtained with a Varian Model A-60 spectrometer.

All microanalyses were conducted by Spang Microanalytical Laboratories of Ann Arbor, Michigan.

3.2 Syntheses

3.2.1 Derivatives of 5-Methyl-2-Hydroxyazobenzene

Derivatives of 5-methyl-2-hydroxyazobenzene were prepared by diazo coupling of appropriate anilines with p-cresol (153). Details for the preparation of 5-methyl-2-hydroxyazobenzene (I) are given below:

p-Cresol (25 g, 270 mmoles) was added to 180 ml of 10% sodium hydroxide in a 1-ℓ beaker. Aniline (25g, 270 mmoles) and 300 g of ice were placed into a second 1-ℓ beaker which was immersed in a methanol-ice bath. To this mixture was added with stirring 80 ml of concentrated hydrochloric acid.[a] Sodium nitrite (19.5 g, 280 mmoles) dissolved in 40 ml of distilled water was added dropwise with stirring at a rate such that a reaction temperature of less than 0° was maintained. The mixture was tested from time to time with starch-iodide paper for excess nitrous acid.[b] Sodium nitrite addition was discontinued with about 2 ml of solution remaining when after 20 minutes, a positive (blue) test was observed.

---

[a] A heterogeneous mixture resulted when p-nitroaniline was used in the preparation of III. Addition of sodium nitrite, however, gave a solution of the diazonium salt and a small quantity of insoluble material which was removed by filtration.

[b] Diluting each drop of test solution with 5-6 drops of distilled water was made necessary by the high acid concentration, which tended to give a false test.

About 200 g of ice was added to the beaker of p-cresol, which was placed in an ice bath. The cold diazonium solution was introduced with rapid stirring over a 10 minute period, and the mixture was stirred for 1 hour and allowed to stand an additional 3 hours in the cold ($< 10°$). The brown precipitate was filtered with suction, washed with cold water, and air dried to give 41.5 g (72%) of impure material. The crude product was recrystallized twice from benzene-ligroin (50/50) and once from hexane to give 5-methyl-2-hydroxyazobenzene (I) red prisms, mp 107-108.5°; lit. (99) 108°.

The melting points of two derivatives of 5-methyl-2-hydroxyazobenzene prepared by the above procedure for use in this study are: 3′,5-Dimethyl-2-hydroxyazobenzene (II), mp 99-101°, lit. (100) 98-100; and 4′-nitro-5-methyl-2-hydroxyazobenzene (III), mp 184-190°, lit. (101) 191°.

3.2.2 Derivatives of 2-Hydroxyazobenzene-5-Sodium Sulfonate

Derivatives of 2-hydroxyazobenzene-5-sodium sulfonate were prepared by diazo coupling of appropriate anilines with 1-phenol-4-sodium sulfonate (154). Details for the preparation of 2′-methoxy-2-hydroxyazobenzene-5-sodium sulfonate (IX) are given below:

o-Anisidine (30 ml, 34 g, 275 mmoles), which had been recently distilled (bp 220-221°), and 300 g of ice were placed in a 1-ℓ beaker which was immersed in a methanol-ice bath. To this mixture was added with stirring 80 ml of concentrated hydrochloric acid. To the heterogeneous mixture, which resulted from the separation of o-anisidine hydrochloride, was added dropwise with stirring

sodium nitrite (19.5 g, 280 mmoles) dissolved in 40 ml of distilled water. The mixture was tested periodically with starch-iodide paper for excess nitrous acid.[b] Sodium nitrite addition was stopped with about 2 ml of solution remaining when a positive (blue) test was observed. The cold ($<0°$), red diazonium solution was filtered[c] and added rapidly with stirring to a solution of 1-phenol-4-sodium sulfonate (52.5 g, 270 mmoles) and sodium carbonate (35 g) in 200 ml of distilled water at room temperature in a 1-ℓ beaker. Ether was added to reduce foaming, and the solution was stirred for several hours. The brown precipitate was filtered with suction and pressed as dry as possible. The damp, crude product was crystallized from 75% ethanol and dried in air to give 35 g (37%) 2′-methoxy-2-hydroxyazobenzene-5-sodium sulfonate (IX).

Derivatives used in subsequent kinetic studies were crystallized at least three times from 50% ethanol. The derivatives, their percent purity as determined by titration of the phenolic proton (see 3.3.5 below), and their microanalyses are: 2-Hydroxyazobenzene-5-sodium sulfonate (V),[d] 94%, Anal. Calcd. for $C_{12}H_9O_4N_2SNa$: C, 48.0; H, 3.0; N, 9.35; S, 10.6. Found: C, 48.3; H, 2.8; N, 9.49; S, 10.5. 4′-Nitro-2-hydroxyazobenzene-5-sodium sulfonate

---

[c]Filtration was made necessary by the presence of a small quantity of green-blue precipitate which may have been introduced with the ice.

[d]Crude material prepared by Mr. Glenn Prestwich, California Institute of Technology.

(VII), 96%, Anal. Calcd. for $C_{12}H_8O_6N_3SNa$: C, 41.7; H, 2.3; N, 12.2. Found: C, 40.8; H, 2.4; N, 12.0. 4′-Methoxy-2-hydroxyazobenzene-5-sodium sulfate (VIII), 90%, Anal. Calcd. for $C_{13},H_{11}N_2O_5SNa$: C, 47.4, H, 3.33. Found: C, 46.4; H, 3.53.

3.2.3 2-Methoxyazobenzene

A procedure outlined by Sciarini (155) for the preparation of 2-methoxyazobenzene was followed with slight modification. Nitrobenzene was reduced with zinc dust by standard methods (156) to give nitrosobenzene, mp 61-62°, in 51% yield. The freshly prepared nitrosobenzene (50 g, 455 mmoles) was mixed with 200 ml of cold absolute ethanol and 165 ml of glacial acetic acid. To this initially inhomogeneous mixture was added with cooling freshly distilled o-anisidine (51 ml, 56 g, 455 mmoles). During the addition, which produced a red-brown solution, a temperature of between 5 and 10° was maintained. The solution was stirred at room temperature an additional 30 minutes and stored several days in a refrigerator. Solvent was removed by rotary evaporation, and the remaining liquid was steam distilled. About 150 g of sodium chloride was added to each 2-ℓ portion of steam distillate before extraction with 3 × 100 ml of ethyl acetate. Combined extracts from 25 ℓ of steam distillate were washed with distilled water and dried over anhydrous sodium sulfate. Solvent removal by rotary evaporation gave 31.7 g (33%) of 2-methoxyazobenzene as a red oil.

3.2.4 2-Hydroxyazobenzene (IV)

A method (157) for demethylating anisole with aluminum chloride was adapted to prepare 2-hydroxyazobenzene. 2-Methoxyazobenzene (26.4 g, 125 mmoles) was dissolved in 155 ml of thiophene-free benzene. After being dried over anhydrous sodium sulfate, the solution was added dropwise with cooling and rapid stirring to a suspension of anhydrous aluminum chloride (16.5 g, 125 mmoles), from a previously unopened bottle, in 180 ml of dry benzene. The reaction was performed usinga pressure-equalizing addition funnel attached to a two-neck flask (500 ml) with a reflux condenser fitted with a calcium chloride drying tube. The dark-red, heterogeneous mixture was heated to reflux with stirring for 7 hours and allowed to stand overnight at room temperature. The mixture was poured over 300 g of ice, 50 ml of 6 N hydrochloric acid was added, and the layers were separated. The reaction flask retained about 10 g of base-insoluble black residue which was discarded. The organic layer was washed with 2 × 300 ml of 2 N hydrochloric acid and 300 ml of distilled water. The solution was dried over anhydrous sodium sulfate, and the solvent was removed by rotary evaporation to give 9.6 g of a red solid, mp 60-70°. The wide melting range was probably due to the presence of starting material.

To isolate the desired product, a portion of the crude material (6.9 g) was dissolved in 280 ml of 10% sodium hydroxide. The solution was filtered and the filtrate was extracted with 25 ml of ether. After being acidified with concentrated hydrochloric acid, the aqueous

layer was extracted with ether. Evaporation of the ether gave 4.9 g of product, mp 77-80°. This was combined with 2.5 g of material isolated from other fractions and the whole was recrystallized from ligroin to give 5.3 g of 2-hydroxyazobenzene (IV), red-orange needles, mp 81-82°, lit. (158) 82-83°, 21% yield from 2-methoxyazobenzene.

3.2.5 2′,4′,6′-Trideuterio-2-Hydroxyazobenzene-5-Sodium Sulfonate (VI)

2,4,6-Trideuterioaniline (3.5 g, 36 mmoles), which had become dark brown on standing, was diazotized and coupled with 1-phenol-4-sodium sulfonate (7.35 g, 37 mmoles). The procedure used has been discussed (see 3.2.2 above). The crude product was stirred in boiling 50% ethanol, and the solution was filtered hot to remove a substantial quantity of brown solid. Solvent was evaporated from the filtrate, and the residue was recrystallized from a minimum of 50% ethanol to give 1.9 g (17%) 2′,4′,6′-trideuterio-2-hydroxyazobenzene-5-sodium sulfonate (VI).

The above material was compared with its nondeuterated analog (V) by recording the spectrum of identical[e] (in mg per ml) solutions of each in the presence and in the absence of copper ion. The two compounds were indistinguishable in the region from 700 to 350 mμ.

---

[e]The 1% difference in molecular weights was ignored.

3.2.6 Derivatives of 5-Methyl-2,2′-Dihydroxyazobenzene

3.2.6.1 Synthesis by Demethylation with Aluminum Chloride: Appropriate o-anisidines were diazotized and coupled with p-cresol according to the general procedure given in 3.2.1. A single crystallization of the crude products gave, from ethanol, 2′-methoxy-5-methyl-2-hydroxyazobenzene (XV), red needles, mp 118.5-120°, lit. (100) 120°; and 2′-methoxy-5,5′-dimethyl-2-hydroxyazobenzene, red plates, mp 133-134°; and, from benzene, 2′-methoxy-5-methyl-4′-nitro-2-hydroxyazobenzene, brown powder, mp 185-187°.

Demethylation of these derivatives with anhydrous aluminum chloride gave corresponding 2,2′-dihydroxyazobenzenes. Details for the preparation of three derivatives of 2,2′-dihydroxyazobenzene (XI, XII, XIII) follows:

5,5′-Dimethyl-2,2′-Dihydroxyazobenzene (XI): 2′-Methoxy-5,5′-dimethyl-2-hydroxyazobenzene (5.5 g, 21 mmoles) was dissolved in 250 ml of reagent benzene. The solution was dried over anhydrous sodium sulfate for 3 hours and decanted into a 500-ml two-neck flask with a reflux condenser fitted with a calcium chloride drying tube. Anhydrous aluminum chloride (7.2 g, 54 mmoles) was added through the open neck of the flask to the magnetically stirred solution. The open neck was stoppered, and the mixture was refluxed for 5.5 hours. The aluminum complex of the product was precipitated by pouring the reaction mixture over 250 g of ice. The precipitate was isolated by suction filtration and washed with water.

An attempt was made to isolate the metal-free product by dissolving the complex in a minimum of 20% sodium hydroxide and reacidifying the solution with concentrated hydrochloric acid. The red precipitate which formed did not melt below 290°. This material, which was most probably unchanged aluminum complex, was redissolved in 20% sodium hydroxide. The solution was diluted with several volumes of water and acidified with concentrated hydrochloric acid. This time the yellow-orange precipitate of the metal-free dye formed. Additional product was precipitated by acidifying the aqueous layer of the two-phase filtrate obtained above after the reaction mixture had been poured over ice. The combined precipitates gave 3 g (60%) of crude product, which gave, after two recrystallizations from 95% ethanol, 0.30 g of 5,5′-dimethyl-2,2′-dihydroxyazobenzene (XI), yellow-needles, mp 217-219°, lit. (105) 219-220°.

4′-Nitro-5-Methyl-2,2′-Dihydroxyazobenzene (XIII). 2′-Methoxy-5-methyl-4′-nitro-2-hydroxyazobenzene (5.2 g, 19 mmoles) was converted to an aluminum complex of its 2′-hydroxy analog (XIII) by adapting the above procedure. The complex was dissolved in 20% sodium hydroxide, and the solution was filtered. The filtrate was diluted with an equal volume of water and made acidic by rapidly adding concentrated hydrochloric acid with stirring. The red precipitate was isolated by suction filtration. It was washed with water, crystallized from 95% ethanol, and recrystallized from benzene to give 0.73 g (14%) 4′-nitro-5-methyl-2,2′-dihydroxyazobenzene (XIII), red-orange, amorphous solid; mp 215-223°.

Anal. Calcd. for $C_{13}H_{11}O_4N_3$: C, 57.2; H, 4.03. Found: C, 57.1; H, 4.06.

5-Methyl-2,2′-Dihydroxyazobenzene (XII). 2′-Methoxy-5-methyl-2- hydroxyazobenzene (5.0 g, 21 mmoles) was demethylated with anhydrous aluminum chloride in the manner described above. A gelatinous, red precipitate of the aluminum complex of the product was isolated by suction filtration. The metal-free dye was obtained in two portions. One fraction was isolated by first dissolving about half of the complex in concentrated sodium hydroxide. The mixture was filtered, and the filtrate was poured over ice. Rapid addition of concentrated hydrochloric acid to the filtrate caused the separation of an orange precipitate, which was isolated by filtration to give 1.3 g of crude material, mp 158-165°. This was crystallized from 95% ethanol to give 0.50 g of yellow needles, mp 162-163°.

The second half of the aluminum complex was dissolved in 20% sodium hydroxide, and the solution was poured over ice. Rapid addition of concentrated hydrochloric acid to the above solution regenerated some red precipitate, which may have been an aluminum complex; but by diluting the mixture with water, a yellow-orange precipitate was formed. The crude material was isolated by filtration, washed with water, and crystallized from 95% ethanol to give 1.2 g of yellow needles, mp 160-163°. The two batches were combined and recrystallized from 95% ethanol to give 1.01 g (21%) 5-methyl-2,2′-dihydroxyazobenzene (XII), yellow needles, mp 164-166°. Anal. Calcd. for $C_{13}H_{12}O_2N_2$: C, 68.5; H, 5.28. Found: C, 68.0; H, 5.30.

3.2.6.2 Synthesis by Nucleophilic Displacement of Chloride 5′-Nitro-5-Methyl-2,2′-dihydroxyazobenzene (XIV).

2-Chloro-5-nitroaniline was diazo coupled with p-cresol according to the general procedure given in 3.2.1. The crude product was crystallized from 95% ethanol to give 2′-chloro-5′-nitro-5-methyl-2-hydroxyazobenzene (XVI), red-brown plates, mp 184-187°.

2′-Chloro-5′-nitro-5-methyl-2-hydroxyazobenzene (XVI, 4.6 g, 16 mmoles) was dissolved in 50 ml of reagent dimethylsulfoxide. Sodium hydroxide (1.2 g) and 10 ml of water were added, and the deep-purple solution was stirred in an oil bath at 90° for 1 hour. The reaction mixture was diluted with an equal volume of water, and concentrated hydrochloric acid was added to precipitate the product. The crude material was isolated by suction filtration, washed with water, and crystallized from 95% ethanol to give an amorphous yellow-brown solid, mp 235-240°. Recrystallization of this material from benzene gave 0.88 g (20%) 5′-nitro-5-methyl-2,2′-dihydroxyazobenzene (XIV), mp 248-253°. Anal. Calcd. for $C_{13}H_{11}O_4N_3$: C, 57.2; H, 4.03. Found: C, 57.2; H, 4.05.

3.2.7 Derivatives of 2,2′-Dihydroxyazobenzene-5-sodium sulfonate

For our purposes it was sufficient to obtain mixtures of 2-hydroxy and 2,2′-dihydroxy derivatives. These were prepared by hydroxylating 2-hydroxyazobenzene-5-sodium sulfonates with hydrogen peroxide in the presence of cupric ion. Details for synthesis and isolation of these mixtures are given below:

4′-Nitro-2,2′-Dihydroxyazobenzene-5-Sodium Sulfonate (XIX). 4′-Nitro-2-hydroxyazobenzene-5-sodium sulfonate (VII, 1.0 g, 2.8 mmoles) and cupric sulfate pentahydrate (1.4 g, 5.6 mmoles) were added to 2 ℓ of distilled water in a 4-ℓ flask. The solution was adjusted to pH7 with sodium hydroxide, and 5 ml of 30% hydrogen peroxide was added. The solution was readjusted from pH 6.5 to pH7, and the mixture was stirred for 4 hours at room temperature. The solvent was removed by rotary evaporation to give a flaky, black copper complex, which was converted to a yellow precipitate of the copper-free sulfonic acid by adding 10 ml of concentrated hydrochloric acid. The precipitate was isolated by suction filtration and washed with water. To convert the sulfonic acid to its sodium salt, the above product was dissolved in 15 ml of 1% sodium hydroxide. The solution was filtered and neutralized with dilute hydrochloric acid. The precipitated salt was isolated by filtration and crystallized from ethanol-water to give 0.30 g of an amorphous red-brown solid which analyzed (see 3.4.5 below) as 41% 4′-nitro-2,2′-dihydroxyazobenzene-5-sodium sulfonate (XIX).

4′-Methoxy-2,2′-Dihydroxyazobenzene-5-Sodium Sulfonate (XX). 4′-Methoxy-2-hydroxyazobenzene-5-sodium sulfonate (VIII, 1.0 g, 3.0 mmoles) and cupric sulfate pentahydrate (1.4 g, 5.6 mmoles) were added to 2 ℓ of distilled water in a 4-ℓ flask. This caused a flocculent precipitate to form. Hydrogen peroxide (5 ml, 30%) was added to the mixture, which was stirred overnight to give a dark solution. Rotary evaporation of solvent gave the copper complex of the product.

Copper-free material was formed by mixing the complex with 10 ml of concentrated hydrochloric acid and 10 ml of water. The precipitated dye was isolated by suction filtration to give 0.23 g of product, which was converted to its sodium salt by dissolving the isolated substance in a minimum of 30% sodium hydroxide and neutralizing the resultant solution with dilute hydrochloric acid. The gelatinous precipitate was isolated and crystallized from 75% ethanol to give 81 mg of golden-brown needles, which analyzed (see 3.4.5) as 94% 4′-methoxy-2,2′-dihydroxyazobenzene-5-sodium sulfonate (XX).

4′,6′-Dideuterio-2,2′-Dihydroxyazobenzene-5-Sodium Sulfonate (XVIII). 2′,4′,6′-Trideuterio-2-hydroxyazobenzene-5-sodium sulfonate (VI, 0.263 g, 0.87 mmole) and cupric nitrate (0.535 g, 2.2 mmoles) were added to 500 ml of distilled water in a 1-ℓ flask. The solution was adjusted to pH7 with dilute sodium hydroxide both before and after the addition of 3 ml of 30% hydrogen peroxide. After being stirred at room temperature for 40 minutes, the reaction mixture was passed over a cation exchange column (40 g of Amberlite IR-120, acidic form). The eluant was neutralized as it came off the column by continuous dropwise addition of sodium hydroxide. Approach to neutrality could be detected by color changes in the solution. When all of the dye had been eluted, the solution was adjusted to pH7, and the solvent was removed by rotary evaporation. About half of the crude material was crystallized from isopropyl alcohol-water (1:1) to give 90 mg of bright, yellow crystals which analyzed (see 3.4.5) as 73% 4′,6′-dideuterio-2,2′-dihydroxy-

azobenzene-5-sodium sulfonate (XVIII).

3.2.8 2,2′-Dihydroxyazobenzene (X)

2,2′-Dihydroxyazobenzene was prepared from its copper complex, which was synthesized by a slight variation of a procedure outlined by Freeman and White (105). The copper catalyst was prepared as follows: Copper sulfate pentahydrate (28.5 g, 115 mmoles) was dissolved in 100 ml of hot water. The solution was cooled and treated with 30 ml of concentrated ammonium hydroxide to form the soluble ammonia complex, which was reduced to the colorless copper (I) species with hydroxylamine hydrochloride (7 g, 100 mmoles) dissolved in 20 ml of water.

o-Aminophenol (11 g, 100 mmoles) and sodium nitrite (7g, 100 mmoles) were dissolved in 250 ml of 5% sodium hydroxide. The solution was filtered, and the filtrate was cooled in an ice-salt bath. Concentrated hydrochloric acid was added dropwise with stirring at such a rate that the temperature remained below 0°. The addition of acid was stopped at 40 ml; for starch-iodide test paper (3.2.1) indicated the presence of excess nitrous acid. There was some uncertainty in the test due to the dark color of the reaction mixture.

The cold diazonium solution was immediately added with stirring over a period of 7 or 8 minutes to freshly prepared copper catalyst solution (see above) contained in a beaker, which was immersed in an ice bath. Some foaming was observed, and the reaction mixture was allowed to stand with occasional stirring for 2 hours. The solution was filtered with suction to give 14 g of copper complex as a black

powder after drying and light crushing. Uncomplexed impurities were removed by slurrying the powder in 200 ml of t-butanol. The slurry was filtered, and the solid was dissolved in 250 ml of concd. hydrochloric acid. After being stirred 2 hours with slight warming, the solution was poured into 250 ml of ice water. The gelatinous, brown-black precipitate was isolated by filtration and washed with water. The crude product was stirred in boiling benzene with a little added butanol. The hot mixture was filtered to remove a considerable quantity of insoluble black material. The filtrate was cooled slowly and suction filtered to give 1.5 g of yellow-orange needles, mp 163-172°. Another 0.7 g of product was recovered by evaporation of the filtrate. The two product fractions were combined and recrystallized twice from benzene to give 1.5 g (14%) of 2,2′-dihydroxyazobenzene (X), mp 174-175°, lit. (105) 172-172.7°.

3.2.9 2,4,6-Trideuterioaniline

2,4,6-Trideuterioaniline was prepared according to the method of Best and Wilson (159). Aniline hydrochloride (8.2 g) was dissolved in 10 ml of deuterium oxide (99.7%). The solution was refluxed for 24 hours, and the solvent was removed by rotary evaporation. A fresh 10 ml aliquot of deuterium oxide was added to the flask, and the sequence was repeated for a total of six equilibrations. The final reaction mixture was neutralized with about 70 ml of 1N sodium hydroxide. The neutral solution was saturated with salt and diluted to 150 ml with saturated salt solution. The mixture was steam distilled, and the product was extracted from the distillate with ether.

The ether solution was dried over anhydrous sodium sulfate, and the solvent was removed to give 4.3 g (70%) of 2,4,6-trideuterioaniline. The pmr spectrum (CCl_4) gave two singlets, peak area 1:1, at 3.4 and 6.95 δ.

3.2.10 Cupric bis-2-Benzeneazo-4-Methyl Phenolate (XXI)

5-Methyl-2-hydroxyazobenzene (I, 0.213 g, 1.0 mmole), cupric acetate (0.200 g, 1.0 mmole), and sodium methoxide (0.053 g, 0.98 mmole) were stirred into a beaker with 100 ml of reagent methanol. The precipitated complex was isolated by suction filtration, washed with methanol, and dried. The brown precipitate of cupric bis-2-benzeneazo-4-methyl phenolate was not crystallized, mp 237-242°, lit (100) 242°.

3.3 Determination of Formation Constants

Formation constants, K_1 and K_2, for complexes in aqueous media, of cupric ion and derivatives of 2-hydroxyazobenzene-5-sodium sulfonate were determined using pH titration methods described by Irving and Rossotti (116).

3.3.1 Titration Procedure.

Titrations of samples at constant ionic strength (0.26 M sodium perchlorate) were done in beakers covered by a rubber sheet with openings for an electrode, a buret tip, and a tube used to admit a stream of dry nitrogen freed of carbon dioxide by passage over Ascarite. An internally standardized Beckman glass electrode connected to a Leeds and Northrup pH meter was used to measure pH. Standardized sodium hydroxide was added from a 1-ml micro-buret

(0.005 ml per division), whose tip was positioned near the beaker wall just above the liquid level. (It was found that tip immersion gave unstable readings near the end-point). Any liquid adhering to the tip was added to the solution by touching the tip against the beaker wall. The solution was maintained at 30.0° with a constant temperature bath; and a water-driven magnet (G. F. Smith Co.) was used in conjunction with a Teflon stirring bar to stir the solution continuously. At moderate stirring speeds, pH readings were stable and reproducible. Beckman pH 7.0 buffer was used to standardize the meter before each titration. Samples were immersed in the bath, stirring was begun, and nitrogen was passed over the solution for 20-30 minutes before the first reading was made. In order to permit the system to reach equilibrium, a period of 15-30 seconds elapsed after each addition of base before the pH meter was read. Data for each set of three titrations, which were needed to determine the formation constants, were gathered within a 24 hour period.

3.3.2 Stock Solutions

Stock solutions used in preparation and titration of samples were made as follows:

Sodium perchlorate (2.6 M) was prepared by weighing anhydrous reagent sodium perchlorate (322 g) into a 1-ℓ flask and diluting to the mark with distilled water.

Cupric nitrate (5.0×10^{-3} M) was prepared by quantitatively transferring reagent grade cupric nitrate trihydrate (1.201 g) to a 1-ℓ volumetric flask and diluting to volume with distilled water.

Ligand (10^{-4}-10^{-3} M) solutions were prepared by dissolving weighed amounts of thrice recrystallized dye, dried at 120° for 2 hours, in distilled water and diluting to volume in a volumetric flask.

Perchloric acid (approx. 0.02 N) was prepared by diluting 1:10 a solution made by diluting 20 ml of 60% reagent perchloric acid to exactly 1 liter.

Sodium hydroxide (IN) was prepared free of carbonate with boiled, distilled water by standard procedures (160). A 0.1 N sodium hydroxide solution 0.265 M in perchlorate, which was used in determination of formation constants, was prepared by pipetting 50 ml of IN stock sodium hydroxide and 50 ml of 2.6 M stock sodium perchlorate into a 500-ml volumetric flask and diluting to volume with boiled, distilled water. This solution was stored in an ordinary volumetric flask, and, as a consequence, a 10% change in normality was noted over 13 months.

3.3.3 Titration Samples

A set of three samples was titrated to determined formation constants with cupric ion for each ligand in the sulfonic acid series. Stock solutions were pipetted into volumetric flasks and diluted to volume with distilled water. The contents of the flask were emptied into a clean, dry beaker for titration. A single pipet was used with each stock solution to assure that each sample contained exactly the same quantities of perchloric acid and sodium perchlorate. The first sample in each set contained only perchloric acid and sodium perchlorate. The second sample was the same as the first, but for an aliquot

of ligand. The third sample was the same as the second, except for an added aliquot of cupric ion. All samples were diluted to the same volume. Important quantities are summarized in Table 21.

Table 21. Aliquot Sizes and Final Volumes of Titration Samples

Compound	4′ - Substituent	2.6 M $NaClO_4$ ml	O.O2N $HClO_4$	Ligand ml	10^{-3}M Cu^{++} ml	Final Volume ml
V	Hydrogen	10.0	10.0	70.0	10.0	100
VII	Nitro	50.0	10.0	50.0	10.0	500[a]
VIII	Methoxy	25.0	10.0	50.0	10.0, 15.0	250[a]

[a]Solubility limitations made necessary larger volumes.

3.3.4 Data Handling

Raw data were recorded as values of pH vs. buret readings. The increment of sodium hydroxide added was varied from 0.5 ml to 0.01 ml in regions of sharp pH increase. Plots were made of pH vs. ml of sodium hydroxide added for each set of data. Smooth curves were drawn through data points with the aid of a French curve, and volume differences ($\Delta V, \Delta V'$) at constant pH were determined from the graph at intervals of 0.1 pH unit (see Figure 8). Computation of formation constants from values of ΔV, $\Delta V'$, and constants of the system (see below 3.3.5) by the least squares method (116) was

accomplished with the aid of an Olivetti-Underwood Programma 101 Desk Computer.

3.3.5 Standardization of Solutions

In addition to the values of ΔV and $\Delta V'$, calculation of the formation constant required a knowledge of pKa of the ligand and of concentrations of cupric ion, sodium hydroxide, and ligand. The necessary quantities were calculated on the basis of dilution factors and the normality of stock solutions. The latter were determined as follows:

Sodium hydroxide was standardized against weighed samples of dried primary standard grade potassium acid phthalate (160) within a few days of a titration experiment. Normalities were taken to be the average of at least two determinations. End points were determined potentiometrically or with phenolphthalein.

Ligand concentrations were determined by titration of the phenolic proton with standardized sodium hydroxide. Solution pH was measured as a function of volume of base added. These data and data for a blank titration were plotted. End points were taken to be the points of maximum slope for difference curves between ligand and blank.

Dissociation constants, pKa's, were determined for each ligand from the above graphs by noting the pH at the half equivalence point.

Cupric ion concentration was determined by titrating aliquots of stock solution against standard sodium thiosulfate. Thiosulfate was standardized against weighed samples of primary standard grade

potassium dichromate (160).

3.4 Kinetic Method

3.4.1 Systems in Acetic Acid

The kinetic behavior of the reaction in glacial acetic acid of hydrogen peroxide with 2-hydroxyazobenzenes in the presence of cupric ion was observed spectrophotometrically. Solutions of ligand in the presence of cupric ion were prepared by weighing samples of recrystallized ligand and of cupric acetate into volumetric flasks and diluting to the mark with glacial acetic acid. Samples of the order of 10 ml were pipetted into 125-ml erlenmeyer flasks immersed in a 37° constant temperature bath. When thermal equilibrium had been achieved, the reaction was started by adding, with swirling, a 1-ml aliquot of 30% hydrogen peroxide. The time of addition was noted, and, at intervals of about a minute, 100 λ aliquot samples of the reaction mixture were removed and diluted to 10 ml (volumetric flask) with glacial acetic acid. The time of dilution was recorded, and the absorbance of the sample was measured at two wavelengths. Concentrations of species in solution as a function of time were calculated from these data using measured extinction coefficients (see 2.4.1)

3.4.2 Systems in Buffered Aqueous Solution

Reaction mixtures less peroxide were prepared by pipetting aliquots of buffer, sodium perchlorate, ligand, and cupric nitrate into 10-ml volumetric flasks and diluting to volume with distilled water. The ionic strength of these solutions was comparable with that used in determination of formation constants. To make kinetic measurements

exactly 2.5 ml of the above solution was pipetted into a clean, dry, quartz spectral cell (1 cm). The absorbance of the solution was measured at 500 or 525 mμ. Hydrogen peroxide (100 λ, 3%) was added to initiate the reaction from a 0.5-ml syringe with a Teflon "needle". In early experiments mixing was achieved by inverting the cell several times. The delay time from addition of peroxide to activation of the spectrophotometer pen was generally less than 10 seconds by this method. In later experiments delay times of about 4 seconds were achieved by adding the peroxide with the aid of a teflon plunger. Peroxide solution was pipetted onto the top surface of the plunger. Peroxide could be added and mixed with the solution in the cell in one step by moving the plunger up and down a few times. Air bubbles, which sometimes formed when mixing was achieved by inversion, were avoided too. The delay time was noted for all runs, and the absorbance was recorded as a function of time at constant wavelength. Absorbance values at zero time were computed from the dilution factor and the absorbance reading before peroxide addition. A check of pH before and after peroxide was added showed no change. Kinetic constants were determined by graphical estimation of initial slopes of absorbance vs. time curves.

3.4.3 Buffers

Buffers used in this study have been described by Good et al. (125). HEPES buffer, pH 7.5. N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid (11.9 g, 50 mmoles) was dissolved in 25 ml of distilled water. Half an equivalent of sodium hydroxide (24.3 ml,

0.97 N) was added, and the buffer solution was transferred to a 100-ml volumetric flask. Distilled water was added to the mark to give 0.5 M buffer of pH 7.5 at 30°.

MES buffer, pH 6.0, 0.5 M, was prepared by the above procedure from 2-(N-morpholino)ethanesulfonic acid monohydrate (10.65 g, 50 mmole) and half an equivalent of sodium hydroxide. A small quantity of fluffy, white precipitate was removed by filtration.

3.4.4 Determination of Peroxide Content (161)

The peroxide titer of reaction mixtures was determined iodometrically. Aliquots of the reaction mixture (1 ml) were pipetted into 20 ml of glacial acetic acidchloroform (3:2) to which 2 ml of saturated potassium iodide had been added. The mixture was swirled and allowed to stand 5 minutes. Distilled water (75 ml) was added and the solution was titrated with 0.1 N sodium thiosulfate to the starch endpoint.

3.4.5 Titration of 2,2′-Dihydroxyazobenzenes with Cupric Ion

Milligram samples of the 2,2′-dihydroxyazobenzene sodium sulfonate were weighed (Cahn Electrobalance) and transferred to volumetric flasks. A few drops of dilute hydrochloric acid were added, and the samples were diluted to volume with distilled water to give solutions near pH 3. At pH 3 complexation of cupric ion with the dihydroxy ligand could be observed without interference by complexation with any monohydroxy ligand present.

Exactly 2.5 ml of each of the above solutions were pipetted into clean, dry spectral cells (1 cm). Samples were titrated in the cell

with 1.00×10^{-3} M cupric nitrate, which was prepared by diluting 200 ml of standardized (see 3.3.2) cupric nitrate (5.00×10^{-3} M) to 1-ℓ with distilled water. Absorbance at 500 mμ was measured as a function of added volume of cupric nitrate. A plot was made of absorbance, after correction for dilution by titrant, vs. volume of cupric nitrate (see Figure 1). End-points were assumed to be the points of intersection of straight lines drawn through the two linear segments of the data curve.

3.5 Preparative Thin Layer Chromatography

Product (41 mg) from the hydroxylation of 2′, 4′, 6′-tri-deuterio-2-hydroxyazobenzene-5-sodium sulfonate (see 3.2.5) was dissolved in a minimum volume of 95% ethanol. The solution was applied in many steps with a linear applicator to an 8 × 8 inch glass plate coated with 0.5 mm of silica gel (Brinkmann, silica gel F_{254}). The chromatogram was developed with a solvent system prepared from 150 ml of ethanol, 10 ml of concentrated ammonium hydroxide, and 20 ml of ether. The material on the developed plate had separated into several broad bands. The product contained in each band was isolated by slurrying the silica gel scraped from each region with ethanol. Two major compounds were recovered by evaporation of ethanol which had been separated from the silica gel. (1) The material (23 mg) from the region of r_f 0.2. This component showed a λ_{max} in the presence of cupric ion at 494 mμ and was assumed to be the 2, 2′-dihydroxyazobenzene derivative. (2) The material (12 mg) isolated from the band at r_f 0.8. This compound did not complex copper in acid solution and had a spectrum identical to starting material.

Appendix I: Derivation of Job's Equation[a]

The following treatment shows how Job's method is developed for a simple case. If we consider that only a single complex can form and that this process is unaffected by pH (or is studied in a buffer system) the complexation reaction may be written:

$$A + nB \rightleftharpoons AB_n$$

This system is then studied using solutions in which the sum of the number of moles of A and B present is a constant, M. These are prepared by making solutions which contain (1 - x)M moles of A per liter and xM moles of B per liter. The concentrations of A, B, and AB_n will be designated C_1, C_2, and C_3, respectively and these are related by the equations:

$$C_1 = M(1 - x) - C_3$$

$$C_2 = Mx - nC_3$$

While these solutions are commonly made up by mixing (1 - x)V ml of an M molar solution of A with xV ml of an M molar solution of B to give a final volume of solution V, the occurrence of any volume change does not invalidate the use of the method of continuous

---

[a]From Mark M. Jones, "Elementary Coordination Chemistry," 282, Prentice-Hall, Englewood Cliffs, N.J., 1964.

variations. It merely makes this particular method of preparing the isomolar solutions unsuitable for that system. If we assume that activities can be replaced by concentrations, we may write the equilibrium constant for the complexation reactions as:

$$K = \frac{C_3}{C_1 \cdot C_2^n}$$

or

$$KC_1C_2^n = C_3$$

Differentiating this expression with respect to x allows the determination of the relationship between the maximum value of C_3 and x:

$$K\left(\frac{dC_1}{dx} \cdot C_2^n + nC_2^{n-1} C_1 \frac{dC_2}{dx}\right) = \frac{dC_3}{dx}$$

For a maximum value of C_3, dC_3/dx must equal zero so

$$C_2^n \frac{dC_1}{dx} + nC_1C_2^{n-1} \frac{dC_2}{dx} = 0$$

now when dC_3/dx is zero,

$$\frac{dC_1}{dx} = -M \text{ and } \frac{dC_2}{dx} = M$$

or

$$(-M)C_2^n + nC_2C_2^{n-1}(M) = 0$$

$$nC_1 = C_2$$

so

$$nM(1 - x) - nC_3 = Mx - nC_3$$

$$n = \frac{x}{1 - x}$$

So by determining at what value of x a maximum in C_3 occurs allows the establishment of n.

Appendix II: pH Titration Data

Run 33c

$N^0 = 0.098$ $T_M^0 = 0.997 \times 10^{-4}$ $V^0 = 100$ ml

$T_L^0 = 4.90 \times 10^{-4}$ $K_A = 5.01 \times 10^7$

Titration Data

A		B		B cont'd		C	
ml	pH	ml	pH	ml	pH	ml	pH
0.00	2.86	0.00	2.91	2.28	8.09	0.00	2.86
0.50	2.99	0.50	3.04	2.31_5	8.20	0.50	2.99
1.00	3.15	1.00	3.21	2.35	8.36	1.00	3.18
1.50	3.47	1.50	3.56	2.39	8.55	1.50	3.51
1.65	3.67	1.65	3.74	2.42_5	8.74	1.65	3.71
1.75	3.88	1.75	4.00	2.46	8.96	1.75	3.99
1.80	4.09	1.80	4.18	2.49_5	9.11	1.80	4.15
1.82_5	4.21	1.83	4.31	2.53_5	9.34	1.82_5	4.28
1.85	4.39	1.85	4.47	2.57_5	9.49	1.85	4.41
1.87_5	4.79	1.87_5	4.80	2.60	9.52	1.87	4.56
1.89	5.46	1.90	5.65			1.89	4.71
1.90	7.00	1.91	6.09			1.91	4.87
1.91	7.89	1.92_5	6.42			1.93_6	5.11
1.92	8.38	1.94	6.63			1.96	5.28
1.93	8.58	1.96	6.82			1.99	5.42
1.95	8.81	1.98	7.00			2.02	5.63
1.99	9.11	2.00	7.11			2.05	5.83
2.02_5	9.28	2.02_5	7.21			2.075	6.11
2.05	9.35	2.05	7.32			2.10	6.38
2.10	9.49	2.07_5	7.41			2.12	6.65
		2.10	7.50			2.14	6.89
		2.13	7.59			2.16	7.11
		2.16	7.69			2.18	7.26
		2.19	7.79			2.20	7.39
		2.22	7.88			2.22_5	7.50
		2.25	7.99			2.25_5	7.69

Meter checked OK
at pH 7.0

Appendix II continued

Run 97

$N^0 = 0.0955$ $T_M^0 = 6.00 \times 10^{-5}$ $V^0 = 250$ ml

$T_L^0 = 1.09 \times 10^{-4}$ $K_A = 5.61 \times 10^7$

Titrations

A		B		C	
ml	pH	ml	pH	ml	pH
0.00	3.19	0.00	3.21	0.00	3.21
1.00	3.15	1.00	3.54	1.00	3.54
1.49	3.95	1.50	3.99	1.50	4.00
1.64	4.24	1.65	4.29	1.65	4.29
1.74	4.62	1.75	4.78	1.75	4.69
1.79	5.19	1.77_5	5.06	1.77_5	4.90
1.84	6.39	1.79	5.27	1.80	5.17
1.85	6.89	1.80	5.43	1.82	5.31
1.86	7.39	1.81_5	5.68	1.84	5.49
1.87	7.70	1.83	6.00	1.86	5.59
1.88	7.90	1.84_5	6.33	1.88	5.70
1.89	8.07	1.86	6.61	1.90	5.81
1.90_5	8.20	1.87_5	6.86	1.92_5	5.99
1.93	8.39	1.89	7.06	1.95	6.11
1.96	8.58	1.91	7.24	1.97_5	6.25
1.99	8.68	1.93	7.39	2.00	6.38
2.04	8.82	1.97	7.61	2.02_5	6.57
2.09	8.97	1.98_5	7.69	2.05	6.69
2.14	9.09	2.00	7.76	2.07	6.81
2.19	9.18	2.02_5	7.88	2.09	6.99
2.28	9.25	2.05	7.99	2.11	7.19
		2.07_5	8.09	2.13	7.38
		2.10	8.18	2.17	7.72
		2.13	8.30	2.19	7.90
		2.16	8.40	2.21	8.06
		2.19	8.49	2.23	8.15
		2.22_5	8.59	2.25	8.25
		2.26	8.66	2.27	8.32
		2.30	8.78	2.29	8.41
		2.35	8.89		
		2.40	8.99		

Appendix II continued

Run 100

$N_0 = 0.0955$ $T_M^0 = 2.00 \times 10^{-5}$ $V^0 = 500$ ml

$T_L^0 = 4.75 \times 10^{-5}$ $K_A = 1.26 \times 10^7$

Titrations

A		B		C	
ml	pH	ml	pH	ml	pH
0.00	3.43	0.00	3.45	0.00	3.41
1.00	3.78	1.00	3.77	1.00	3.73
1.50	4.22	1.50	4.19	1.50	4.15
1.65	4.49	1.65	4.41	1.65	4.38
1.70	4.63	1.70	4.51	1.70	4.48
1.72_5	4.71	1.72_5	4.60	1.72_5	4.55
1.75	4.80	1.75	4.69	1.75	4.62
1.77_5	4.97	1.77_5	4.80	1.77_5	4.72
1.80	5.11	1.80	4.95	1.80	4.82
1.82	5.22	1.82_5	5.17	1.82_5	5.01
1.84	5.34	1.85	5.36	1.85	5.16
1.86	5.49	1.87_5	5.59	1.87_5	5.29
1.88	5.65	1.90	5.82	1.90	5.43
1.90	5.82	1.92_5	6.11	1.92_5	5.58
1.92	6.04	1.95	6.37	1.95	5.69
1.94	6.39	1.97_5	6.55	1.97_5	5.81
1.96	6.69	2.00	6.71	2.00	5.96
1.98	7.02	2.02_5	6.86	2.02_5	6.10
2.00	7.48	2.05	7.05	2.05	6.22
2.02	7.81	2.07_5	7.18	2.07_5	6.34
2.04	8.09	2.10	7.30	2.10	6.50
2.06	8.22	2.12_5	7.48	2.12_5	6.68
2.08	8.38	2.15	7.61	2.15	6.87
2.10	8.45	2.17_6	7.80	2.17_5	7.11
		2.20	8.00	2.20	7.35
		2.22_5	8.16	2.22_5	7.53
		2.25	8.30	2.25	7.71
		2.27_5	8.40	2.27_5	7.89
		2.30	8.51	2.30	8.01
		2.32_5	8.60	2.32_5	8.12
		2.35	8.66	2.35	8.20
		2.37_5	8.71		
		2.40	8.79		

Appendix III-A: Proof of Bjerrum's Equation for the Case N = 2

In the case where N = 2, Bjerrum's equation becomes

$$\bar{n} + (\bar{n} - 1)K_1[L] + (\bar{n} - 2)K_1K_2[L]^2 = 0$$

We want to show that this equation is true when

$$K_1 = \frac{CuL}{(Cu)(L)}$$

$$K_2 = \frac{CuL_2}{(CuL)(L)}$$

$$\bar{n} = \frac{CuL + 2CuL_2}{T_M}$$

and

$$T_M = Cu + CuL + CuL_2$$

Begin by dividing the original equation by $K_1[L]$

$$\frac{\bar{n}}{K_1L} + \bar{n} - 1 + (\bar{n} - 2)K_2L = 0$$

Substitute for the value of $\bar{n}$

$$\frac{CuL + 2CuL_2}{K_1(L)(T_M)} + \frac{CuL + 2CuL_2}{T_M} + \frac{K_2(L)(CuL) + 2K_2(L)(CuL_2)}{T_M} = 1 + 2K_2(L)$$

Multiply through by T_M

$$\frac{CuL}{K_1(L)} + \frac{2CuL_2}{K_1(L)} + CuL + 2CuL_2 + K_2(L)(CuL)$$

$$+ 2K_2(L)(CuL_2) = T_M + 2K_2(L)T_M$$

Make the substitutions

$$\frac{CuL}{K_1L} = Cu \quad \text{and} \quad K_1L = \frac{CuL}{Cu}$$

$$K_2(L)(CuL) = CuL_2 \quad \text{and} \quad K_2(L) = \frac{CuL_2}{CuL}$$

from the equilibrium expressions. Thus

$$Cu + 2(CuL_2)\left(\frac{Cu}{CuL}\right) + CuL + 2CuL_2 + CuL_2 + 2(CuL_2)\left(\frac{CuL_2}{CuL}\right)$$

$$= T_M + 2T_M\left(\frac{CuL_2}{CuL}\right)$$

Combine terms to give

$$Cu + CuL + 3CuL_2 + 2(CuL_2)\left\{\frac{Cu}{CuL} + \frac{CuL_2}{CuL} - \frac{T_M}{CuL}\right\} = T_M$$

since $Cu + CuL_2 - T_M = -CuL$, the above becomes

$$Cu + CuL + 3CuL_2 + 2(CuL_2)(-1) + Cu_T$$

$$Cu + CuL + CuL_2 = T_M$$

QED

Appendix III-B: Proof of Equation 61

We wish to show that

$$\bar{n} + (\bar{n}' - 1)(K_1 L) + (\bar{n}' - 2)(K_1 K_2 L^2) - \Delta\,(\bar{n}' - 1)(L) = 0$$

Given that

$$K_1 = \frac{CuL}{(Cu)(L)}, \qquad K_2 = \frac{CuL_2}{(CuL)(L)}, \qquad K_{Cu}^{H} = \frac{(CuOH)(H)}{Cu}$$

$$\bar{n}' = \frac{CuOH + CuL + 2CuL_2}{T_M},$$

$$T_M = Cu + CuOH + CuL + CuL_2$$

and that

$$\Delta = \frac{(K_{Cu}^{H}/H)\,(1 - K_1 K_2 L^2)}{(\bar{n}' - 1)(L)(1 + K_1 L + K_1 K_2 L^2 + K_{Cu}^{H}/H^+}$$

Begin by making substitutions into the expression for T_M from the equilibrium expressions.

$$T_M = Cu + (K_{Cu}^{H}/H)(Cu) + K_1(L)(Cu) + K_1 K_2(L^2)(Cu)$$

The ratio $\frac{CuOH}{T_M}$ can be expressed as

$$\frac{CuOH}{T_M} = \frac{K_{Cu}^{H}/H}{1 + K_1 L + K_1 K_2 L^2 + K_{Cu}^{H}/H}$$

Substitute this expression into Δ to obtain

$$\Delta = \frac{CuOH}{T_M} \cdot \frac{(1 - K_1K_2L^2)}{(\bar{n}' - 1)(L)}$$

Divide the original expression by K_1L, and substitute for Δ to obtain

$$\frac{\bar{n}'}{K_1L} + \bar{n} - 1 + (\bar{n}' - 2)(K_2L) - \frac{CuOH}{T_MK_1L} + \frac{CuOHK_2L}{T_M} = 0$$

Substitute for $\bar{n}'$, and multiply by T_M

$$\frac{CuOH + CuL + 2CuL_2}{K_1L} + CuOH + CuL + 2CuL_2 - T_M$$

$$+ (CuOH + CuL + 2CuL_2)K_2L - 2T_MK_2L$$

$$- \frac{CuOH}{K_1L} + CuOH\ K_2L = 0$$

Combine terms

$$\frac{CuL + 2CuL_2}{K_1L} + CuOH + CuL + 2CuL_2 - T_M$$

$$+K_2L(2CuOH + CuL + 2CuL_2 - 2T_M) = 0$$

Add the equation $K_2L(CuL) = CuL_2$.

$$\frac{CuL + 2CuL_2}{K_1L} + CuOH + CuL + 2CuL_2 - T_M$$

$$+ \ 2K_2L(CuOH + CuL + CuL_2 - T_M) = CuL_2$$

From the expression for T_M, note that

$$-Cu = (CuOH + CuL + CuL_2 - T_M)$$

Substitute this expression and simplify to obtain

$$\frac{CuL + 2CuL_2}{K_1L} + CuOH + CuL + CuL_2 - T_M - (2K_2L)(Cu) = 0$$

Finally make the following substitutions from the equilibrium equations

$$K_1L = \frac{CuL}{Cu} \qquad \text{and} \qquad K_2L = \frac{CuL_2}{CuL}$$

to give

$$Cu + 2CuL_2 \left(\frac{Cu}{CuL}\right) + CuOH + CuL + CuL_2 - 2\left(\frac{CuL_2}{CuL}\right)(Cu) = T_M$$

which simplifies to

$$Cu + CuOH + CuL + CuL_2 = T_M$$

QED

Appendix IV: Iterative Solution to Equation 62

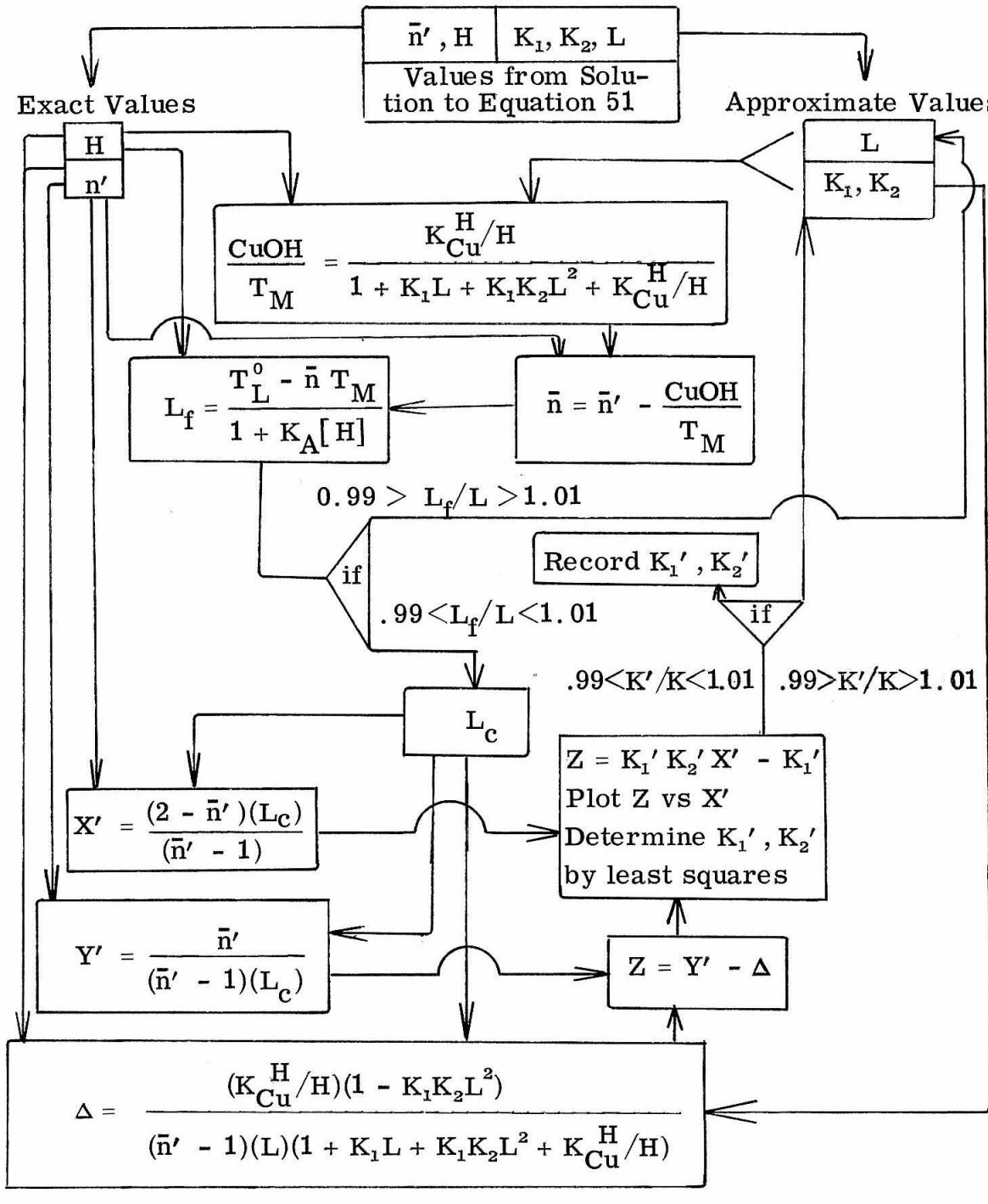
In the process of solving equation 51, a set of $\bar{n}$ and L values were generated as a function of pH and approximate values of K_1 and K_2 were determined. The derivation in section 2.3.3.4 shows that for $\bar{n}_A > 0.95$, the computed $\bar{n}$ values are, more correctly, $\bar{n}'$ values, and that

$$\bar{n} = \bar{n}' - \frac{CuOH}{T_M}$$

We note that (equation 48)

$$L = \frac{T_L^o - \bar{n} T_M^o}{1 + K_A[H]}$$

from which it is obvious that, in addition to the Δ correction to equation 51, the original values of X and Y must be corrected for errors in the determination of L. The iterative process is described in the block diagram below.


$\bar{n}'$, H
K_1, K_2, L
Values from Solution to Equation 51
Exact Values
Approximate Values
H
n′
L
K_1, K_2
$\frac{CuOH}{T_M} = \frac{K_{Cu}^H/H}{1 + K_1L + K_1K_2L^2 + K_{Cu}^H/H}$
$L_f = \frac{T_L^0 - \bar{n}\,T_M}{1 + K_A[H]}$
$\bar{n} = \bar{n}' - \frac{CuOH}{T_M}$
$0.99 > L_f/L > 1.01$
if
$.99 < L_f/L < 1.01$
Record K_1', K_2'
if
$.99 < K'/K < 1.01$
$.99 > K'/K > 1.01$
L_c
$Z = K_1' K_2' X' - K_1'$
Plot Z vs X′
Determine K_1', K_2'
by least squares
$X' = \frac{(2 - \bar{n}')(L_c)}{(\bar{n}' - 1)}$
$Y' = \frac{\bar{n}'}{(\bar{n}' - 1)(L_c)}$
$Z = Y' - \Delta$
$\Delta = \frac{(K_{Cu}^H/H)(1 - K_1K_2L^2)}{(\bar{n}' - 1)(L)(1 + K_1L + K_1K_2L^2 + K_{Cu}^H/H)}$

Appendix V: Data and Results Used in Final Iteration to Calculate Values of K_1 and K_2 Reported in Table 8.

Run 97

$N_0 = 0.0955$ $\quad T_M^0 = 6.00 \times 10^{-5}$ $\quad V^0 = 250$ ml

$T_L^0 = 1.09 \times 10^{-4}$ $\quad K_A = 5.61 \times 10^{7}$

$K_1 = 2.7 \times 10^{5}$ $\quad K_1K_2 = 1.93 \times 10^{11}$ $\quad K_{Cu}^H = 1.59 \times 10^{-7}$

pH	$[H] \times 10^{+7}$	$\Delta V' \times 10^{+2}$ ml	$\bar{n}'$	$\bar{n}$	L	K_1L	$K_1K_2L^2$	$\frac{K_{Cu}^H}{[H]}$	$X' \times 10^6$	$Y' \times 10^{-5}$	$\Delta \times 10^{-5}$
5.4	39.8	3.2	0.204	0.170	4.3	0.116	0.037	0.040	-0.99	-5.80	-0.93
5.5	31.6	4.0	0.255	0.215	5.4	0.145	0.056	0.050	-1.27	-6.32	-0.938
5.6	25.1	5.1	0.325	0.277	6.4	0.174	0.080	0.063	-1.60	-7.45	-1.00
5.7	20.0	6.6	0.420	0.364	7.7	0.208	0.115	0.079	-2.10	-9.30	-1.12
5.9	12.6	8.9	0.566	0.490	11.0	0.324	0.278	0.126	-3.68	-11.8	-1.21
6.0	10.0	10.0	0.571	0.487	14.0	0.378	0.378	0.159	-5.25	-12.7	-0.664
6.1	7.9	11.6	0.752	0.658	15.3	0.411	0.450	0.200	-7.69	-19.7	-1.41

Appendix V continued

Run 96

$N_0 = 0.0955$ $T_M^0 = 4.00 \times 10^{-5}$ $V^0 = 250$ ml

$T_L^0 = 1.09 \times 10^{-4}$ $K_A = 5.6 \times 10^7$

$K_1 = 3.0 \times 10^5$ $K_1K_2 = 6.0 \times 10^{11}$ $K_{Cu}^H = 1.59 \times 10^{-7}$

pH	$[H] \times 10^{+7}$	$\Delta V' \times 10^{+2}$ ml	$\bar{n}'$	$\bar{n}$	L	K_1L	$K_1K_2L^2$	$\frac{K_{Cu}^H}{[H]}$	$X' \times 10^6$	$Y' \times 10^{-5}$	$\Delta \times 10^{-5}$
5.2	63.1	2.5	0.239	0.217	2.83	0.085	0.048	0.025	-0.65	-11.10	-0.95
5.3	50.1	3.0	0.286	0.250	3.50	0.105	0.074	0.032	-0.84	-11.45	-0.98
5.4	39.8	3.6	0.344	0.313	4.30	0.127	0.107	0.040	-1.08	-12.2	-1.08
5.5	31.6	4.2	0.401	0.376	5.30	0.159	0.168	0.050	-1.42	-12.6	-1.00
5.6	25.1	5.1	0.487	0.445	6.43	0.193	0.248	0.063	-1.90	-14.7	-0.96
5.7	20.0	6.2	0.592	0.544	7.70	0.232	0.357	0.079	-2.67	-18.8	-0.97
5.8	15.9	7.1	0.678	0.622	8.50	0.255	0.432	0.100	-3.49	-24.8	-1.15
6.3	5.0	14.0	1.337	1.263	20.1	0.602	2.42	0.317	3.95	19.7	-1.51
6.5	3.2	16.3	1.556	1.476	26.6	0.805	4.26	0.501	2.08	10.5	-1.69

Appendix V continued

Run 100

$N_0 = 0.0955$ $T_M^0 = 2.00 \times 10^{-5}$ $V^0 = 500$ ml

$T_L^0 = 4.78 \times 10^{-5}$ $K_A = 1.26 \times 10^{7}$

$K_1 = 2.9 \times 10^{5}$ $K_1K_2 = 1.54 \times 10^{11}$ $K_{Cu}^{H} = 1.59 \times 10^{-7}$

pH	$[H] \times 10^{+7}$	$\Delta V' \times 10^{+2}$	$\bar{n}'$	$\bar{n}$	$L \times 10^{-7}$	K_1L	$K_1K_2L^2$	$\frac{K_{Cu}^{H}}{H}$	$X' \times 10^{6}$	$Y' \times 10^{-5}$	$\Delta \times 10^{-5}$
5.1	79.4	2.1	0.200	0.183	4.34	0.126	0.029	0.020	-0.975	-5.78	-0.47
5.2	63.1	2.5	0.238	0.218	5.35	0.155	0.044	0.025	-1.238	-5.85	-0.48
5.3	50.1	2.9	0.276	0.251	6.59	0.191	0.067	0.032	-1.571	-5.79	-0.49
5.4	39.8	3.7	0.353	0.324	7.97	0.231	0.097	0.040	-2.029	-6.89	-0.51
5.5	31.6	4.4	0.420	0.385	9.66	0.280	0.144	0.050	-2.63	-7.50	-0.52
5.6	25.1	5.4	0.516	0.480	11.7	0.344	0.212	0.063	-3.58	-9.09	-0.55
5.7	20.0	6.5	0.620	0.584	13.8	0.389	0.299	0.079	-5.01	-11.85	-0.48
5.8	15.9	7.6	0.726	0.679	16.2	0.470	0.404	0.100	-7.51	-16.21	-0.68

Appendix V continued

Run 104

$N_0 = 0.0955$ $T_M^0 = 2.00 \times 10^{-5}$ $V^0 = 500$ ml

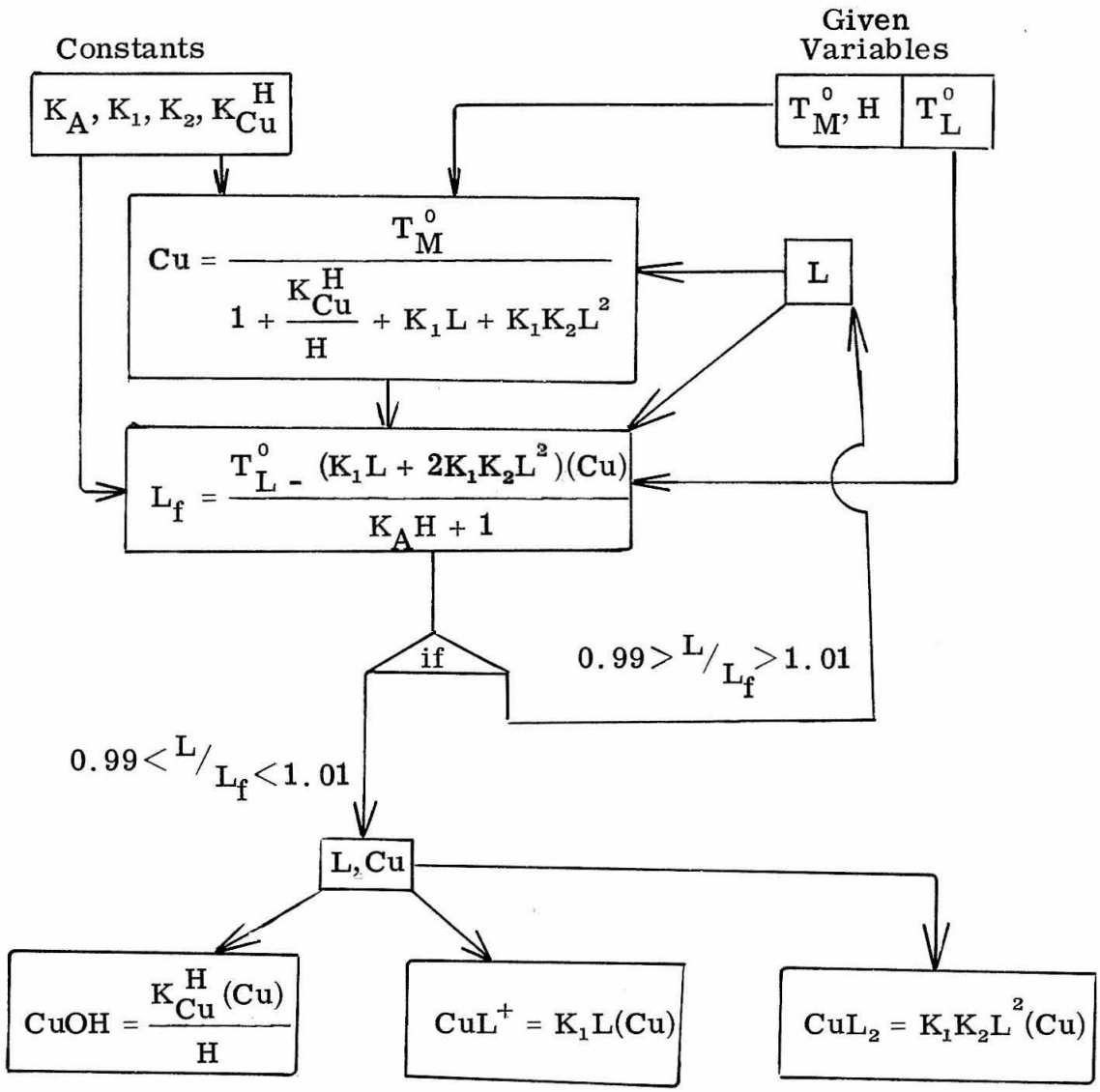
$T_L^0 = 4.78 \times 10^{-5}$ $K_A = 1.26 \times 10^7$

$K_1 = 3.5 \times 10^5$ $K_1K_2 = 0.885 \times 10^{11}$ $K_{Cu}^H = 1.59 \times 10^{-7}$

pH	$[H] \times 10^{+7}$	$\Delta V' \times 10^{+2}$	$\bar{n}'$	$\bar{n}$	$L \times 10^{-7}$	K_1L	$K_1K_2L^2$	$\frac{K_{Cu}^H}{H}$	$X' \times 10^6$	$Y' \times 10^{-5}$	$\Delta \times 10^{-5}$
5.1	79.4	2.0	0.191	0.191	4.35	0.152	0.017	0.020	-0.974	5.42	-0.47
5.2	63.1	2.5	0.238	0.218	5.44	0.191	0.026	0.025	-1.26	-5.75	-0.47
5.4	39.8	3.3	0.315	0.287	8.25	0.289	0.064	0.040	-2.02	-5.57	-0.47
5.5	31.6	3.8	0.362	0.328	10.10	0.353	0.090	0.050	-2.60	-5.82	-0.47
5.6	25.1	4.7	0.448	0.409	12.10	0.424	0.129	0.063	-3.40	-6.71	-0.51
5.7	20.0	5.5	0.525	0.481	14.6	0.511	0.190	0.079	-4.54	-7.56	-0.52
5.8	15.9	6.7	0.639	0.590	17.0	0.595	0.254	0.100	-6.43	-10.40	-0.63
5.9	12.6	7.5	0.716	0.659	20.5	0.717	0.371	0.126	-9.25	-12.30	-0.61

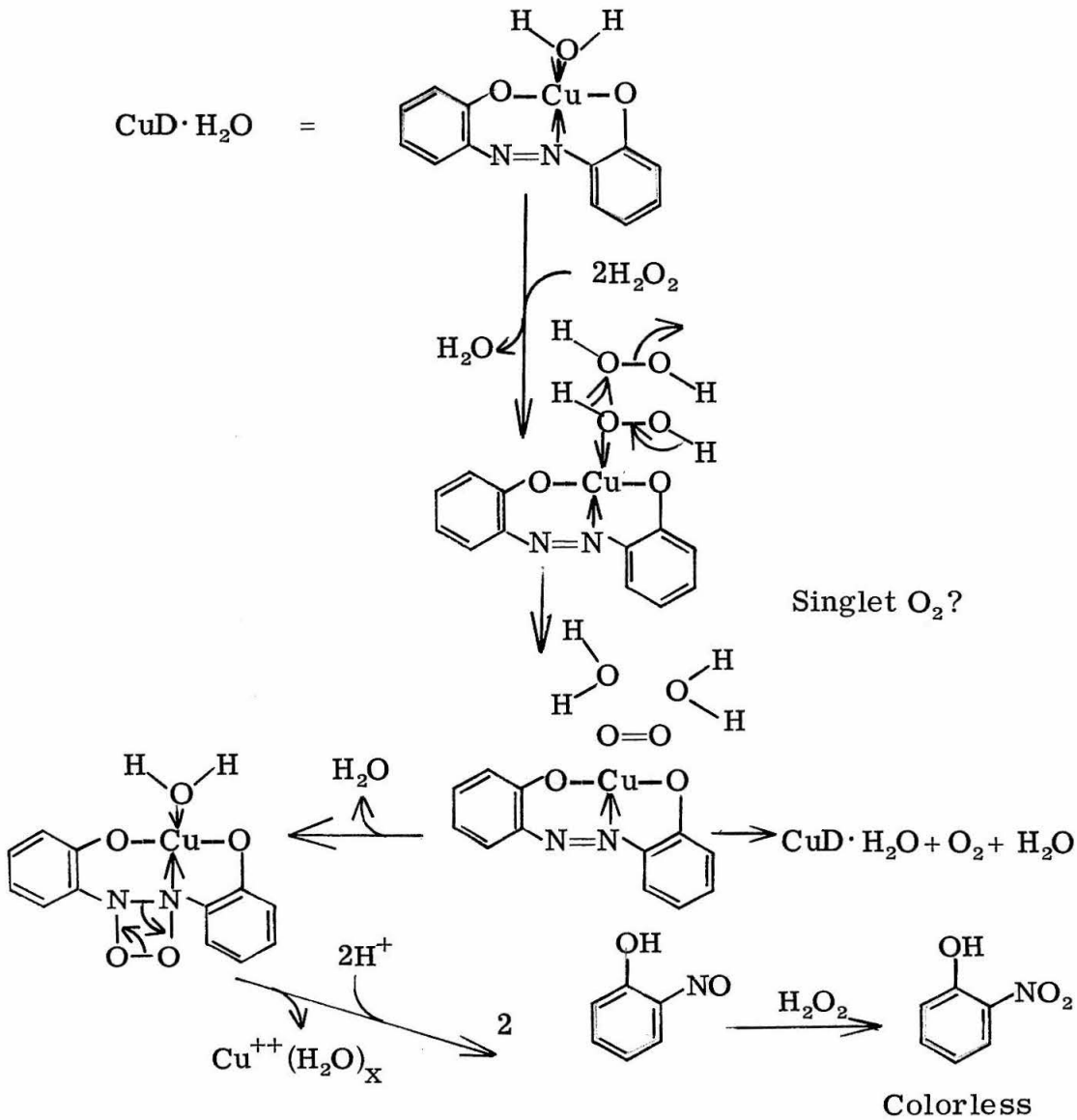
Appendix VI. Iterative Method of Determining Species Concentration:

Equations 74 and 75 can be solved for [L] and [Cu] by an iterative procedure. Concentrations of various copper complexes are then calculated using equilibrium expressions and [L], [Cu]. The method is described in the following block diagram.



Appendix VII. A Mechanism for the Destruction of the 2, 2′ - Dihydroxyazobenzene Copper Complex by Hydrogen Peroxide.

A possible explanation for the observed decomposition of CuD by peroxide (section 2.4.1.2) is given in Scheme



Scheme

We suggest that copper catalyzed decomposition of hydrogen peroxide is capable of producing singlet oxygen in a manner similar to that proposed for the generation of singlet oxygen by alkaline solutions of hydrogen peroxide and hypochlorous acid Precedence for peroxide decomposition by copper complexes in the manner shown is found in the experiments of Sigel et al. on the decomposition of hydrogen peroxide by cupric ion-bipyridyl complexes (see Scheme XVII, section 1.6.2.3). Addition of singlet oxygen to double bonds to form four membered rings has been discussed (162). The formation of nitrophenols from nitrosophenols by hydrogen peroxide is a known reaction (163).

REFERENCES

(1) R. W. F. Hardy and R. C. Burns, Ann. Rev. Biochem., 37, 331 (1968).

(2) A. L. Lenninger, "Bioenergetics," W. A. Benjamin, New York, 1965.

(3) H. R. Mahler and E. H. Cordes, "Biological Chemistry," 561, Harper and Row, New York, 1966.

(4) O. Hayashi, Ann. Rev. Biochem., 31, 21 (1962).

(5) O. Hayashi and M. Nozaki, Science, 164, 389 (1969).

(6) H. S. Mason, W. L. Fowlks, and E. Peterson, J. Amer. Chem. Soc., 77, 2914 (1955); O. Hayashi, M. Katagiri, S. Rothberg, ibid., 5450.

(7) O. Hayashi, Ann. Rev. Biochem., 38, 21 (1969).

(8) Mahler and Cordes, "Biological Chemistry," 565.

(9) B. C. Saunders, H. G. Holmes-Siedle, and B. P. Stark, "Peroxidase," Butterworths, London, 1964.

(10) D. R. Buhler and H. S. Mason, Arch. Biochem. Biophys., 92, 424 (1961).

(11) M. K. Roach, W. N. Reese, and P. J. Creaven, Biochem. Biophys. Res. Comm., 36, 596 (1969). K. Lippel and J. F. Mead, Biochem. Biophys. Acta, 152, 669 (1968).

(12) S. Kaufman, W. F. Bridgers, F. Eisenberg, and S. Friedman, Biochem. Biophys. Res. Comm., 9, 497 (1962).

(13) K. I. Kivirikko and D. J. Prockop, Proc. Nat. Acad. Sci., 57, 782 (1967).

(14) S. Udenfriend, Science, 152, 1335 (1966).

(15) H. Maeno and P. Feigelson, Biochem. Biophys. Res. Comm., 21, 297 (1965).

(16) J. B. Lombardini, T. P. Singer, and P. B. Boyer, J. Biol. Chem., 244, 1172 (1969).

(17) S. Kawai, T. Oshims, and F. Egami, Biochem. Biophys. Acta, 104, 316 (1965).

(18) D. M. Ziegler and F. H. Pettit, Biochemistry, 5, 2932 (1966).

(19) Mahler and Cordes, "Biological Chemistry," 516.

(20) J. Peterson, E. J. McKenna, R. W. Estabrook, and M. J. Coon, Arch. Biochem. Biophys., 131, 245 (1969).

(21) O. Hayashi, Ed., "Oxygenases," Academic Press, 24, New York, 1962.

(22) C. Chen and C. Lin, Biochem. Biophys. Acta., 184, 634 (1969). Ibid., 170, 366 (1968).

(23) M. Hamberg and B. Samulsson, Biochem. Biophys. Res. Comm., 21, 531 (1965).

(24) S. Bergstrom, Science, 157, 382 (1967).

(25) E. J. Corey, W. E. Russey, P. R. Ortiz de Montellano, J. Amer. Chem. Soc., 88, 4750 (1966).

(26) P. D. G. Dean, P. R. Ortiz de Montellano, K. Bloch, and E. J. Corey, J. Biol. Chem., 242, 3014 (1967).

(27) H. Nakano, C. Takemoto, H. Sato, and B. Tamaoki, Biochem. Biophys. Acta, 152, 186 (1968).

(28) R. T. Williams, "Detoxication Mechanisms," 2nd Ed., Wiley, New York, 1959.

(29) D. W. Robbins, Ann. Rep. Chem. Soc., 67, 445 (1965).

(30) Hayashi, "Oxygenases," 260.

(31) Saunders et al., "Peroxidase," 126.

(32) Ibid., 184-185.

(33) W. E. Knox, Biochem. Biophys. Acta., 14, 117 (1954).

(34) G. A. Hamilton, in "Advances in Enzymology", Vol. 32, 55, F. F. Nord, Ed., Interscience, New York, 1969.

(35) S. Diner, in "Electronic Aspects of Biochemistry," 239, B. Pullman, Ed., Academic Press, New York, 1964.

(36) H. Mager and W. Berends, Biochem. Biophys. Acta, 118, 440 (1966).

(37) K. Trefzer and S. Fallab, Helv. Chim. Acta, 48, 945 (1965).

(38) H. J. Bright, B. J. B. Wood, and L. L. Ingram, Ann. N.Y. Acad. of Sci., 100, 965 (1963).

(39) M. Ardon, "Oxygen," 90, W. A. Benjamin, New York, 1965.

(40) P. B. Chock, R. B. K. Dewar, J. Halpern, and L. Yoong Wong, J. Amer. Chem. Soc., 91, 82 (1969).

(41) W. C. Schumb, C. N. Satterfield, and R. L. Wentworth, "Hydrogen Peroxide," Reinhold, New York, 1955.

(42) S. Yamamoto, H. Takeda, Y. Maki, O. Hayashi, J. Biol. Chem., 244, 2951 (1969).

(43) A. H. Soloway, J. Theor. Biol., 13, 100 (1966).

(44) W. Ferrical, D. R. Kearns, and P. Radlick, J. Amer. Chem. Soc., 91, 3396 (1969).

(45) E. McKeowan and W. A. Waters, J. Chem. Soc., B, 1040 (1966).

(46) R. H. Steel and L. C. Cusachs, Nature, 213, 800 (1967).

(47) E. Frieden, S. Osaki, and H. Kobayashi, J. Gen. Physiol., 49, 213 (1965).

(48) S. Senoh, H. Kita, and M. Kamimoto, in "Biological and Chemical Aspects of Oxygenases," 378, K. Bloch and O. Hayashi, Ed., Maruzen, Tokyo, 1966.

(49) S. Takemori, H. Yasuda, K. Mihara, K. Suzuki, and M. Katagiri, Biochem. Biophys. Acta, 191, 58 (1969).

(50) H. Kita, Y. Miyake, M. Kamimoto, S. Senoh, and T. Yamano, J. Biochem. (Tokyo), 66, 45 (1969).

(51) R. E. McMahon, H. R. Sullivan, J. C. Craig, and W. L. Pereira, Arch. Biochem. Biophys., 132, 575 (1969).

(52) D. M. Jerina, J. W. Daly, and B. Witkop, J. Amer. Chem. Soc., 89, 5488 (1967).

(53) J. Daly, D. Jerina, and B. Witkop, Arch. Biochem. Biophys., 128, 517 (1968).

(54) D. W. Russell, E. E. Conn, A. Sutter, and H. Grisebach, Biochem. Biophys. Acta, 170, 210 (1968).

(55) G. Guroff, J. Daly, D. Jerina, J. Renson, B. Witkop, and S. Udenfriend, Science, 157, 1524 (1967).

(56) D. Jerina, J. Daly, W. Landis, B. Witkop, and S. Udenfriend, J. Amer. Chem. Soc., 89, 3347 (1967).

(57) G. Guroff, M. Levitt, J. Daly, and S. Udenfriend, Biochem. Biophys. Res. Comm., 25, 253 (1966).

(58) G. Guroff and J. Daly, Arch. Biochem. Biophys., 122, 212 (1967).

(59) H. Theorell, Enzymologia, 10, 250 (1942).

(60) W. A. Schroeder, J. R. Shelton, J. B. Shelton, B. Robberson, G. Apell, Arch. Biochem. Biophys., 131, 653 (1969).

(61) P. Nicholls and G. R. Schonbaum, in "The Enzymes," 2nd Ed., Vol 8, 222, P. Boyer, H. Lardy, and K. Myrback, Ed., Academic Press, New York, 1963.

(62) Saunders et al., "Peroxidase," 129.

(63) A. B. Deisseroth and A. L. Dounce, Arch. Biochem. Biophys., 131, 30 (1969).

(64) Saunders et al., "Peroxidase," 34.

(65) K. G. Paul, in "The Enzymes," 2nd Ed., Vol. 8, 259, P. Boyer, H. Lardy, and K. Myrback, Ed., Academic Press, New York, 1963.

(66) Mahler and Cordes, "Biological Chemistry," 591.

(67) Saunders et al., "Peroxidase," 64.

(68) Ibid., 208.

(69) K. G. Paul, "The Enzymes", 272, 274.

(70) I. Yamazaki, in "Biological and Chemical Aspects of Oxygenases," 433, K. Bloch and O. Hayashi, Ed., Maruzen, Tokyo, 1966.

(71) S. Udenfriend, C. Clark, J. Axelrod, and B. B. Brodie, J. Biol. Chem., 208, 731 (1954).

(72) Hamilton, "Advances in Enzymology," 84.

(73) G. Hamilton, J. Amer. Chem. Soc., 86, 3391 (1964).

(74) M. Viscontini, Fortschritte der Chem. For., 9, 605 (1968).

(75) Hamilton, "Advances in Enzymology," 80.

(76) A. Bobst and M. Vis contini, Helv. Chim. Acta, 49, 884 (1966).

(77) R. O. C. Norman and J. R. Lindsay Smith, in "Oxidases and Related Redox Systems," 131, T. King, H. Mason, and M. Morrison, Ed., Wiley, New York, 1965.

(78) A. W. Wahlefeld, L. Jaenicke, and G. Hein, Ann., 715 52 (1968).

(79) R. M. Acheson and C. M. Hazelwood, Biochem. Biophys. Acta, 42, 49 (1960).

(80) V. Ullrich, D. Hey, and Hj. Staudinger, Biochem. Pharm., 16, 2237 (1967).

(81) M. B. Dearden, C. R. E. Jefcoate, and J. R. Lindsay Smith, in "Oxidation of Organic Compounds-III," 260, Vol 77, "Advances in Chemistry Series," R. Gould, Ed., American Chemical Society, Washington, D.C., 1968.

(82) V. H. Staudinger and V. Ullrich, Z. Naturforsh., 19B., 877 (1964).

(83) R. D. Gray, J. Amer. Chem. Soc., 91, 56 (1969).

(84) G. S. Hammond and C. Wu, in "Oxidation of Organic Compounds-III," 186, Vol 77, "Advances in Chemistry Series," R. Gould, Ed., American Chemical Society, Washington, D.C., 1968.

(85) T. Shiga and A. Isomato, J. Phys. Chem., 73, 1139 (1969).

(86) J. H. Fendler and G. L. Gasowski, J. Org. Chem., 33, 2755 (1968).

(87) C. Jefcoate and R. O. C. Norman, J. Chem. Soc., B, 48 (1968).

(88) W. Armstrong, Can. J. Chem., 47, 3737 (1969).

(89) G. Hamilton, J. Friedman, and P. Campbell, J. Amer. Chem. Soc., 85, 5266 (1966).

(90) G. Hamilton, J. Hanifin, and J. Friedman, J. Amer. Chem. Soc., 85, 5269 (1966).

(91) Hamilton, "Advances in Enzymology," 77.

(92) J. Schubert, V. Sharma, E. White, and L. Bergelson, J. Amer. Chem. Soc., 90, 4476 (1968).

(93) H. Sigel, C. Flierl, and R. Griesser, J. Amer. Chem. Soc., 91, 1061 (1969).

(94) E. Erlenmeyer, C. Flierl, and H. Sigel, J. Amer. Chem. Soc., 91, 1061 (1969).

(95) H. Pfitzner, Angew. Chem., 64, 397 (1952).

(96) H. Pfitzner and H. Baumann, Angew. Chem., 70, 232 (1959).

(97) Z. Yoshida, K. Kazama, and R. Oda, Kogyo Kagaku Z. asshi, 62, 1399 (1959).

(98) H. Zollinger, "Azo and Diazo Chemistry," 210, Interscience, New York, 1961.

(99) R. J. P. Hazard, J. M Cheymal, A. Sekera, P. Crabrier, and J. deAntoni, French Patent, 1,336,070 (1963). [C.A. 60, P2958 (1964)].

(100) Y. Hayakawa, Sci. Repts. Saitama Univ., Ser. A 2, 145 (1957).

(101) M. L. Ernsberger and W. R. Brode, J. Org. Chem., 6, 331 (1941).

(102) W. McPherson and H. J Lucas, J. Am. Chem. Soc., 31, 281 (1909).

(103) J. W. Compton and L. M. Liggett, J. Am. Oil Chemists Soc., 28, 81 (1951).

(104) J. B. Conant and M. F. Pratt, J. Am. Chem. Soc., 48, 2468 (1926).

(105) D. C. Freeman and C. E. White, J. Org. Chem., 21, 379 (1956).

(106) E. C. Hornig, Ed., "Organic Syntheses," Vol. III, 754, Wiley, New York,

(107) Ibid., 586.

(108) L. H. Long and G. F. Freeguard, Nature, 207, 403 (1966).

(109) L. Friedman and A. Sheckter, J. Org. Chem., 25, 877 (1960).

(110) J. A. J. Jarvis, Acta. Cryst., 14, 961 (1961).

(111) H. K. D. Drew and J. K. Landquist, J. Chem. Soc., 292 (1938).

(112) V. S. Sharma, Biochem. Biophys. Acta., 148, 37 (1967).

(113) F. J C. Rossotti and H. Rossotti, "The Determination of Stability Constants," 279, McGraw Hill, New York, 1961.

(114) Mark M. Jones, "Elementary Coordination Chemistry," 282, Prentice-Hall, Englewood Cliffs, N.J., 1964.

(115) W. C. Vosberg and G. R. Cooper, J. Am. Chem. Soc., 63, 437 (1941).

(116) H. M. Irving and H. S. Rossotti, J. Chem. Soc., 3397 (1953) and 2904 (1954).

(117) Rossotti and Rossotti, 83.

(118) S. Craberek, R. C Courtney, and A. E. Martell, J. Amer. Chem. Soc., 74, 5057 (1952).

(119) H. Sigel, R. Greisser, and D. B. McCormick, Arch. Biochem. Biophys., 134, 217 (1969).

(120) H. Sigel, Angew Chem. (International Ed.), 8, 167 (1969).

(121) H. Kido, Sci. Repts. Saitama Univ., 2A, 157 (1957).

(122) C. K. Jorgensen, "Inorganic Complexes," 57, Academic Press, London, 1963.

(123) H. Irving and D. H. Mellor, J. Chem. Soc., 5237 (1962).

(124) H. Irving, J. Chem. Soc., 4056 (1962).

(125) N. E. Good, G. D. Winget, W. Winter, T. N. Connolly, S. Izawa, and R. M. M. Singh, Biochem., 5, 467 (1966).

(126) D. Perrin, J. Chem. Soc., 3189 (1960).

(127) W. S. Schumb, C. N. Satterfield, R. L. Wentworth, "Hydrogen Peroxide", 392, Reinhold, New York, 1955.

(128) V. M. Dzimoko and K. A. Dunaevskaya, Z. Obsh. Khim., 31, 3385 (1961).

(129) H. Zollinger, in "Advances in Physical Organic Chemistry", Academic Press, V. Gold, Ed., Vol. 2, 162-197 (1964).

(130) L. Melander, "Isotope Effects on Reaction Rates", Ronald Press, New York, 1960.

(131) M. E. Kurz, P. Kovacic, A. K. Bose, I. Kogajevsky, J. Amer. Chem. Soc., 90, 1815 (1969).

(132) J. R. Lindsay-Smith and R. O. C. Norman, J. Chem. Soc., 2897 (1963).

(133) C. R. E. Jefcoate and R. O. C. Norman, J. Chem. Soc., B, 48 (1968).

(134) T. Nambara, M. Numazawa, S. Akiyama, Chem. Pharm. Bull., 17, 2394 (1969).

(135) R. D. Chambers, P. Goggin, W. K. R. Musgrave, J. Chem. Soc., 1804 (1959).

(136) C. Buehler and H. Hart, J. Amer. Chem. Soc., 85, 3177 (1963).

(137) G. W. Kirby and L. Ogunkoya, J. Chem. Soc., 6914 (1965).

(138) A. P. Best and C. L. Wilson, J. Chem. Soc., 28 (1938).

(139) V. Gold and D. P. N. Satchell, J. Chem. Soc., 3623 (1955).

(140) L. G. Sillen and A. E. Martell, Eds., "Stability Constants of Metal-Ion Complexes", 723, Chemical Society of London, Sp. Pub. No. 17, 1964.

(141) J. O. Edwards, in "Peroxide Reaction Mechanisms", J. O. Edwards, Ed., 94, Interscience, New York, 1962.

(142) R. O. C. Norman and J. R. Lindsay-Smith in "Oxidases and Related Redox Systems", T. S. King, H. S. Mason, and M. Morrison, Eds., 131, Wiley, New York, 1965.

(143) R. O. C. Norman and R. Taylor, "Electrophilic Substitution in Benzenoid Compounds", 283-301, Elsevier Publishing, New York, 1965.

(144) R. O. C. Norman and G. K. Radda, Proc. Chem. Soc., 138, 1962.

(145) M. Anbar, D. Meyerstein, and P. Netta, J. Phys. Chem., 70, 2660, 1966.

(146) J. O. Edwards, "Peroxide Reaction Mechanisms", 67.

(147) G. E. Dunn, Can. J. Chem., 44, 1261 (1966).

(148) N. A. Vysotskaya and A. I. Brodskii, J. Gen. Chem. USSR, 32, 2241 (1962).

(149) W. A. Waters, "Mechanisms of Oxidation of Organic Compounds", 134, Wiley, New York, 1964.

(150) W. Brackman and E. Havinga, Rec. Trav. Chim., 74, 1107 (1955).

(151) L. Ingraham, "Biochemical Mechanisms", 72, Wiley, New York, 1962.

(152) M. R. Okun, 7th International Cell Conference, Seattle, 1969. [Reported in Chem. Eng. News, Sept. 22, 1969].

(153) (a) C. Liebermann and S. Kostanecki, Chem. Ber., 17, 130 (1884). (b) H. Gilman, Ed., "Organic Syntheses", Vol. I, 49, Wiley, New York, 1941.

(154) G. Schultz and E. Ichenhaeuser, J. prakt. Chem., [2], 77, 100 (1908).

(155) L. J. Sciarini, Arch. Biochem. Bioph., 71, 437 (1957).

(156) E. C. Horning, Ed., "Organic Syntheses", Vol. III, 668, Wiley, New York, 1955.

(157) G. Baddeley, J. Chem. Soc., 330 (1944).

(158) W. McPherson and H. J. Lucas, J. Am. Chem. Soc., 31, 281 (1909).

(159) A. P. Best and C. L. Wilson, J. Chem. Soc., 239 (1946).

(160) W. C. Pierce, E. L. Haenish, and D. T. Sawyer, "Quantitative Analysis", 4th Ed, Wiley, New York, 1958.

(161) N. Rabjon, Ed., "Organic Syntheses", Col. Vol. IV, 319, Wiley, New York, 1963.

(162) C. S. Foote, Accounts of Chemical Research, 1, 104 (1968).

(163) G. Travagli, Atti. Accad. sci. Ferrarg, 27, 3 (1949). [C.A. 45, 7544 e (1951)].

INDEX

Schemes

Tables

Figures

ABSTRACTS OF PROPOSITIONS

Proposition I: Experiments are proposed to test the applicability of the concepts of aromaticity to transition metal chelates. Specifically we propose a nuclear magnetic resonance study of a series of metal carbonyl chelates of bidentate trialkyl phosphines in an effort to find evidence for possible ring currents in the chelated rings.

Proposition II: A study of the decomposition of hydrogen peroxide by catalase and copper chelates in the presence of limonene is proposed to determine whether or not the oxygen molecules are generated in the singlet state.

Proposition III: Experiments are proposed employing C^{14}-labelled catechol and phenol to provide information concerning aspects of tyrosinase function which are presently being debated. The proposed experiments are designed to determine the number of active sites in the enzyme and to shed additional light on the nature of tyrosinase isozymes.

Proposition IV: A number of deuterium labelling experiments are proposed to provide additional evidence concerning the mechanism of the reaction of organolithium reagents with 1-bicyclo-[3.1.0]-hexyl-trimethylammonium bromide. The proposed experiments are

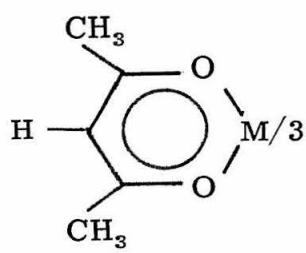
designed to distinguish between several possible elimination mechanisms giving highly strained intermediates and/or substitution by direct displacement.

Proposition V: Almost no information is available concerning the photochemistry of transition metal chelates in which the absorption bands of the organic ligand are irradiated. An investigation of the effect of complexing metal ions on the photochemical rearrangement of azoxybenzene derivatives is proposed to provide some information in a long neglected area.

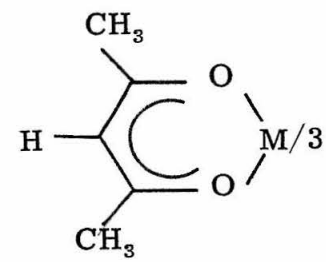
PROPOSITION I

Aromaticity and Ring Currents in Transition Metal Chelates

Since a number of transition metal complexes of acetyl acetone (acac) have been observed to undergo a variety of electrophilic substitution reactions at the unique ring carbon, it has been thought that perhaps some acac complexes have aromatic character (1). The suggestion has been made that the six π electrons of the acetylacetonate ligand overlap with vacant d-orbitals of the transition metal ion to form a molecular orbital in which metal electrons are delocalized over the ligand (2). The X-ray crystal structure of $Co(acac)_2 \cdot 2H_2O$ showed that the chelate ring was essentially planar and that bond distances were symmetrical (3). Such a structure, although consistent with an aromatic formulation (I), is also consistent with a structure in which electron delocalization is essentially confined to the ligand (II).



I

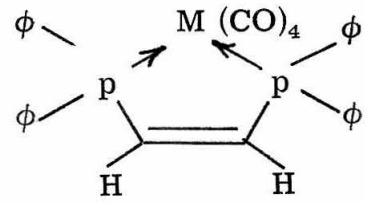


II

By analogy with numerous studies of carbocyclic and heterocyclic systems, one might hope to distinguish between I and II on the basis of the presence or absence of ring currents (4) induced by an external magnetic field and detected in the proton magnetic resonance shifts of the ring hydrogens. The existence of ring currents in systems with $(4n + 2)\ \pi$ electrons has been most dramatically demonstrated by the nmr spectrum of [18] annulene where the chemical shift difference between the shielded (internal) and deshielded (external) protons is 10.7 τ (5). The results of an nmr study of a series of 10 diamagnetic acac complexes made several years ago by Cotton (6) showed that the chemical shifts of the ring protons were, in all cases, essentially the same as the chemical shift for acetylacetone itself (6). These results imply that II is a better representation of the transition metal acetylacetonates, and that acac complexes cannot be classified as aromatic.

The absence of a ring current in acac complexes is not surprising in view of the fact that the acetylacetonate anion is a donor ligand and not likely to accept or delocalize electrons from the metal ion. On theoretical grounds, a complex between a donor-acceptor ligand and a transition metal atom would be a better candidate for the kind of electron delocalization normally associated with aromaticity. Such a ligand is trivalent phosphorous, which is an excellent donor-acceptor ligand because it has an unshared pair of electrons for donation and empty 3d orbitals capable of bonding by overlap with metal, d, electrons (7).

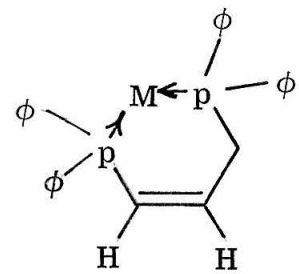
In a recent study of the carbonyl complexes of Cr, Mo, and W with cis-bis-1,2-(di-phenylphosphino)ethylene (III), King and Eggers (8) have given nmr evidence for the existence of a ring-current in the 5-membered chelate ring. King and Eggers suggest that the downfield shift of the olefinic protons (relative to the phenyl protons) in the complex may be due to an induced ring current in the potentially aromatic chelate ring (i.e., the ring has 6 π electrons).



III

We propose that the work of King and Eggers be extended to analogous systems in an effort to determine whether ring currents can be induced in chelate rings with bidentate phosphorous ligands.

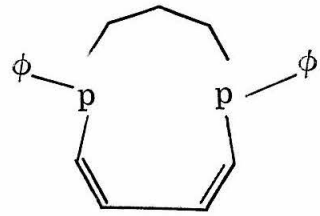
By employing known (8) synthetic methods, it should be possible to use cis 1,3-dichloropropene (9) to prepare complex (IV) which has an additional methylene group in the chelate ring. The methylene group would effectively eliminate the possibility of extended delocalization in the ring and any induced ring currents. If the downfield shift observed by King and Eggers is due to a ring current, then a much smaller shift to lower field should be observed in IV. If a shift of



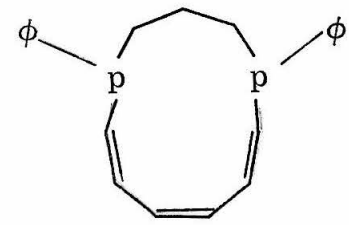
IV

similar magnitude is observed in IV, then an explanation other than ring currents must be sought.

Should the evidence from an nmr study of IV tend to support the ring current explanation, then we propose to investigate the possibility (1), that the concept of aromaticity is generally applicable to transition metal chelates of trivalent phosphorous ligands with metal carbonyls and (2), that Huckel's Rule applies. The proposed study is immediately complicated by the fact that chelates are not likely to form with ring sizes greater than six (10). Thus the diphosobutadiene analog of IV would not be likely to form a stable chelate. We suggest that this problem might be overcome by attempting to prepare ligands which would allow the formation bicyclic chelate rings. Specifically, we propose the use of ligands V and VI, both of which might be expected to form chelates analogous to III. The complexes of V and VI would result in chelate rings with 2 and 4 more π electrons respectively than chelate III. If III is aromatic and Huckel's Rule



V



VI

holds, then a ring current should be observed in VI but not in V. The results of these and similar studies would provide information about the nature of the molecular orbitals which result from π bonding of d orbitals.

References

(1) J. P. Collman, Angew. Chem., 77, 154 (1965).

(2) G. M. Badger, "Aromatic Character and Aromaticity", 36, Cambridge University Press, London, 1969.

(3) G. J. Bullen, Acta Cryst., 12, 703 (1959).

(4) Ibid., G. M. Badger, p. 61.

(5) L. M. Jackman et al., J. Amer. Chem. Soc., 84, 4307 (1962).

(6) R. H. Holm and F. A. Cotton, J. Amer. Chem. Soc., 80, 5658 (1958).

(7) L. Orgel, "An Introduction to Transition Metal Chemistry", 147, Wiley, New York, 1966.

(8) R. B. King and C. A. Eggers, Inorg. Chim Acta, 2, 33 (1968).

(9) W. C. Wolfe, H. M. Doukas, and J. S. Ard, J. Amer. Chem. Soc., 76, 627 (1954).

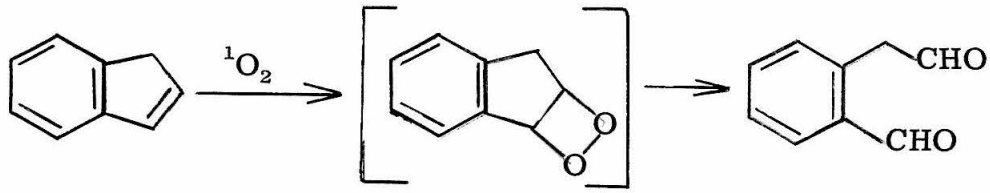
(10) F. P. Dwyer and D. P. Mellor, "Chelating Agents and Metal Chelates", 20, Academic Press, New York, 1964.

(11) K. Issleiband and F. Krech, Chem. Ber., 94, 2656 (1961).

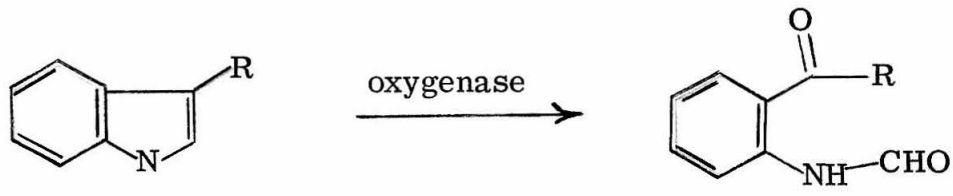
PROPOSITION II

An Investigation into the Possibility that Singlet Oxygen is formed by the Decomposition of Hydrogen Peroxide by Transition Metal Chelates and Catalase

Ground state oxygen is a triplet molecule and as such is normally unreactive with respect to ground state singlet organic compounds. Recently studies have come to light regarding the reactive properties of excited singlet oxygen (1). The suggestion has been made by a number of authors that perhaps the "activated" oxygen in oxygenase reactions is similar to singlet oxygen (1-4). The proposal that singlet oxygen is involved in biological oxygenations is especially appealing in the case of those dioxygenase reactions which can be envisioned to derive from cyclic peroxide intermediates. We note, for example, the strong formal analogy between the results of a recent study (5) of the photooxygenation of indene (equation 1) and the conversion of tryptophan to formylkynurenine (6) by tryptophan oxygenase (equation 2). Proposals regarding the biological source of the singlet oxygen have not been forthcoming.

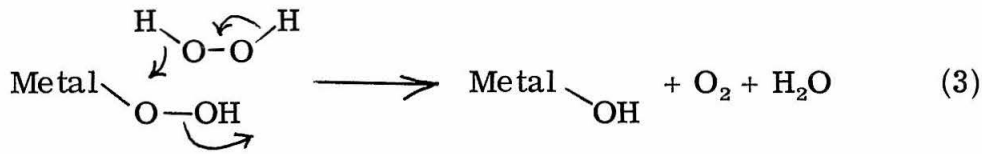


(1)



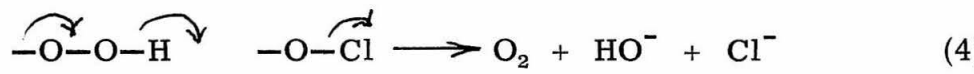
(2)

Recent studies of the decomposition of hydrogen peroxide by catalase (7) and cupric ion chelates (8) suggests that the predominant mode of decomposition is heterolytic rather than homoltyic (equation 3).



(3)

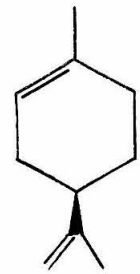
We are struck by the similarity of equation 3 and the proposed mechanism for the decomposition of hydrogen peroxide with alkaline hypochlorite (9) (equation 4).



(4)

The latter reaction has been shown to give exactly the same reaction products with olefins as singlet oxygen produced photochemically (1).

We propose that a study be made to determine whether the oxygen liberated by catalase and various metal chelates is singlet oxygen. In particular, we propose a study of the decomposition of hydrogen peroxide by catalase and catalase models in the presence of limonene, whose reaction products with singlet oxygen have been well characterized (1). The identification of singlet oxygen in these



limonene

systems would be helpful in determining the direction of future studies of the activation of oxygen by enzymatic systems.

References

(1) A. H. Soloway, J. Theor. Biol., 13, 100 (1966).

(2) C. S. Foote, Accounts of Chemical Research, 1, 104 (1968).

(3) R. H. Steel and L. C. Cusacks, Nature, 213, 800 (1967).

(4) C. Chen and C. Lin, Biochem. Biophys. Acta, 184, 634 (1969). Ibid., 170, 366 (1968).

(5) W. Fenical, D. R. Kearns, P. Radlick, J. Am. Chem. Soc., 91, 3396 (1969).

(6) H. Maeno and P. Feigelson, Biochem. Biophys. Res. Comm., 21, 297 (1965).

(7) A. B. Deisseroth and A. L. Dounce, Arch. Biochem. Biophys., 131, 30 (1969).

(8) H. Sigel, C. Flierl, and R. Griessen, J. Amer. Chem. Soc, 91, 1061 (1969).

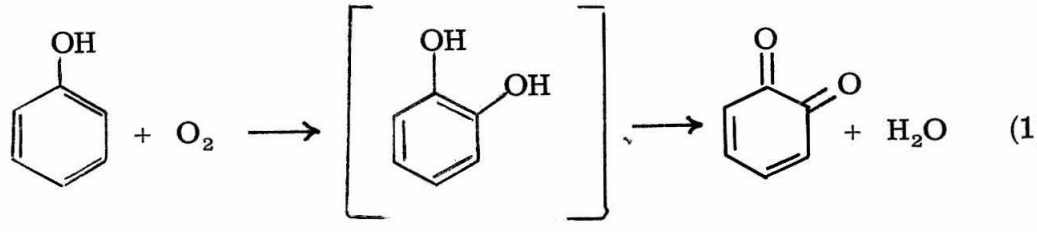
(9) E. McKeown and W. A. Water, J. Chem. Soc. B, 1040 (1966).

PROPOSITION III

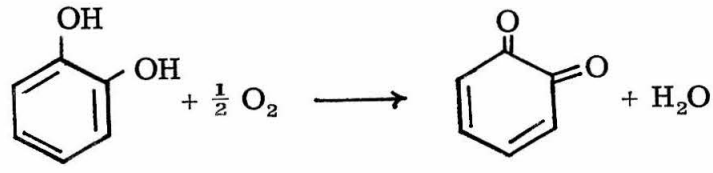
Multiple Forms of Tyrosinase

Tyrosinase (o-diphenol oxidoreductase E.C. 1.10.3.1) is an enzyme which is widely distributed in both plants and animals. In animals tyrosinase plays a role in the formation of melanin pigment, and much of the interest in the animal enzyme has evolved due to extensive studies of the tyrosinase system by researchers in genetics, cancer, and developmental biology. Highly purified forms of tyrosinase are more readily available from plants however. The plant enzyme is commonly known as phenolase or phenol oxidase, and it is known to participate in the formation of colored materials as, for example, in the browning of apples (1). The discussion which follows deals specifically with the plant enzyme, but the general features of the plant system are found in the animal enzyme as well.

Tyrosinase contains copper and is but one of a large number of metalloenzymes now known to be directly involved with oxygen metabolism. Tyrosinase has been found to have two distinct functions, both of which result in the reduction of atmospheric oxygen. The two functions are illustrated in equations 1 and 2.



(1)



(2)

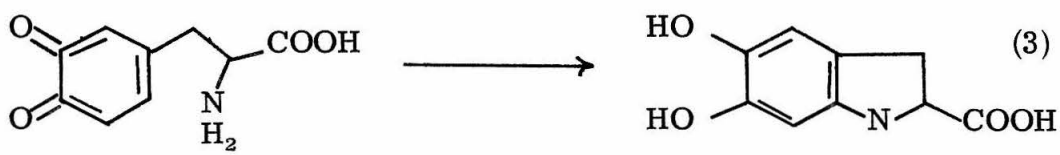
For historical reasons, reactions 1 and 2 have been called the cresolase and the catecholase activities respectively (1). Various mechanisms have been proposed to account for observations regarding the dual activity of tyrosinase, and lively debate over their relative merits continues unabated (2). One proposal suggests that only the catecholase function is enzymatic and that ortho hydroxylation of the monophenol occurs by way of the o-dihydroxy phenol present in the system (1a). Kertesz still holds to this notion even though, as Mason has pointed out (2), the nonenzymatic proposal is neither consistent with a number of experiments nor with modern notions about biological processes. If we limit consideration to mechanisms in which both reactions are enzymatic, then the mechanistic debate is limited to the question of whether the enzyme has one active site or two. Since the diphenol product of cresolase activity cannot be isolated (its oxidation to quinone is too rapid) there is also a question as to whether the presumed intermediate of monophenol oxidation (see equation 1) is ever freed from the enzyme surface (3, 4).

Directly related to the question of tyrosinase active sites are the observations that the cresolase/catecholase activity ratio varies considerably from one biological source to another and between different

enzyme preparations from the same source (5). One possible explanation for these results comes from the discovery of multiple forms of tyrosinase in highly purified preparations from mushroom (6) and Neurospora (7). More recent studies have confirmed the existence of four distinct tyrosinase isozymes (8) and the early results (6) which showed that the amino acid content of the four forms was similar. The bulk of the evidence supports the idea that the observed isozymes are tetrameric aggregates (MW $\cong$ 125, 000) of polypeptide units with MW $\cong$ 30, 000 (8). Furthermore, analysis of the copper content of the tetramer implies that each monomer unit has one copper atom (6, 7), therefore, if one assumes that a copper atom is required at the active site, it is reasonable to suggest that tyrosinase is composed of two unique monomers which have distinctly different catecholase/ cresolase activities and that the tyrosinase variations result from random aggregation of these subunits. Several papers (6, 8, 9) have been written in support of this idea, however, the exact nature of the isozymes is still unclear, and the possibility exists that at least some of the forms are artifacts of the isolation procedure (8).

One of the most unusual aspects of tyrosinase behavior is the observation that the enzyme is irreversibly inactivated during the course of its reaction with many phenols and cresols (10). This has been found to be a general phenomenon for tyrosinase which, in addition to bein g inactivated, becomes incorporated into the pigmented structural protein coat in insects (11).

Several years ago, Ingraham and Wood (12) gave evidence for the formation of a compound between C^{14} labelled phenol substrate and mushroom tyrosinase. The nature of the reaction inactivation process suggests that the compound forms at the active site (13), and a very likely possibility is that the highly reactive quinones formed during the catechol oxidation inactivate the enzyme by reacting with a protein moiety in a manner similar to the internal Michael Addition observed during the oxidation of tyrosine, equation 3 (12, 14).



(3)

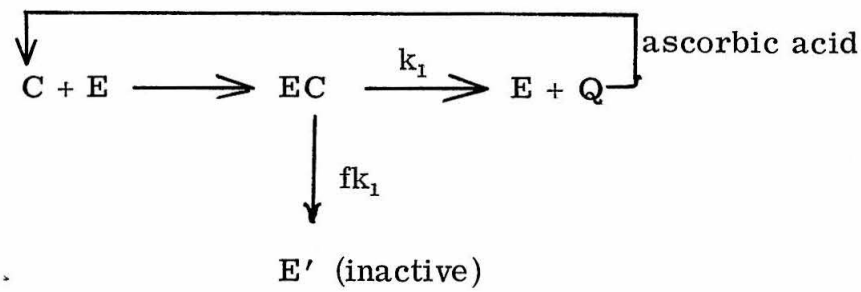
Experiments are proposed using C^{14}-labelled phenol or catechol in an effort to probe further the mechanism of tyrosinase action and the nature of the observed isozymic forms.

(1) Experiment with C^{14}-labelled phenol and unlabelled catechol to determine whether o-dihydroxyphenol is released from the enzyme surface: An experiment is proposed in which C^{14}-labelled phenol of high specific activity is incubated with tyrosinase and an excess of unlabelled catechol. After an appropriate length of time, the reaction is quenched, and the catechol is isolated and examined for radioactivity. The absence of radioactivity in the isolated catechol would prove that the conversion of phenol to quinone (equation 1) proceeds without the release of the intermediate from the enzyme. If the phenol is converted directly to quinone, then it should be possible to

resolve the question of of the number of active sites by the following experiment.

(2) Separate experiments with C^{14} phenol and C^{14} catechol to determine the number of active sites: Incubate two identical preparations of mushroom tyrosinase, (A) with C^{14} phenol and C^{12} catechol, and (B) with C^{12} phenol and C^{14} catechol. Allow sufficient time for reaction inactivation to take place. Isolate the inactive enzymes, and examine the tryptic digests of both by two-dimensional chromatography. The formation of a metabolite-enzyme compound would be demonstrated by the identification of an altered peptide containing radioactive label. Two unique active sites should give different altered peptides with radioactive label when the two digests are compared. Equivalent or nearly equivalent sites would be expected to give comparable incorporation patterns.

(3) Inactivation rate experiments with C^{14} catechol to determine the nature of tyrosinase isozymes: The available evidence suggests that tyrosinase inactivation can be described by equation 4. Under normal assay conditions ascorbic acid is present which immediately regenerates catechol (5), so that $[EC]$ may be assumed to remain constant. Under these conditions the rate constant for the formation of quinone, k_1, is proportional to the rate constant for inactivation, fk_1. We propose to measure the rate of inactivation by observing the rate of incorporation of C^{14} labelled catechol into enzyme. We suggest that this labelling technique be used to determine the catecholase activity of tyrosinase isozymes in the crude enzyme preparation. By



(4)

where:

C = catechol	EC = enzyme-catechol complex
Q = quinone	E′ = enzyme-quinone compound
E = enzyme	f = fraction of C to Q conversions which give inactive enzyme

this method the complications inherent in studying enzyme activities in preparations which have been subjected to numerous manipulations during isolation are avoided. The specific experiment proposed is as follows:

To incubation tubes containing the standard assay mixture and C^{14} catechol add identical quantities of crude mushroom tyrosinase. At appropriate times during the incubation add a 100-fold excess of unlabelled catechol to essentially quench the labelling process. Allow the enzyme to inactivate (i.e., form the enzyme-catechol compound) and proceed to isolate inactivated isozymes by adapting methods known to separate the various forms. Dialyze and wash the isolated material

to remove any unbound radioactive label. Measure the quantity of incorporated label per mg protein and determine the relative values of fk_1 for each form.

Since the relative values of fk_1 are proportional to the relative activities of the protein in the original, crude mixture, we suggest that if the reported differences in activity are real, the values of fk_1 will vary from one isozyme to the other. If, on the other hand, the rates of incorporation in each of the forms is the same, we must conclude that there is but a single enzyme in the original mixture, and that the observed variations are really artifacts of the isolation procedure.

References

(1) For reviews see: (a) D. Kertesz and R. Zito, in "Oxygenases", O. Hayashi, Ed., Academic Press, New York, 1962. (b) H. S. Mason, Ann. Revs. of Biochem., 34, 595 (1965).

(2) H. S. Mason, in "Biological and Chemical Aspects of the Oxygenases", 287, K. Bloch and O. Hayaishi, Eds., Maruzen Co. Ltd., Tokyo, 1966.

(3) F. E. Aerts and R. E. Vercauteren, Enzymologica, 28, 1, (1964).

(4) S. Osaki, Arch. Biochem. Biophys., 100, 378 (1963).

(5) C. R. Dawson and R. J. Magee, in "Methods in Enzymology", 817, S. P. Colowick and N. O. Kaplan, Eds., Vol. 2, Academic Press, New York, 1955.

(6) S. Bouchillous, D. McMahill, and H. S. Mason, J. Biol. Chem., 238, 1699 (1963).

(7) M. Fling, N. H. Horowitz, and S. F. Heinemann, J. Biol. Chem., 238, 2045 (1963).

(8) R. L. Jolley, R. M. Nelson, and D. A. Robb, J. Biol. Chem., 244, 3251 (1969).

(9) D. B. Hoffman, Ph.D. Thesis, Columbia Univ., 1967. [From Dissertation Abstracts, 23B, 536 (1967)].

(10) J. M. Nelson and C. R. Dawson, Adv. in Enzymol., 4, 99 (1944).

(11) H. K. Mitchell, U. M. Weber, and G. Schaar, Genetics, 57, 357 (1967).

(12) B. J. B. Wood and L. L. Ingraham, Nature, 205, 291 (1965).

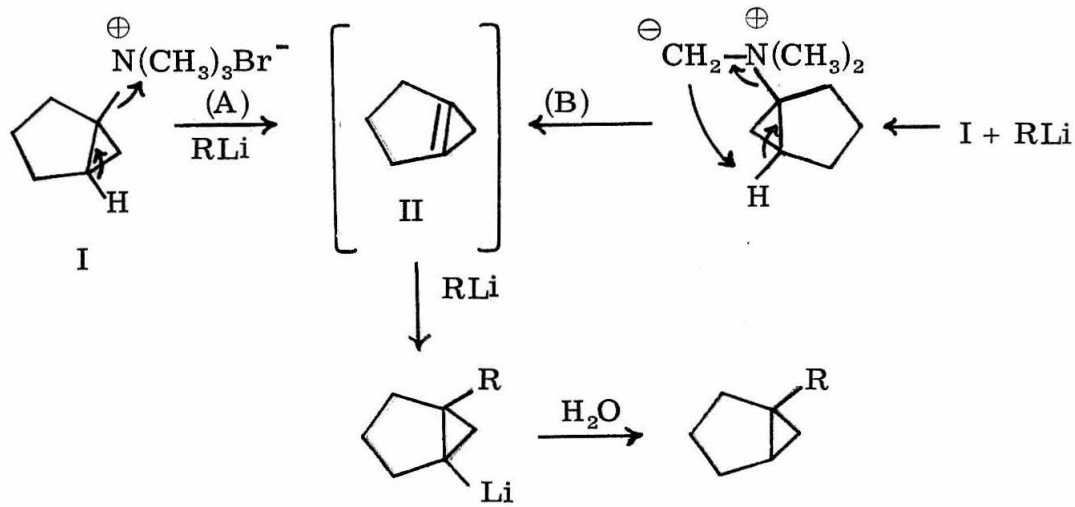
(13) B. J. Ludwig and J. M. Nelson, J. Amer. Chem. Soc., 61, 2601 (1939).

(14) H. S. Mason, Adv. in Enzymol., 16, 105 (1955).

PROPOSITION IV

An Experiment to Test the Proposed Transient Existence of Bicyclo-[3.1.0]-Hexene-$\Delta^{1,5}$.

Several years ago, Blanchard, Simmons, and Taylor (1) presented the results of a study of the reaction of methyl and phenyl lithium with 1-bicyclo-[3.1.0]-hexyltrimethylammonium bromide (I). Two possible reaction paths were discussed.



III R = CH_3

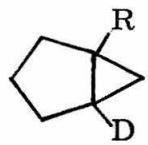
IV R = Ph

Reaction paths A and B both implied the transient existence of a highly strained cyclopropene intermediate, bicyclo-[3.1.0]-hexene-$\Delta^{1,5}$ (II). The reaction proceeds quite cleanly with methyllithium; and the yield of III, isolated by flash distillation, was reported to be 60% based on methyllithium. Of the two reaction paths given, Blanchard, Simmons, and Taylor (1) favored path B over path A in view of the known reactivity of quaternary methyl groups (2). No other reaction paths were discussed with the exception of the possible direct displacement of trimethylamine by alkyllithium. Direct displacement was considered highly unlikely, however, in view of the steric requirements of the displacement of bulky trimethylamine.

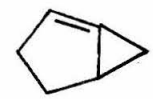
To our knowledge, the suggested route to III and IV via II has gone unchalleneged and untested. We feel that II is unusual enough, even as a transient intermediate, to merit further study; in addition, we remain skeptical about the proposed elimination scheme and we suggest that other possibilities be investigated. Specific experiments are proposed below.

(1) Work up the reaction mixture with D_2O: In studying the reaction of I with organo lithium reagents, Blanchard, Simmons, and Taylor (1) worked up their reaction mixtures with water. We propose that deuterium oxide be used in the work up and that the products be analyzed by nmr and mass spectroscopy in an effort to determine the extent and location of any deuterium in the final product. The presence or absence of deuterium in the final product would help to distinguish between the elimination and direct displacement

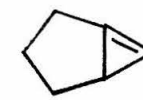
mechanisms. If deuterium is found in the product, its position would shed light on the direction of elimination. Blanchard, Simmons, and Taylor have considered only 1-5 elimination which would require the formation of deuterio derivative V. We wish to suggest that there is a likelihood that elimination (if it occurs) proceeds 1-2 or (less likely) 1-6 to give intermediate VI and/or VII.



V

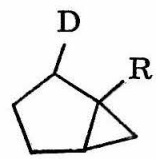


VI

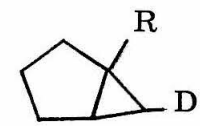


VII

If VI or VII were intermediates, one might a priori expect addition products in which the methyl and phenyl groups are at positions other than the bridgehead. Although this is probably the reason why elimination via VI and/or VII was not considered, we suggest that the addition of methyl or phenyl lithium to VI or VII might be expected to give III and IV for two reasons: (A) The bridgehead carbon is sterically accessable to the organolithium reagent. Organolithium reagents have been shown to have very pronounced steric requirements in addition to activated olefins (3); and (B) the bond angles in VI and VII would be expected to result in decreased electron density at the bridgehead carbon favoring attack by the nucleophilic carbon of the organolithium reagent. With these considerations, we propose that evidence for deuterated derivatives VIII and IX be examined.

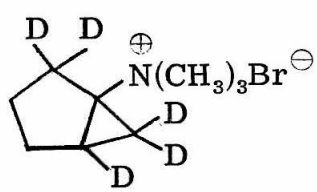


VIII

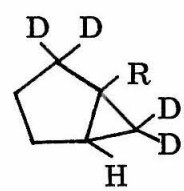


IX

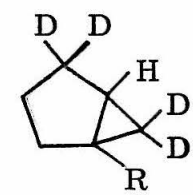
(2) Study the elimination reaction with pentadeuterio derivative, X: Further information regarding the elimination reaction would be gained by a study of the interaction of pentadeuterio derivative, X with phenyllithium. The direction of elimination could be studied by observing the deuterium substitution pattern in the product. Compound X itself should be fairly easy to prepare beginning with 2, 2, 5, 5-tetradeuterocyclopentanone (4) and dideuterochloromethyl iodide (5). The general preparative methods have been discussed (5).



X



XI



XII

The intermediacy of II as a discrete entity would require the formation of XI and XII as products in equal amounts. Elimination via XIII or IX would be expected to give rather different products.

An experiment with X would also be of value in distinguishing between elimination by paths A and B since the trimethyl amine

liberated in the reaction must contain deuterium if path B is operative (6).

(3) Attempt to trap the intermediate olefin with cyclopentadiene: Finally, we propose that the reaction be studied in the presence of cyclopentadiene which would be expected to trap cyclopropene (II) as a Diels-Alder adduct. This procedure has been used to trap cyclopropenes generated in the presence of organolithium reagents (7).

We suggest that, taken together, the results of these experiments will provide good evidence about the mechanism of the stated reaction and the likelihood of the proposed transient existence of bicyclohexene, II.

References

(1) E. P. Blanchard, H. E. Simmons, and J. S. Taylor, J. Org. Chem., 30, 4321 (1965).

(2) G. Wittig and M H. Wetterling, Ann., 557, 193 (1947).

(3) B. A. Borek and H. Waelsch, J. Amer. Chem. Soc., 75, 1771 (1953).

(4) A. C. Cope, J. Lazar, N. A. LeBel, and D. L. Ross, J. Org. Chem., 27, 2627 (1962).

(5) E. P. Blanchard, J. Org. Chem., 28, 1397 (1963).

(6) A. C. Cope, N. A. LeBel, D. T. Moore, W. R. Moore, J. Amer. Chem. Soc., 83, 3861 (1961).

(7) R. M. Magid and J. G. Welch, J. Amer. Chem. Soc., 90, 5211 (1968).

PROPOSITION V

A Study of the Effect of Complexing Metal Ions on the Photochemical Rearrangement of Azoxybenzene Derivatives

The last ten years has seen the coming of age of mechanistic investigations aimed at understanding the behavior of organic molecules in photochemically excited states. Much of the progress in this field has come as a result of newly developed techniques and fruitful cooperation between experimental and theoretical chemists. A prime example of the value of such cooperation is the recent exposition of the Woodward-Hoffman Rules for electrocyclic reactions (1). The last few decades have also seen a rebirth of interest in the chemistry of coordination compounds. To a considerable degree, interest and growth in coordination chemistry has come about as a result of the stimulus provided by the development first of ligand-field theory and later of molecular orbital theory.

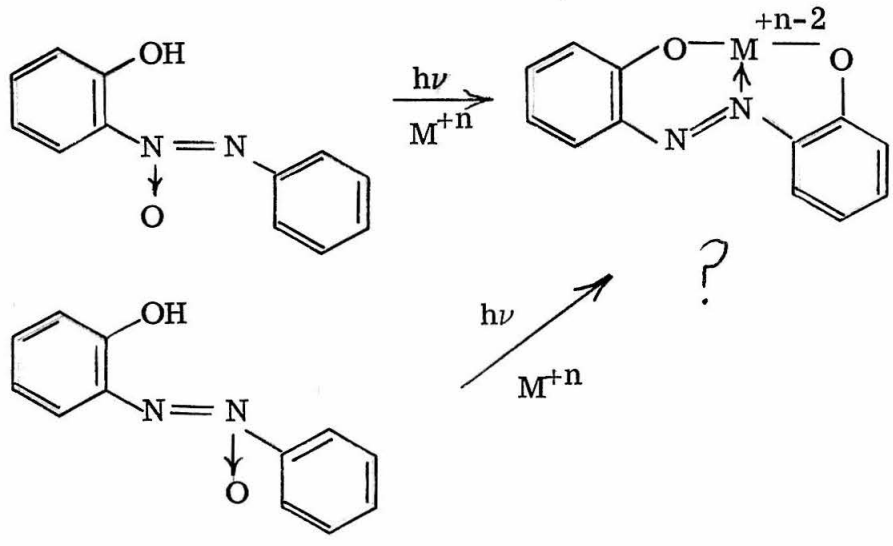
Up to the present time much of the experimental work in coordination chemistry has centered around the measurement of spectroscopic and magnetic properties and studies of elementary thermal processes, ligand exchange and oxidation-reduction for example. Studies of the reactions of photochemically excited states, on the other hand, have been limited. Recent reviews (2, 3) of the subject of inorganic photochemistry have indicated that there are a great many difficulties in the field, and that for the *most part*

quantitative studies have been confined to octahedral complexes, particularly those of Cr(III) and Co(III) whose energy levels and ground state chemistry are fairly well understood (2). Even so, the Cr(III) systems, for example, are quite complex and the results are subject to many interpretations. This problem is well illustrated in a recent paper on the photo-aquation of $Cr(NH_3)_6^{3+}$ by Adamson (1).

Wehry (3) has pointed out the virtual absence of information regarding the significance of intermolecular energy transfer processes in simple coordination compounds. This plus the fact that very little seems to be known about the interaction of complexing metals with excited ligands means that our ability to understand the processes basic to photosynthesis are severely limited.

In view of the virtual absence of photochemical studies in this area, we propose a study of the influence of complexing ions [i.e., Cr(III), Co(III), Cu(II)] on the photochemical rearrangement of azoxybenzene derivatives (5). In particular, we suggest a study of the effect of metal ions on the rearrangement of 2-hydroxyazoxybenzene derivatives which might be expected to give stable 2, 2′-dihydroxyazobenzene complexes as a result of the rearrangement.

The nature of the complexes of 2-hydroxyazoxybenzenes is unknown, however, it is known that metal ions form stable complexes with N-oxides (6). We propose to determine the nature of possible metal complexes with the azoxybenzene derivatives and to investigate quantitative aspects of the reaction at different wavelengths, if indeed the reaction occurs at all. It is conceivable that the metal ion will



inhibit the reaction completely by providing a mode of deexcitation not open to the free ligand. We suggest that this would be interesting in itself and that potential differences between different metal ions would be even more interesting. We also propose a study of the effect of various photosensitizers on the rearrangement in the presence and absence of complexing ions.

References

(1) (a) R. B. Woodward and R. Hoffmann, J. Amer. Chem. Soc., 87, 395 (1965). (b) J. J. Vollmer and K. L. Servis, J. Chem. Ed., 45, 214 (1968).

(2) D. Valentine, Adv. in Photochem., 6, 123 (1968).

(3) E. L. Wehry, Quart. Revs. (London), 21, 213 (1967).

(4) A. W. Adamson, J. Phys. Chem., 71, 799 (1967).

(5) M. M. Shemyakin, T. E. Agadyhanyan, V. I. Maimind, Proc. Acad. Sci. USSR, 135, 1295 (1960).

(6) J. V. Quagliano, J. Amer. Chem. Soc., 83, 3770 (1961).