PURIFICATION, PHYSICOCHEMICAL PROPERTIES AND KINETIC MECHANISM OF A STEROID DIOXYGENASE

by HSIN-HSIUNG TAI

(Under the supervision of Professor Charles J. Sih)

3,4-Dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione-4,5-dioxygenase was purified to a homogeneous state from Nocardia restrictus. The molecular weight of the enzyme was estimated to be around 280,000 from sedimentation equilibrium and sedimentation velocity-diffusion data. The physicochemical properties described include: sedimentation coefficient ($S^{o}_{20,w}$ = 10.28 S); diffusion coefficient ($D^{o}_{20,w}$ = 3.42 x 10^{-7} $cm^2 sec^{-1}$); Stokes' radius (a = 62.5 Å); frictional ratio (f/f_o = 1.47); and apparent partial specific volume ($\bar{v}$ = 0.733 ml g^{-1}). The enzyme has an ultraviolet absorption maximum at 280 mμ, and the specific absorbance ($E^{0.1\%}_{1\ cm}$) is 0.93. Quantitative analytical data show 1.13 g-atom of ferrous iron and 9 half-cystinyl residues per mole of the enzyme.

Metal-chelating agents, including o-phenanthroline, 8-hydroxyquinoline and α,α'-dipyridyl, inhibited the enzyme, and the inhibition was noncompetitive with respect to both organic substrate and molecular oxygen. Sulfhydryl inhibitors also inhibited the enzyme at the concentration of 1 mM.

Substrate specificity studies indicated that substitution of an alkyl group at a position adjacent to the dihydroxyl group was required for maximal activity. Introduction of a bulky alkyl group at other positions rendered the substrate less reactive.

Kinetic studies with steroid dioxygenase gave intersecting initial velocity plots that conform to a sequential mechanism. Linear noncompetitive product inhibition patterns were observed with respect to either substrates indicating the formation of a dead-end complex. A structural analog of the organic substrate inhibited the enzyme competitively with respect to the organic substrate as expected, but inhibited the enzyme uncompetitively with respect to molecular oxygen. These results are consistent with an ordered Bi-Uni mechanism where molecular oxygen is added first, followed by organic substrate, and the product is then released. Detailed analysis of the kinetic data suggest the existence of a dead-end enzyme-oxygen-product complex.

APPROVED _____
Professor of Pharmaceutical Biochemistry

DATE March 5, 1970

PURIFICATION, PHYSICOCHEMICAL PROPERTIES AND KINETIC MECHANISM OF A STEROID DIOXYGENASE

by

HSIN-HSIUNG TAI

A thesis submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

(Pharmaceutical Biochemistry)

at the

UNIVERSITY OF WISCONSIN

1970

ACKNOWLEDGEMENTS

The author wishes to express his deep appreciation and gratitude to Professor Charles J. Sih for his advice, guidance and financial support during the course of this investigation.

The author also is indebted to Professor W. W. Cleland for helpful discussions and the use of kinetic programs.

Thanks are also due to Dr. I. Y. Huang for his help on amino acid composition analysis.

Finally, appreciation is expressed to my wife, Chen, whose encouragement and understanding has made the attainment of this goal possible.

TABLE OF CONTENTS

INTRODUCTION

Among the most important biological conversions in the initial stages of metabolism of many substrates are those in which molecular oxygen is incorporated into substrate molecules. The enzymes that participate in these oxygen-fixing reactions are generally termed oxygenases (1). For convenience, oxygenases can be divided into two groups. Those which catalyze the incorporation of a single atom of molecular oxygen into substrates are termed monooxygenases (2), while those catalyzing the incorporation of both atoms of molecular oxygen into substrates are referred to as dioxygenases (2).

$$S + O_2 \longrightarrow SO_2$$

Dioxygenases

$$S + O_2 + H_2A \longrightarrow SO + H_2O + A$$

Monooxygenases

Here S is the substrate and H_2A is the reductant.

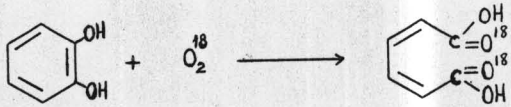
Monooxygenases are involved in the metabolism of a wide variety of substrates whereas dioxygenases are primarily concerned with the transformations of aromatic amino acids and phenolic compounds (3). More recently, several mammalian dioxygenases have been shown to participate in the biosynthesis of hormones (4),

vitamins (5,6) and other physiologically active substances (7). However, the concentration of these enzymes in tissues is low and, therefore, it is usually difficult to obtain mammalian dioxygenases in large quantities. On the other hand, dioxygenases in microorganisms are often inducible and thus serve as a more suitable starting material for enzyme studies. In fact, several microbial dioxygenases have been highly purified, and some of them have even been crystallized. Although the general properties and the reaction mechanisms of dioxygenases have been studied in some detail, the kinetic mechanism—namely the order of addition of organic substrate and oxygen to the enzyme, has been a controversial issue.

This thesis is divided into two parts: Part one deals with the purification and physicochemical properties of a steroid dioxygenase; Part two is concerned with an analysis of the kinetic mechanism of this enzyme by means of steady state kinetic studies.

LITERATURE

During the course of investigation on the metabolism of various aromatic compounds, especially aromatic amino acids, a number of enzymes have been described which catalyze oxidative cleavage of the rings of these aromatic compounds. Hayaishi and collaborators first demonstrated the novel characteristic of such enzymic reactions through the use of the heavy oxygen isotope, O^{18} (2). They showed that the cleavage of catechol by the enzyme pyrocatechase involved the utilization of molecular oxygen and not the oxygen of water. Subsequently, it was established that both oxygen atoms in the product were derived from the same oxygen molecule (8).



Although dioxygenases are also capable of catalyzing the cyclization of unsaturated fatty acids (4) or the cleavage of alicyclic rings, the majority of dioxygenases thus far discovered are concerned with the fission of aromatic rings or "phenolytic" (3).

Phenolytic dioxygenases may be divided into three subclasses according to their substrate structures:

I. Catechol Dioxygenases

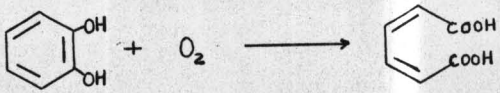
When o-dihydroxyphenyl compounds are cleaved by the action of dioxygenases, two modes of ring fission have been demonstrated: intradiol or extradiol types (9,10). Iron has been found to be a sole cofactor in these dioxygenases, and its role in these reactions has been extensively studied (9-13). Regardless of the valency of the iron, it was suggested that iron plays a catalytic role by interacting with substrates (10, 11, 13, 14).

A. Intradiol type (Pyrocatechase type)

This type of enzyme catalyzes ring fission between two adjacent carbon atoms bearing hydroxyl groups. Two enzymes of this type have been extensively studied.

1. Pyrocatechase (Catechol 1,2-dioxygenase)

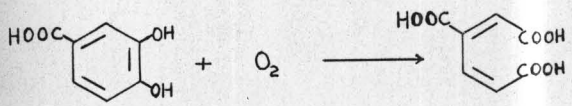
Pyrocatechase was first isolated from Pseudomonas in 1950 and was shown to produce cis,cis-muconic acid from catechol (15).



Purification of this enzyme has been obtained by several groups from different sources (16-20). Pyrocatechase was found to contain 2 g-atoms of iron per mole of the enzyme, but the molecular weight (M.W.) of the enzyme varied from different sources. For example, 95,000 from P. arvilla (19), 100,000 from P. fluorescens (20) and 78,000 from B. fuscum (17). The enzyme shows a distinct red color with a broad absorption peak at 440 mμ and a sharp electron spin resonance (ESR) signal at g = 4.2, which were interpreted to arise from ferric iron bound to the enzyme (10,11,19, 21). Titration of the enzyme with the substrate, catechol under anaerobic conditions, suggested that 2 moles of catechol combined with the enzyme (14). A reaction mechanism has been proposed by Hayaishi (2,10) involving a four-membered ring peroxide intermediate and subsequent shifting of electrons results in the ring fission product. Km's for catechol and oxygen were found to be 5×10^{-7} M and 2×10^{-5} M, respectively (18).

2. Protocatechuate 3,4-dioxygenase

Protocatechuate 3,4-dioxygenase, first studied by Stanier and Ingraham in 1954 (22), catalyzes a similar oxygenation reaction with protocatechuate to form β-carboxy-cis,cis-muconic acid.



A number of investigators have described various methods of purifying the enzyme from Pseudomonas (22,23), Nocardia (24) and Neurospora (25). Crystallization of this enzyme has been achieved by Hayaishi's group from P. aeruginosa (26,27).

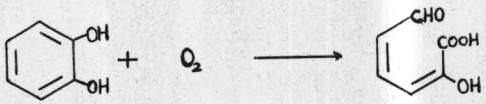
Protocatechuate 3,4-dioxygenase resembles pyrocatechase in many aspects such as the visible spectrum, the ESR spectrum and the valence state of iron (14,26,28). The enzyme from P. putida has a M.W. around 420,000 (23), while the crystalline one from P. aeruginosa has a M.W. estimated to be at 700,000 (27). The crystalline enzyme was found to contain 8 g-atoms of iron per mole of the enzyme. Both electron microscopy and dissociation in dilute alkali indicated 8 subunits (9,14). The enzyme exhibited high specificity with respect to protocatechuate, while catechol or its alkyl derivatives were slightly attacked (27). Km's for protocatechuate and O_2 were found to be 3.0×10^{-5} M and 4.3×10^{-5} M (27).

B. Extradiol type (Metapyrocatechase type)

This type of enzyme catalyzes ring fission of catechol or its derivatives between a hydroxylated carbon and an adjacent carbon atom bearing hydrogen or other groups.

1. Metapyrocatechase (Catechol 2,3-dioxygenase)

This type of enzyme was first described by Dagley and Stopher in 1959 (29) and was found to catalyze the conversion of catechol to α-hydroxymuconic-ϵ-semialdehyde.



The enzyme from P. arvilla was found to be extremely unstable in the presence of air by Hayaishi's group and attempts to purify it appeared to be hopeless (30). However, they later reported that low concentrations of organic solvents such as 10% acetone could protect the enzyme from inactivation. This key observation has led to the crystallization of this

dioxygenase (31,32). Subsequently purification from other sources was also reported (33). The crystalline enzyme has a M.W. of 140,000 (32) and contains 3 g-atoms of iron per mole (9,10). Dissociation in dilute alkali, N-terminal determination and titration with substrate under anaerobic conditions all indicated that the enzyme consisted of 2 to 3 subunits (9,14). All the iron in the native enzyme was shown to be in the divalent state by ESR and colorimetric determinations (10,34). Rapid inactivation of the enzyme activity appeared to be due to oxidation of ferrous iron to the ferric form, which could be reactivated by incubation of the enzyme with ferrous iron and a reducing agent under anaerobic conditions (34). Inactivation could be prevented in the presence of the substrate, catechol (34). The enzyme contains 12 half-cystine residues of which 4 were readily titratable by p-hydroxymercuribenzoate (PHMB) with no concommitant loss of activity. Chelating agents and nitrogenous bases protected the enzyme not only against oxidizing agents, but also against inhibition by sulfhydryl reagents. In addition, these compounds acted as competitive inhibitors with respect to the organic substrate (18,34). All these findings suggested that iron is not only involved in the activation of molecular oxygen, but also in the binding of the

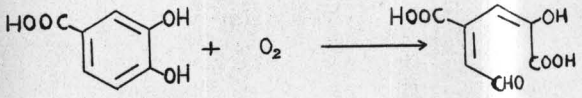
substrate, catechol (14,26,34). This hypothesis gained further support from ESR studies (10,26). Km's for both catechol and oxygen were estimated to be 3×10^{-6} M and 7×10^{-6} M, respectively (18).

As to the reaction mechanism, Hayaishi proposed that oxygen is activated at the ferrous site to form the perferryl iron complex, (Fe^{III}-O_2) which reacts with catechol to form a peroxide intermediate; subsequent intramolecular rearrangements lead to the formation of the final product (2,10,26). This sequence of interactions between substrates and enzyme was recently questioned by the same group (14,28), since organic substrate can combine with the enzyme in the presence or absence of oxygen, and oxygenated intermediate of the enzyme has never been detected as a discernible entity. As with the case of tryptophan pyrrolase (34), this enzyme appears to combine with tryptophan first increasing the reactivity of heme, and it then reacts with oxygen. On the basis of these observations, Hayaishi forwarded a general mechanism for dioxygenases whereby substrate combines with the enzyme first followed by molecular oxygen (14,28).

2. Protocatechuate 4,5-dioxygenase

Dagley and Patel (35) described the oxidation of protocatechuate by an iron dependent enzyme. The

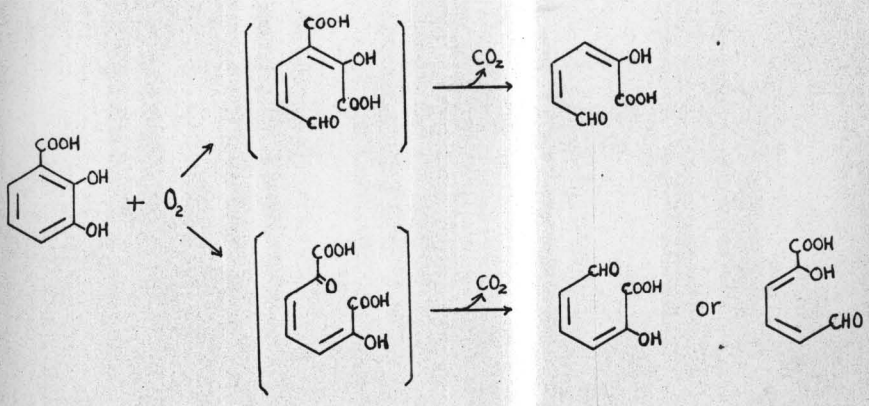
pyrocatechase type cleavage of protocatechuate was characteristic of the entire fluorescent group whereas metapyrocatechase type cleavage was confined to some nonfluorescent organisms (36). The enzyme described by Dagley and Patel catalyzes the conversion of protocatechuate to γ-carboxy-α-hydroxy cis,cis-muconic ϵ-semialdehyde (37).



Studies on this enzyme have been attempted by a few groups (38-41). This enzyme has been found to be extremely unstable (40). Dagley's group have studied some of the factors that influenced the activity of this enzyme and have obtained a nearly homogeneous preparation (41). Molecular weight was estimated to be 140,000. The enzyme lost activity rapidly when diluted. Inactivation was partially prevented by L-cysteine. Apparent Km for protocatechuate was 4.6×10^{-5} M; Km for oxygen was 3.03×10^{-4} M (41).

3. 2,3-Dihydroxybenzoate dioxygenase

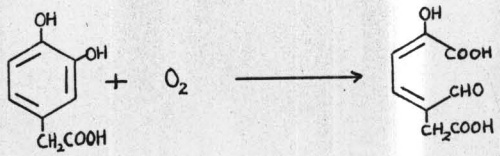
This enzyme was first studied by Ribbons in Pseudomonas fluorescens (42). Extracts of this strain catalyzed the rapid oxidation of 2,3-dihydroxybenzoate to α-hydroxymuconic-ϵ-semialdehyde with evolution of carbon dioxide. The site of ring cleavage and the order of oxygenation and decarboxylation remain to be clarified (43).



Purification was achieved up to 15-fold by Ribbons, et al. (43). The enzyme was inactivated rapidly by air, but the activity may be restored by anaerobic incubation of the enzyme with reducing agents or ferrous iron. The enzyme was noncompetitively inhibited by α,α'-dipyridyl and was competitively inhibited by catechol and its derivatives with respect to the substrate. The Km value for 2,3-dihydroxybenzoate was 7.5×10^{-6} M (43).

4. 3,4-Dihydroxyphenylacetate-2,3-dioxygenase

Senoh, et al. first succeeded in the isolation and crystallization of this enzyme from p-hydroxyphenylacetate adapted cells of P. ovallis (44-46). This dioxygenase catalyzed the formation of α-hydroxy-δ-carboxymethyl muconic-ϵ-semialdehyde by the oxidative ring fission of 3,4-dihydroxyphenylacetic acid at the 2,3-position.



The M.W. of this enzyme was around 100,000 and it was dissociable into 3 subunits by prolonged storage under air or by titration with PHMB (12). The enzyme

contained 4-5 g-atoms of iron per mole and 11 cysteine residues, of which 6 residues were easily titrated with PHMB (12,46). o-Phenanthroline and 8-hydroxyquinoline caused appreciable inhibition, which suggested the presence of ferrous iron at the active center (12). ESR and chemical analysis all confirmed this finding (12).

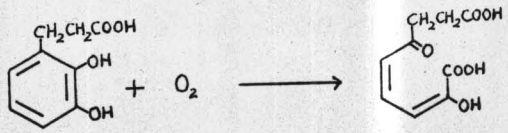
The release of iron from the enzyme and the decrease in enzyme activity corresponded to the degree of modification with PHMB. This finding indicated the presence of sulfur-iron linkage which may be responsible for the aggregation of the subunits (12).

The enzyme possessed fairly broad substrate specificity for 4-substituted catechol derivatives. The presence of a carboxyl group and increasing the length of the side chain of the substrate enhanced oxidation (12).

A reaction mechanism similar to that of metapyrocatechase was proposed (12).

5. 2,3-Dihydroxy-β-phenylpropionate-1,2-dioxygenase

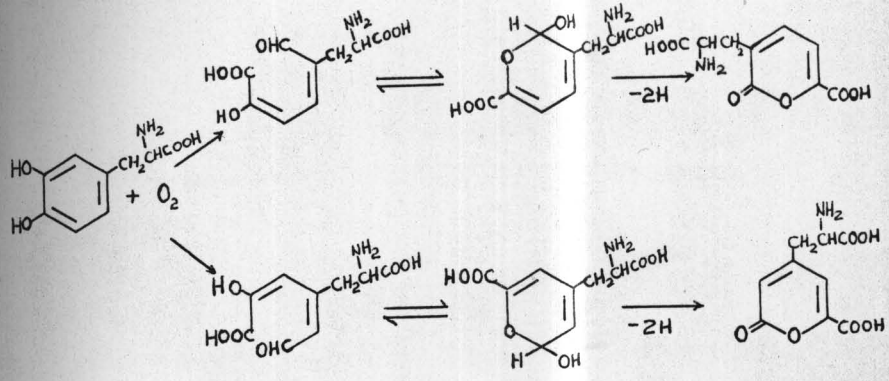
This enzyme, first described by Dagley from Achromobacter (47), catalyzed the following reaction:



The enzyme had been purified 18-fold and displayed an absolute requirement for Fe^{++} (47,48); its M.W. was around 70,000 as estimated by the Sephadex method. The catalyzed reaction depended strongly on the order of addition of the reactants. Ferrous iron must be added before the organic substrate to assure an immediate and rapid oxidation (48). Initial velocity studies were conducted on the partially purified enzyme. An intersecting pattern was observed. Substrate inhibition was observed with molecular oxygen but not with the organic substrate. These data led Dagley, et al. (48) to propose a preferred sequence of substrate addition whereby the organic substrate interacted with the enzyme first followed by oxygen, and the product was then released.

6. 3,4-Dihydroxyphenylalanine (DOPA)-2,3-dioxygenase and 4,5-dioxygenase

These two metapyrocatechase type enzymes were first demonstrated by Senoh, et al. in their studies on the biosynthesis of stizolobic and stizolobinic acids in plants (49,50).



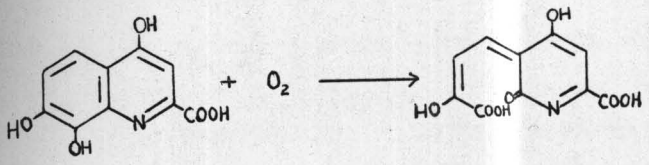
Piattelli, et al. also investigated the biogenesis of the pigments of Centrospermae. They showed that L-3,4-dihydroxyphenylalanine-4,5-dioxygenase was involved in the initial stages of their biogenesis (51-54).

Thus far, no purification of these enzymes was reported.

7. 7,8-Dihydroxykynurenate 8,9-dioxygenase

First description of this enzyme appeared in the studies of the metabolism of tryptophan in Pseudomonas by Hayaishi (55,56). The enzyme catalyzes

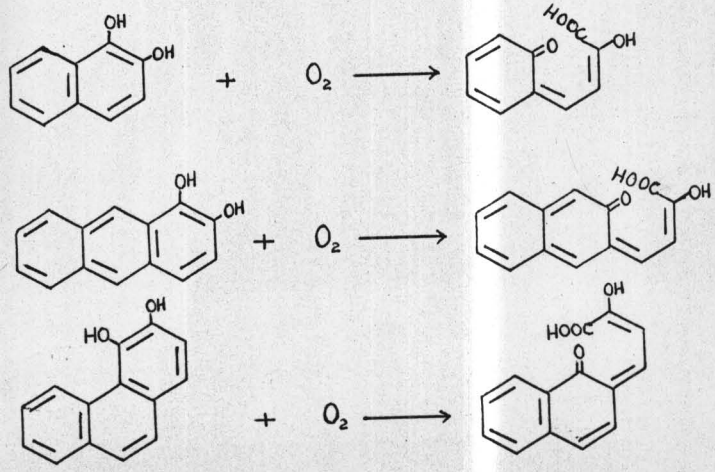
the following conversion:



Further attempts in the purification of this enzyme have not been conducted.

8. Dihydroxypolynuclear aromatic dioxygenase(s)

Crude extracts of a Pseudomonas sp. catalyzed the ring fission of the following polynuclear aromatic compounds. The positions of cleavage have been confirmed by the isolation and characterization of reaction products (57,58).

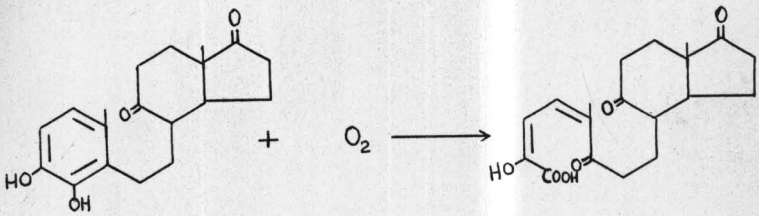


Rogoff (59) suggested that different enzyme systems were involved with angular phenanthrenes, linear arranged naphthalenes, and anthracenes, based on the electron densities at carbon atoms. On the other hand, Evans, et al. (57) proposed that all these ring fission reactions were catalyzed by one enzyme based on studies on enzyme induction.

So far, Evans, et al. have demonstrated the absolute requirement for Fe^{++}, and inactivation can be reactivated by $NaBH_4$ under anaerobic conditions.

9. 3,4-Dihydroxy 9,10-secoandrosta-1,3,5(10)-triene-9,17-dione 4,5-dioxygenase

This enzyme was first observed by Sih, et al. (60) in their studies of steroid metabolism by microorganisms. The enzyme catalyzes the following ring opening reaction:



Crude extracts prepared from N. restrictus has been shown to cleave the substrate at the 4,5-position

and the product formed exhibited absorption maxima at 325 and 393 mu (60,61).

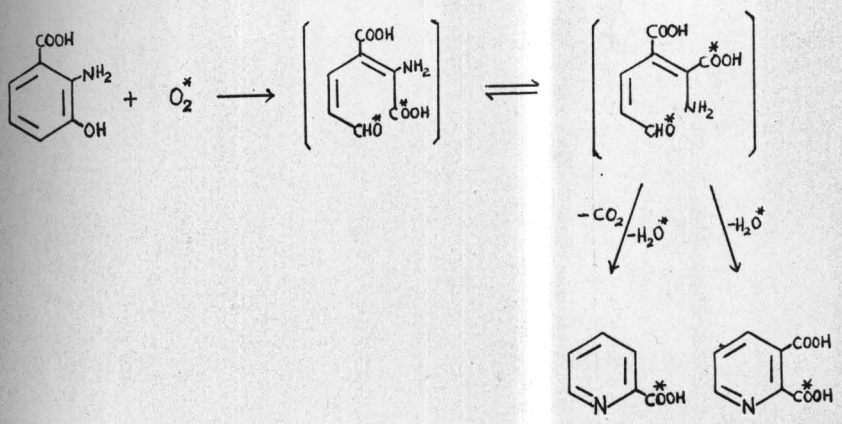
The reaction was stimulated by the presence of ferrous iron. Aside from steroidal substrates, a variety of catechol derivatives were studied. Only 3-isopropyl catechol and 3-tertiary butyl-5-methyl catechol were significantly metabolized (61).

II. Phenolic Dioxygenases

Enzymes of this group normally attack phenolic substrates or their derivatives. They are usually present in mammalian liver or kidney. Some have also been found in microorganisms. They absolutely require ferrous iron for activation.

1. 3-Hydroxyanthranilate dioxygenase

The enzyme catalyzed the ring fission of 3-hydroxyanthranilate between carbons 3 and 4 to yield α-amino-β-carboxymuconic semialdehyde which was not isolated because of ring closure to form either picolinic acid (enzymatically) or quinolinic acid (spontaneously) (62-64).



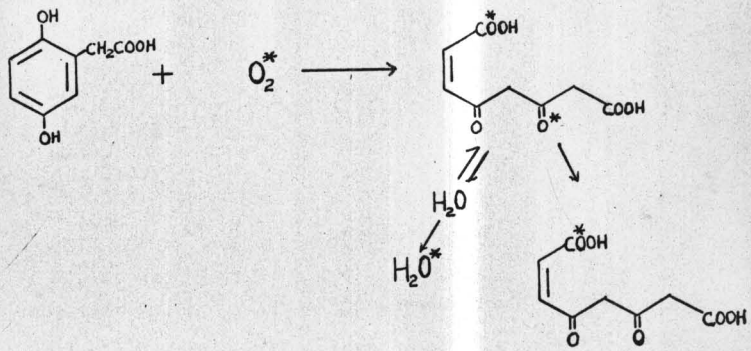
Purification of this enzyme was reported by several groups from beef liver (65-69), and from beef kidney (70). But no homogeneous enzyme has been obtained. They all showed an absolute requirement for ferrous iron and the loss of activity was mainly due to the oxidation of bound ferrous iron.

Kinetic studies of this dioxygenase was conducted by Henderson, et al. (70). Initial velocity studies showed a parallel pattern which suggested an irreversible step interrupting the sequential addition of the two substrates. Picolinic and quinolinic acids

produced competitive inhibition patterns with respect to 3-hydroxyanthranilate, but uncompetitive inhibition patterns with respect to oxygen. In addition, o-phenanthroline showed competitive inhibition with respect to 3-hydroxyanthranilate. The order of addition of substrates apparently could not be deduced from these kinetic data. Km's for oxygen and 3-hydroxyanthranilate were 3.1×10^{-4} M and 4×10^{-5} M, respectively (70).

2. Homogentisate dioxygenase

The enzyme catalyzed the conversion of homogentisic acid to maleylacetoacetate. The product found had only one atom of atmospheric oxygen incorporated, for the carbonyl oxygen at C_3 in the product exchanged with water very rapidly (71).



The enzyme was found both in microorganisms and in mammals. Crystalline enzyme has been obtained from P. fluorescens and the M.W. was estimated to be 380,000 (72). Although no homogeneous enzyme has been reported from mammalian sources, the bulk of information available for this enzyme was derived from studies with the mammalian liver enzyme.

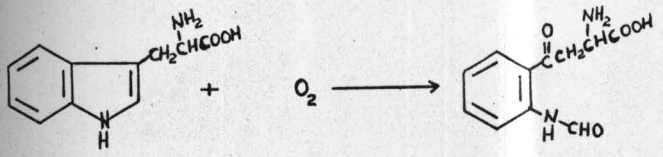
The ferrous iron requirement, originally discovered by Suda, et al. (73), was studied extensively by Tokuyama (74-76), Flamm and Crandall (77) and by Takemori, et al. (78). The involvement of -SH residues in the active center as ferrous mercaptans and oxygen was activated by this complex were proposed by Crandall, et al. (77,79) from kinetic studies. Mercurials showed competitive inhibition with respect to ferrous iron and noncompetitive inhibition with respect to homogentisic acid. Ferrous iron showed competitive activation with respect to oxygen (77,79). The Km for homogentisic acid was 6×10^{-4} M (in bacteria) (72), and the Km for oxygen was 1×10^{-3} M (in mammals) (77).

III. Other Aromatic Dioxygenases

1. Tryptophan pyrrolase (Tryptophan oxygenase)

Tryptophan pyrrolase is a heme-containing dioxygenase which catalyzes the insertion of molecular

oxygen into the pyrrole ring of tryptophan, yielding L-formylkynurenine as the reaction product (80-83).



Purification of this enzyme has been attempted both from animal and microbial sources. Homogeneous preparations have been obtained both by Hayaishi (84) and by Feigelson (85) from different species of Pseudomonas. More extensive characterization of this enzyme has been done by Feigelson (85). The enzyme has a M.W. around 120,000 and consisted of 4 subunits. Quantitative analytical data showed 1 mole of ferriprotoporphyrin IX and only a trace amount of copper and nonheme iron per mole of the enzyme (85).

Feigelson, et al. have also demonstrated the allosteric properties of the enzyme:

1) Catalytic activity was a sigmoidal function of the tryptophan concentration (86).

2) Apparent Km for oxygen was minimal at high tryptophan concentrations. In the presence of 2-methyltryptophan, apparent Km for oxygen was minimal and was independent of tryptophan concentration (87).

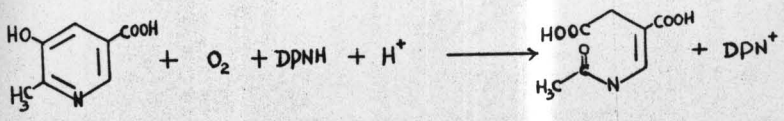
3) Increased affinity for CO, CN^- in the presence of tryptophan or its analog (88-89).

4) High concentration of sodium dodecyl sulfate was required to dissociate the enzyme when tryptophan or its analog was present (85).

Increased reactivity of heme toward heme-binding substances in the presence of tryptophan prompted Hayaishi's group to demonstrate the existence of an oxygenated intermediate. Both spectral and rapid kinetic measurements suggested the presence of this intermediate (28). The Km for oxygen was 4.2×10^{-5} M (87).

2. <u>2-Methyl-3-hydroxypyridine-5-carboxylic acid-2,3-dioxygenase</u>

The enzyme was shown to catalyze the following reaction:



This enzyme was discovered by Snell's group in their studies on the oxidative degradation of vitamin B_6 by bacteria (90). The homogeneous enzyme has a M.W. of 166,000 and contains 2 moles of FAD per mole (91). No metal ions have been found in significant quantities. Spectral measurements and initial velocity kinetic studies provided evidence that the reaction proceeded in a concerted fashion via a ternary complex of dioxygenase, NADH and organic substrate; NADPH was almost as effective as NADH. Among several pyridine analogs tested, only 5-pyridoxic acid was able to replace the natural substrate but was very much less active (91).

A similar dioxygenase, cleaving 5-pyridoxic acid in preference to any pyridine analogs but being specific for NADPH was demonstrated by the same group (92,93). But no purification studies have been conducted.

A brief summary of the physicochemical properties of the well characterized dioxygenases is listed in Table I.

TABLE I

SUMMARY OF PHYSICOCHEMICAL PROPERTIES OF SOME DIOXYGENASES

Subclass of Phenolytic Dioxygenases	Type	Name of Dioxygenase	M.W.	$S_{20,w}$ (Svedbergs)	$D_{20,w}$ ($\times 10^{7}$ cm^{2} sec^{-1})	Cofactor	Iron Content (g-atom mole^{-1})	Number of Subunits	Km for Substrate (M)	Km for Oxygen (M)
Catechol Dioxygenases	Intradiol	Pyrocatechase	95,000	4.31	4.5	Fe^{+++}	2	-	5×10^{-7}	2×10^{-5}
		Protocatechuate 3,4-dioxygenase	700,000	19.4	-	Fe^{+++}	7-8	8	3×10^{-5}	4.3×10^{-5}
	Extradiol	Metapyrocatechase	140,000	5.54	3.92	Fe^{++}	3	2-3	3×10^{-6}	7×10^{-6}
		Protocatechuate 4,5-dioxygenase	140,000	5.22	3.39	Fe^{++}	-	-	4.6×10^{-5} (app.)	3.03×10^{-4}
		3,4-Dihydroxy-phenylacetate 2,3-dioxygenase	100,000	7.19	-	Fe^{++}	4-5	3	-	-
		2,3-Dihydroxy-phenylpropionate 1,2-dioxygenase	70,000	-	-	Fe^{++}	-	-	5×10^{-5}	1.2×10^{-4}
		Steroid dioxygenase	286,000	10.28	3.42	Fe^{++}	1	-	3.7×10^{-4} (3-IPC)	1.8×10^{-4}
Phenolic Dioxygenases		3-Hydroxyanthranilate dioxygenase	-	-	-	Fe^{++}	-	-	4×10^{-5}	3.1×10^{-4}
		Homogentisate dioxygenase	380,000	11.8	-	Fe^{++}	-	-	6×10^{-4}	-
Other Aromatic Dioxygenases		Tryptophan pyrrolase	120,000	6.26	4.78	Fe^{+++} Proto-porphyrin IX	1	4	-	4.2×10^{-5}
		2-Methyl-3-hydroxypyridine-5-carboxylic acid 2,3-dioxygenase	166,000	7.30	-	FAD	-	-	4.8×10^{-5}	-

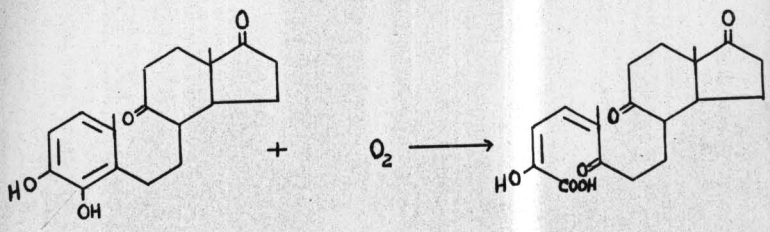
PART I

PURIFICATION AND PHYSICOCHEMICAL PROPERTIES OF STEROID DIOXYGENASE

INTRODUCTION

A number of investigators have described various methods of purifying the extradiol type of dioxygenases from different species of Pseudomonas (32,33,41,43,46) and Achromobacter (47). Only metapyrocatechase (32) and 3,4-dihydroxyphenylacetate-2,3-dioxygenase (46) were crystallized and well characterized. These two enzymes show no significant absorption in the visible range and contain nonheme iron which appears to be in the divalent state (10,12).

Previous studies in this laboratory (61) have demonstrated the involvement of an extradiol type of steroid dioxygenase in the degradative pathway of steroid metabolism in microorganisms. This enzyme has been shown to catalyze the following conversion:



In order to deduce the reaction mechanism of this steroid dioxygenase, it is necessary to first purify this enzyme and examine its physical and chemical properties.

EXPERIMENTAL PROCEDURE

1. Materials

Cultures of Nocardia restrictus (ATCC 14887) were grown in a medium containing 2% dextrose, 1% yeast extract and 0.8% nutrient broth. The enzyme was induced with progestrone after 30 hours of growth, and the cells were harvested no later than 15 hours after induction (usually a 10 to 15 hour induction yielded the highest specific activity). Harvested cells could be stored at -20°C without significant loss of activity.

Chemicals. - 3,4-Dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione was synthesized by the procedure of Sih, et al. (94). Catechol and 8-hydroxyquinoline were products of Eastman Kodak Company. 3-Methylcatechol, 4-methylcatechol, 3-isopropylcatechol, 4-isopropylcatechol, 4-tert-octylcatechol, 3-methyl-5-tert-octylcatechol, and 3-tert-butyl-5-methylcatechol were purchased from Gallard-Schlesinger Chemical Manufacturing Corporation, Garden City, New York. Iodoacetamine, α,α'-dipyridyl, o-phenanthroline and dithiothreitol (DTT) were obtained from Calbiochem. Sodium mersalyl, sodium iodoacetate, N-ethylmaleimide, 3,4-dihydroxyphenylalanine, p-hydroxymercuribenzoate, sodium ethylenediamine-tetraacetate, 5,5'-dithio-bis-(2-nitrobenzoic acid)

(DTNB) and diethylaminoethyl cellulose (DEAE, 0.92 meq/gm) were products of Sigma Chemical Company. α-Naphthoquinoline and m-phenanthroline were obtained from Chemical Procurement Laboratories, Inc., Sodium azide was a product of Matheson Coleman and Bell, Inc.. Potassium cyanide was obtained from Fisher Scientific Company. Nonionic cellulose was a product of Bio-Rad Laboratories. Calcium phosphate gel-cellulose was prepared according to the procedure of Kosicki (95).

2. Methods

Enzyme Assay. - 3-Isopropylcatechol (3-IPC) was routinely used as the substrate because of the scarce supply of the natural seco-steroid substrate and the enzyme has a very low Km for the seco-steroid. The enzyme activity was measured by the rate of product formation at 393 mμ (absorption maximum of the reaction product) in a Gilford Spectrophotometer model 240 equipped with an automatic recording apparatus. The assay system contained: 1 μmole of 3-IPC, a suitable amount of the enzyme, in 3 ml of 0.05 M potassium phosphate buffer, pH 7.5. One unit of enzyme activity was defined as that amount of enzyme causing the formation of 1 μmole of product per minute at 20°C, under the above assay conditions. Specific activity was expressed as the number of enzyme units per mg of protein.

Protein Determination - Protein concentration was determined by the Biuret method (96), the Lowry method (97) and direct spectrophotometric measurement (98). Crystalline bovine serum albumin was used as the protein standard.

Disc-Gel Electrophoresis - Polyacrylamide disc-gel electrophoresis was carried out according to Davis (99) in an electrophoresis apparatus (analytical model, Canalco), with 7.5% polyacrylamide gel at pH 8.4. Gels were stained for protein with Coomassie Brilliant Blue R 250 and destained in 7% acetic acid as described by Chrambach, et al. (100).

Amino Acid Analysis - The homogeneous enzyme was desalted by passing through a Sephadex G-25 column, equilibrated with deionized water. Samples of desalted enzyme were lyophilized and hydrolyzed in 6 N HCl at 110°C in evacuated, sealed Pyrex tubes for 24 or 72 hours. The samples were then placed in a flash evaporator to remove the HCl and the residue was dissolved in 1 ml of 0.2 N sodium citrate buffer, pH 2.2. A 0.3 ml portion was analyzed in a Beckman Spinco model 120B amino acid analyzer according to the method of Moore, et al. (101). The threonine and serine values were calculated by extrapolation to zero time. The amide nitrogen value was estimated in a

similar manner. Cystine was determined after converting cystine to cysteic acid by performic acid oxidation (102). Tryptophan was determined by the method of Beaven and Holiday (103) and the method of Edelboch (104).

Iron Assay - Iron content was determined quantitatively in a Perkin-Elmer model 303 atomic absorption spectrometer at 2483 Å. Standard curves were established by the use of a primary standard obtained from Gentec Hospital Supply Co., Milwaukee, Wis.. Iron content was also determined colorimetrically with the o-phenanthroline method (105).

Optical Spectra - The spectrum of the homogeneous enzyme was measured at room temperature with a Cary model 15 recording spectrophotometer.

Gel Permeation Chromatography - The diffusion coefficient $D_{20,w}$, of the homogeneous enzyme was determined chromatographically according to the procedure of Ackers (106). A column (1.9 x 75.7 cm) of Sephadex G-200 was equilibrated at 4°C against 0.05 M potassium phosphate buffer, pH 7.5, and calibrated with blue dextran 2,000 (ca. 2 mg) and beef liver catalase (0.3 mg). Steroid dioxygenase (5 mg) was then applied onto the column along with these standard proteins in 1 ml and the column was eluted with the same buffer. The eluate was collected in 3 ml fractions at a flow

rate of 12 ml per hour. Catalase was assayed by the reduction of absorbancy at 240 mμ spectrophotometrically (107). The void volume, V_o, the imbibed volume, V_i, and the elution volume, V_e, were employed to calculate a mean effective pore radius (r) of the column. Since the Stokes' radius (a) of catalase is known, r can be readily calculated by the molecular sieve equation of Ackers (106).

$$\frac{V_e - V_o}{V_i} = (1 - \frac{a}{r})^2 \left[1 - 2.104(\frac{a}{r}) + 2.09(\frac{a}{r})^3 - 0.95(\frac{a}{r})^5\right]$$

The gel pore radius, r, and the experimental value of $V_e - V_o/V_i$ for steroid dioxygenase were then employed to calculate the Stokes' radius, a, of the enzyme. The diffusion coefficient, D, was calculated from the Stokes' radius by use of the Stokes-Einstein equation (108).

$$D = \frac{KT}{6\pi\eta a}$$

where K is the Boltzman constant, T is the absolute temperature and η is the viscosity.

Analytical Ultracentrifugation - Velocity, equilibrium, zonal sedimentation and diffusion experiments were performed in a Spinco model E analytical ultracentrifuge, equipped with RTIC units. The An-D rotor was used for all experiments.

a. Sedimentation velocity - The sedimentation velocity was performed in a 12-mm double-sector synthetic boundary cell at a rotor speed of 59,780 rpm according to the procedure of Schachman (109). Schlieren optics was used for the photography of the sedimenting boundaries. The photographic plates were developed and read with a Gaertner comparator. Sedimentation coefficients were calculated from plots of log r versus time, where r is the radial distance from center of rotation to boundary.

b. Sedimentation equilibrium - Sedimentation equilibrium experiments were performed according to the meniscus depletion method of Yphantis (110). All runs were made in standard double-sector cells with 12-mm aluminum filled epoxy resin centerpieces and sapphire windows. Equilibrium was assumed to be attained when the fringe displacement between any two radial distances remained constant for 5 to 10 hours as described by Tanford (111). After equilibrium was attained, the Rayleigh interferographs were photographed with the use of Kodak spectroscopic II-G plates. Measurements of vertical fringe displacements, f, against radial distance, r, were made with a Gaertner Comparator. The positions of 5 fringes, 3 black and 2 white, were measured at each value of r and an average displacement,

f, of 5 fringes was used to calculate molecular weight, by the following equation:

$$\overline{M}w = \frac{2RT}{\omega^2(1 - \overline{v}\rho)} \frac{d\ln f}{dr^2}$$

where $\overline{v}$ is the partial specific volume of the enzyme, ρ is the solvent density, and ω is the angular velocity.

c. Zonal sedimentation (Vinograd sedimentation) - Analytical zone sedimentation was performed according to the method of Vinograd, et al. (112). Samples (20 µl) were placed in the Vinograd cell-sample chamber. After the cells were assembled, 0.5 ml of the medium of sedimentation was placed in the main chamber. The medium consisted of 0.5 KCl in 0.05 M phosphate buffer, pH 7.5. The rotor was accelerated to 59,780 rpm. Ultraviolet absorption pictures were taken at 8 minute intervals at 280 mµ with a high intensity ultraviolet light source. The plates were developed and traced with a densitometer. The sedimentation coefficient was calculated from the slope of the plot of log r versus time.

d. Diffusion - Diffusion studies were done in low speed runs (10,589 rpm) with the use of the valve type synthetic boundary cell. At this speed the boundary did not move appreciably. The apparent diffusion coefficient,

D_{app}, was calculated by the maximum ordinate-area method from the following relationship (113).

$$D_{app} = \frac{1}{4\pi t} \left(\frac{A}{k H_{max}}\right)^2$$

where A is the area under the gradient curve as measured on the photographic plate at time, t. H_{max} is the maximum height of that curve. k is the magnification factor along the radial coordinate. Area measurements were calculated by numerical integration on the assumption that the area within each narrow segment was equal to that of a rectangle whose height was the average value of the two sides of the segment. Usually 15 to 20 narrow segments were taken.

e. Frictional ratio - Frictional ratio, f/f_o of proteins was calculated from the following equation (114):

$$\frac{f}{f_o} = \frac{a}{\left(\frac{3\bar{v}M}{4\pi N}\right)^{1/3}}$$

where N is the Avogadro number.

RESULTS

Purification of Steroid Dioxygenase

All steps of the purification procedure described herein were carried out at 4°C unless otherwise specified. The various buffers, used routinely, included 10% acetone to stabilize the activity of the enzyme. The results of a typical purification protocol are presented in Table II.

Step 1: Preparation of cell-free extract - Frozen N. restrictus cell paste (500 g) was suspended in 750 ml of 0.01 M potassium phosphate buffer, pH 7.5. The suspension in 70 ml portions was sonicated for 10 minutes in an ice-salt bath with a Branson Sonifier (20 KC). The crude extract was freed of whole cells and debris by centrifugation at 27,000 x G for 15 minutes.

Step 2: Acetone fractionation - Acetone, precooled to -20°C, was added to the cell-free extract to give a concentration of 55% (v/v). The precipitate was then removed, by centrifuging the mixture at 12,000 x G for 10 minutes at -10°C. The clear supernatant was brought up to 66% with respect to acetone concentration. Centrifugation at 12,000 x G at -10°C for 5 minutes yielded the active precipitate. A second acetone

TABLE II

PURIFICATION OF STEROID DIOXYGENASE FROM *NOCARDIA RESTRICTUS*

Fraction	Volume (ml)	Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Recovery (%)
1. Crude extract	900	31,833.0	4,592.7	0.14	100
2. Acetone fractionation (55% - 66%)	49	2,121.6	3,304.8	1.56	72
3. Freezing and thawing	47	1,756.6	3,121.7	1.78	68
4. DEAE-cellulose chromatography	500	238.3	1,377.0	5.78	30
5. Calcium phosphate-cellulose chromatography	260	106.8	642.3	6.02	14

fractionation could be repeated if needed. The precipitate was then dissolved in 50 ml of 0.01 M potassium phosphate buffer, pH 7.5, containing 10% acetone, and the insoluble materials were removed by centrifugation. This acetone fraction was kept frozen overnight.

Step 3: DEAE-cellulose chromatography - The frozen acetone fraction was then thawed, and any residual precipitate was removed by centrifugation. The clear supernatant was then applied onto a DEAE-cellulose column (3.1 x 25 cm), previously equilibrated with 0.05 M potassium phosphate buffer, pH 7.5, containing 10% acetone (hereafter referred to as acetone buffer). The column was then washed with 2% $(NH_4)_2SO_4$ in acetone buffer, adjusted to pH 7.5 with 2.5 N NH_4OH until the eluate was virtually protein-free (around 8 column volumes). A linear gradient was used with 450 ml of buffer in both vessels. The mixing vessel contained 2.5% $(NH_4)_2SO_4$ in acetone buffer, pH 7.5, and the reservoir contained 6.5% $(NH_4)_2SO_4$ in acetone buffer, pH 7.5. Fractions of 20 ml, at a flow rate of 80 ml/hr, were collected. Active fractions were combined and concentrated by the addition of $(NH_4)_2SO_4$ to 60% saturation. The precipitate was dissolved in 10 ml of 0.001 M potassium phosphate buffer, pH 6.8, containing 10% acetone and was then desalted by passing through a

Sephadex G-25 column, equilibrated with the same buffer.

Step 4: Calcium phosphate gel-cellulose chromatography - The combined desalted DEAE-cellulose fraction was then applied onto a calcium phosphate gel-cellulose column (2.5 x 19 cm), previously equilibrated with 0.001 M potassium phosphate buffer, pH 6.8, containing 10% acetone. The gel to protein ratio was 10. After washing the column with 100 ml of 0.005 M potassium phosphate buffer, pH 6.8, containing 10% acetone, the column was eluted with a gradient consisting of 200 ml of 0.005 M potassium phosphate buffer, pH 6.8, and 200 ml 0.035 M potassium phosphate buffer, pH 6.8. Both buffers contained 10% acetone, and 10 ml fractions were collected at a flow rate of 60 ml/hr. The active fractions were combined and concentrated with $(NH_4)_2SO_4$ as above. The precipitate was dissolved in 5 ml of acetone buffer and stored in a freezer at -20°C.

Homogeneity - Disc electrophoresis was run for every step in the purification procedure as shown in Figure 1. The last step had only a single zone with a trace of contaminant at the front. Electrophoresis on cellulose polyacetate strips at pH 7.0 and 8.6, both showed single bands (Figure 2). The Schlieren patterns obtained during a sedimentation velocity experiment in

Figure 1. Disc electrophoresis of steroid dioxygenase. The steroid dioxygenase (75 μg) in the calcium phosphate gel fraction, DEAE-cellulose fraction, acetone fraction and 200 μg of crude extract (from left to right) were separately applied onto a column (0.5 x 5.5 cm) of polyacrylamide gel, and electrophoresis was carried out for 60 minutes at a constant current of 3.3 ma. Direction of migration was toward the anode (bottom).

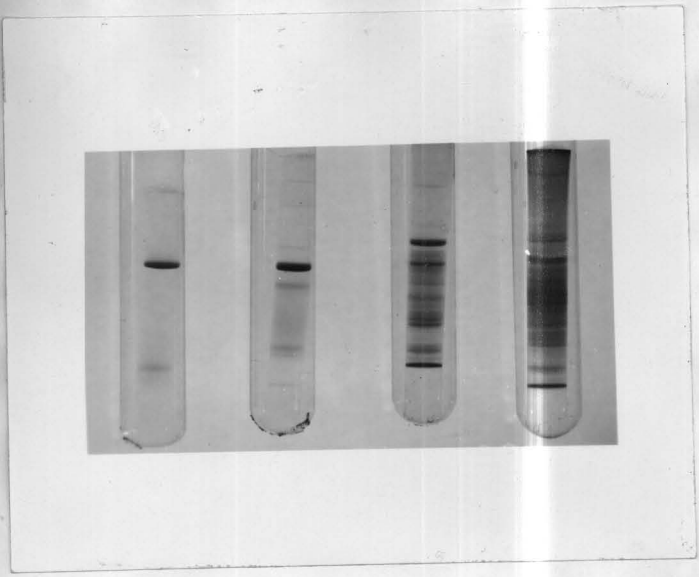
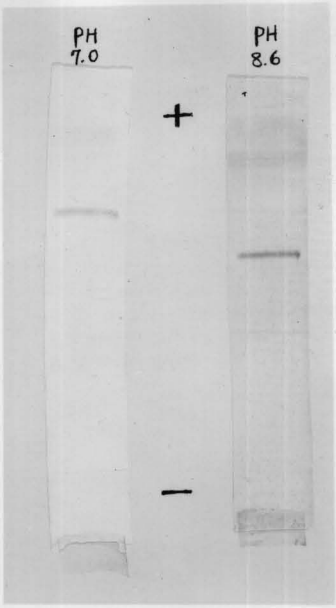


Figure 2. Electrophoresis on cellulose polyacetate strips. Purified steroid dioxygenase (25 μg) was applied onto cellulose polyacetate strips (2.5 x 15 cm) at the center. Electrophoresis was carried out at 200 volts for 45 minutes in 0.05 M sodium phosphate buffer, pH 7.0, and for 30 minutes in 0.05 M tris-versene-borate buffer, pH 8.6. The strips were stained with 0.25% amido black in 7% acetic acid.


PH
7.0
PH
8.6
+
—

the analytical ultracentrifuge were shown in Figure 3. A single, symmetrical boundary was observed.

Physical Properties of Steroid Dioxygenase

Diffusion coefficient by gel permeation chromatography - The elution profiles of blue dextran, catalase and steroid dioxygenase on a column of Sephadex G-200 (1.9 x 75.7 cm) are depicted in Figure 4. The elution volume, V_e, of blue dextran (72 ml) corresponds to the void volume, V_o, of the column. The elution volume of the standard enzyme catalase, in combination with its known Stokes' radius, a, gave the value of 220.7 Å for the column parameter; r, the effective gel pore radius. Using this column calibration parameter, together with the elution volume of the enzyme (105 ml), a value of 62.5 Å was obtained for the Stokes' radius, a, of steroid dioxygenase. Substitution of this value into the Stokes-Einstein equation, a value of 3.42×10^{-7} $cm^2 sec^{-1}$ was obtained for the diffusion coefficient, $D_{20,w}$. $D_{20,w}$, calculated by this method is essentially equal to $D^o_{20,w}$ (85).

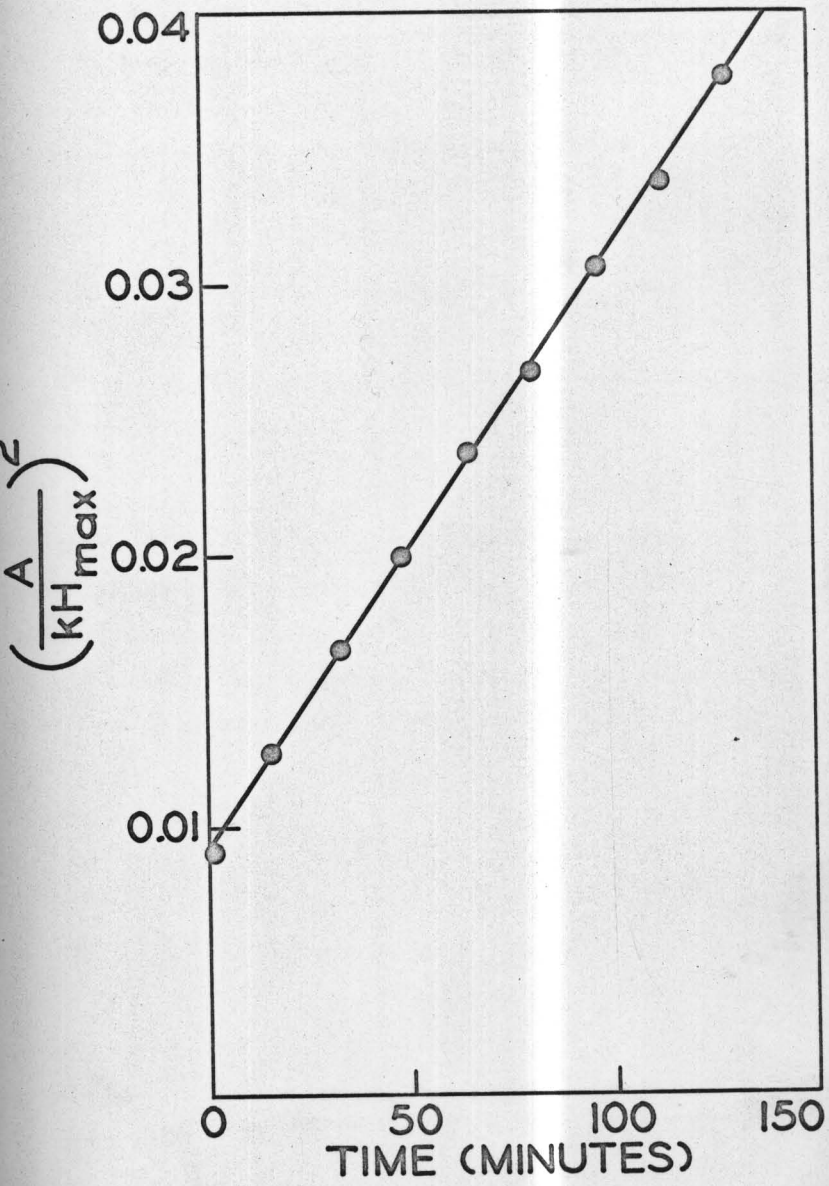
Diffusion coefficient by analytical ultracentrifugation - The diffusion coefficient of the enzyme was measured at three different concentrations. A typical measurement is shown in Figure 5. The diffusion coefficient decreased with increasing protein

Figure 3. Sedimentation velocity of steroid dioxygenase. The purified enzyme (8 mg per ml) in 0.05 M potassium phosphate buffer, pH 7.5, was centrifuged at 59,780 rpm and the Schlieren patterns were recorded (from left to right) at 8, 16, 24, 32, and 40 min after full speed was reached.

Figure 4. The determination of the diffusion coefficient by gel permeation chromatography. Blue dextran (1), steroid dioxygenase (2), and catalase (3) were applied onto a column of Sephadex G-200 (1.9 x 75.7 cm) in 0.05 M potassium phosphate buffer, pH 7.5. Blue dextran was detected by monitoring the absorbancy at 280 mμ (•). Catalase was assayed by measuring the reduction of absorbancy at 240 mμ in the presence of hydrogen peroxide (▲). Steroid dioxygenase was detected by the standard assay method (▲).

OPTICAL DENSITY AT 280mμ (○)
0.7
0.6
0.5
0.4
0.3
RELATIVE ACTIVITY (▲,△)
1.00
0.80
0.60
0.40
0.20
20
40
60
80
100
120
140
ELUATE VOLUME (ML.)
2
3

Figure 5. Determination of diffusion coefficient by ultracentrifugal method. Purified steroid dioxygenase (6.78 mg per ml) was centrifuged at 10,589 rpm for 130 min. $(\frac{A}{k H_{max}})^2$ was plotted against t (min).


0.04
0.03
0.02
0.01
$\left(\frac{A}{kH_{max}}\right)^2$
0
50
100
150
TIME (MINUTES)

concentrations. The concentration dependence, calculated by the least squares method, is given by the following expression:

$$D_{20,w} = D^{o}_{20,w} (1 - 0.0238\ C)$$

where C is the concentration in mg/ml. $D^{o}_{20,w}$ was found to be 3.55×10^{-7} $cm^2 sec^{-1}$.

Figure 6 shows the diffusion coefficient dependence on protein concentration.

Sedimentation coefficient - The sedimentation velocity of the purified enzyme was also measured at three different concentrations. The sedimentation coefficient again decreased with increasing protein concentration. The concentration dependence, calculated by the least squares method, is given by the following expression:

$$S_{20,w} = S^{o}_{20,w} (1 - 0.033\ C)$$

where C is the concentration in mg/ml. $S_{20,w}$ was found to be 10.35×10^{-13} sec, the value of the sedimentation coefficient at infinite dilution. Figure 7 shows the sedimentation coefficient dependence on protein concentrations. The $S_{20,w}$ value can be alternatively obtained by analytical zonal sedimentation velocity

Figure 6. Diffusion coefficient dependence on concentration.

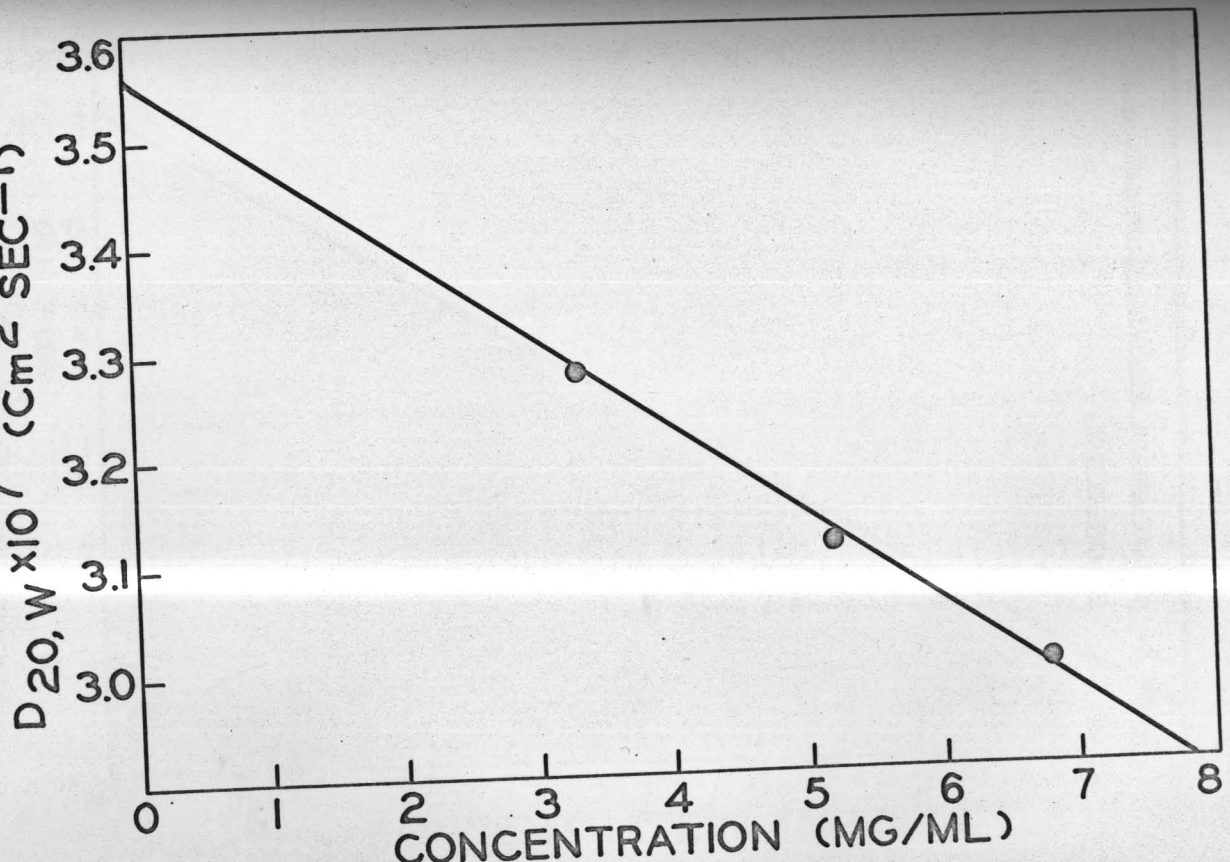
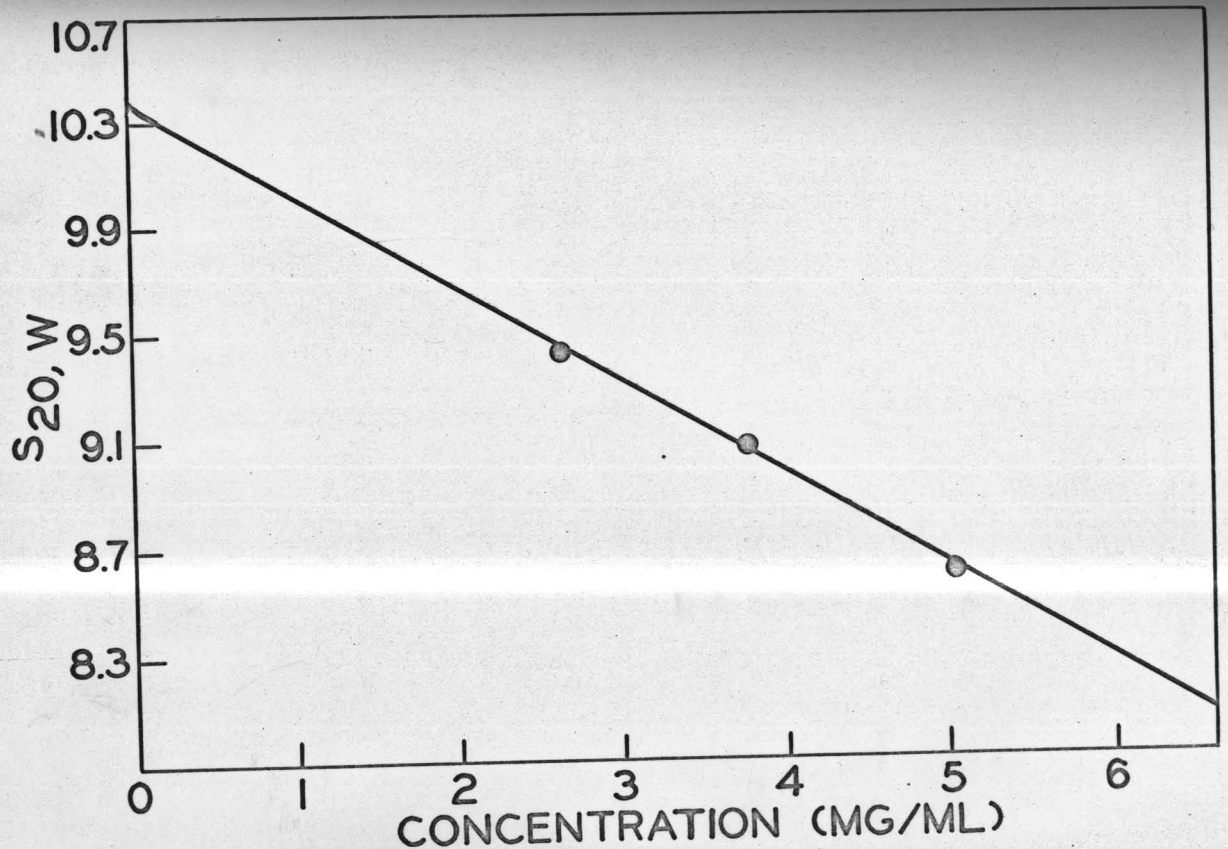

3.6
3.5
3.4
3.3
3.2
3.1
3.0
$D_{20,W} \times 10^7$ (Cm^2 SEC^{-1})
0
1
2
3
4
5
6
7
8
CONCENTRATION (MG/ML)

Figure 7. Sedimentation coefficient dependence on concentration.


10.7
10.3
9.9
9.5
9.1
8.7
8.3
S20, W
0
1
2
3
4
5
6
CONCENTRATION (MG/ML)

method in a single experiment (115). Figure 8 shows the plot from this experiment. The value, 10.28×10^{-13} sec, was in good agreement with the extrapolated value of $S_{20,w}$, calculated from the results of the synthetic boundary experiments.

Partial specific volume - Partial specific volume was estimated from the amino acid composition by the method of Cohn and Edsall (116) and was found to be 0.733 ml per gram.

Molecular weight by sedimentation velocity-diffusion - Substitution of experimentally determined values of 10.28×10^{-13} sec and 3.42×10^{-7} $cm^2 sec^{-1}$ for the sedimentation coefficient, $S_{20,w}$, and the diffusion coefficient, $D_{20,w}$, respectively, as well as 0.733 ml g^{-1} for the partial specific volume into the classical Svedberg equation (117), a value of 280,000 for the molecular weight, $M_{S.D.}$ of the native enzyme was obtained.

Molecular weight by sedimentation equilibrium - Figure 9 shows the plot of log f versus r^2, derived from a typical sedimentation equilibrium experiment on the purified enzyme. Two such experiments on different preparations at enzyme concentrations of 0.44 mg/ml and 0.66 mg/ml gave figures of 278,000 and 294,000, respectively. An average value of 286,000 serves as an

Figure 8. Determination of sedimentation coefficient by Vinograd sedimentation velocity method. Log r was plotted against t (min).

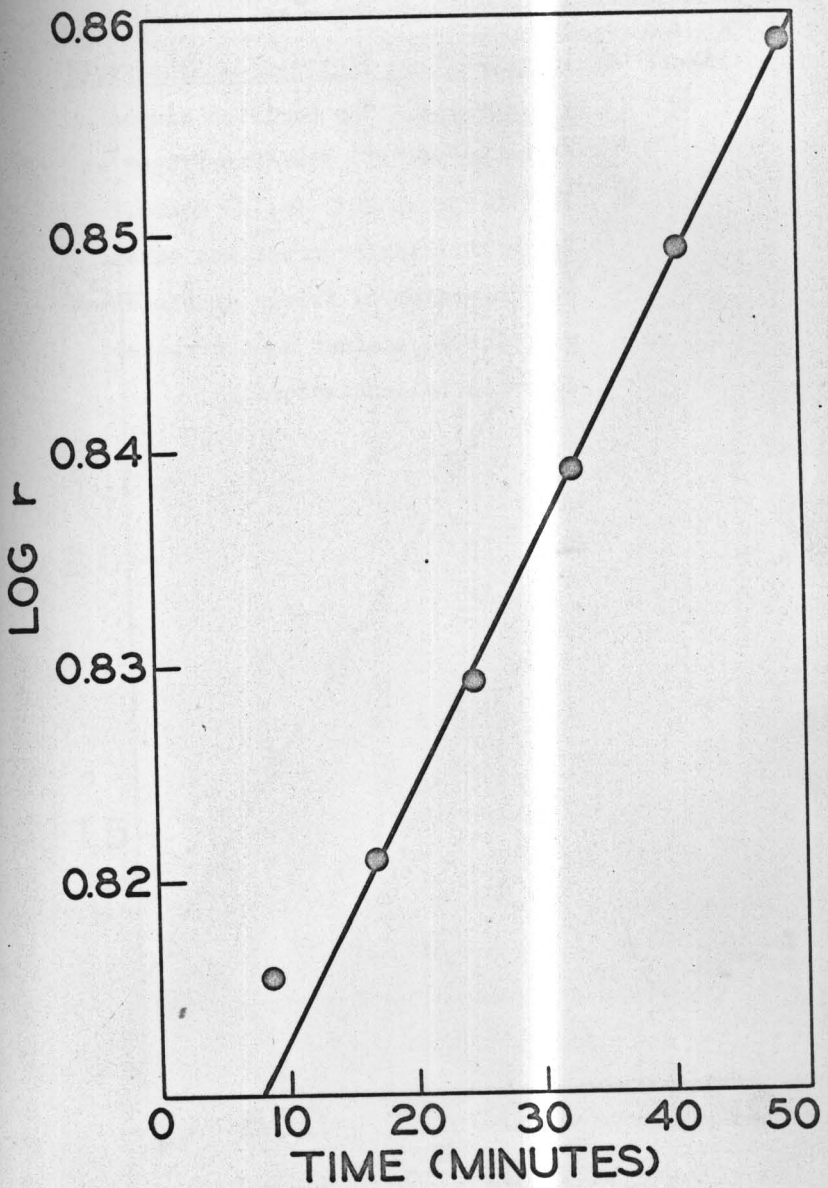
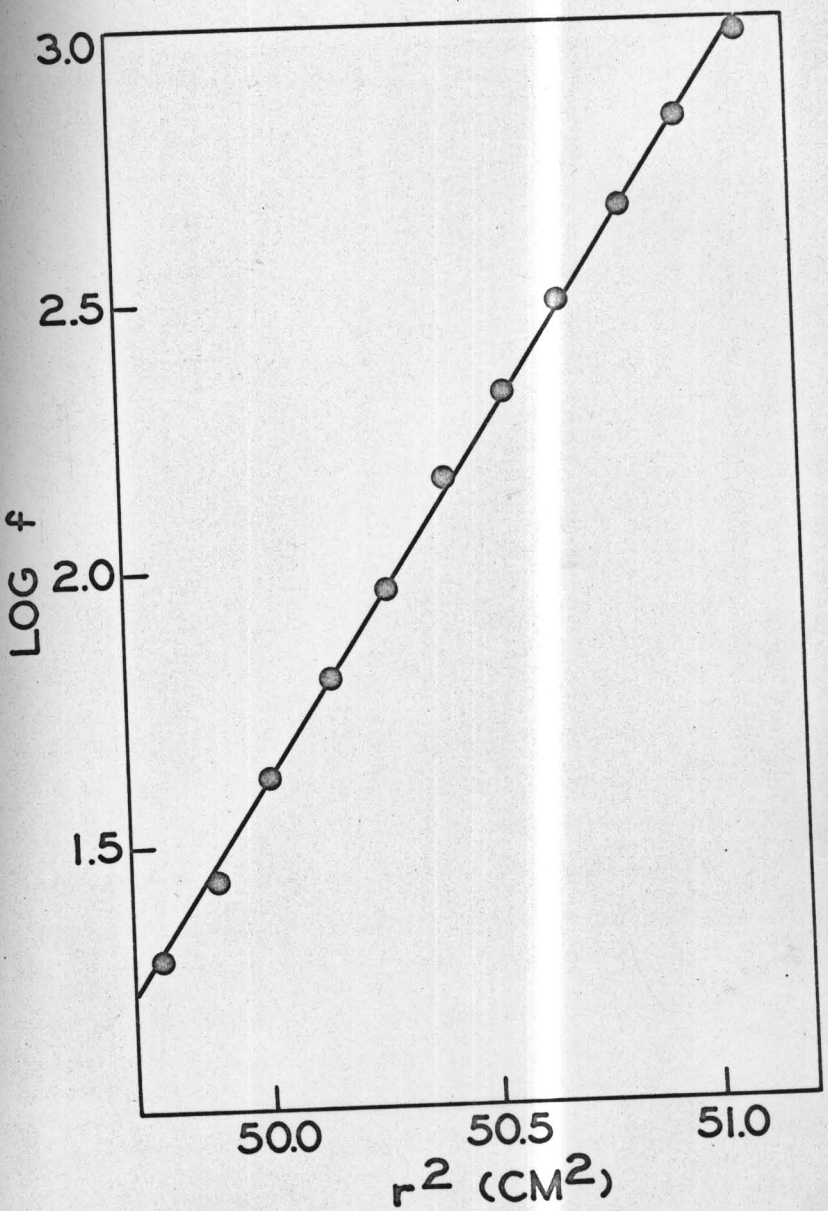

0.86
0.85
0.84
0.83
0.82
LOG r
0
10
20
30
40
50
TIME (MINUTES)

Figure 9. Sedimentation equilibrium of steroid dioxygenase. The purified enzyme (0.44 mg per ml) was centrifuged at 13,410 rpm at 20°C for 20 hours. Rayleigh interferograph was taken. The logarithm of fringe displacement was plotted against square radial distance in centimeters.


3.0
2.5
2.0
1.5
LOG f
50.0
50.5
51.0
r2 (CM2)

estimate for the anhydrous molecular weight, Mequil, of the native steroid dioxygenase.

Molecular shape - With the use of the experimentally established values of 62.5 Å and 286,000 for the Stokes' radius and molecular weight, respectively, the molar frictional ratio, f/f_o, of the native enzyme was calculated to be 1.47. Assuming a degree of hydration of approximately 20% (118), a value of 8 can be calculated for the axial ratio, p, of an assumed prolate ellipsoidal model for the steroid dioxygenase (119,120). Thus, this protein appeared to fit the category of a compact, moderately asymmetric macromolecule, into which most globular proteins fall.

Chemical Studies of Steroid Dioxygenase

Stability - Crude enzyme and acetone fractions were very unstable in the presence of air. Almost 50% of the activity was lost within a day at 4°C. The presence of a low concentration of an organic solvent such as acetone, ethanol and glycerol partially protected the enzyme from inactivation by air. Half-life of the enzyme could be prolonged to 3 days in the presence of 10% of these organic solvents. Although higher concentration of glycerol (20%) protected the enzyme from inactivation for longer periods at 4°C (the half-life was 5 days), the high viscosity of glycerol caused

difficulties in column chromatographic procedures. The enzyme was found to be much more stable in the purified form. Storage at -20°C in the presence of 10% acetone proved to be the best way to keep the enzyme in an active form for relatively longer periods.

Effect of pH - Oxygen uptake assay of the enzyme activity at various pH values is shown in Figure 10. The enzyme had a broad pH optimum from pH 5.8 to pH 7.5.

Effect of sulfhydryl inhibitors - As shown in Table III, the enzyme was not markedly inhibited immediately by SH inhibitors at concentrations of 1 mM. However, prolonged incubation with sodium iodoacetate or p-hydroxymercuribenzoate produced significant inhibition of dioxygenase activity.

Effect of metal-chelating agents - The effects of various ferrous and ferric ion chelating agents on the steroid dioxygenase activity are shown in Table IV. No appreciable inhibition at 1 mM concentration of various chelating agents was observed immediately. On the other hand, considerable inhibition of the dioxygenase activity was noted with 8-hydroxyquinoline and o-phenanthroline after prolonged contact with the enzyme. This observation suggested that the participating metal may be iron in the ferrous form. Double reciprocal plots showed that o-phenanthroline exhibited noncompetitive

Figure 10. Effect of pH on steroid dioxygenase activity. Steroid dioxygenase activity was measured with a Gilson model KM oxygraph. Reaction mixture contained 1 μmole 3-IPC, 75 μg of enzyme and respective buffer in a final volume of 2.5 ml. The reaction was run at 20°C. 0.05 M Acetate buffer was used for pH 4.5-5.5; 0.05 M potassium phosphate buffer was used for pH 6.0-8.0; and 0.05 M tris-HCl buffer was used for pH 8.0-8.5. The substrate was not stable above pH 8.5.

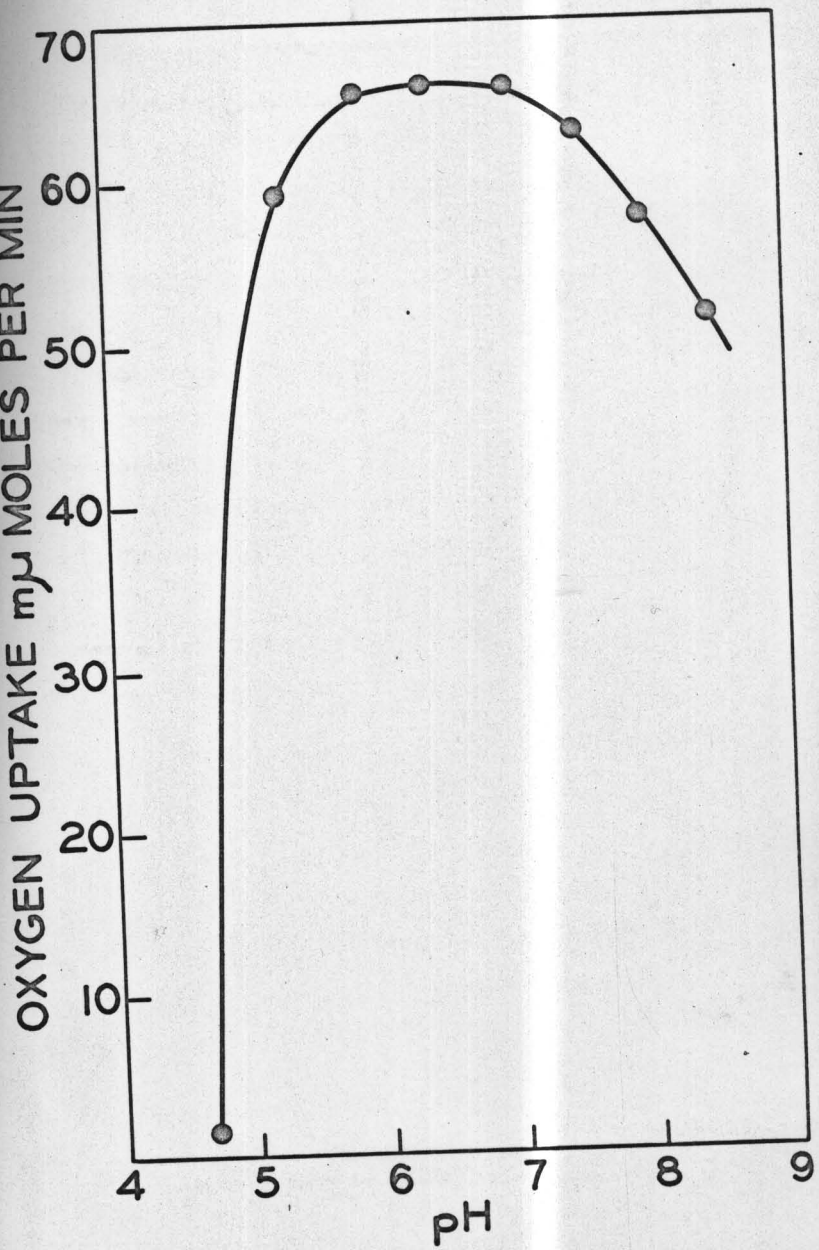

OXYGEN UPTAKE mμ MOLES PER MIN
70
60
50
40
30
20
10
4
5
6
7
8
9
pH

TABLE III

EFFECT OF -SH INHIBITORS ON STEROID DIOXYGENASE ACTIVITY

Additions	Activity	
	Incubation for 0 min (%)	Incubation for 10 min (%)
None	100.0	100.0
p-Hydroxymercuribenzoate	65.7	29.3
Sodium mersalyl	54.3	41.8
Iodoacetamide	89.8	62.3
Sodium iodoacetate	84.0	13.4
N-Ethylmaleimide	80.0	76.0

Incubation of the enzyme with 1 mM of each of the compounds listed in the standard reaction mixture without 3-IPC. After incubation, the reaction was initiated by the addition of 3-IPC, Enzyme (26 μg) was used.

TABLE IV

EFFECTS OF METAL-CHELATING AGENTS ON STEROID DIOXYGENASE ACTIVITY

Additions	Activity: Incubation for 0 min (%)	Activity: Incubation for 10 min (%)
None	100.0	100.0
α,α'-Dipyridyl	98.7	61.3
o-Phenanthroline	90.8	32.3
8-Hydroxyquinoline	89.2	9.7
α-Naphthoquinoline (0.5 mM)	104.0	103.0
m-Phenanthroline	95.5	83.2
Sodium azide	93.6	96.8
Potassium cyanide	95.5	96.8
Ethylenediaminetetra-acetate	98.7	96.8

Incubation of the enzyme with 1 mM of each of the compounds listed in the standard reaction mixture without 3-IPC. After incubation, the reaction was initiated by the addition of 3-IPC. Enzyme (23 μg) was used.

inhibition with respect to both organic substrate (Figure 11) and molecular oxygen (Figure 12).

Substrate specificity - Several catechol derivatives were tested for their reactivity with the enzyme as shown in Table V. Only 3-isopropylcatechol and 3-t-butyl-5-methylcatechol were attacked at a marked rate by the enzyme. This confirmed our previous observations using crude extracts (61).

Absorption spectrum of homogeneous enzyme - The absorption spectrum of homogeneous enzyme (Figure 13) showed no significant peaks in the visible region, and no evidence was found for the presence of heme. The spectrum is similar to that of metapyrocatechase, reported by Hayaishi (32). The absorption maximum at 280 mμ is characterized by a specific absorbance value ($E_{1\ cm}^{0.1\%}$) of 0.93.

Amino acid composition - The amino acid composition data, obtained as the mean of 24-hour and 72-hour hydrolysates of a homogeneous preparation of the enzyme, are summarized in Table VI. It is noteworthy that this high molecular weight enzyme contained only 9 residues of either cysteine or cystine equivalent.

Iron determination - A homogeneous preparation of the enzyme was passed through a Sephadex G-25 column (1.1 x 10 cm), equilibrated against deionized water, to

Figure 11. o-Phenanthroline inhibition with 3-IPC as the variable substrate. The reaction mixture consisted of 23 µg of enzyme, molecular oxygen concentration was 2.80×10^{-4} M, and the indicated concentrations of o-phenanthroline and 3-IPC in a total volume of 3 ml of 0.05 M potassium phosphate buffer, pH 7.5, at 20°C. —▲—▲— 8×10^{-3} M; —■—■— 5×10^{-3} M; —•—•— none.

150
100
50
0
10
20
30
$\frac{1}{v} \times 10^{-6}$ (MOLE^{-1} MIN)
$\frac{1}{3\text{-IPC}} \times 10^{-3}$ (M^{-1})

Figure 12. o-Phenanthroline inhibition with oxygen as the variable substrate. The system contained 27 μg of enzyme protein, 3-IPC, at the concentration of 1.11×10^{-4} M, and the indicated concentrations of o-phenanthroline and molecular oxygen in a final volume of 3 ml of 0.05 M potassium phosphate buffer, pH 7.5, at 20°C. —▲-▲— 8×10^{-3} M; —■-■— 5×10^{-3} M; —•-•—none.

$\frac{1}{v} \times 10^{-6}$ (MOLE^{-1} MIN)
150
100
50
0
5
10
$\frac{1}{O_2} \times 10^{-3}$ (M^{-1})

TABLE V

SUBSTRATE SPECIFICITY OF STEROID DIOXYGENASE

Compounds	Relative V_{max}	Km (M)
3-Isopropylcatechol	100.0	3.7×10^{-4}
3-Tertiary-butyl-5-methyl-catechol	82.4	1.8×10^{-3}
3-Methylcatechol	7.4	4.5×10^{-4}
4-Methylcatechol	4.3	3.4×10^{-4}
Catechol	0.6	1.3×10^{-4}
3-Methyl-5-tertiary-octyl-catechol	0	-
4-Tertiary-octylcatechol	0	-
4-Isopropylcatechol	0	-
3,4-Dihydroxyphenylalanine	0	-

Various catechol derivatives at various concentrations were assayed in the standard reaction mixtures. V_{max} and Km were then calculated from their respective Lineweaver and Burk plots. Relative V_{max} for 3-IPC was taken as 100. Enzyme (50 µg) was used.

Figure 13. Absorption spectrum of steroid dioxygenase. Absorption spectrum of the enzyme was measured with a Cary 15 spectrophotometer after passing the enzyme through a Sephadex G-25 column, equilibrated with 0.05 M potassium phosphate buffer, pH 7.5. The same buffer was used as the blank.

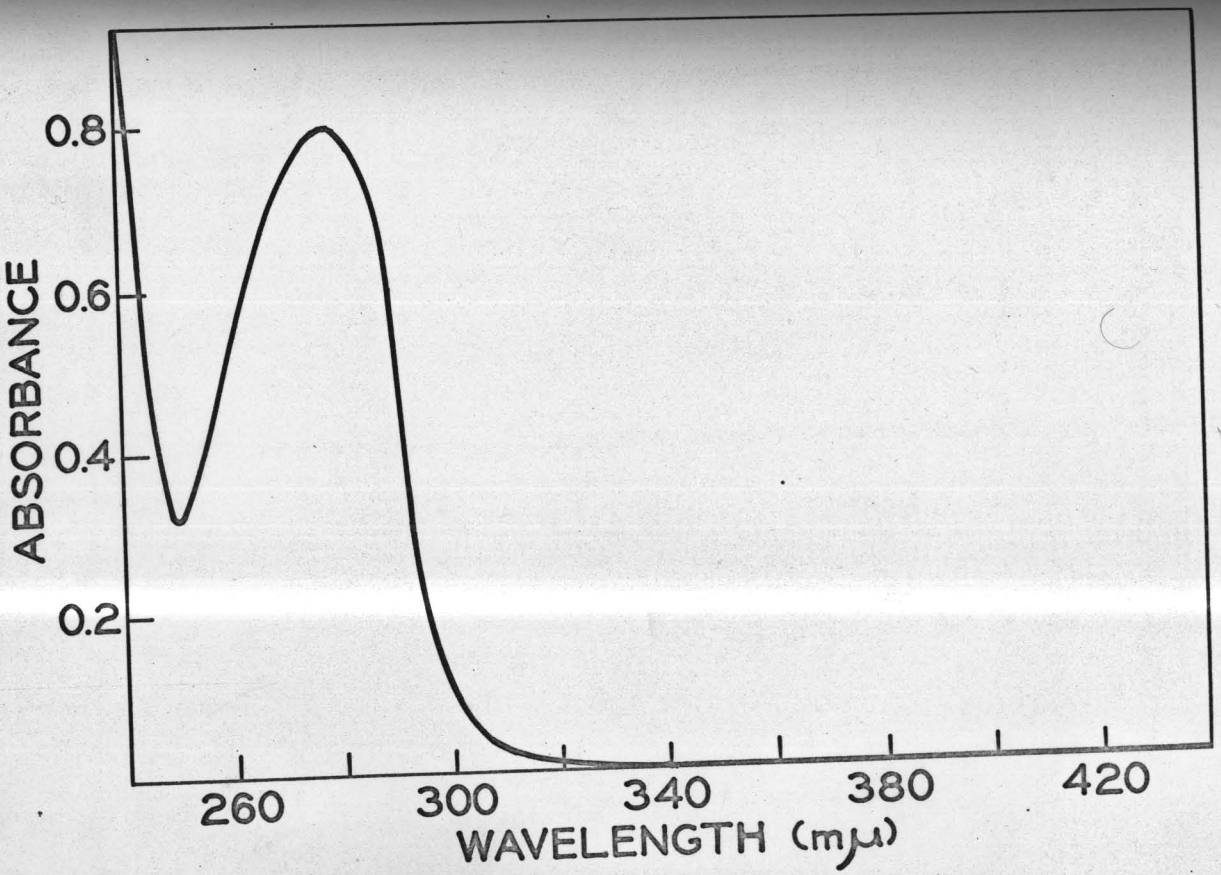

ABSORBANCE
0.8
0.6
0.4
0.2
260
300
340
380
420
WAVELENGTH (mμ)

TABLE VI

AMINO ACID COMPOSITION OF STEROID DIOXYGENASE

The purified enzyme (1.29 mg) was hydrolyzed for 24 hours and 72 hours in 1 ml 6 N HCl, respectively. Corrected composition was used to calculate the nearest integer for M.W. of 286,000.

Amino Acid Residue	24 hrs (μmole/mg)	72 hrs (μmole/mg)	Corrected Composition (μmole/mg)	Nearest Integer for M.W. of 286,000
Lysine	0.142	0.144	0.143	41
Histidine	0.252	0.257	0.254	73
Ammonia[a]	0.430	0.492	0.399	114
Arginine	0.475	0.477	0.476	136
Aspartic acid	0.771	0.749	0.763	218
Threonine[a]	0.479	0.431	0.502	144
Serine[a]	0.296	0.252	0.318	91
Glutamic acid	0.716	0.705	0.710	203
Proline	0.451	0.444	0.447	128
Glycine	0.866	0.854	0.860	246
Alanine	0.751	0.742	0.746	213
Half cystine[c]	0.031		0.031	9
Valine[b]	0.845	0.881	0.881	252
Methionine	0.108	0.106	0.107	31
Isoleucine[b]	0.237	0.246	0.246	70
Leucine	0.657	0.655	0.656	188
Tyrosine[a]	0.175	0.173	0.176	50
Phenylalanine	0.428	0.427	0.428	122
Tryptophan[d]			0.127	36
TOTAL				2365

[a]Corrected to zero time.

[b]Taken at 72 hrs due to slow liberation.

[c]Performic acid oxidation data.

[d]Spectrophotometric data.

remove any salts and contaminating metal ions. It was then analyzed for iron. Using a modified o-phenanthroline method, a value of 1.13 g-atom of iron per mole of the enzyme was obtained, which agreed well with the figure of 1.10 g-atom of iron per mole of the enzyme, obtained by atomic absorption spectroscopic measurements.

A summary of the physicochemical properties of steroid dioxygenase is listed in Table VII.

TABLE VII

SUMMARY OF PHYSICOCHEMICAL PROPERTIES OF STEROID DIOXYGENASE

$S^{o}_{20,w}$, Sedimentation coefficient (sec x 10^{13})	10.28
$D^{o}_{20,w}$, Diffusion coefficient ($cm^2 sec^{-1}$ x 10^7)	3.42
$M_{S.D.}$, Molecular weight by sedimentation velocity-diffusion	280,000
M_{equil} Molecular weight by equilibrium sedimentation	286,000
a, Stokes' radius (Å)	62.5
f/f_o, Frictional ratio	1.47
$\bar{v}$, Apparent partial specific volume (ml g^{-1})	0.733
$E^{0.1\%}_{1\ cm}$, Specific absorbance at 280 mu	0.93
Number of g-atom of Fe^{++} per mole of enzyme	1.13

DISCUSSION

The steroid dioxygenase from N. restrictus has been purified virtually to a homogeneous state. No gross signs of heterogeneity in preparations of the enzyme were detected by sedimentation velocity analysis in the analytical ultracentrifuge or electrophoresis on both polyacrylamide gel and cellulose polyacetate strips.

Purification of metapyrocatechase type of dioxygenase was greatly hampered by the instability of the enzyme, until Hayaishi, et al. (32) reported that 10% acetone in buffers was successful in protecting metapyrocatechase from inactivation. Although most extradiol type of dioxygenases could not be stabilized by acetone, we have observed that acetone was successful in partially protecting the steroid dioxygenase from inactivation. Although glycerol was a better stabilizer, its high viscosity prevented its use in column chromatography. The purification procedure herein described could be accomplished within two days and the purified enzyme could then be kept at -20°C for longer periods with a minimum loss of activity.

Molecular weight determinations by sedimentation equilibrium and sedimentation velocity-diffusion methods agreed very well. Sephadex G-200 chromatography was used to estimate the diffusion coefficient instead of

molecular weight mainly because of the inaccuracy of the method. As pointed out by Siegel and Monty (121), the empirical method of Andrews (122) gives good correlation with the molecular weight of a protein only when the standard and unknown proteins employed have quite similar frictional ratios and partial specific volumes. More recently, it has been shown that molecular sieve data are a measure of the Stokes' radius of a macromolecule, rather than its molecular weight (106,123). Once the Stokes' radius is determined from molecular sieve data, it can be used to calculate the diffusion coefficient. The diffusion coefficient obtained in this way was in good agreement with that obtained from the ultracentrifugal method.

The sedimentation coefficient, obtained by analytical zone and synthetic boundary sedimentation velocity methods, was also in close agreement. This suggested the applicability of the more economic and less time-consuming former method for the determination of $S^{o}_{20,w}$ of the protein. However, the usefulness of this method is limited by the fact that proteins of low molecular weight (such as lysozyme or myoglobin) sediment so slowly that diffusion would make it impossible to locate the peak accurately by the time the band of sedimenting protein had moved significantly.

The cause of the instability of the enzyme is not well understood. Some purified enzyme preparations contained low amounts of iron and also possessed low specific activities; and the addition of ferrous iron to these preparations could increase the enzyme activities. Thus, the inactivation may be due to the loss of loosely bound ferrous iron during column chromatography, or due to the oxidation of bound ferrous iron to the ferric form which then may be released from the enzyme as reported by others (13).

The iron content of the homogeneous enzyme was found to be 1 g-atom per mole of the enzyme. This was the highest value we have ever observed. Whether this figure corresponds to the natural content of the enzyme cannot be as yet ascertained since iron may be lost during purification as mentioned above. Hayaishi, et al. (10,13) have recently reported that the iron content and the specific activity of metapyrocatechase can be varied under different growth conditions. Although we have not examined the relationship between iron content and growth conditions, higher iron content might be obtained for this high molecular weight enzyme if the microorganisms were cultivated in a more suitable medium.

Based on the observations of several dioxygenases, Hayaishi, et al. (14) have suggested that the number of

iron atoms in the dioxygenase coincided with the number of subunits. Although this assumption was found to be true for pyrocatechase, metapyrocatechase and protocatechuate 3,4-dioxygenase, some exceptions were noted. Crystalline 3,4-dihydroxyphenylacetate-2,3-dioxygenase possessed three subunits in its native state but contained 4-5 g-atom of iron per mole of the enzyme (12, 46). Subunit structure of steroid dioxygenase has not yet been extensively studied. Preliminary experiments revealed that the enzyme could be dissociated in 0.05 N NaOH into identical subunits having sedimentation coefficient of 2.6 S. Reconstitution of the purified enzyme in the presence of DTT and ferrous iron showed the iron content could be as high as 7-8 g-atom per mole of the enzyme but the specific activity could not be increased proportionally. It is difficult to imagine that all of the reconstituted iron atoms in dioxygenases participate catalytically. It appears more likely that only one atom of ferrous iron possesses catalytic function and the rest participate in the aggregation of subunits or in some other capacity, as in the case of 3,4-dihydroxyphenylacetate-2,3-dioxygenase (12).

Hayaishi, et al. (13) found the inactivation of metapyrocatechase produced by oxidizing agents and sulfhydryl inhibitors, could be prevented by the presence of substrate. They suggested that the

substrate binding site might be closely associated with ferrous iron and sulfhydryl groups. However, the steroid dioxygenase did not respond to sulfhydryl inhibitors immediately and even after prolonged incubation the enzyme activity was not completely abolished.

Titration of the native enzyme with DTNB, a specific reagent for -SH groups, revealed very few exposed -SH groups, which suggested the possible existence of an iron mercaptide linkage in the active center.

α-Naphthoquinoline and m-phenanthroline were found to be very potent inhibitors of metapyrocatechase, and the inhibitions were found to be competitive with respect to the substrate, catechol. Although these nitrogenous bases are very poor iron chelators, their inhibitory capacity was even more pronounced than that of o-phenanthroline. This led Hayaishi to propose that substrate binding was stabilized through some sort of hydrophobic interaction. However, in the case of steroid dioxygenase, no apparent inhibition could be observed with these two compounds at concentrations of 1 mM. o-Phenanthroline, instead of inhibiting the enzyme competitively with the organic substrate as in the case of metapyrocatechase, inhibited the enzyme noncompetitively with respect to

both the organic substrate and molecular oxygen. Thus, the mechanism of inhibition by o-phenanthroline appeared to differ for the two enzymes. The noncompetitive inhibition, produced by o-phenanthroline, suggested the presence of two different types of combining sites on the enzyme. One type is affected by the interacting substrate while the other type, being at a remote distance from the substrate combining sites, is unaffected.

Substrate specificity studies have yielded some information on the structural requirements for enzyme action. Substitution of an alkyl group at position 3 of catechol was required for maximal activity, which differed from most of the metapyrocatechase type of dioxygenases. Introduction of a bulky alkyl group at positions 4 or 5 rendered the substrate molecule considerably less reactive. Furthermore, the size of the alkyl substituent at position 3 appeared to be also important. Apparently, the bulkier the side chain was, the more reactive the substrate became. This is reflected from the finding that the natural steroidal substrate which possesses a very bulky hydrophobic side chain, has a very low Km value. We can, therefore, visualize the substrate binding site as a hydrophobic cavity which directs the side chain of the substrate to situate in such a manner that the two hydroxyl groups

are oriented toward the catalytic center resulting in ring fission.

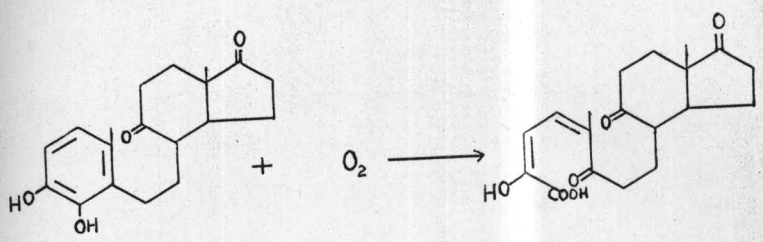
PART II

KINETIC STUDIES OF STEROID DIOXYGENASE

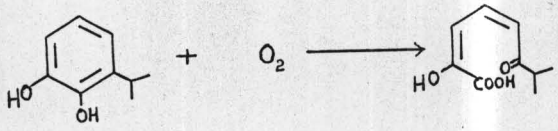
INTRODUCTION

The kinetic properties of 3-hydroxyanthranilate dioxygenase was first examined by Henderson, et al. (70), using initial velocity and inhibition studies. However, no definitive conclusions were drawn as to the order of the addition of the substrates to the enzyme. Recently, Dagley, et al. (48) examined the rate of interaction of 2,3-dihydroxy-β-phenylpropionate dioxygenase with its substrates and proposed that the organic substrate is added to the enzyme before molecular oxygen. This proposition was reached by these workers on the basis that molecular oxygen produced substrate inhibition whereas the organic substrate did not. Although Hayaishi (11) first postulated that oxygen is added to the extradiol type of dioxygenase prior to the organic substrate, more recent studies by this group (14,28) suggest that the reversed order of addition is more likely. In view of the conflicting views, this investigation was undertaken to define the sequence of substrate addition through the use of product and dead-end inhibition studies.

The steroid dioxygenase catalyzes the following reaction:



As described in Part I, this enzyme is capable of catalyzing the oxidative cleavage of several substituted catechols besides its natural seco-steroidal substrate. Among these, 3-IPC was the most reactive artificial substrate.



Since the seco-steroidal substrate has a very low Km and in limited supply, 3-IPC was chosen as the organic substrate for all our subsequent kinetic studies.

EXPERIMENTAL PROCEDURE

1. Materials

Chemically pure 3-isopropylcatechol and 4-isopropylcatechol were purchased from Gallard-Schlesinger Chemical Manufacturing Corp.. o-Phenanthroline was a product of Calbiochem. Purified enzyme was prepared as described in Part I.

2. Methods

a. Preparation of stock solution of enzyme - Enzyme solution was passed through a Sephadex G-25 column, equilibrated with 0.05 M potassium phosphate buffer, pH 7.5 to remove acetone and was diluted with the same buffer to required concentration. The diluted enzyme was stable at 0°C at least during the experimental period.

b. Substrate and inhibitor concentrations - The absorption maxima and the molar extinction coefficients of 3-isopropylcatechol and 4-isopropylcatechol were 273 mμ ($\epsilon_M = 2.24 \times 10^3$), 278 mμ ($\epsilon_M = 2.24 \times 10^3$), respectively (124).

c. Protein concentration - Protein was estimated spectrophotometrically by using the formula $1.55\ OD_{280} - 0.76\ OD_{260}$ = mg/ml (98) or by the Biuret method (96).

d. Preparation of reaction product - Acetone fraction was first prepared from crude extracts of N. restrictus and was then passed through a Sephadex G-25 column, equilibrated with 0.05 M potassium phosphate buffer, pH 7.5, to remove colored, low molecular weight materials. 3-IPC (13.5 mg), dissolved in 2 ml 0.05 M potassium phosphate buffer, pH 7.5, was incubated with 2 ml of Sephadex-treated acetone fraction (27 mg protein) at 30°C with continuous shaking. A 0.03 ml aliquot of the reaction mixture was removed occasionally and diluted to 3 ml with the same buffer to check the increase in absorbancy at 393 mμ until the theoretical value was reached (i.e., all the substrate converted to product). After the incubation period, the reaction mixture was immediately passed through a Sephadex G-25 column, (1.5 x 30 cm), pre-equilibrated with the same buffer, to obtain the pure product. The concentration of the product was determined spectrophotometrically at 393 mμ ($_{M}$ = 1.85 x 10^4). The product was prepared and used immediately.

e. Oxygen concentration - The concentration of dissolved oxygen was measured by a Clark oxygen electrode, fitted with an automatic recording apparatus. The instrument was calibrated by measuring the electrode current, first for a known concentration of O_2.

A blank reading was taken by exhausting oxygen with dithionite. A linear relationship was obtained between concentrations of O_2 and currents. The concentration of O_2 in water exposed to water saturated air at 20°C was taken to be 0.28 mM (125) and in water saturated with pure O_2, 1.25 mM. The concentrations of O_2 required in reactions were obtained by gassing the buffer in situ with O_2-N_2 mixtures to attain the desired recorder readings.

f. Kinetic determinations - The reaction was followed by measuring the appearance of product at 393 mμ with a Gilford Spectrophotometer model 240, attached with an automatic recording apparatus. Most kinetic experiments were carried out using a 3.0 ml reaction mixture in 1 cm silica cuvettes at 20°C. The cuvettes were filled with solutions containing substrates. The reaction was initiated by the addition of 0.03 ml of the enzyme. Initial velocities were determined by measuring the tangent to the recorded curve extrapolated to the time of enzyme addition. Each experimental point was determined in duplicate. Each experiment was repeated twice to establish a consistent pattern (i.e., competitive, noncompetitive, etc.).

g. Data processing - The nomenclature used herein is that of Cleland (126,127). Reciprocal velocities were plotted graphically against the reciprocal of substrate concentrations and any point which deviated greatly from a linear relationship was discarded. The remaining data were fitted to equation (3)

$$v = \frac{VS}{K + S} \qquad (3)$$

Reciprocal plots of initial velocity, product inhibition and dead-end inhibition data were examined to determine the pattern, (i.e., intersecting, competitive inhibition, etc.) and the slopes (K/V) and intercepts were plotted graphically against either the reciprocal of the non-varied substrate concentration (for initial velocity experiments) or the inhibitor concentration (for inhibition experiments) to determine the linearity of these replots. Data conforming to a sequential initial velocity pattern, a linear competitive inhibition pattern, a linear uncompetitive and a linear noncompetitive inhibition pattern were fitted to equations 4, 5, 6, and 7, respectively.

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \qquad (4)$$

$$v = \frac{VA}{K(1 + 1/K_{is}) + A} \qquad (5)$$

$$v = \frac{VA}{K + A(1 + 1/K_{ii})} \qquad (6)$$

$$v = \frac{VA}{K(1 + 1/K_{is}) + A(1 + 1/K_{ii})} \qquad (7)$$

In equations 5 through 7, K_{is} and K_{ii} are apparent inhibition constants for slope and intercept. All fitting of experimental data to the respective equations were carried out by the least squares method, assuming equal variance for the velocities (129). All least square fits reported herein were performed by a digital computer, using the Fortran programs of Cleland (128,130). These programs provide values for the constants in a fitted equation, the standard errors of their estimates, and weighing factors for further analysis.

RESULTS

Initial velocity studies - The initial velocity patterns for the reaction are shown in Figures 1 and 2. When 3-isopropylcatechol (3-IPC) was plotted as the variable substrate with different concentrations of oxygen as the varied fixed substrate (Figure 1), an intersecting pattern was obtained. When oxygen was plotted as the variable substrate, with different concentrations of 3-IPC as the changing fixed substrate, an intersecting pattern was again observed. When the data were fitted to equation 4, the Michaelis constants for 3-IPC (K_a) and oxygen (K_b) were found to be 3.7×10^{-4} M and 1.8×10^{-4} M, respectively. The dissociation constant for 3-IPC (K_{ia}) was 3.64×10^{-4} M. The closeness of values between K_a and K_{ia} is reflected from the fact that the intersecting point is almost on the horizontal axis. The plot also showed substrate inhibition by 3-IPC, which was observable at high 3-IPC concentrations.

Product inhibition studies - Product inhibition patterns and their replots are shown in Figures 3 through 7. With 3-IPC as the variable substrate, and oxygen concentration lower than its K_m, linear noncompetitive product inhibition pattern was observed. (Figures 3 and 5). When the oxygen concentration was

Figure 1. Initial velocity plots with 3-IPC as the variable substrate. The reaction was carried out in 0.05 M potassium phosphate buffer, pH 7.5, in a final volume of 3 ml at 20°C. Oxygen concentration from top to bottom: 8.40×10^{-5} M; 1.67×10^{-4} M; and 2.80×10^{-4} M. Enzyme, 23 µg per cuvette.

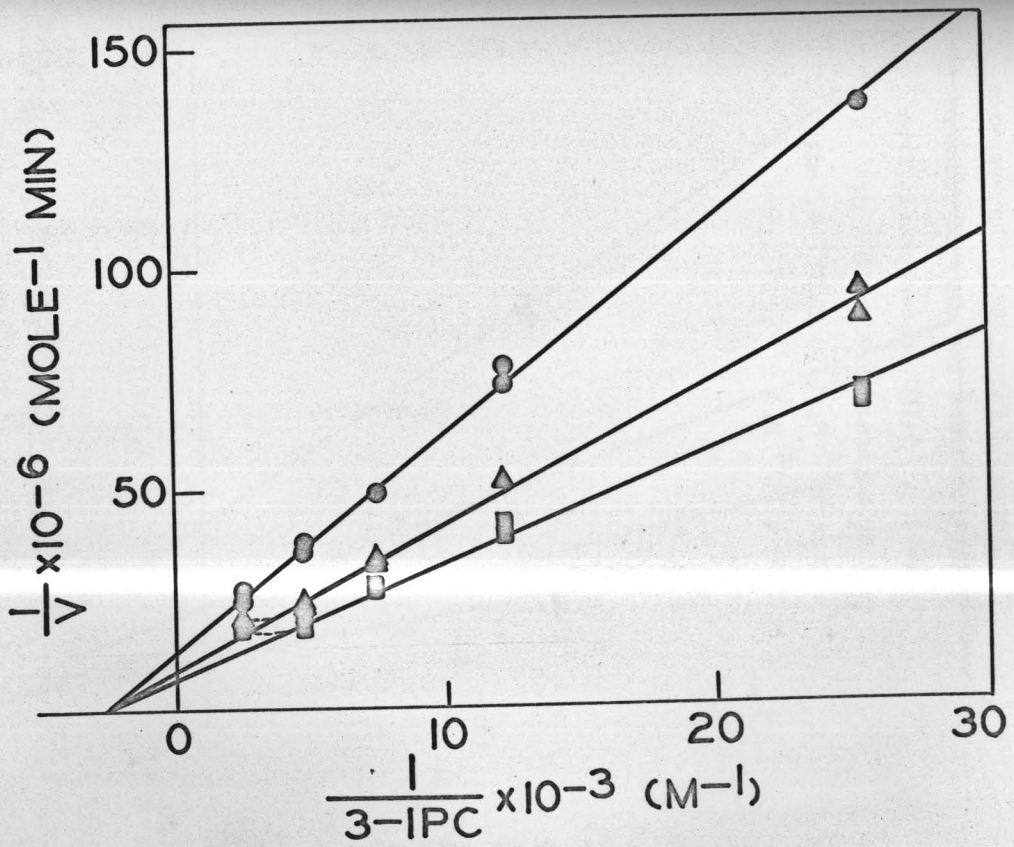

150
100
50
0
10
20
30
$\frac{1}{V} \times 10^{-6}$ (MOLE^{-1} MIN)
$\frac{1}{3\text{-IPC}} \times 10^{-3}$ (M^{-1})

Figure 2. Initial velocity plots with oxygen as the variable substrate. The reaction conditions were the same as those of Figure 1. 3-IPC concentration from top to bottom: 3.90×10^{-5} M; 8.26×10^{-5} M; 1.35×10^{-4} M; and 2.06×10^{-4} M.

150
100
50
0
5
10
$\frac{1}{v} \times 10^{-6}$ (MOLE^{-1} MIN)
$\frac{1}{O_2} \times 10^{-3}$ (M^{-1})

Figure 3. Product inhibition with 3-IPC as the variable substrate at low oxygen concentration (1.4×10^{-4} M). The reaction conditions were the same as those of Figure 1. Concentration of product from bottom to top: 0; 7.10×10^{-5} M; 1.42×10^{-4} M; and 2.13×10^{-4} M. Enzyme, 23 µg per cuvette.

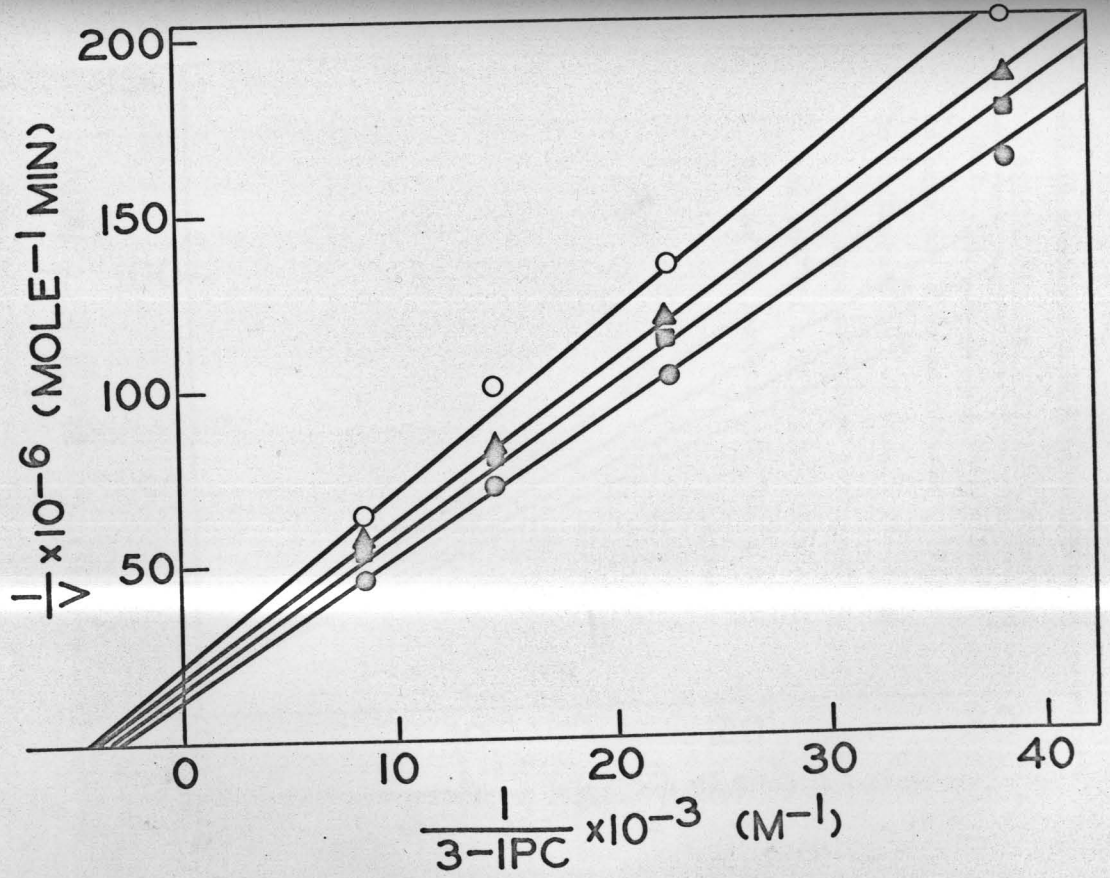

200
150
100
50
$\frac{1}{V} \times 10^{-6}$ (MOLE^{-1} MIN)
0
10
20
30
40
$\frac{1}{3\text{-IPC}} \times 10^{-3}$ (M^{-1})

Figure 4. Product inhibition with 3-IPC as the variable substrate at high oxygen concentration (2.8×10^{-4} M). The reaction conditions were the same as those of Figure 1. Concentration of product from bottom to top: 0; 6.94×10^{-5} M; 1.39×10^{-4} M; and 2.08×10^{-4} M. Enzyme, 50 µg per cuvette.

150
100
50
0
10
20
30
$\frac{1}{V} \times 10^{-6}$ (MOLE^{-1} MIN)
$\frac{1}{3\text{-IPC}} \times 10^{-3}$ (M^{-1})

Figure 5. Replots from Figure 3 of slopes and intercepts versus product concentration.

INTERCEPT x 10-6 (●)
SLOPE x 10-3 (▲)
P x 10^5 (M)
24
20
16
12
8
4
0
6.0
5.0
4.0
3.0
2.0
1.0
10
20

Figure 6. Product inhibition with oxygen as the variable substrate. The reaction systems were similar to those of Figure 1, except 1.95×10^{-4} M of 3-IPC was the fixed substrate. Product concentration from bottom to top: 0; 9.46×10^{-5} M; 1.51×10^{-4} M; and 2.08×10^{-4} M. Enzyme, 27 µg per cuvette.

50
25
0
2.5
5.0
7.5
$\frac{1}{v} \times 10^{-6}$ (MOLE^{-1} MIN)
$\frac{1}{O_2} \times 10^{-3}$ (M^{-1})

Figure 7. Replots from Figure 6 of slopes and intercepts versus product concentration.

SLOPE x10−3 (▲)
6.0
5.0
4.0
3.0
2.0
1.0
INTERCEPT x 10−6 (●)
12
10
8
6
4
2
0
10
20
P x10^5 (M)

raised, the intersecting point lies closer to the vertical axis (Figure 4) reminiscent of a competitive inhibition pattern. With oxygen as the variable substrate the product also gave linear noncompetitive inhibition (Figures 6 and 7).

Dead-end inhibition studies - Since the observed product inhibition pattern failed to yield an unequivocal answer as to the order of the addition of substrates, dead-end inhibition studies were undertaken in an attempt to unravel the problem. The structural analog, 4-isopropylcatechol (4-IPC), was selected as the dead-end inhibitor since it had been established that this compound was not oxidized at a significant rate by the enzyme. Using 3-IPC as the variable substrate, 4-IPC gave a competitive inhibition pattern as expected (Figure 8). However, when oxygen was used as the variable substrate, 4-IPC gave a uncompetitive inhibition pattern (Figure 9).

Figure 8. Dead-end inhibition by 4-IPC with 3-IPC as the variable substrate at fixed concentration of oxygen (2.8×10^{-4} M). The reaction conditions were the same as those of Figure 1. 4-IPC concentration from bottom to top: 0; 1.65×10^{-4} M; 3.30×10^{-4} M; and 6.60×10^{-4} M. Enzyme, 27 µg per cuvette.

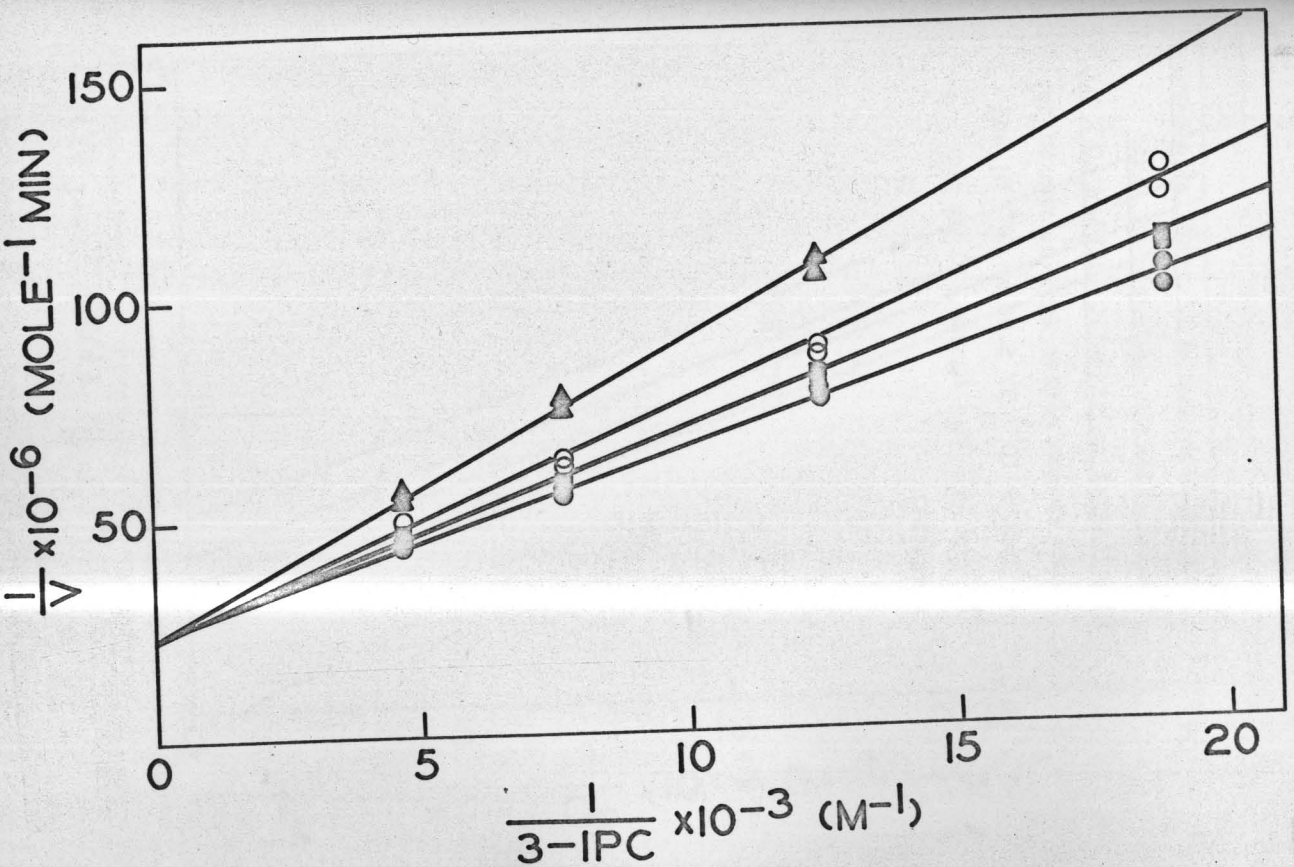
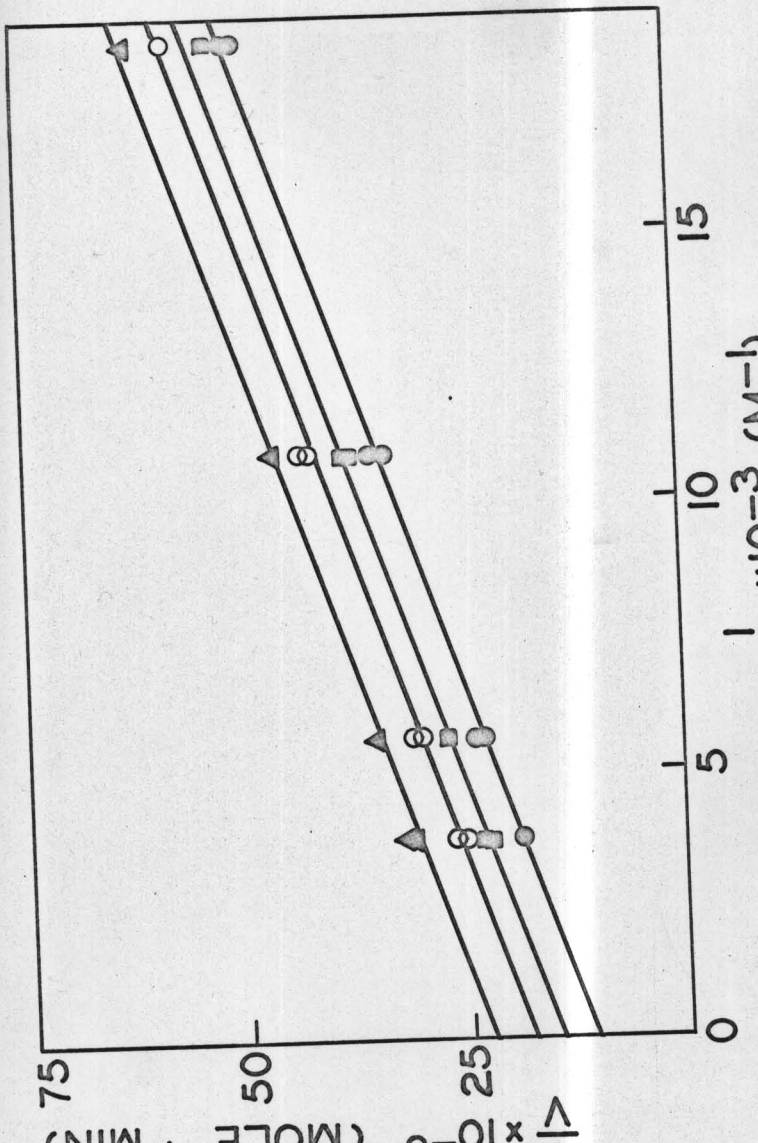
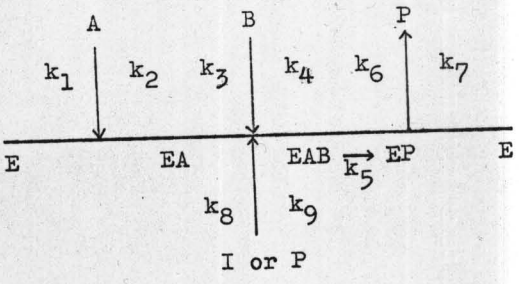

150
100
50
0
5
10
15
20
$\frac{1}{V} \times 10^{-6}$ (MOLE^{-1} MIN)
$\frac{1}{3\text{-IPC}} \times 10^{-3}$ (M^{-1})

Figure 9. Dead-end inhibition by 4-IPC with oxygen as the variable substrate at fixed concentration of 3-IPC (1.03×10^{-4} M). 4-IPC concentration from bottom to top: 0; 3.55×10^{-4} M; 5.68×10^{-4} M; and 8.52×10^{-4} M. Enzyme, 31 µg per cuvette.


75
50
25
0
5
10
15
$\frac{1}{V} \times 10^{-6}$ (MOLE^{-1} MIN)
$\frac{1}{O_2} \times 10^{-3}$ (M^{-1})

DISCUSSION

The initial velocity, product inhibition and dead-end inhibition patterns are consistent with the following sequential ordered Bi-Uni mechanism:



(8)

This mechanism requires that both substrates be added to the enzyme before the product is released, and that the addition of substrates follows an obligatory order. The interconversion of central complexes is virtually irreversible for this type of oxygenation.

Derivation of the steady-state rate equation for mechanism 8 can be obtained from Figure 10, an alternate expression of mechanism 8, by application of the King and Altman method (131).

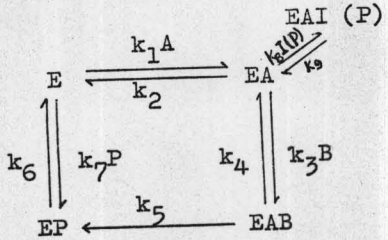


Figure 10

The steady-state rate equation for the above mechanism can be written in the form of equation 9.

$$v = \frac{VAB}{K_{ia}K_b + K_bA + K_aB + AB + \frac{K_{ia}K_b}{K_{ip}}P + \frac{K_a}{K_{ip}}BP + \frac{K_b}{K_{ii}}AI} \qquad (9)$$

By assuming the concentrations of product and inhibitor equal to zero, equation 9 becomes the familiar Bi-substrate sequential initial velocity equation 10.

$$v = \frac{VAB}{K_{ia}K_b + K_bA + K_aB + AB} \qquad (10)$$

Taking the reciprocal form of equation 10 and after rearranging, one obtains equations 11 and 12: B is the changing fixed substrate when A is varied.

$$\frac{1}{v} = \frac{K_a}{V}\left(1 + \frac{K_{ia}K_b}{K_aB}\right)\left(\frac{1}{A}\right) + \frac{1}{V}\left(1 + \frac{K_b}{B}\right) \qquad (11)$$

A is the changing fixed substrate when B is varied.

$$\frac{1}{v} = \frac{K_b}{V}\left(1 + \frac{K_{ia}}{A}\right)\left(\frac{1}{B}\right) + \frac{1}{V}\left(1 + \frac{K_a}{A}\right) \qquad (12)$$

The slope of the two equations is respectively

$$\frac{K_a}{V}\left(1 + \frac{K_{ia}K_b}{K_aB}\right)$$

and

$$\frac{K_b}{V}\left(1 + \frac{K_{ia}}{A}\right)$$

for A or B as the variable substrate. These slopes are a function of the non-varied substrate. Therefore, intersecting reciprocal plots will be observed. Figures 1 and 2 show intersecting patterns for varying 3-IPC and O_2, and thus they are compatible with a sequential mechanism for this enzyme as would be expected for this type of oxygenase reaction. It is noteworthy that $K_{ia}/K_a \simeq 1$, as evidenced by the appearance of the intersecting point virtually being on the horizontal axis. This suggests that the apparent Michaelis constant is independent of the concentration of the fixed substrate used or the apparent Michaelis constant is equal to the limiting Michaelis constant.

It is more convenient to analyze the product and dead-end inhibition data, by converting equation 9 into its reciprocal form (equations 13 and 14).

A varies

$$\frac{1}{v} = \frac{K_a}{V}\left(1 + \frac{K_{ia}K_b}{K_aB}\right)\left(1 + \frac{P}{K_{ip}}\right)\left(\frac{1}{A}\right) + \frac{1}{V}\left[1 + \frac{K_b}{B}\left(1 + \frac{I}{K_{ii}}\right)\right] \qquad (13)$$

B varies

$$\frac{1}{v} = \frac{K_a}{V}\left[\left(1 + \frac{I}{K_{ii}}\right) + \frac{K_{ia}}{A}\left(1 + \frac{P}{K_{ip}}\right)\right]\left(\frac{1}{B}\right) + \frac{1}{V}\left[1 + \frac{K_a}{A}\left(1 + \frac{P}{K_{ip}}\right)\right]$$

(14)

When we are dealing with simple product inhibition, we can set I = 0 in equations 13 and 14 to yield the corresponding equations 15 and 16.

P inhibiting, A varies

$$\frac{1}{v} = \frac{K_a}{V}\left(1 + \frac{K_{ia}K_b}{K_a B}\right)\left(1 + \frac{P}{K_{ip}}\right)\left(\frac{1}{A}\right) + \frac{1}{V}\left(1 + \frac{K_b}{B}\right) \qquad (15)$$

P inhibiting, B varies

$$\frac{1}{v} = \frac{K_b}{V}\left[1 + \frac{K_{ia}}{A}\left(1 + \frac{P}{K_{ip}}\right)\right]\left(\frac{1}{B}\right) + \frac{1}{V}\left[1 + \frac{K_a}{A}\left(1 + \frac{P}{K_{ip}}\right)\right]$$

(16)

Equation 15 predicts linear competitive inhibition at fixed levels of B whereas equation 16 predicts linear noncompetitive inhibition at fixed levels of A. The product inhibition pattern observed in Figure 3 or Figure 6 apparently does not conform to that predicted by equation 15, indicating that the mechanism is considerably more complicated.

Dead-end inhibition by product(s) has been observed in many cases, especially when the product is structurally similar to one of the substrates. Cleland (127,132) has dealt with this type of mixed dead-end and product inhibition kinetically. Thus, it appears that the product not only combines with the enzyme form, E, but is also competing for another enzyme form with the substrate, giving rise to dead-end inhibition.

On the basis of the above considerations, if we assume A is molecular oxygen, B, the organic substrate (3-IPC) and set I = P in equations 13 and 14, we obtain

P inhibiting, A varies

$$\frac{1}{v} = \frac{K_a}{V}\left(1 + \frac{K_{ia}K_b}{K_aB}\right)\left(1 + \frac{P}{K_{ip}}\right)\left(\frac{1}{A}\right) + \frac{1}{V}\left[1 + \frac{K_b}{B}\left(1 + \frac{P}{K_{ip}}\right)\right] \qquad (17)$$

P inhibiting, B varies

$$\frac{1}{v} = \frac{K_b}{V}\left[\left(1 + \frac{P}{K_{ip}}\right) + \frac{K_{ia}}{A}\left(1 + \frac{P}{K_{ip}}\right)\right]\left(\frac{1}{B}\right) + \frac{1}{V}\left[1 + \frac{K_a}{A}\left(1 + \frac{P}{K_{ip}}\right)\right] \qquad (18)$$

Equations 17 and 18 both predict linear noncompetitive inhibition patterns at unsaturated levels of the fixed

substrate. The product inhibition patterns observed in Figures 3 through 7 do conform to the patterns predicted by equations 17 and 18.

At low oxygen concentration (1.4×10^{-4} M), equivalent to 0.8-fold of Km, the pattern is clearly noncompetitive (Figure 3). When the oxygen concentration is raised to 2.8×10^{-4} M, equivalent to 1.6-fold of Km, the pattern now changes over to a competitive pattern. Replot of slopes and intercepts are all linear functions. The symmetry of equations between equation 17 and 18 allows the same interpretation for the reverse order of substrate additions, but the product in this case would be the dead-end inhibitor for E-3-IPC, which is quite unlikely. This possibility is eliminated by the dead-end inhibition studies. Both the slopes and the intercepts are linear functions of the product concentrations rather than parabolic functions, as observed in other examples of mixed dead-end product inhibitions (133-135), because the ring fission reaction is virtually an irreversible process.

For dead-end inhibition, simply set P = 0 into equations 13 and 14.

A varies, B is constant,

$$\frac{1}{v} = \frac{K_a}{V}\left(1 + \frac{K_{ia}K_b}{K_a B}\right)\left(\frac{1}{A}\right) + \frac{1}{V}\left[1 + \frac{K_b}{B}\left(1 + \frac{I}{K_{ii}}\right)\right] \qquad (19)$$

B varies, A is constant,

$$\frac{1}{v} = \frac{K_b}{V}\left[\frac{K_{ia}}{A} + \left(1 + \frac{I}{K_{ii}}\right)\right]\left(\frac{1}{B}\right) + \frac{1}{V}\left(1 + \frac{K_a}{A}\right) \qquad (20)$$

Equation 19 predicts linear uncompetitive inhibition whereas equation 20 predicts linear competitive inhibition. The dead-end inhibition patterns observed in Figures 8 and 9 are consistent with the above predictions only if A is molecular oxygen and B is the organic substrate.

The possibility of the dead-end inhibitor, 4-IPC, combining with the central complex, EAB, is ruled out by the prediction that linear uncompetitive patterns will be observed for either substrates as the variable substrate. The possibility of the product combining with the central complex, EAB, in addition to E, is also ruled out by the prediction that noncompetitive inhibition will be observed with respect to the first substrate added, and uncompetitive inhibition will be observed with respect to the other substrate.

The kinetic mechanism actually can be further substantiated by the use of dead-end inhibitors, competitive to molecular oxygen. This kind of inhibitor should yield noncompetitive patterns with respect to the organic substrate. o-Phenanthroline, a ferrous ion chelator, was expected to compete with

molecular oxygen for the ferrous iron site. However, the observed kinetic pattern is actually noncompetitive with respect to both oxygen and organic substrate, and it remains to be clarified whether o-phenanthroline combines with the ferrous iron site of the enzyme.

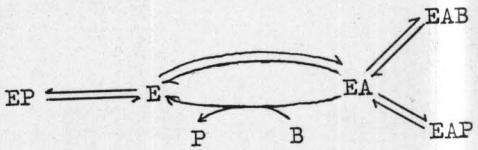
Aside from organic compounds, we have been searching for a gas which would be competitive to oxygen. Carbon monoxide, a ferrous iron binding gas in hemoproteins, was tested, but no inhibition was observed. This is not surprising since steroid dioxygenase is a nonheme iron enzyme, which may differ in behavior from heme-containing oxygenases.

Nitric oxide (NO), has one electron in one of its antibonding orbitals, which is expected to behave very similarly to oxygen (oxygen has two parallel spin electrons in the antibonding orbitals). Recently, Senoh, et al. (136) have demonstrated NO could exhibit similar ESR signal in the presence of organic substrate to that produced by oxygen in their studies with 3,4-dihydroxyphenylacetate-2,3-dioxygenase. Unfortunately, the reaction between NO and O_2 in buffered solutions is so rapid that no steady-state kinetic experiments can be conducted.

In order to prove that the mechanism of steroid dioxygenase is indeed ordered as depicted in mechanism 8, it is necessary to examine all other possible mechanisms

giving a rate equation similar to equation 9 and eliminate them from consideration.

Simple Theorell-Chance mechanism is ruled out simply because no competitive product inhibition pattern is observed with respect to either substrate. Equation 9 is also given by a Theorell-Chance mechanism in which dead-end EAB and EAP ternary complexes are formed. However, they do not participate in the reaction, but can only break down again to EA.



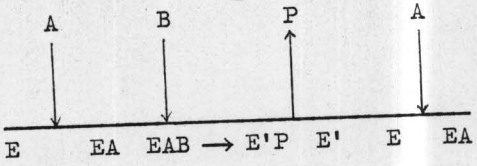
In view of the high improbability of the absence of central complexes, this mechanism seems unlikely. Nevertheless, there is no way of distinguishing this mechanism from the ordered one by steady-state kinetics.

Rapid equilibrium random mechanism is also unlikely because no competitive product inhibition pattern is observed with either substrates as the variable substrate.

Random mechanism is another possibility for this dioxygenase catalyzed reaction. As pointed out by Cleland (126,135), reciprocal plots for the Bi-substrate random mechanism are always 2/1 functions, and the

product inhibition patterns are always noncompetitive with respect to either substrate regardless of the saturation level of the other. Although there are some indications of curvature near the vertical axis in the present case, replots are linear functions of the product, and nearly competitive product inhibition is noted at high concentrations of oxygen. These results are not consistent with a random mechanism at least at the concentration range studied. Most enzymic reactions have some percentage of randomness; evidently, this enzyme is no exception. Particularly at high concentrations of organic substrate, an alternate reaction sequence could be operating. It is probably valid to say for the case discussed here that the mechanism is predominantly ordered and that the contribution of random addition is small.

Another possible ordered mechanism, Iso-ordered Bi-Uni can also be ruled out by the following consideration.



Simple Iso-ordered Bi-Uni mechanism predicts noncompetitive product inhibition with respect to

either substrates as observed, but raising the concentration of oxygen should change the noncompetitive pattern into an uncompetitive pattern, which is not what we observed.

Iso-ordered Bi-Uni mechanism with dead-end complex EAP also predicts noncompetitive inhibition with respect to either substrate, but the inhibition pattern should change to uncompetitive when oxygen concentration is raised, but this again is not what is observed.

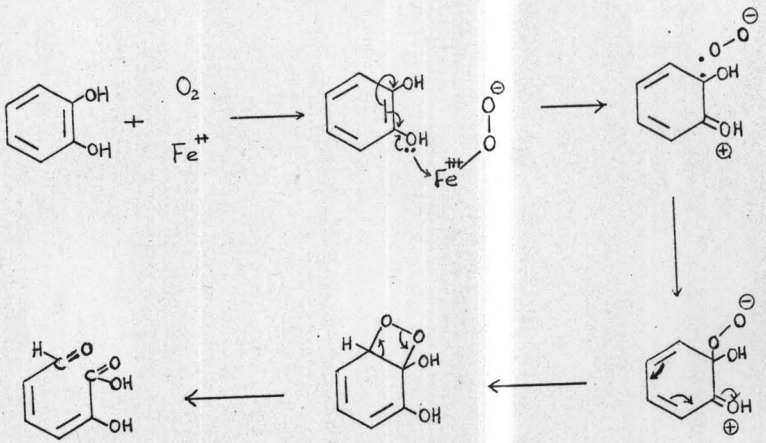
From the above discussions, it is reasonable to conclude that the most probable kinetic mechanism for steroid dioxygenase is an ordered Bi-Uni mechanism where oxygen is added first, followed by the organic substrate, and the product is then released, as depicted in mechanism 8 or Figure 10.

Recently, Hayaishi has suggested that the reverse order of addition of substrates is more likely for dioxygenases in general based on binding experiments for metapyrocatechase (14,28) and fast reaction kinetics for tryptophan pyrrolase (28). As pointed out by Hayaishi (28), the primary site of substrate binding does not seem to involve iron itself and the initial binding of the organic substrate to the enzyme may render the iron more accessible to molecular oxygen. However, this proposition does not necessarily mean that the organic substrate has to be added first at the catalytic site. It is possible that the organic

substrate generates a conformational change, so that the catalytic center can be properly oriented to activate incoming oxygen and substrate. ESR studies with 3,4-dihydroxyphenylacetate-2,3-dioxygenase (12,136) along with binding experiments with metapyrocatechase (14,28) seem to support this observation. The native enzyme does not show any typical signal in either the presence or absence of oxygen, nor does it show any signal ($g = 4.2$) under strictly anaerobic conditions in the presence of the organic substrate. Once the organic substrate is present in excess, introduction of limited amounts of oxygen intermittently could repeatedly exhibit the $g = 4.2$ signal.

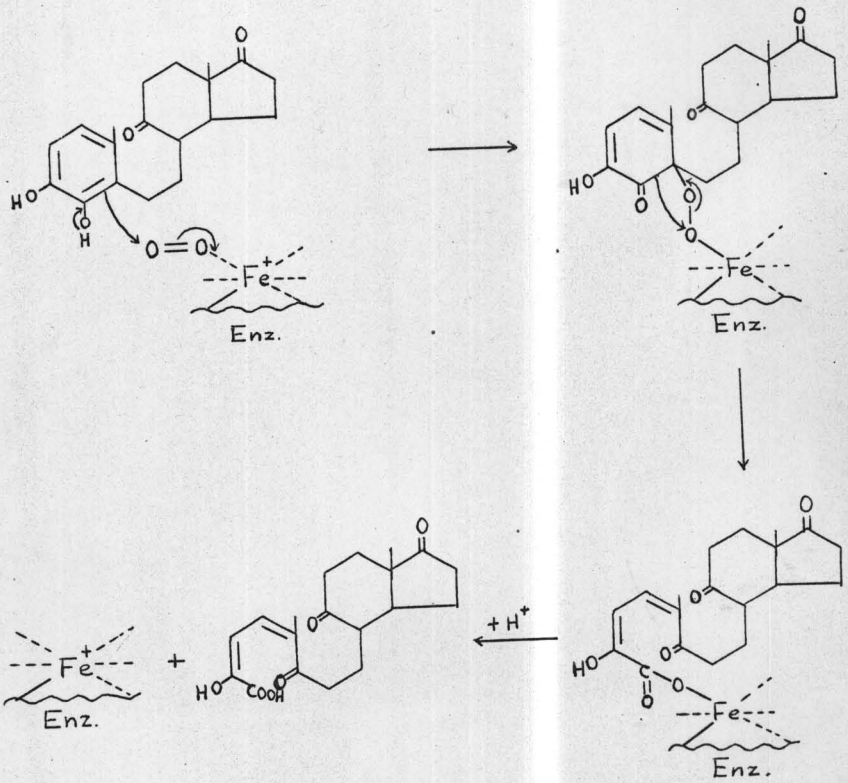
As to the reaction mechanism concerning the activation of oxygen (or substrate), the nature of reaction (free radical or ionic) and the structure of intermediate(s), no definitive conclusion can be drawn as yet.

A majority of dioxygenases are involved in the oxidative cleavage of aromatic nuclei. They contain either heme or nonheme iron in their active center and it has been tacitly assumed that oxygen binds with iron and is activated in some manner. From ESR studies, Hayaishi (2) proposed a radical mechanism for the metapyrocatechase reaction which may be envisaged as follows:



The so-called "active oxygen" in his mechanism is the perferryl iron complex, $Fe^{III}-O_2^-$ (formally Fe^{VI}) and the reaction proceeds via a four-membered ring peroxide intermediate. But Hamilton (137) claims that such an intermediate seems energetically unfavorable, for not only is the aromatic resonance lost but the strain in the four-membered ring would be very large. Based on chemical analogy of intramolecular rearrangement of peresters (138), Hamilton (137) proposed an ionic mechanism involving ferrous iron

acting as an acid in withdrawing electrons from oxygen, which in turn withdraws electrons from aromatic substrates. A similar mechanism is preferred by us, which can be written for steroid dioxygenase, but it does not involve a seven-membered ring lactone intermediate, as suggested by Hamilton.



In view of the resemblances between the action of some dioxygenases and photosensitized oxygenations, an alternative possibility would be the involvement of singlet oxygen as the reactive species (139-143). Although the lowest singlet level, $^1\Delta_g$, lies some 23 Kcal above the ground state (144), it would not be unreasonable to assume that the high energy requirement and spin change could be overcome by complexation of the oxygen with the metal of the enzyme. However, it is difficult to envisage the dissociation of enzymatically bound oxygen in order to generate the highly reactive, singlet oxygen. On the other hand, the enzymatically bound oxygen may well be diamagnetic in character. Collman (145) has shown that diamagnetic oxygen complexes can oxygenate substrates under unusually mild conditions.

REFERENCES

1. Hayaishi, O., Rothberg, S. and Mehler, A. H., Abstracts of the 130th Meeting of the American Chemical Society, Atlantic City, New Jersey, 1956, p. 53C.

2. Hayaishi, O., Proceedings of the 6th International Congress of Biochemistry, Plenary Sessions, New York, N.Y., I.U.B., v. 33, p. 31 (1964).

3. Mehler, A. H., in O. Hayaishi (Editor), Oxygenases, Academic Press, Inc., New York, N.Y., 1962, p. 87.

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

5. Olson, J. A. and Hayaishi, O., Proc. Natl. Acad. Sci., 54, 1364 (1965).

6. Goodman, D. S., Huang, H. S., Kanai, M. and Shiratori, T., J. Biol. Chem., 242, 3543 (1967).

7. Nakajima, H., J. Biol. Chem., 238, 3797 (1963).

8. Itada, N., Biochem. Biophys. Res. Commun., 20, 149 (1965).

9. Nozaki, M., Fujisawa, H. and Kotani, S., Proc. Intl. Congr. Biochem., 7th, Tokyo, 1967, p. 565.

10. Nozaki, M., Kojima, Y., Nakazawa, T., Fujisawa, H., Ono, K., Kotani, S., Hayaishi, O. and Yamano, T., in Biological and Chemical Aspects of Oxygenases, K. Bloch and O. Hayaishi, eds., p. 347, Maruzen Co., Japan, 1966.

11. Nakazawa, T., Kojima, Y., Fujisawa, H., Nozaki, M., Hayaishi, O. and Yamano, T., J. Biol. Chem., 240, PC3224 (1965).

12. Senoh, S., Kita, H. and Kamimoto, M., in Biological and Chemical Aspects of Oxygenases, K. Bloch and O. Hayaishi, eds., p. 378, Maruzen Co., Japan, 1966.

13. Nozak, M., Ono, K., Nakazawa, T., Kotani, S. and Hayaishi, O., J. Biol. Chem., 243, 2682 (1968).

14. Nozaki, M., Nakazawa, T., Fujisawa, H., Kotani, S., Kojima, Y. and Hayaishi, O., Advances in Chemistry Series, 77, 242 (1968).

15. Hayaishi, O. and Hashimoto, K., J. Biochem., 37, 371 (1950).

16. Hayaishi, O., Katagiri, M. and Rothberg, S., J. Biol. Chem., 229, 905 (1957).

17. Nakazawa, H., Inoue, H. and Takeda, Y., J. Biochem., 54, 65 (1963).

18. Hayaishi, O., in T. E. King, H. S. Mason and M. Morrison (Editors), Oxidases and Related Redox Systems, John Wiley and Sons, Inc., New York, N.Y., p. 286 (1965).

19. Kojima, Y., Fujisawa, H., Nakazawa, A., Nakazawa, T., Kanetsuna, F., Taniuchi, H., Nozaki, M. and Hayaishi, O., J. Biol. Chem., 242, 3270 (1967).

20. Nakazawa, A., Kojima, Y. and Taniuchi, H., Biochim. Biophys. Acta, 147, 189 (1967).

21. Watari, H., Nakazawa, T., Yamano, T. and Hayaishi, O., in Biological and Chemical Aspects of Oxygenases, K. Bloch and O. Hayaishi, eds., p. 369, Maruzen Co., Japan, 1966.

22. Stanier, R. Y. and Ingraham, J. L., J. Biol. Chem., 210, 799 (1954).

23. Ornston, L. N., J. Biol. Chem., 241, 3787 (1966).

24. Cain, R. B. and Cartwright, N. J., Biochim. Biophys. Acta, 37, 197 (1960).

25. Gross, S. R., Gafford, R. D. and Tatum, E. L., J. Biol. Chem., 219, 781 (1956).

26. Hayaishi, O., Bacteriol. Rev., 30, 720 (1966).

27. Fujisawa, H. and Hayaishi, O., J. Biol. Chem., 243, 2673 (1968).

28. Hayaishi, O., Ann. N. Y. Acad. Sci., 158, 318 (1969).

29. Dagley, S., Stopher, D. A., Biochem. J., 73, 169 (1959).

30. Kojima, Y., Itada, N. and Hayaishi, O., J. Biol. Chem., 236, 2223 (1961).

31. Nozaki, M., Kagamiyama, H. and Hayaishi, O., Biochem. Biophys. Res. Commun., 11, 65 (1963).

32. Nozaki, M., Kagamiyama, H. and Hayaishi, O., Biochem. Z., 338, 582 (1963).

33. Cain, R. B. and Farr, D. R., Biological and Chemical Aspects of Oxygenases, K. Bloch and O. Hayaishi, eds., p. 125, Maruzen Co., Japan, 1966.

34. Ishimura, Y., Nozaki, M., Hayaishi, O., Tamura, M. and Yamazaki, I., J. Biol. Chem., 242, 2574 (1967).

35. Dagley, S. and Patel, M. D., Biochem. J., 66, 227 (1957).

36. Stanier, R. Y., Palleroni, N. J. and Doudoroff, M., J. Gen. Microbiol., 43, 159 (1966).

37. Dagley, S., Evans, W. C. and Ribbons, D. W., Nature, 188, 560 (1960).

38. Ono, K., Nozaki, M., Hayaishi, O. and Senoh, S., The 17th Symposium on Enzyme Chemistry (at Tokushima, 1965); abstracts of papers, p. 37.

39. Cain, R. B., Nature, 193, 842 (1962).

40. Wheelis, M. L., Palleroni, N. J. and Stanier, R. Y., Arch. Microbiol., 59, 302 (1967).

41. Dagley, S., Geary, P. J. and Wood, J. M., Biochem. J., 109, 559 (1968).

42. Ribbons, D. W., Biochem. J., 99, 309 (1966).

43. Ribbons, D. W., Watkinson, R. J., Advances in Chemistry Series, 77, 252 (1968).

44. Adachi, K., Takeda, Y., Senoh, S. and Kita, H., Biochim. Biophys. Acta, 93, 483 (1964).

45. Kita, H., Kamimoto, M., Senoh, S., Adachi, K. and Takeda, Y., 6th International Congress of Biochem. (New York), Abstracts IV-155, p. 333 (1964).

46. Kita, H., Kamimoto, M., Senoh, S., Adachi, K. and Takeda, Y., Biochem. Biophys. Res. Commun., 18, 66 (1965).

47. Dagley, S., Chapman, P. J. and Gibson, D. T., Biochem. J., 97, 643 (1965).

48. Dagley, S. and Geary, P. J., Biochim. Biophys. Acta, 167, 459 (1968).

49. Senoh, S., Imamoto, S., Maeno, Y., Yamashita, K., Matsui, M., Tokuyama, T., Sakan, T., Komamine, A. and Hattori, S., Tetrahedron Letters, no. 46, 3431, 3439 (1964).

50. Senoh, S., Nippon Kagaku Zasshi (J. Chem. Soc. Japan), 86, 1087 (1965).

51. Piattelli, M. and Minale, L., Phytochemistry, 3, 307, 547 (1964).

52. Piattelli, M., Minale, L. and Prota, G., Phytochemistry, 4, 125 (1965).

53. Piattelli, M., Minale, L. and Prota, G., Tetrahedron, 20, 2325 (1964).

54. Minale, L., Piattelli, M. and Nicolaus, R. A., Phytochemistry, 4, 593 (1965).

55. Kuno, S., Tashiro, M., Tanuichi, H., Horibata, K. and Hayaishi, O., Federation Proc., 20, 3 (1961).

56. Horibota, K., Tanuichi, H., Tashiro, M., Kuno, S., Hayaishi, O., Sakan, T., Tokuyama, T. and Senoh, S., Symposia on Enzyme Chemistry, Nankodo Press, 15, 117 (1961).

57. Evans, W. C., Fernley, H. N. and Griffiths, G., Biochem. J., 95, 819 (1965).

58. Davies, J. I., and Evans, W. C., Biochem. J., 91, 251 (1964).

59. Rogoff, M. H., J. Bact., 83, 998 (1962).

60. Sih, C. J., Wang, K. C., Gibson, D. T. and Whitlock, H. W., Jr., J. Am. Chem. Soc., 87, 1386 (1965).

61. Gibson, D. T., Wang, K. C., Sih, C. J. and Whitlock, H. W., Jr., J. Biol. Chem., 241, 551 (1966).

62. Bokman, A. H. and Schweigert, B. S., Arch. Biochem. Biophys., 33, 270 (1951).

63. Miyake, A., Bokman, A. H. and Schweigert, B. S., J. Biol. Chem., 211, 391 (1954).

64. Mehler, A. H., J. Biol. Chem., 218, 241 (1956).

65. Decker, R. H. and Leach, F. R., Federation Proc., 19, 8 (1960).

66. Iaccarino, M., Boeri, E. and Scardi, V., Biochem. J., 78, 65 (1961).

67. Decker, R. H., Kang, H. H., Leach, F. R. and Henderson, L. M., J. Biol. Chem., 236, 3076 (1961).

68. Vescia, A. and DiPrisco, G., J. Biol. Chem., 237, 2318 (1962).

69. DiPrisco, G., Vescia, A. and Boeri, E., Arch. Biochem., Biophys., 95, 400 (1961).

70. Ogasawara, N., Gander, J. E. and Henderson, L. M., J. Biol. Chem., 241, 613 (1966).

71. Crandall, D. I., Krueger, R. C., Anan, F., Yasunobu, K. and Mason, H. S., J. Biol. Chem., 235, 3011 (1960).

72. Adachi, K., Iwayama, Y., Tauoka, H. and Takeda, Y., Biochim. Biophys. Acta, 118, 88 (1966).

73. Suda, M. and Takeda, Y., J. Biochem. (Tokyo), 37, 381 (1950).

74. Tokuyama, K., J. Biochem. (Tokyo), 46, 1379 (1959).

75. Tokuyama, K., J. Biochem. (Tokyo), 46, 1453 (1959).

76. Tokuyama, K., J. Biochem. (Tokyo), 46, 1559 (1959).

77. Flamm, W. G. and Crandall, D. I., J. Biol. Chem., 238, 389 (1963).

78. Takemori, S., Furuya, E., Mihara, K. and Katagiri, M., in Biological and Chemical Aspects of Oxygenases, K. Bloch and O. Hayaishi, eds., p. 395, Maruzen Co., Japan, 1966.

79. Crandall, D. I., in T. E. King, H. S. Mason and M. Morrison (Editors), Oxidases and Related Redox Systems, John Wiley and Sons, Inc., New York, N.Y., p. 263 (1965).

80. Knox, W. E. and Mehler, A. H., J. Biol. Chem., 187, 419 (1950).

81. Tanaka, T. and Knox, W. E., J. Biol. Chem., 234, 1162 (1959).

82. Feigelson, P. and Greengard, O., J. Biol. Chem., 236, 153 (1961).

83. Greengard, O. and Feigelson, P., J. Biol. Chem., 237, 1903 (1962).

84. Ishimara, Y., Okazaki, T., Nakazawa, T., Ono, K., Nozaki, M. and Hayaishi, O., in K. Bloch and O. Hayaishi (Editors), Biological and Chemical Aspects of Oxygenases, Maruzen Co., Japan, 1966, p. 416.

85. Poillon, W. N., Maeno, H., Koike, K., Feigelson, P., J. Biol. Chem., 244, 3447 (1969).

86. Feigelson, P. and Maeno, H., Biochem. Biophys. Res. Commun., 28, 289 (1967).

87. Feigelson, P., Maeno, H., Poillon, W. and Rosenfeld, H., Proceedings Seventh International Congress of Biochemistry, 1967, III, p. 445.

88. Maeno, H. and Feigelson, P., J. Biol. Chem., 243, 301 (1968).

89. Maeno, H. and Feigelson, P., Biochemistry, 7, 968 (1968).

90. Burg, R. W., Rodwell, V. W. and Snell, E. E., J. Biol. Chem., 235, 1164 (1960).

91. Sparrow, L. G., Ho, P. P. K., Sundaram, T. K., Zach, D., Nyns, E. J. and Snell, E. E., J. Biol. Chem., 244, 2590 (1969).

92. Rodwell, V. W., Volcani, B. E., Ikawa, M. and Snell, E. E., J. Biol. Chem., 233, 1548 (1958).

93. Ikawa, M., Rodwell, V. W. and Snell, E. E., J. Biol. Chem., 233, 1555 (1958).

94. Sih, C. J., Lee, S. S., Tsong, Y. Y. and Wang, K. C., J. Biol. Chem., 241, 540 (1965).

95. Kosicki, G. W., Biochemistry, 7, 4299 (1968).

96. Gornall, A. G., Bardawill, C. S. and David, M. M., J. Biol. Chem., 177, 751 (1949).

97. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., J. Biol. Chem., 193, 265 (1951).

98. Warburg, O. and Christian, W., Biochem. Z., 310, 384 (1941).

99. Davis, B. J., Ann. N.Y. Acad. Sci., 121, 404 (1964).

100. Chrambach, A., Reisfeld, R. A., Wyckoff, M. and Zaccari, J., Anal. Biochem., 20, 150 (1967).

101. Moore, S. and Stein, W. H., in S. P. Colowick and N. O. Kaplan (Editors), Methods in Enzymology, vol. VI, Academic Press, New York, N.Y., 1963, p. 819.

102. Moore, S., J. Biol. Chem., 238, 235 (1963).

103. Beaven, G. H. and Holiday, E. R., Advan. Protein Chem., 7, 319 (1952).

104. Edelhoch, H., Biochemistry, 6, 1948 (1967).

105. Harvey, A. E., Jr., Smart, J. A. and Amis, E. S., Anal. Chem., 27, 26 (1955).

106. Ackers, G. K., Biochemistry, 3, 723 (1964).

107. Beers, R. F., Jr. and Sizer, I. W., J. Biol. Chem., 195, 133 (1952).

108. Gosting, L. J., Advan. Protein Chem., 11, 449 (1956).

109. Schachman, H. K., in S. P. Colowick and N. O. Kaplan (Editors), Methods in Enzymology, vol. IV, Academic Press, New York, N.Y., 1957, p. 32.

110. Yphantis, D. A., Biochemistry, 3, 297 (1964).

111. Kawahara, K. and Tanford, C., Biochemistry, 5, 1578 (1966).

112. Vinograd, J., Bruner, R., Kent, R. and Weigle, J., Proc. Natl. Acad. Sci. U.S., 49, 902 (1963).

113. Ehrenberg, A., Acta Chem. Scand., 11, 1257 (1957).

114. Svedberg, T. and Pedersen, K. O., The Ultracentrifuge, Oxford University Press, New York, N.Y., 1940.

115. Blair, J. E., Lenaz, G. and Haard, N. F., Arch. Biochem. Biophys., 126, 753 (1968).

116. Cohn, E. J. and Edsall, J. T., Proteins, Amino Acids, and Peptides as Ions and Dipolar Ions, Reinhold Publishing Co., New York, N.Y., 1943, p. 370.

117. Svedberg, T. and Pedersen, K. O., The Ultracentrifuge, Oxford University Press, New York, N.Y., 1940.

118. Edsall, J. T., in H. Neurath and K. Bailey (Editors), The Proteins, vol. 1, part B, Academic Press, New York, N.Y., 1953, chapter 7.

119. Perrin, F., J. Phys. Radium, 7, 1 (1936).

120. Westley, F., and Cohen, I., Biopolymers, 4, 201 (1966).

121. Siegel, L. M. and Monty, K. J., Biochem. Biophys. Res. Commun., 19, 494 (1965).

122. Andrews, P., Biochem. J., 91, 222 (1964).

123. Laurent, T. C. and Killander, J., J. Chromatography, 14, 317 (1964).

124. Mason, H. S., J. Am. Chem. Soc., 70, 138 (1948).

125. Wasser, A., Model 777 Laboratory Oxygen Analyzer, p. 12, Fullerton, Calif., Beckman Instruments, Inc.

126. Cleland, W.W., Biochim. Biophys. Acta, 67, 104 (1963).

127. Cleland, W. W., Biochim. Biophys. Acta, 67, 173 (1963).

128. Cleland, W. W., Nature, 198, 463 (1963).

129. Wilkinson, G. N., Biochem. J., 80, 324 (1961).

130. Cleland, W.W., Advan. Enzymol., 29, 1 (1967).

131. King, E. L. and Altman, C. J., J. Phys. Chem., 60, 1375 (1956).

132. Cleland, W. W., Biochim. Biophys. Acta, 67, 188 (1963).

133. Fromm, H. J. and Nelson, D. R., J. Biol. Chem., 237, 215 (1962).

134. Zewe, V. and Fromm, M. J., J. Biol. Chem., 237, 1668 (1962).

135. Wratten, C. C., Cleland, W. W., Biochemistry, 2, 935 (1963).

136. Kita, H., Miyake, Y., Kamimoto, M., Senoh, S. and Yamano, T., J. Biochem. (Tokyo), 66, 45 (1969).

137. Hamilton, G. A., Advan. Enzymol., 32, 55 (1969).

138. Gould, E. S., Mechanism and Structure in Organic Chemistry, Henry Holt and Company, New York, N.Y., 1959, p. 633.

139. Yoshida, Z., and Kato, M., J. Chem. Soc. Japan, Pure Chem. Sect., 75, 106, 109, 112 (1954).

140. Hayaishi, O., Rothberg, S., Mehler, A. H. and Saito, Y., J. Biol. Chem., 229, 889 (1957).

141. Harkness, D. R., Tsai, L. and Stadtman, E. R., Arch. Biochim. Biophys., 108, 323 (1964).

142. Matsuura, T., Matsushima, H. and Sakamoto, H., J. Am. Chem. Soc., 89, 6370 (1967).

143. Baldwin, J. E., Basson, H. H. and Krauss, H., Chem. Commun., p. 84 (1968).

144. Foote, C. S., Accounts Chem. Res., 1, 104 (1968).

145. Collman, J. P., Accounts Chem. Res., 1, 136 (1968).