Spring 1978

THE MECHANISM OF ACTION AND THE STATE OF COPPER IN HEXOSE OXIDASE

DANIEL ARTHUR KERSCHENSTEINER

THE MECHANISM OF ACTION AND THE STATE OF COPPER IN HEXOSE OXIDASE

by

DANIEL ARTHUR KERSCHENSTEINER

B.S., Syracuse University, 1971
M.S., Syracuse University, 1973

A THESIS

Submitted to the University of New Hampshire

In Partial Fulfillment of

The Requirements for the Degree of

Doctor of Philosophy

Graduate School

Department of Biochemistry

May, 1978

This thesis has been examined and approved.

Gerald L. Klippenstein

Thesis director, Gerald L. Klippenstein
Assoc. Prof. of Biochemistry

Miyoshi Ikawa

Miyoshi Ikawa, Prof. of Biochemistry

James A. Stewart

James A. Stewart, Assoc. Prof. of Biochem.

N. Dennis Chasteen

N. Dennis Chasteen, Assoc. Prof. of Chemistry

Edward K. Tillinghast

Edward Tillinghast, Assoc. Prof. of Zoology

Jan 13, 1978

Date

ACKNOWLEDGEMENTS

I wish to thank Dr. Gerald L. Klippenstein for his confidence in my ability and his encouragement and advice throughout the laboratory work and writing of this manuscript.

Recognition is also gratefully extended to Milt Misogianes for performing the atomic absorption analyses and to Nestor Holyk who was unselfish in performing the EPR experiments.

Special thanks are due to Dr. N. Dennis Chasteen for help in interpreting the EPR spectra and for providing the theoretical basis for copper dimer transitions.

This work was supported in part by a grant from the Agricultural Experiment Station, H-188.

Dedicated with love and appreciation to my wife, Martha

TABLE OF CONTENTS

TABLES

LIST OF FIGURES

Abstract

Hexose oxidase was purified from an aqueous extract of Chondrus crispus by first co-precipitating the enzyme with carrageenan with 2% $CaCl_2$. The precipitate was resolubilized with 2 M NaCl and the carrageenan selectively removed as its insoluble cetyltrimethylammonium complex. The hexose oxidase that remained was purified by DEAE-cellulose chromatography using a 0-0.5 M NaCl gradient or pH gradient of 6.8 - 4.0. A 54 fold purification with a recovery of 1% was obtained. A fraction having a specific activity of 36 U/mg showed one major band after disc gel electrophoresis. This band corresponded with a zone which stained for hexose oxidase activity with a specific stain developed for this purpose.

The enzyme had a molecular weight of 140,000 as determined from gel filtration, and contained two subunits of about 70,800 as determined from SDS-polyacrylamide gel electrophoresis. The isoelectric point of the protein was pH 4.40. The enzyme contained only 4% by weight carbohydrate. Trace metal analysis by atomic absorption showed a minimum of 4.0 mol copper and 5.3 mol iron per mol protein.

The kinetics of enzyme catalysis was studied polarographically in the presence and absence of each product. The data was consistent with a ping pong bi bi mechanism of hexose oxidation.
The kinetic constants determined from this analysis were:

$$K_m^{O_2},\ app = 22\text{-}33\ ppm$$

$$K_m^{glu} = 11\ mM$$

The inhibition constants determined with H_2O_2 were:

$$K_i^{O_2} = 1.4 \text{ mM}$$

$$K_i^{glu} = 3.0 \text{ mM}$$

The inhibition constants determined with D-glucono-δ-lactone were:

$$K_i^{O_2} = 0.44 \text{ M}$$

$$K_i^{glu} = 0.30 \text{ M}$$

The state of copper was determined by EPR analysis. A narrow signal of 79 G was observed at g = 2.075 with no hyperfine structure. The signal was considered to be either Cu (II) in the perpendicular region of the spectrum with hidden hyperfine structure, or exchange or weak dipole-dipole coupled copper ions. The copper ions are separated by 7.9 Å if dipolar coupling is presumed. The evidence for a dimeric structure came from spectral features which included an uv absorption at 330 nm and an EPR shoulder suggestive of a pair of perpendicular absorbances. By attributing the pair of resonances to coupled copper (II) ions, a very small D splitting of 2.81 x 10^{-3} cm^{-1} was calculated which could arise from electron-electron dipolar and/or exchange interactions.

Under an atmosphere of N_2 followed by glucose addition, the copper signal diminished by 47% (68 ± 2.4 to 36 ± 1), while the iron signal (g = 4.26) remained essentially unchanged (22 to 20). Copper

(II) was considered to be changing to Cu (I) under these conditions.

A mechanism was proposed which was consistent with the data obtained from the physical-chemical study and kinetic determinations. Oxygen binds to the copper dimers which are in the univalent state with the formation of peroxo-ion and 2 Cu(II). Hydrogen peroxide is released upon donation of protons from groups located on the protein. Glucose binds to the enzyme next, reducing the pairs of copper (II) to pairs of Cu (I) as was suggested by the diminished EPR signal shown under these conditions. The protons are replaced on the protein with D-glucono-δ-lactone formation, completing the cycle.

INTRODUCTION

A. Copper-Containing Proteins and Oxygen

Metalloproteins are particularly well-suited for the study of the mechanism of enzyme action since the unique physical-chemical characteristics of the metal ion constitute intrinsic probes in the region of the protein where it is bound (Vallee and Williams, 1968). Because most metal ions in these enzymes take an active part in the action of the enzyme, studies to determine the metal ion's environment, valence, and ligands at rest and during active catalysis, will enable certain predictions to be made about the order of events which make up the mechanism of the enzyme. Some studies can be carried out in the absence of substrate, thus eliminating the effects induced by the substrate binding, so that only the region in the enzyme involved directly with catalysis, the active center, is scrutinized, as the metal ion labels this site.

Many enzymes require metal ions either as a tightly bound cofactor or as a complex with substrate. Over 30% of the enzymes which had been studied up to 1964 have a metal ion requirement or are metalloproteins (Dixon and Webb, 1964). The most frequently observed metal ion cofactors are transition group elements, like copper and iron. These two metals serve as important cofactors for enzymes and proteins which utilize oxygen, the oxidases, oxygenases and oxygen-carriers. Their usefulness in enzymes can be attributed to the favorable chemical properties of metal ions in these reactions, the availability of copper and iron in sea water and the role each has played in the adaptation of organisms to an aerobic atmosphere (Friedean and Hsieh, 1976).

A listing of the more well-known copper containing proteins, their function and copper content (from Malmström, et al., 1975), shows that these proteins may contain more than one copper atom/mol protein and are active in the utilization of oxygen, as O_2 carriers, or as catalysts of oxidation reactions employing oxygen as the electron acceptor:

Protein	Function	Number of Copper atoms
Hemocyanin	O_2 transport	2/subunit
Superoxide dismutase	O_2^- dismutation	2
Cytochrome c oxidase	Oxidation of cytochrome c; terminal enzyme in respiration	2
Laccase	Oxidation of organic substrates	4
Tyrosinase		4-6
Galactose oxidase		1
Stellacyanin	Electron transport	1

Transition metal ions, such as copper, function in enzymes involved with oxygen reactions because they are capable of changing the electronic structure of oxygen, reducing the strength of the bond between the oxygen atoms. Oxygen possesses unusual stability because the molecule is held together by one two-electron bond and also by two three-electron bonds (Ingraham, 1966). To be used in oxidations the molecule must be activated in some way, weakening the strong bonds. One way in which metal ions such as copper can serve as activators is by partially or completely donating a readily available electron to the oxygen molecule. Ingraham (1966) notes that when a metal-oxygen complex is formed the oxygen donates a pair of electrons to the metal and the

metal donates an electron pair from a filled d-shell producing a double bond. As a result the oxygen atoms are now held together by just one single bond which is more readily broken. More importantly, the overall charge of the complex is positive and so electron addition for redox catalysis is favored. Transition metal ions, then, activate oxygen by forming complexes in which the electronic structure of oxygen is changed. Once activated, the addition of electrons for the purpose of reducing oxygen is favored by the net-positive charge of the complex.

Copper-containing enzymes which use oxygen as an electron acceptor can be classified as either producing hydrogen peroxide in a two electron reduction, or producing water in a four-electron change. Galactose oxidase catalyzes a two electron reduction of oxygen in which galactose is oxidized in the C-6 position with the formation of peroxide. The blue oxidases such as laccase catalyze the reduction of oxygen to water through a four electron oxidation of its phenolic substrate. There are some copper enzymes, such as tyrosinase, which catalyze a cleavage of molecular oxygen in which one atom is reduced to water while the other atom becomes incorporated into a phenolic product. Enzymes which catalyze this type of reaction are called mixed-function oxidases (Hayashi and Hashimoto, 1950).

A widely studied group of copper enzymes are the so-called blue oxidases. The enzymes, ascorbic acid oxidase, laccase and ceruloplasmin and the oxygen carrier protein hemocyanin, are examples of blue copper proteins. Upon deoxygenation or introduction of the second substrate to these proteins, the blue color bleaches (Lontie and Vanquickenborne, 1974). This phenomenon was interpreted

as a change occurring in the valence state of copper from Cu(II) to Cu(I) under reducing conditions and was supported by experiments using valence-specific copper-chelating agents (Dawson, 1966). The copper in these proteins is viewed as storing and conducting electrons from one substrate to the other.

The development of probing magnetic techniques, such as electron paramagnetic resonance spectroscopy (EPR) has enabled researchers to study the state of copper in these proteins with regard to its electronic and magnetic properties. Three different forms of copper (II) have been found in one enzyme, laccase, as a result of EPR study (Malmström, et al., 1968).

The copper in blue proteins as compared with simple chelates of copper was first thought to be different when it was found that the color was 5-10 times more intense than that of model complexes (Ingraham, 1966). It was concluded that the chemistry of copper found in the blue proteins is unlike any simple copper complex.

Studies of the magnetic properties of copper protein in the EPR analysis of laccase by Malmström and Vänngård and coworkers, uncovered much information about the state of the four coppers in this enzyme. The copper in this enzyme was viewed as consisting of one-half Cu(II) and one-half Cu(I), (Levine, 1966), since EPR integration studies showed that half the copper in laccase could be detected as Cu(II) while the other half was not detectable, (Malkin, et al., 1969), and assumed to be Cu (I), the most common diamagnetic form of copper (Malmström, 1965). From anaerobic titrations, however, it was found that laccase could accept four electrons, as many as it had copper ions, indicating that all the copper must be Cu(II) (Fee, et

al., 1969). Furthermore, the copper that was detected in EPR analysis consisted of two types. This conclusion was based on the magnetic splitting parameters of each form (Broman, et al., 1962). One form, called Type I copper, was found to be responsible for the intense blue color in these enzymes. It had a small hyperfine splitting constant indicative of severe electron delocalization (Malmström, et al., 1968). Type 2 copper had properties very similar to small chelates and could bind anionic intermediates such as peroxo anion in the action of laccase (Andreasson and Vänngård, 1970). Some maintain, however, that Type 2 copper is derived from denatured enzyme (Nakamura, 1976). Two ions failed to give an EPR signal and were thought at first to be Cu(I). This is not the only form of copper which is incapable of producing an EPR signal (Ochiai, 1977). Copper with a short relaxation time or an easily saturated transition resulting from copper being in a specific environment would not be expected to give an EPR signal. Antiferromagnetically coupled copper or low spin Cu(III) would give the same result. The EPR-nondetectable copper in laccase was found to be a pair of magnetically coupled Cu(II) ions. This conclusion was based on the discovery that these two EPR-silent ions in the enzyme accepted electrons in pairs (Malmström, et al., 1968). Laccase has a large absorption in the region of 330 nm, a feature consistent with a copper dimer (Malkin, et al., 1969). In addition, when laccase and other dimer-containing proteins were reacted with NO a broad absorption EPR spectrum at g=2 with forbidden transitions containing 7 hyperfine lines was produced, all of which are diagnostic of magnetically coupled copper (Mason, 1976). Thus three forms of copper were found in laccase: Type 1 was found to be responsible for

the intense blue color, Type 2 was found to be involved in anion binding, and Type 3 was a copper dimer which acted as an electron pair acceptor. Copper dimers have since been discovered in other blue oxidases, such as ascorbic acid oxidase (Lee and Dawson, 1973), and ceruloplasmin (Frieden and Hsieh, 1976), along with the other two copper forms. This suggests they are general features of the blue copper oxidases (Malmström, et al., 1975).

A copper dimer structure has been found in nonblue copper-containing proteins of which tyrosinase is one example. This multi-copper enzyme failed to show an EPR spectrum (Kertesz, 1966). But the enzyme absorbed light at 330 nm (Mason, 1976) and produced a spectrum attributable to dimeric copper upon reaction with nitrous oxide (Schoot Witerkamp and Mason, 1973). The existence of copper dimers in the blue and nonblue oxidases suggests that copper dimers may be a common feature in the transfer of pairs of electrons in these enzymes.

Another form of copper, Cu(III), has been implicated in the action of galactose oxidase, an enzyme which contains only one copper atom. On the basis of redox titrations, Hamilton (1976) has proposed that Cu(III) may be formed from Cu(I) in the resting enzyme by a two electron transfer. Occasionally, Cu(II) is formed when superoxide "leaks" out of the active enzyme, and this form is the only form detected by EPR spectroscopy.

An unusual copper-cysteine structure has been suggested by Williams and his coworkers (Byers, et al., 1973) to explain some features of blue oxidases. In this structure, a pair of Cu(I) atoms are coupled to a pair of cysteine sulfhydryls. Electron transfer would occur through the reversible cystine/cysteine formation. The evidence

against the involvement of this form in these blue oxidases has been provided by amino acid analysis which showed no differences in cysteine/cystine ratio between reduced or oxidized forms of the enzyme.

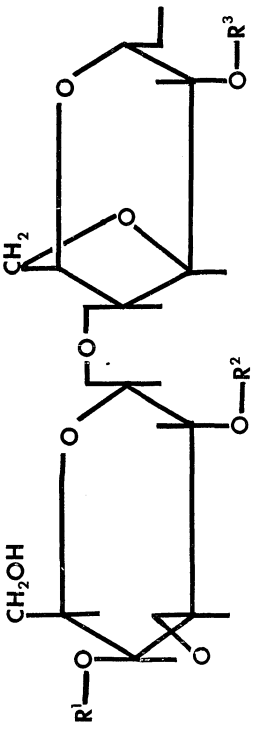
From this discussion of the various forms of copper that have been detected or postulated as working in the copper-containing proteins, it is apparent that the state of copper in these proteins is much more complex than was thought before the development of EPR analysis of biological systems. Much work is required before it becomes possible to understand the function that copper plays in these enzymes.

B. Hexose Oxidase and Carrageenan

Carrageenan is a polysulfated galactan which makes up a large part of the dry weight of Chondrus crispus. Because of its solubility and anionic nature, carrageenan was seen to influence the behavior of proteins in an aqueous extract of red algae. Since the purification of the multicopper enzyme, hexose oxidase (E.C. 1.1.3.5) was affected by the presence of this carbohydrate, a brief introduction of the properties and forms that this polysaccharide may be appropriate.

Carrageenan is a cell wall polysaccharide which makes up between 30 and 80% of the dry weight of Chondrus, depending upon the season (Whistler and Smart, 1953). The carbohydrate has been studied extensively and structural analysis indicates a backbone structure consisting of two galactose residues linked alternately $\alpha(1\text{-}4)$ and $\beta(1\text{-}3)$, with sulfate esters present on carbons 2 or 4 or both, depending on the class. It is widely recognized that there are at least three main classes of carrageenan found in the red algae: κ (kappa), ι

Figure 1: Repeating structure of carrageenan. The molecule consists of alternating $\alpha(1-4)$ and $\beta(1-3)$ linkages of D-galactose and 3-6 anhydro D-galactose units. Substituents R_1, R_2, or R_3 are either sulfate groups or protons depending upon the class of polysaccharide, κ, ι or λ. (From Haus, 1974.)



	k	ℓ	λ
R^1	SO_3H	SO_3H	H
R^2	H	H	SO_3H
R^3	H	SO_3H	SO_3H

(iota), and λ (lambda) carrageenans. These three classes differ in the degree of sulfation and in the amount of 3, 6 anhydro-D-galactose found in the 1-4 linked residue. These forms are illustrated in Figure 1. Carrageenan in fact does not consist of three homogeneous macromolecular species but is rather a family of polysaccharides which has a more or less continuous variation in molecular weight, extent of sulfation, and fraction of D-galactose residues in the 3, 6 anhydro form (Haus, 1974). These characteristics suggest a variation in properties and chemistry for the mixture of carrageenan as would be found in a Chondrus extract.

Carrageenan appears to exist in an ion-exchange equilibrium with the surrounding sea water (Haus, 1974). Like another polyanionic carbohydrate, agar, it is water soluble and capable of forming gels under certain conditions. The degree and position of the sulfate groups in the carbohydrate backbone have been shown to affect the gel-forming and ion-exchange ability of the polysaccharide (Haus, 1974). For example, 0.25% KCl will precipitate the κ-carrageenan fraction but λ-carrageenan remains in solution. Taken as a family of polysaccharides then, carrageenan as is found in Chondrus extracts can have a wide range of species which differ in ion-exchange capacity and gel-forming ability, making attempts to remove it from solution particularly difficult.

Bean and Hassid (1958) discovered an enzyme in Iridophycus flaccidum with properties similar to those of hexose oxidase and any carrageenan which may have been present in extracts of this algae would have been removed by the barium-methanol step used in their fractionation procedure.

The purpose of this work was to purify and characterize the enzyme hexose oxidase. The purification of the enzyme took advantage of the coprecipitation of the enzyme with carrageenan. Effort was then made to characterize the protein and to study the mechanism of action of the enzyme by kinetic analysis and by probing the active site by EPR spectroscopy.

MATERIALS AND METHODS

A. Preparation of Hexose Oxidase.

1. Algae Preparation

Chondrus crispus was collected in the intertidal zone at Hilton Park in Newington, New Hampshire. The freshly picked fronds were taken directly to the lab, sorted, and rinsed under cold running tap water for about thirty minutes. The washed material was then dried on a rack in a fume hood under a flow of air. The crisp, dried (about 16% moisture by weight) algae was ground to about 0.5 cm flakes with a Waring blender before storage in a freezer at -20°C.

2. Algae Extraction

One hundred grams of dried Chondrus was prepared for aqueous extraction by regrinding in a blender with pressure applied by hand using a dry sponge. This finely ground material was added to 1 liter of 10 mM sodium phosphate, pH 6.8. After stirring for at least 15 hours with an overhead stirrer at a speed fast enough to keep the flakes suspended, the brownish suspension was filtered under gentle suction through two layers of cheesecloth into a chilled filter flask. It was then centrifuged at 11,000 x g for 30 minutes at 4° C to remove insoluble material.

3. Carrageenan Precipitation

The bright red supernatant with a typical volume of 550 to 650 ml was placed in an ice-cold beaker and solid $CaCl_2 \cdot 2H_2O$ (J. T. Baker) was added gradually with stirring to a final concentration of 2% w/v. Throughout this addition, the pH was carefully maintained at

6.8 by addition of 1 M NaOH. After allowing to stand for 10 minutes, the precipitate which contained the enzyme was collected by centrifugation at 11,000 x g for 5 minutes. Normally, about 20 to 30 grams wet weight of precipitate was recovered. The supernatant, containing most of the red pigmented protein phycoerythrin, was discarded.

4. Precipitate Wash and Resuspension

The precipitate was washed with 50 ml of 0.1 M sodium phosphate, pH 6.8 for 10 minutes to remove easily solubilized contaminants. The pH of the slurry was monitored and maintained at 6.8. The suspension was centrifuged at 12,000 x g and the resulting supernatant, which contained little activity, was discarded while the washed precipitate was reextracted twice with 50 ml portions of 2 M NaCl dissolved in the 0.1 M sodium phosphate buffer. The suspension was centrifuged at 26,000 x g for 10 min and the two supernatants pooled. Twenty-five ml of 1.0% cetyltrimethylammonium bromide (CTAB Br, J. T. Baker) in water was added to the combined supernatants to 0.2% w/v, and allowed to stand for 15 minutes. The resulting precipitate of carrageenan was removed by centrifugation at 26,000 x g. The yellow-orange supernatant was dialyzed against two liters of 10 mM sodium phosphate pH 6.8, with two changes.

5. DEAE-cellulose Chromatography

After dialysis, the solution was centrifuged at 26,000 x g for 15 min to remove residual precipitate. The clear supernatant was then applied to a 1.5 x 12 cm column of Whatman DE-52 DEAE-cellulose (Reeve Angel) at a flow rate of 1.0 ml/min and washed with 0.1 M sodium phosphate

buffer, pH 6.8. After the sample was applied, the column was washed with this buffer until the effluent had no absorbance at 280 nm. Elution of the column was accomplished either with a pH gradient or a salt gradient established by 250 ml each of 0.1 M sodium phosphate buffer, pH 6.8, and 250 ml of 0.05 M sodium citrate, pH 4.0 or by a 0-0.5 M linear gradient of sodium chloride in 0.1 M sodium phosphate, pH 6.8 (500 ml total volume) respectively. A flow rate of 0.33 ml/min was used in the elution and 5 ml fractions were collected. Protein content in each fraction was estimated from the absorbance at 280 nm, the pH or NaCl conductance was measured, and hexose oxidase activity determined as described below. The most active fractions, generally those eluting between pH 5.9 and 4.9 or at 0.1 M NaCl were pooled and the pH adjusted to 6.2 with the addition of 0.2 M disodium hydrogen phosphate. The hexose oxidase solution was concentrated to about 10 ml by pressure ultrafiltration with an Amicon concentrator using a PM-10 membrane and N_2 at 20 psi. The concentrated hexose oxidase solution was dialyzed against 1 liter of 10 mM sodium phosphate, pH 6.2. The dialysis casing (Spectrapore, National Scientific) was boiled for 2 hr prior to use.

A. Analysis and Characterization of Hexose Oxidase.

1. Assay of Hexose Oxidase Activity

The activity of hexose oxidase was measured polarographically by the rate of oxygen conversion to hydrogen peroxide using glucose as the second substrate. A Clark-type oxygen electrode and meter, model 54 RC (Yellow Springs Instrument Co.), connected to a Heath chart

recorder was used. The sensor was covered by a thin (0.0005-0.001 inch) Teflon FEP membrane and fitted into a reaction vessel of standard kind for oxygen uptake studies but was custom made of glass to accept the larger size sensor. The reaction chamber, 4.86 ml in volume, was sealed with a ground glass stopper. The contents of the chamber were stirred at a constant rate with the aid of a magnetic micro stirring bar. The reaction chamber was enclosed by a water jacket and maintained at 25°C by a Haake-FJ circulating water bath. Before use, the meter was calibrated by setting it at 8.4 ppm when the cell contained distilled water which had been saturated with air at 25° under 1 atmosphere pressure. Buffered glucose substrate was placed in a glass cylinder located in the constant temperature water bath and water-saturated air was allowed to equilibrate the solution at constant temperature. Five ml of substrate solution was added to the reaction chamber, stirred at constant speed, and the excess removed by replacing the glass stopper. The chamber was examined for air bubbles, then the stopper was removed, 0.1 ml of substrate solution withdrawn and quickly replaced by 0.1 ml of enzyme solution. The dilution this makes to any substrate or inhibitor concentration present is near 2%. The stopper was replaced, the chamber reexamined for air bubbles, stirring speed checked, and the chart recorder started. One unit of enzyme activity is equivalent to an initial rate of oxygen consumption of one ppm min^{-1} mg^{-1}. Glucose concentrations used in this work are expressed as total D-glucose without regard to α- or β- anomer proportions.

2. Kinetic Analysis

Kinetic analysis of the enzyme reaction was determined using partially purified enzyme. The initial velocity of oxygen conversion was measured as described before. Normally, membranes capable of detecting oxygen in the 0-20 ppm range were used. In those experiments in which the concentration of oxygen was fixed at the level of water-saturated air, high sensitivity membranes (0-10 ppm) were used. For experiments which called for variable oxygen concentrations, pure oxygen was metered into the buffered glucose solution mixed with water-saturated air so that O_2 concentrations between 8.4 to 20 ppm were obtained.

Buffers used for studying the effect of pH on the rate were 0.1 M phosphate and citrate-phosphate, made in accordance with Gomori (1955). Solid glucose was added to a concentration of 0.1 M and the pH of each solution measured before use.

Inhibition of the rate of the forward reaction was done using hydrogen peroxide (30% w/v, Mallinckrodt) and D-glucono-δ-lactone (Sigma). In those experiments utilizing hydrogen peroxide, a stock solution of 1.8 M H_2O_2 in water was used to make 0.5, 1.0 and 2.0 mM H_2O_2 in 10 ml of 0.1 M buffered substrate solution. The final concentration of H_2O_2 was determined from its extinction coefficient of 43.6 M^{-1} cm^{-1} at 240 nm (Beers and Sizer, 1952). Not more than 20 µl of stock peroxide solution was added to 10 ml of substrate solution, so dilution was negligible. In those inhibition experiments utilizing D-glucono-δ-lactone, solid D-glucono-δ-lactone was added with stirring to 10 ml of 0.2 M buffered substrate solution to final concentrations of 0.05 M, 0.1 M and 0.2 M with no regard to volume changes. Five ml

of this solution was pipetted into the reaction chamber and the assay started as previously described. The time from the mixing of the inhibitor to the beginning of the recording trace was 0.8 min, during which the pH of the solution dropped from 6.21 to 6.14, from the spontaneous hydrolysis of the lactone.

To obtain an accurate measurement of the initial velocity the enzymatic conversion of oxygen to hydrogen peroxide was recorded at 1 cm/minute chart speed for about 5 minutes. The best straight line was drawn through the initial part of the record and two successive points of intersection were found between the line and the corners of the chart paper grid. The slope of the line was calculated by dividing the oxygen converted to the nearest tenth ppm, by the change in time to the nearest tenth of a minute.

3. Disc gel Electrophoresis

Analytical disc gel electrophoresis was carried out according to Davis (1964) using 7% acrylamide gels. Samples of protein were mixed with 40% sucrose and layered on the stacking gel; this in turn was layered with reservoir buffer which contained bromphenol blue as the tracking dye. Gels were run at 2-3 mA per gel tube until the dye front was 1 cm from the bottom. A temperature of 10°C was maintained by a flow of cold tap water throughout the run. The gels were taken out of their glass tubes and the position of the tracking dye was marked by a needle puncture. Gels were stained for either protein with 0.25% Coomassie Brilliant Blue in methanol: acetic acid: water (1:1:5), activity, or carbohydrate (vide infra).

4. Activity Stain

The activity of hexose oxidase was localized and quantitated on polyacrylamide gels by the following technique. Following electrophoresis the gels were immersed in 5 ml of 0.1 M glucose solution which had been saturated with oxygen. One tenth ml of a 1 mg/ml solution of horseradish peroxidase in water was then added to each tube. The tubes were mixed on a vortex mixer, and then 1 ml of 3 mg/ml solution of o-dianisidine·2 HCl in water was added. Upon development of the color (in about 30 minutes, depending on the activity applied), the incubation mixture was poured off and the colorimetric reaction halted by the addition of 7% acetic acid. The yellow-gold bands, stable to acetic acid, were quantitated with a scanning densitometer.

5. Molecular Weight Determination by Gel Filtration with Sephacryl S-200

Sephacryl S-200 (Pharmacia) was washed with 0.1 M sodium phosphate, pH 6.8, and the slurry poured into a 1.5 cm column to a final height of 96.5 cm. The gel was packed and equilibrated with a downward flow of the sodium phosphate buffer at a rate of 15 ml/hr or 8.2 ml/hr-cm^2. Calibration of the column was accomplished by measuring the elution volume of the following proteins: Bovine γ-globulin fraction (169,000), Aspergillus niger glucose oxidase (160,000), glucose-6-phosphate dehydrogenase (104,000), human hemoglobin (64,500), horseradish peroxidase (40,000), and α-chymotrypsinogen A (25,700).

6. SDS-Polyacrylamide gel Electrophoresis

This procedure was done as described by Weber and Osborn (1969). The concentration of acrylamide in the gels was 10% and samples and standards used were made up in SDS/2-mercaptoethanol

buffer and incubated at 42°C for 4 hours. After incubation, the proteins were mixed with bromphenol blue tracking dye and glycerol and this mixture was layered atop the SDS gels, and electrophoresis carried out at 4 mA per tube for 9 hours. The gels were sliced in the middle of the tracking dye front and stained for 15 minutes in hot 0.25% Coomassie Brilliant Blue. The gels were destained in a transverse destainer and the residual stain removed by diffusion in 7% acetic acid for several days. The following proteins, with their molecular weights, were used as molecular weight markers: Bovine serum albumin (68,000), glutamate dehydrogenase (53,000), pepsin (35,000) and α-chymotrypsinogen A (25,700).

7. Isoelectric Focusing on Acrylamide Gels

Isoelectric focusing on acrylamide gels was based on the method of Haglund (1971). Gels were 7% in acrylamide and 4% in ampholyte (Ampholine, LKB). Gelling solution (1.90 ml) was mixed with a 0.1 ml solution of protein before polymerizing with ultraviolet light. The gels were run for 6 hours at 400 V using 0.02 M NaOH as the catholyte solution and 0.01 M phosphoric acid as the anolyte solution. Afterward, the gels were either stained for activity or sliced into 0.5 cm sections in order to determine the pH profile.

8. Carbohydrate Analysis

Total reducible carbohydrate was determined by the method of Dubois et. al. (1956). Two ml of an aqueous sample containing between 10 to 70 µg of carbohydrate was mixed with 50 µl of 80% aqueous phenol. Five ml of concentrated sulfuric acid was added and after allowing to

stand for 1 hour, the absorbance at 490 nm was read. Glucose at concentrations of 10-70 µg/ml were used as standards.

9. Protein Analysis

Protein concentrations were determined by the method of Lowry et. al. (1951) using bovine serum albumin as a standard.

10. Electron Paramagnetic Resonance (EPR)

EPR data was obtained on a Varian Model E-9 spectrometer at X-band (9.5 KHz) frequencies and 100-KHz field modulation. The g_m values were determined from the resonance position of solid diphenyl picrylhydrazyl (DPPH) used as an external standard. A solution of hexose oxidase was placed into 3 mm diameter quartz sample tubes to a height of about 2 cm and frozen by placing in liquid nitrogen. The tube was then positioned in a sample Dewar which contained liquid nitrogen and was placed in the sample cavity of the EPR spectrometer. All spectra were recorded at 77° K.

For anaerobic EPR experiments, oxygen was removed by bubbling N_2 through a solution of hexose oxidase for about 10 minutes after which solid glucose was added. The EPR tube was flushed with N_2 and deaereated enzyme solution was injected into the tube by a syringe. The solution of hexose oxidase was diluted by about 5% with the addition of solid glucose.

RESULTS

A. PURIFICATION of HEXOSE OXIDASE

1. Effects of Salts and pH on Enzyme Precipitation

Effect of $CaCl_2$. It was observed that when $CaCl_2$ was added to an aqueous extract of Chondrus crispus there was a coprecipitation of the polysaccharide carrageenan and hexose oxidase. The effect of increasing concentrations of $CaCl_2 \cdot 2H_2O$ from 0.1% to 5.0% on the quantity of enzyme recovered in the resulting precipitate and supernatant was studied. The results (Figure 2) indicated that an increasing amount of enzyme was taken out of solution with an increasing calcium chloride concentration, up to 2.0% $CaCl_2 \cdot 2H_2O$. Above 2% calcium chloride, no further enzyme was precipitated. The recovery of enzyme, as judged by the nearly quantitative yield from the sum of the activities in the precipitate and supernatant, ruled out enzyme inactivation by calcium. The precipitate which contained hexose oxidase with the highest specific activity and in the best yield was produced by 2.0% $CaCl_2 \cdot 2H_2O$.

Effect of pH on enzyme precipitation. The pH at which the precipitation was performed was shown to influence the recovery of the enzyme. The effect of $CaCl_2$ concentrations from 1% to 30% at pH 4.9 (Figure 3) showed that much less enzyme was precipitated at this pH than at pH 6.5. At pH 4.9, 5% $CaCl_2 \cdot 2H_2O$ precipitated only 35% of the total activity versus 87% for an analogous preparation done at pH 6.5 (Figure 2). More enzyme was precipitated at pH 4.9 as more $CaCl_2 \cdot 2H_2O$ was added, but the recovery was far less than that recovered at pH 6.5.

Figure 2: The effect of increasing concentration of $CaCl_2 \cdot 2H_2O$ upon the recovery of hexose oxidase activity in Chondrus extracts. In this experiment, 25 ml of a 10,000 x g extract having a total activity of 27.5 U was used. Solid $CaCl_2 \cdot 2H_2O$ was added to the desired concentration at pH 6.5 and the resulting precipitate was separated from the bulk solution by centrifugation at 26,000 x g. The precipitate was brought to 10 ml with 0.1 M sodium phosphate buffer, pH 6.8 and the total activity and protein in this and the supernatant were determined. The solid squares in the illustration represent the activity measured in the precipitate; the open squares, that which is in the supernatant.

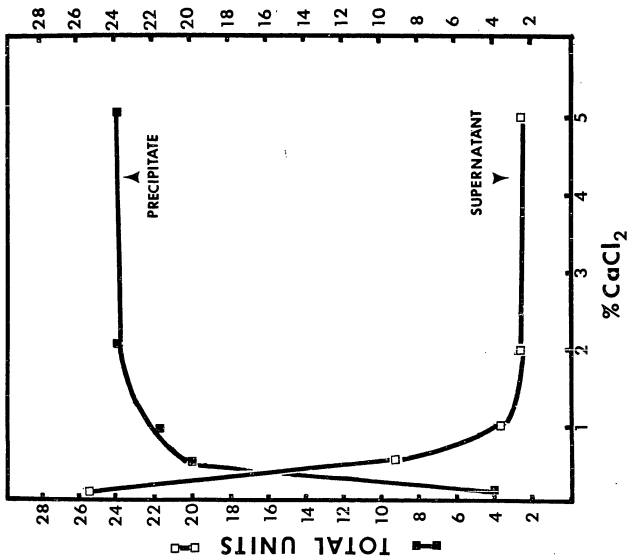


Figure 3: The effect of increasing concentrations of $CaCl_2$ on the recovery of activity in extracts of Chondrus done at pH 4.9. Experimental conditions are the same as in Figure 2, except the pH of the extract was maintained at pH 4.9 with 1 M KOH. Solid squares represent total activity recovered in the precipitate. Open squares represent the activity recovered in the supernatant.

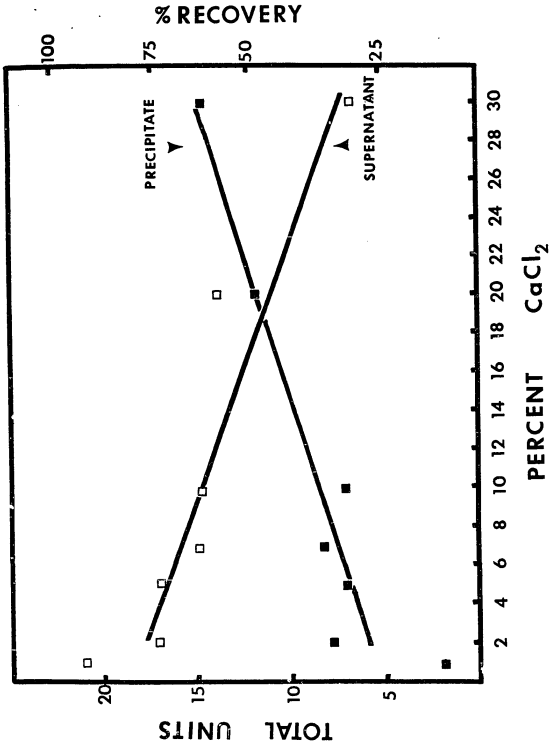


Figure 4: The effect of pH on the amount of hexose oxidase precipitated by $CaCl_2$. The effect of the pH of the Chondrus extract on the quantity of enzyme precipitated by 5% $CaCl_2 \cdot 2H_2O$ was determined as in Figure 2, except the pH of the extract was adjusted from 3 to 10 with 1 M HCl or 1 M NaOH. The total amount of activity in the precipitate is shown in solid squares. The total amount of activity found in the supernatant is shown by the open squares.

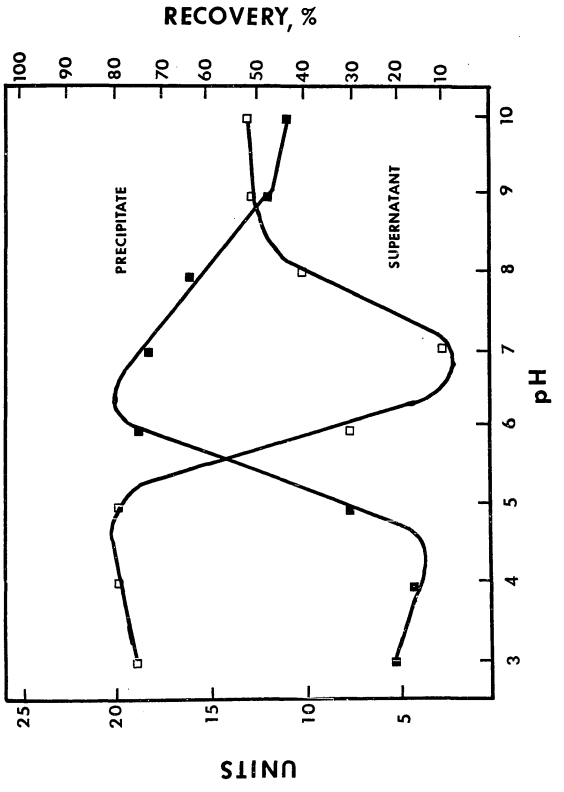
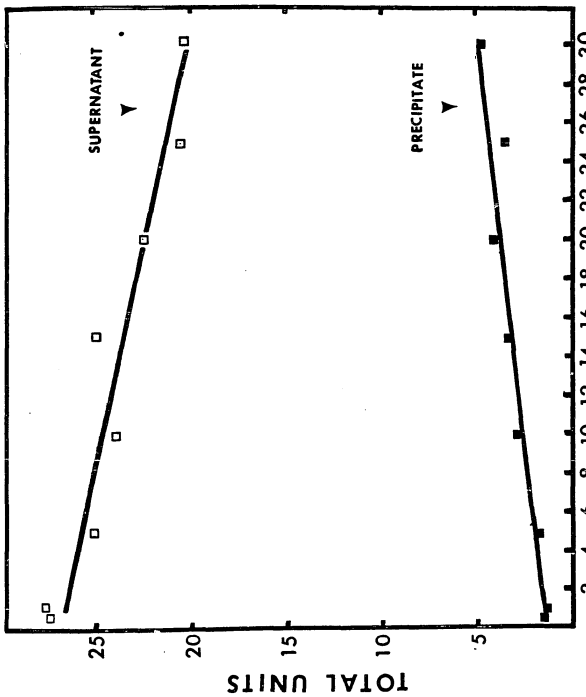


Figure 5: The effect of increasing KCl concentrations on the amount of enzyme precipitated from Chondrus extracts. The experimental conditions were the same as those used in Figure 2 except that solid KCl was added to the extract from 0.5% to 30% final concentration. The pH of the extract was maintained at 6.5 with 1 M KOH. Solid squares represent the total units recovered in the precipitate. Open squares represent the total units that remain in the supernatant.



The effect of pH was examined more closely in an experiment in which the amount of enzyme activity precipitated by 5% $CaCl_2 \cdot 2H_2O$ was determined at pH values between 3 and 10 (Figure 4). The result showed that optimum recovery was produced between pH 6 and 7. It was important to maintain the pH in the optimum range since the pH dropped to 5 after addition of $CaCl_2$ to the slightly buffered Chondrus extract.

Effect of KCl. The effect of increasing concentrations of KCl on the precipitation of hexose oxidase was examined and the results illustrated in Figure 5. Although KCl was found to precipitate the polysaccharide at pH 6.5, the results show that little enzyme was precipitated by this salt. Thus at concentrations of up to 30% KCl (w/v), only 5 units out of a total of 28 were precipitated. Changing the pH of the extract to 11 or 4.9 produced no increase in the amount of enzyme precipitated by 10% KCl (data not shown).

2. Seasonal Variation of Activity

A seasonal variation of hexose oxidase contained in Chondrus extracts was observed during these studies. In two similar extracts, one made with algae collected during July and one with October algae, it was found that in the October sample a total of 680 units of enzyme were extracted while the July extract contained 300 units of activity per 100 grams of ground algae. Also, the October sample had a better recovery of enzyme which was precipitated by $CaCl_2$. An enzyme recovery of 75-87% was obtained with the October sample but no more than 30-40% was recovered in the July sample. Seasonal variations in the amount of carrageenan found in Chondrus have been observed by Butler (1936), who measured a high of 82% carrageenan content by weight in July to a

low of about 60% content in the period from January to April. Thus there appears to be seasonal variability for both carrageenan and hexose oxidase content in Chondrus extracts.

3. Resolubilization

Once precipitated by $CaCl_2$, the enzyme was very difficult to resolubilize. Various salts, buffers and detergents were tested for their ability to resolubilize hexose oxidase in a given quantity of $CaCl_2$ precipitate. It was shown that the best solubilizer was 0.1 M sodium phosphate which contained 2 M NaCl. It is likely that this combination of salts functions by removing calcium ions from solution as calcium phosphate while carrageenan redissolves as its sodium salt.

4. Cetyltrimethylammonium bromide (CTAB) Fractionation

The resolubilization of enzyme activity from the $CaCl_2$ precipitate could be part ion-exchange and part resolubilization of the carrier carrageenan from the insoluble calcium salt to the soluble sodium form. The successful purification of the enzyme required the selective removal of carrageenan. It is known that detergent cations can selectively remove carrageenan from a mixture of agar, pectin and other gel-forming polysaccharides (Scott, 1960), and an experiment was performed to determine if the cationic detergent cetyltrimethylammonium bromide (CTAB) could effectively eliminate the polysaccharide from a solution containing hexose oxidase. Preliminary experiments indicated that carrageenan could indeed be precipitated at the NaCl resolubilization step, but whether coprecipitation of the enzyme and polysaccharide reoccurred depended upon the ionic strength of the solution. Experiments performed at low ionic strength (0.01 M sodium phosphate) showed that

Table I

The Effect of 0.20% Cetyltrimethylammonium bromide on the Removal of Carrageenan from Resolubilized $CaCl_2$ Precipitate at High Ionic Strength

FRACTION	VOLUME	PROTEIN	CARBO-HYDRATE	ACTIVITY	TOTAL PROTEIN	TOTAL CARBO-HYDRATE	TOTAL ACTIVITY	SPECIFIC ACTIVITY	RECOVERY	FOLD PURIFICATION
	ml	mg/ml	mg/ml	units/ml	mg	mg	units	units/mg	%	
NaCl re-suspension, 2 M in NaCl	100	1.05	1.47	1.0	105	147	100	0.95	-	-
0.20% CTAB supernatant, after dialysis against sodium phosphate, 0.01 M, pH 6.8	138	0.38	0.24	0.60	50	31	78	2.6	78	3

TABLE II: THE PURIFICATION OF HEXOSE OXIDASE

Fraction	Volume	Protein	Total Protein	Carbo-hydrate	Total Carbo-hydrate	Activity	Total Activity	Specific Activity	Recovery	Fold Purification
	ml	mg/ml	mg	mg/ml	mg	units/ml	units	units/ml	%	
10% Extract	700	1.7	1180	1.72	1200	0.6	420	0.62	-	-
2% $CaCl_2$ supernatant ca.	700	1.04	730	1.34	940	0.05	35	-	-	-
2% $CaCl_2$ precipitate,	80	2.90	232	3.20	260	3.8	304	1.30	72	2.1
after suspension Wash, supernatant	61	0.78	46	1.30	78	0	-	-	-	-
1st NaCl suspension	60	2.60	157	3.20	194	4.5	270	2.0	64	3.2
1st NaCl resol., supernatant	50	1.62	81	2.10	106	3.4	170	2.1	-	3.4
2nd NaCl resol., suspension	60	1.44	87	0.98	59	1.4	84	1.0	-	-
2nd NaCl resol., supernatant	50	0.40	20	0.34	17	0.7	35	1.8	-	-
Combined supernates	85	1.16	99	1.47	125	2.3	196	2.0	47	3.2
0.20% CTAB precipitate	10	-	-	12.0	120	1.8	18	-	-	-
0.20% CTAB supernatant	110	0.68	75	0.05	5.5	1.7	187	2.5	45	4.0
DEAE-cellulose I	1.3	2.24	3	N.D.	N.D.	2.0	26	9	6	15
DEAE - II cellulose	1.0	0.12	0.12	N.D.	N.D.	4.0	4	34	1.3	54

low concentration of CTAB were effective in removing some carrageenan, but higher concentrations of the detergent resulted in precipitation of hexose oxidase. Further experiments demonstrated that CTAB was capable of precipitating carrageenan at high ionic strength, even in the presence of 2 M NaCl which was used to resolubilize the enzyme found in the $CaCl_2$ precipitate. The high sodium chloride concentration had the advantage of "salting in" the proteins. Table I lists the results of one experiment performed in this manner; in this instance, 93% of the carbohydrate was removed while 78% of the activity from the previous step remained soluble. An additional benefit derived from this step was the moderate purification of hexose oxidase as a result of the elimination of a small amount of contaminating proteins.

5. DEAE-cellulose Chromatography

Following the removal of carrageenan by CTAB treatment, the partially purified hexose oxidase could be further fractionated by ion-exchange chromatography using DEAE-cellulose. From some preliminary experiments, using fractions containing redissolved carrageenan-hexose oxidase, it was determined that the polyanionic carbohydrate must be removed before a consistent DEAE-fractionation could be made. For example, the concentration of NaCl needed to elute the enzyme from DEAE-cellulose was determined in separate fractionations of carrageenan-containing hexose oxidase to be 0.12 M, 0.15 M, 0.20 M, and 0.33 M NaCl in 0.1 M sodium phosphate, pH 6.8. Varying elution patterns were also observed when a pH gradient of pH 6.8 to 2.2 was used. Depending on the preparation used, some enzyme could be eluted at pH 4.9 while at other times no enzyme at all could be eluted under the same conditions.

In most cases, the yield of enzyme following DEAE-cellulose fractionation was poor.

Ion-exchange chromatography of the CTAB-treated hexose oxidase produced a more consistent fractionation, with improved yields. Two elution programs, one a 0-0.5 M NaCl gradient in the first fractionation and the other, a pH gradient established by 250 ml each of 0.1 M sodium phosphate buffer pH 6.8 and 0.05 M sodium citrate, pH 4.0 for the second fractionation (Table II) were required to obtain a pure preparation of enzyme. The enzyme under these conditions eluted at a NaCl molarity of 0.11 M and a pH value of 5.4. The highly purified enzyme which was recovered after this fractionation had 13 times the specific activity as the fraction which was applied to the column (Table II).

The purity of the enzyme was assessed by the one band produced in disc gel electrophoresis of the enzyme having a specific activity of 36 U/mg (Figure 6). The band corresponded to a band stained for activity, having an R_f of 0.51.

6. Activity Stain for Hexose Oxidase.

A specific staining procedure was developed for detecting and quantitating hexose oxidase following disc gel electrophoresis based on the method described by Manwell and Baker (1963). The procedure uses the chromogen o-dianisidine, which, as it is oxidized following catalysis by horseradish peroxidase, becomes insoluble and fixes at the site of peroxide production in disc gels. Thus when a gel containing hexose oxidase was placed in staining solution, a thin band of precipitated, oxidized chromogen was detected at the location of hexose

oxidase. Figure 7 shows a photograph of a series of gels which contain various quantities of enzyme. Figure 8 is a plot of the standard curve which was made from measuring the absorbance throughout the length of the gel of each band by a scanning densitometer versus the amount of activity contained in the gel. The resulting straight line indicated that the stained area is linearly related to the activity contained in the gel. In addition, this procedure can be used to localize and/or quantitate any peroxide-producing enzyme in gels. This specific activity stain, for example, was used to locate hexose oxidase in the determination of its isoelectric position (pI_e) in polyacrylamide pH gradient gels (Figure 24).

Figure 6: Disc gel electrophoresis of purified hexose oxidase. Hexose oxidase was purified according to the procedure outlined in "Results". After disc gel electrophoresis in 7% gels, the first gel, containing 83 µg of enzyme at 36 U/mg was stained for protein using coomassie blue, while the second gel, containing 8.3 µg of protein was stained for activity as described previously in Materials and Methods.

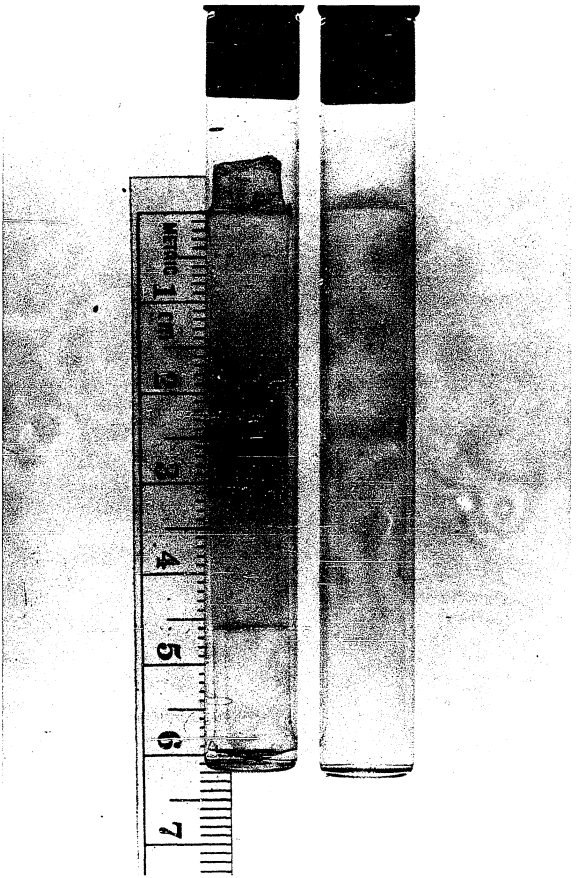


Figure 7: Hexose oxidase activity stain of disc gels. Gel number 1 contained 430 µg of protein (0.167 U of activity) and was stained for protein. Gels 2-7, contained 0, 0.033, 0.067, 0.10, 0.133, and 0.167 U of activity, respectively, and were stained for activity as described previously. The specific activity of the enzyme used was 0.37 U/mg.

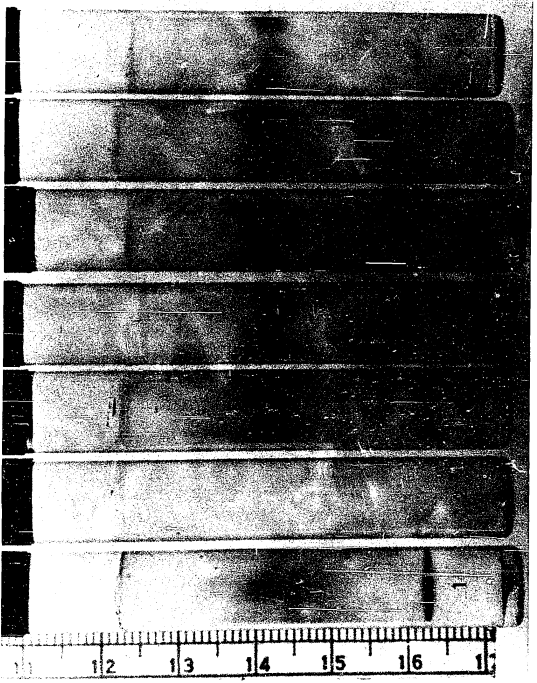
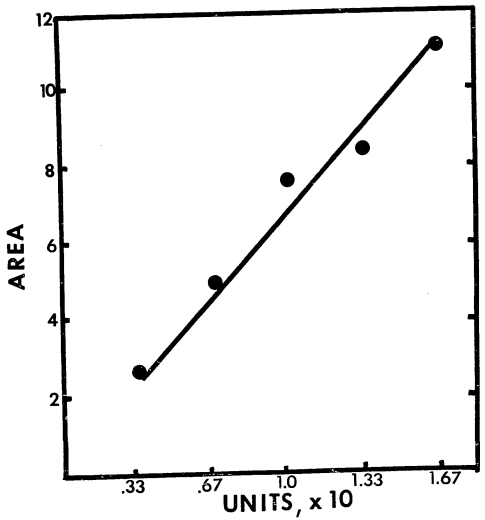


Figure 8: Standard curve of the quantity of stain from the area of densitometric tracing versus the amount of activity applied. The area under the densitometer tracing of the gels, shown in Figure 7 is expressed as $O.D._{500}$ x 0.01 cm chart units.



B. KINETICS

Kinetic determinations were made using separate preparations of partially purified hexose oxidase with the assay system described in Materials and Methods.

1. pH Dependence of the Hexose Oxidase Reaction.

A plot of activity measured at pH values from 5.9 to 6.9 in phosphate and phosphate-citrate buffers (Figure 9) produced a curve with a peak at pH 6.05. The shallow pH-rate profile suggests conformational changes or changes in state of protonation of groups on the protein which are remote from the active site (Cleland, 1977).

2. Initial Velocity Pattern of Hexose Oxidase with Varied Glucose Concentrations and at Constant Oxygen Concentration.

Glucose concentrations ranging from 4.0 mM to 100 mM were made in 0.1 M sodium phosphate, pH 6.2, and the initial velocity of the enzyme-catalyzed reaction measured at constant initial oxygen concentration of 8.4 ppm. A Lineweaver-Burke treatment of the data is shown in Figure 10. The apparent K_m for glucose was 10.4 mM.

3. Initial Velocity Pattern of Hexose Oxidase with Varied Oxygen Concentration and Glucose at Constant Non-Saturating Concentrations.

The initial rates of the enzyme reactions were determined using glucose concentrations near the apparent K_m while the concentrations of O_2 were varied. Figure 11 is a plot of the data in double-reciprocal form. The series of parallel lines obtained is indicative of a ping-pong type of reaction mechanism in which one substrate is bound and a product released before a second substrate is bound and

Figure 9: The effect of pH on the initial activity of hexose oxidase. The initial activity of hexose oxidase was determined with 0.1 M glucose concentration in 0.1 M sodium phosphate (solid circles) and 0.1 M sodium citrate (solid squares) at various pH values. The enzyme preparation used had a specific activity of 2.5 U/mg.

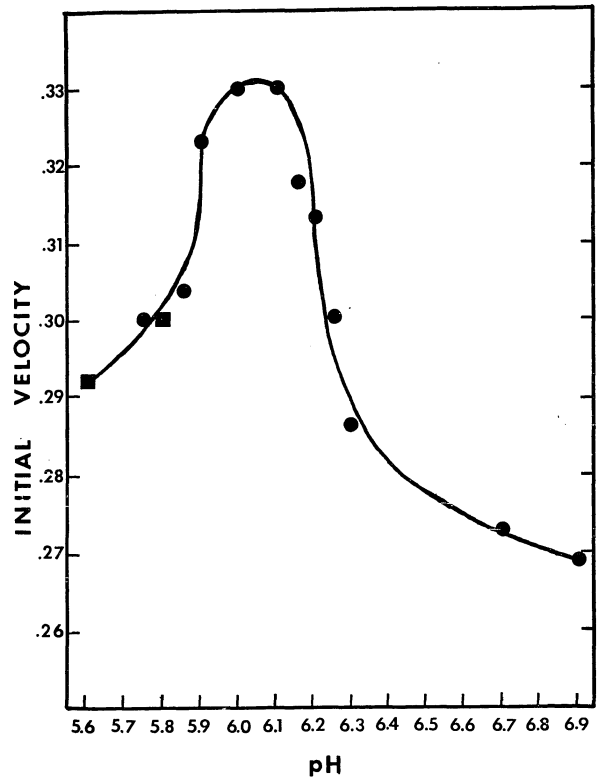


Figure 10: The effect of varied glucose concentrations on the initial velocity of hexose oxidase at constant initial oxygen concentration. Glucose solutions at concentrations of 100, 20, 10, 6, 6, 5, and 4 mM were allowed to equilibrate overnight to assure complete mutarotation. The concentration of oxygen initially was 8.4 ppm. Hexose oxidase specific activity was 8.5 U/mg.

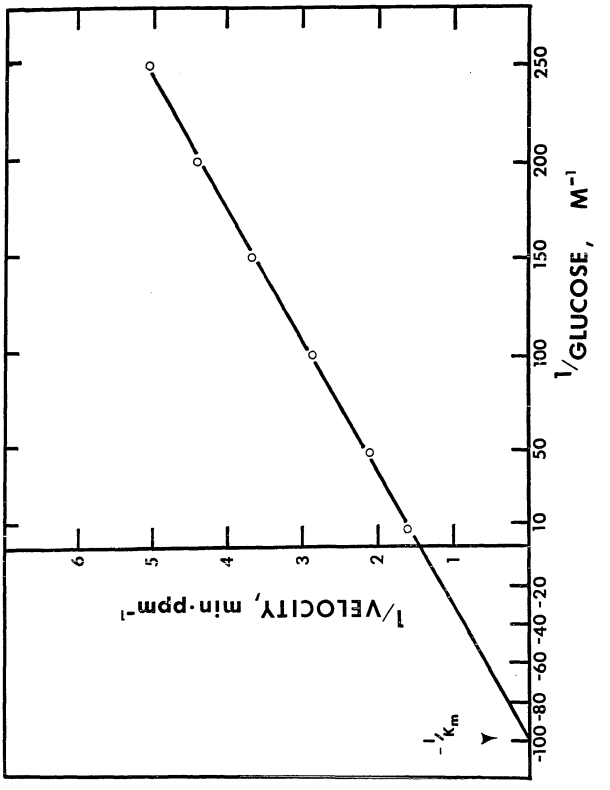


Figure 11: The effect of varied oxygen on the initial rate of enzyme catalysis with constant glucose. The open circles represent the result using 100 mM glucose, the open squares that using 10 mM glucose and the open triangles that with 5 mM glucose. Hexose oxidase used in this study had a specific activity of 1.0 U/mg.

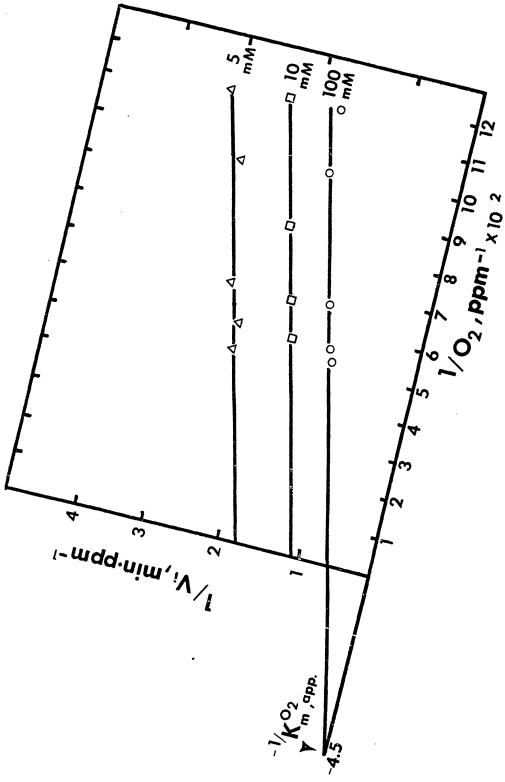
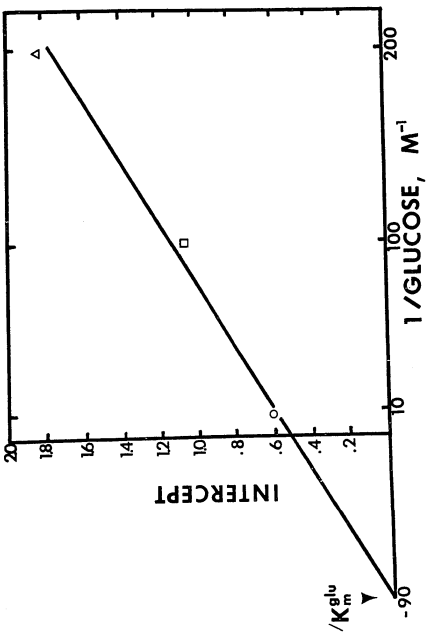


Figure 12: The plot of the y-intercepts of Figure 11 versus the reciprocal of glucose concentration.



the other product released from the enzyme. Since hexose oxidase catalyzes a two substrate-two product reaction, the mechanism is referred to as ping pong bi bi according to Cleland (1963).

An apparent K_m of 22 ppm for oxygen was determined from the line constructed from the results of the experiment using 100 mM glucose concentration (Figure 11, open circle). The y-intercepts of the lines plotted in Figure 11 can be replotted to obtain an estimate of the true K_m of glucose. A value of 11 mM was calculated from the intersection of this replotted line with the x-axis (Figure 12), in good agreement with the value obtained from the double reciprocal plot in which the apparent K_m was estimated at 10.4 mM glucose (Figure 10).

4. Product Inhibition: The Effect of Hydrogen Peroxide on the Initial Velocity of the Enzyme Reaction.

The effect of hydrogen peroxide upon the initial velocity of the enzyme reaction was measured according to the procedure described previously. Figure 13 is a plot of the data in double reciprocal form. The series of lines which converge at a point on the ordinate represents a pattern of competitive inhibition between glucose and hydrogen peroxide. Glucose and hydrogen peroxide, then, either compete for a common enzyme form or each is connected in a reversible way to a common intermediate form of the enzyme. This result is consistent with the proposed ping pong mechanism with glucose and hydrogen peroxide competing directly for one enzyme form.

The same data, when plotted according to Dixon (1953), (Figure 14) depicts a series of lines converging at a point above the abscissa. The results further support competitive inhibition occurring with

Figure 13: The effect of hydrogen peroxide on the initial rate of the forward reaction, with varied glucose concentration. A fixed initial oxygen concentration of 8.4 ppm and varied glucose concentration of 100, 33.3, 20, 10, and 5 mM were used. Hexose oxidase having a specific activity of 1.5 U/mg was used.

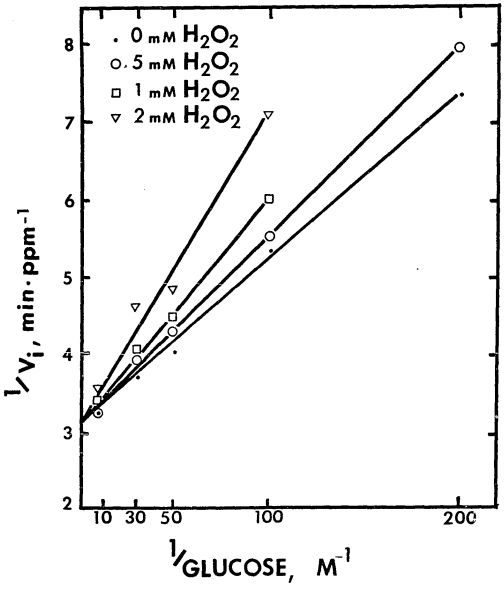


Figure 14: A replot of the data in Figure 13 according to Dixon (1953). The graph is a plot of the reciprocal of the initial velocity versus the inhibitor concentration. Oxygen concentration initially was a constant 8.4 ppm. The dots represent data obtained with glucose at 100 mM concentration, the open circles with glucose at 33.3 mM, the open squares at 20 mM and the open triangles at 10 mM concentration.

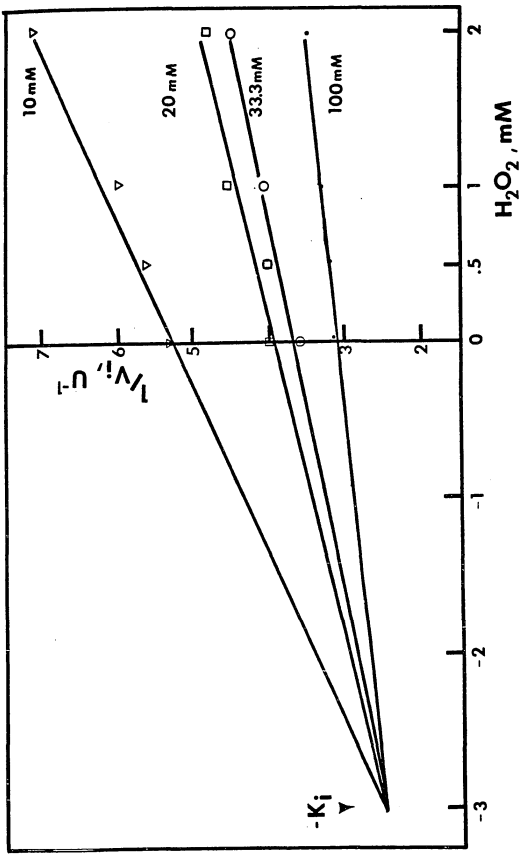
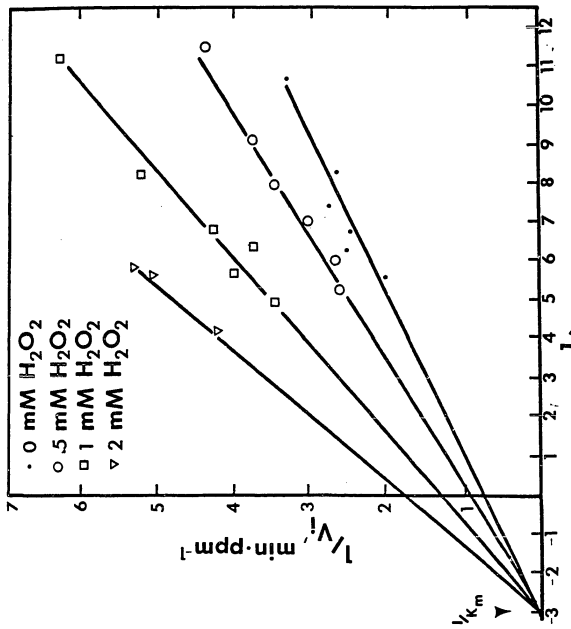


Figure 15: Product inhibition by hydrogen peroxide on the initial rate of the forward hexose oxidase reaction with varied oxygen concentrations. The initial rate of the enzyme reaction was measured at fixed (100 mM) glucose concentration while the oxygen concentration was varied between 8.4 and 20 ppm. Hexose oxidase having a specific activity of 3.5 U/mg was used in this analysis.



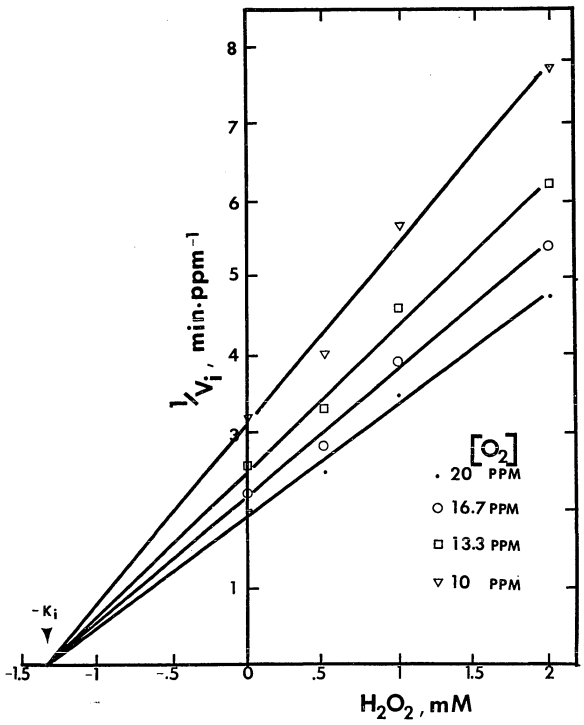
glucose in the presence of hydrogen peroxide, and is consistent with the ping pong mechanism of action of the enzyme. The point at which these lines cross in the x-coordinate gives a measure of the inhibition constant of peroxide for this substrate: A value of 3.0 mM H_2O_2 was estimated from this treatment.

5. Product Inhibition: The Effect of Hydrogen Peroxide on the Forward Reaction Rate at Constant, Saturating Glucose Concentration with Varied Oxygen Concentrations.

Hydrogen peroxide was allowed to inhibit the forward reaction while glucose was maintained at a constant saturating concentration while the concentration of oxygen was varied. The result of this experiment is shown in Figure 5. The x-intercept of the intersecting lines provides an additional measure of the apparent K_m for oxygen. $K_m^{O}2$ was estimated to be 33 ppm for this series. Because the oxygen electrode assay system employed in this measurement of the initial rates of the enzyme reaction was incapable of reading above 20 ppm oxygen, the extrapolation of the experimentally determined line to a point on the x-axis was long. This may explain the variation in the valves of 22 to 33 ppm for the apparent $K_m^{O}2$. Nevertheless, the lines obtained from this treatment tend to converge at a point on the x-axis and indicate a noncompetitive pattern of inhibition. In this case, hydrogen peroxide lowered the proportion of central complexes that result in products by causing partial reversal of the reaction (Cleland, 1977). The result is consistent with the proposed ping pong mechanism.

A plot of this data by the method of Dixon (1953) (Figure 16) produced a value of 1.4 mM for the hydrogen peroxide inhibition constant

Figure 16: A replot of the data of Figure 15 according to Dixon (1953). The dots represent the values with oxygen at 20 ppm, the open circles with oxygen at 16.7 ppm, the open squares with oxygen at 13.3 ppm and the open triangles at 10 ppm.



with respect to oxygen. Complete inhibition of the reaction was observed for the assay at low oxygen concentration and 2.0 mM H_2O_2.

6. Product Inhibition: The Effect of D-Glucono-δ-lactone on the Forward Reaction Rate at Constant Initial Oxygen Concentration with Varied Glucose Concentration.

The effect of D-Glucono-δ-lactone on the reaction rate determined at various glucose concentrations is shown in Figure 17. The lines for the inhibited and uninhibited enzyme reactions converge at a point on the x-axis, forming a noncompetitive pattern of inhibition. This result is consistent with the ping pong mechanism already proposed, for in this case D-glucono-δ-lactone caused partial reversal of the reaction which results in less central complexes forming products. (Cleland, 1977).

A Dixon replot, shown in Figure 18, gave the value of 0.3 M as the inhibition constant of D-glucono-δ-lactone with respect to glucose.

7. Product Inhibition: The Effect of D-glucono-δ-lactone on the Forward Reaction Rate at Constant, Saturating Glucose Concentration with Varied Oxygen Concentration.

The effect of D-glucono-δ-lactone on the initial velocity of hexose oxidase under conditions of constant glucose and varied oxygen was determined and the result plotted in double reciprocal form (Figure 19). The lines converging at a point on the y-axis form a competitive inhibition pattern of D-glucono-δ-lactone with respect to oxygen. This is again consistent with the proposed mechanism as oxygen and the lactone compete for the same form of enzyme.

Figure 17: The effect of D-glucono-δ-lactone on the initial rate of the enzyme reaction with initial oxygen at 8.4 ppm and varied glucose concentration. Hexose oxidase with a specific activity of 2.5 U/mg was used.

The line drawn through the dots in the figure is the uninhibited reaction, the open triangles were with 0.05 M lactone, the open circles were with 0.10 M lactone and the open squares were with 0.20 M lactone.

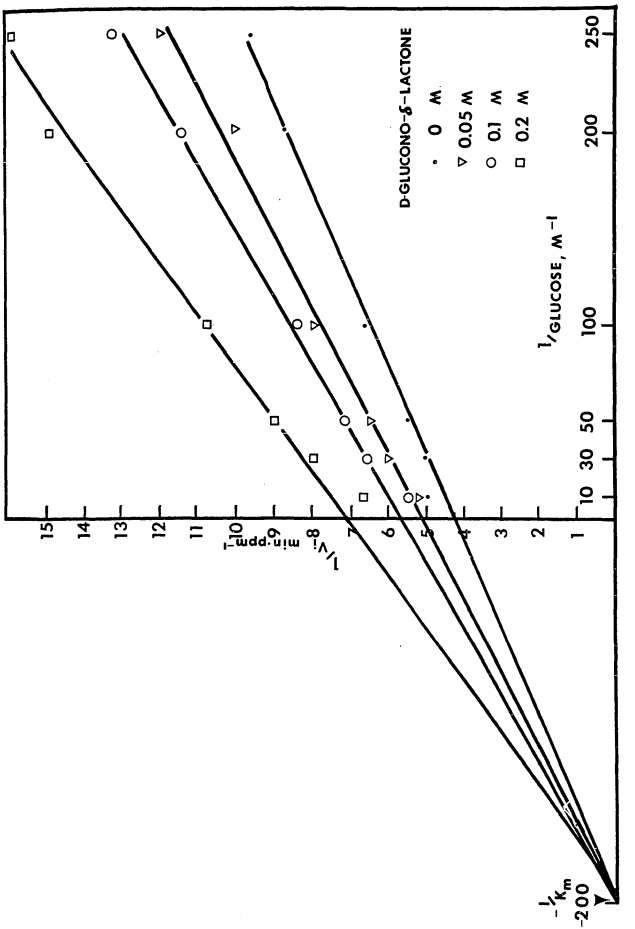


Figure 18: A replot of the data of Figure 18 according to Dixon (1953). The data was obtained as described in the legend for Figure 17.

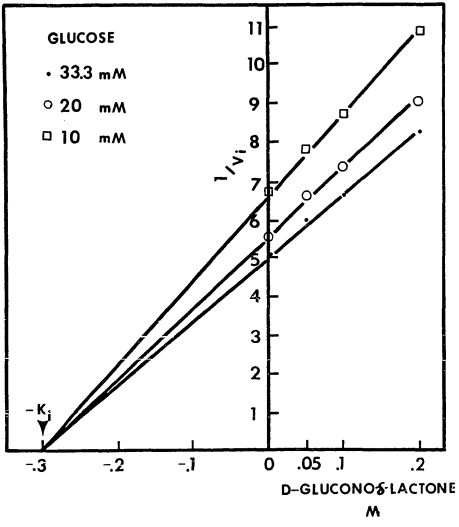


Figure 19: The effect of D-glucono-δ-lactone on the initial velocity of the enzyme reaction at saturated glucose concentration and varied oxygen concentrations. The concentration of oxygen was varied between 8.4 ppm and 20 ppm. The result of 0.1 M (open circles) and 0.2 M (open squares) D-glucono-δ-lactone are illustrated.

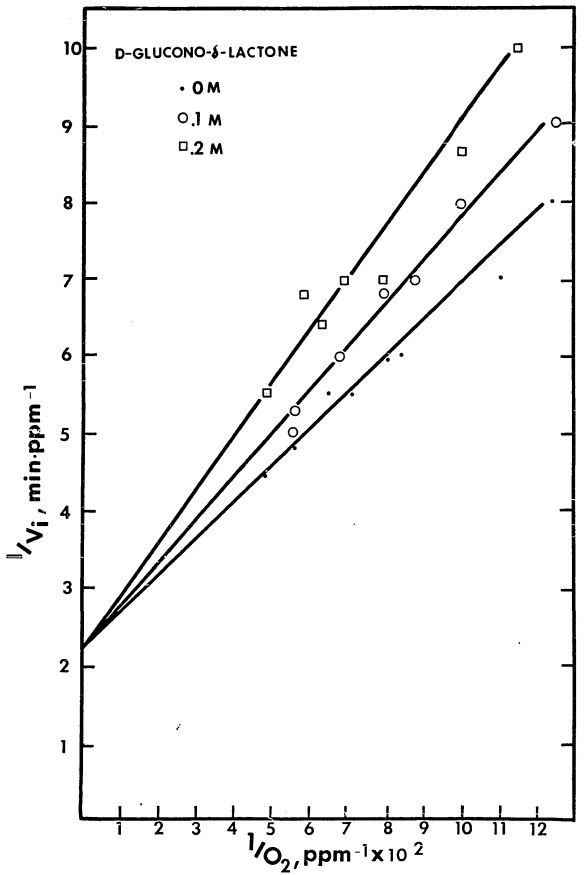
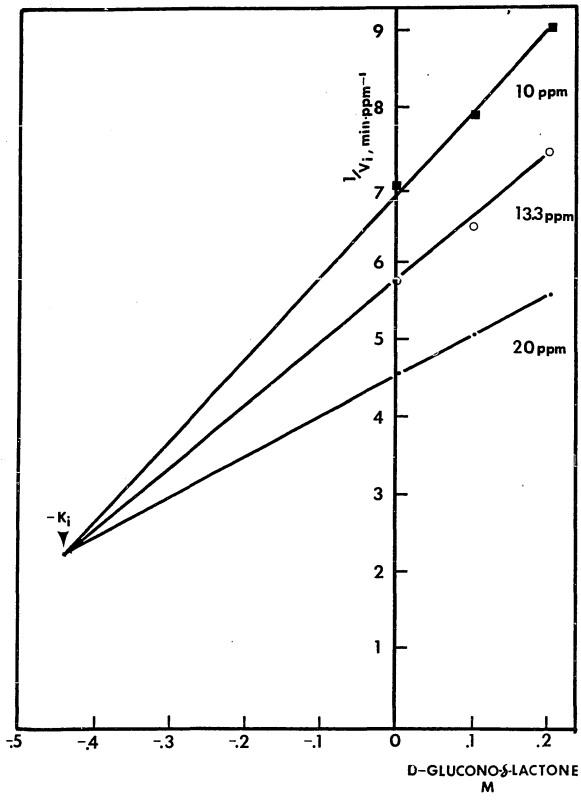
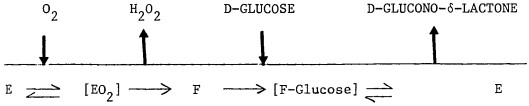


Figure 20: A replot of the data given in Figure 19, according to Dixon (1953). The dots represent the values obtained with oxygen concentration at 20 ppm, the open circles with oxygen at 13.3 ppm, and the solid squares that with 10 ppm oxygen.



A Dixon plot of the same data gave 0.44 M as an estimate of the K_i for this inhibitor for oxygen (Figure 20).

From these kinetic studies, the order of addition and release of substrates and products by Chondrus hexose oxidase has been established as a ping pong bi bi mechanism. This mechanism can be depicted using the known substrates and products as follows:



The diagram above is one of two ways of depicting a mechanism of this kind. The other way, in which glucose is added first, is equally probable to the one given above.

A table of the patterns of inhibition and the estimated inhibition constants found for each product and substrate is given below:

TABLE III

Inhibition and Inhibition Constants of Hexose Oxidase

SUBSTRATE	PRODUCTS: H_2O_2	PRODUCTS: D-GLUCONO-δ-LACTONE
O_2	NONCOMPETITIVE K_i = 1.4 mM	COMPETITIVE K_i = 0.44 M
GLUCOSE	COMPETITIVE K_i = 3.0 mM	NONCOMPETITIVE K_i = 0.30 M

C. PHYSICAL-CHEMICAL CHARACTERIZATION

1. Molecular Weight Determination by Gel Filtration

An estimate of the molecular weight of hexose oxidase was made by gel filtration using Sephacryl S-200 Superfine as the chromatographic media. The elution volume of protein standards was plotted against the proteins' molecular weight (Figure 21). By comparing its elution volume with those of the standard proteins the molecular weight of hexose oxidase was estimated at 140,000.

2. SDS-polyacrylamide Gel Electrophoresis

To determine the subunit composition and size for Chondrus hexose oxidase, the enzyme was subjected to SDS-polyacrylamide gel electrophoresis. The position of the band in the gel is related to its size and the molecular weight of the banded protein can be estimated from a standard curve constructed by plotting mobilities of proteins which had been treated similarly, versus the log of their molecular weight. Hexose oxidase produced one sharp band when subjected to SDS-polyacrylamide electrophoresis (Figure 22), which corresponded to a molecular weight of 70,800 (Figure 23). Since the enzyme had a native molecular weight of 140,000 as determined by gel filtration analysis, it appears that native hexose oxidase contains two subunits with a molecular weight of about 70,000.

3. Isoelectric Point of Hexose Oxidase

The isoelectric point of hexose oxidase was determined by isoelectric focusing in polyacrylamide gels followed by staining for hexose oxidase activity as described in Materials and Methods. The

Figure 21: Molecular weight determination of hexose oxidase by gel filtration using Sephacryl S-200 Superfine. The proteins used as standards were Bovine-γ-globulin (169,000), open triangle; glucose oxidase (160,000), solid square; Glucose-6-phosphate dehydrogenase (104,000), open square; hemoglobin (64,500), open circle; horseradish peroxidase (40,000), closed triangle; and α-chymotrypsinogen A (25,700), solid circle. Hexose oxidase was found to elute at a volume marked as an "x" on the calibration line. A void volume, V_o, of 60 ml was determined from the elution volume of Blue Dextran.

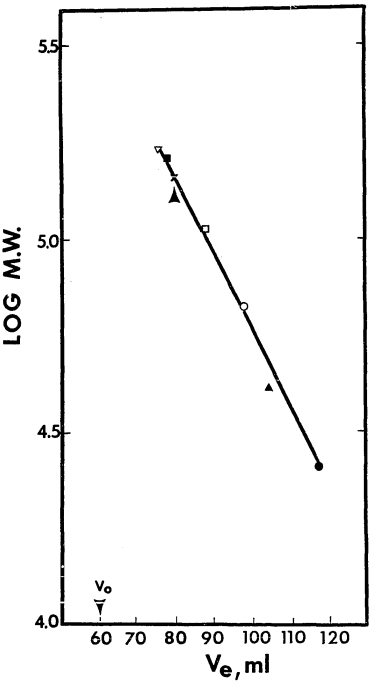


Figure 22: SDS-polyacrylamide gels containing hexose oxidase and molecular weight standards. The first gel contained 14 µg of 36 U/mg hexose oxidase which had been treated as described previously in Materials and Methods. The second gel contained 10 µg each of BSA (68,000), glutamate dehydrogenase (53,000), pepsin (35,000), chymotrypsinogen A (25,700), lysozyme (14,300) and cytochrome c (11,700). The bottom of each gel was sliced at the center of the tracking dye front.

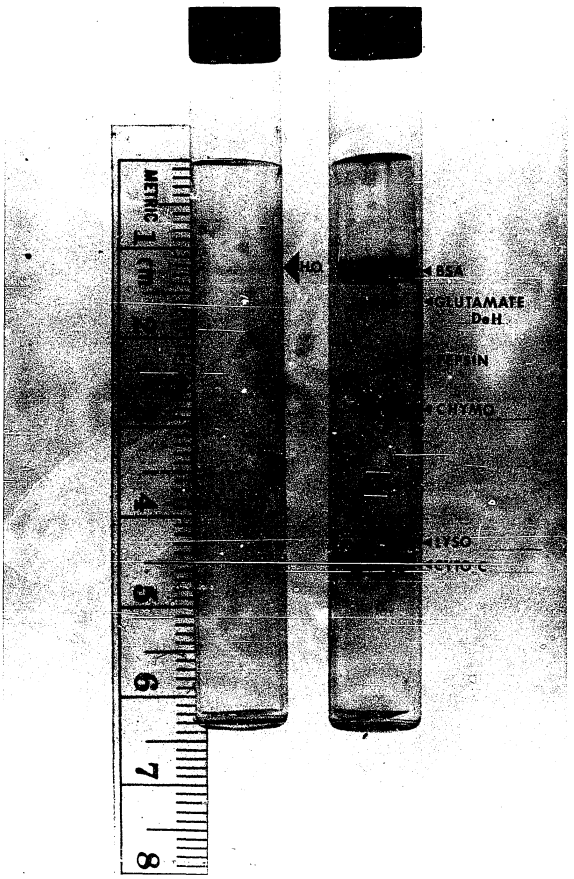


Figure 23: SDS-polyacrylamide gel electrophoresis. Standard curve of the log molecular weight of each of the standard proteins versus their retardation factor in the gel (R_f). The solid square represents BSA; the solid triangle, glutamate dehydrogenase; the open circle represents pepsin while the open square, chymotrypsinogen. The arrow marks the R_f of hexose oxidase.

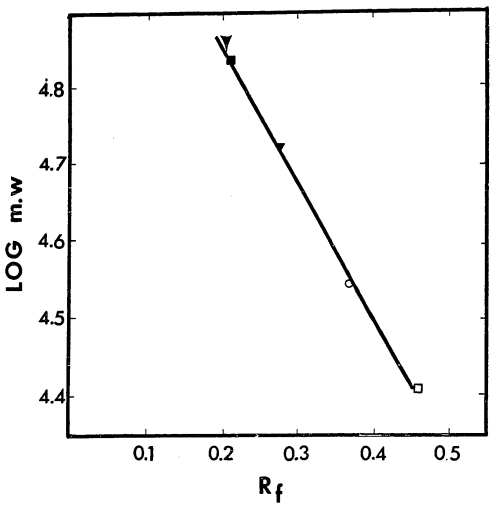


Figure 24: Isoelectric focusing of hexose oxidase in polyacrylamide gels. A total of 0.2 U of hexose oxidase was applied and the enzyme located by the specific activity stain. The specific activity of hexose oxidase was 1.4 U/mg.

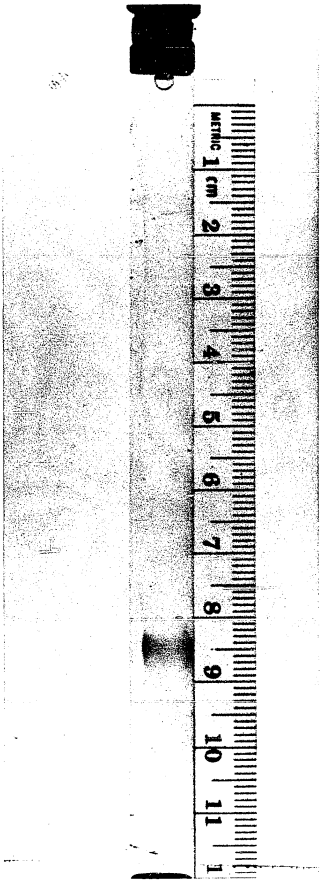
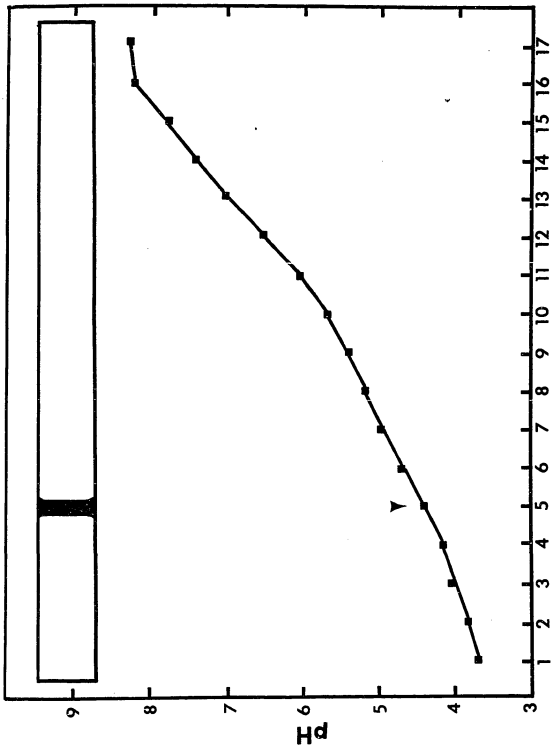


Figure 25: Isoelectric focusing of hexose oxidase in polyacrylamide gels. The isoelectric point of hexose oxidase was determined by applying the specific activity stain technique to hexose oxidase after isoelectric focusing in polyacrylamide gels (see Materials and Methods). The arrow marks the migration position of hexose oxidase. The pI_e of the enzyme was determined by comparing its position in the gel with the pH of that section of the gel.



pI_e for hexose oxidase was found to be pH 4.40 (Figures 24 and 25).

4. Carbohydrate Content of Hexose Oxidase

The amount of carbohydrate contained in a hexose oxidase preparation was determined as the quantity of reducible sugars associated with a known quantity of protein. A sample of 83 µg of protein was found to contain 3.4 µg of carbohydrate; thus the enzyme contained 3.4/86.4 or 4.0% by weight of carbohydrate.

5. Copper Content of Hexose Oxidase

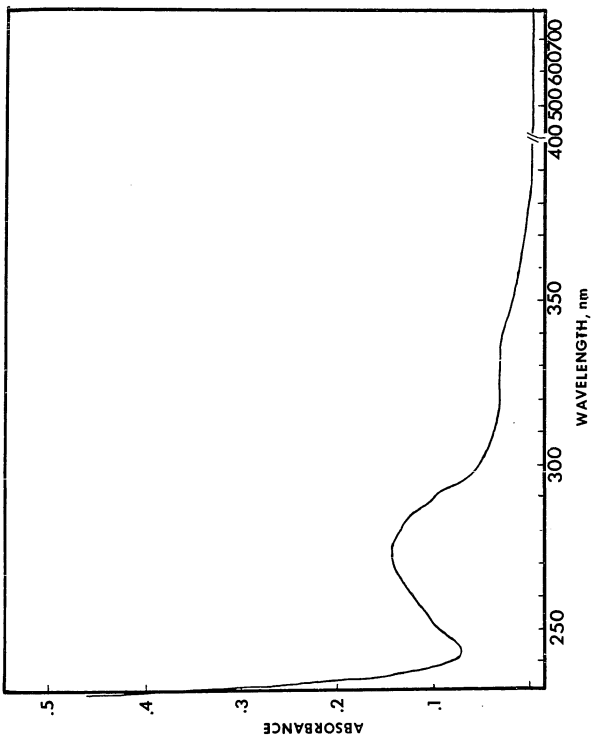
The amount of copper contained in hexose oxidase was determined by atomic absorption analysis. A sample of concentration 83 µg/ml with a specific activity of 36 U/mg was found to contain 0.15 ppm copper. This corresponds to a minimum value of 4.0 mol copper per mol protein, since the concentration of copper was 0.15×10^{-3} g/l ÷ 63.54 g/mol = 2.36×10^{-6} mol copper, and the concentration of protein was 0.083 g/l ÷ $1.4 \times 10^{+5}$ g/mol or 5.93×10^{-7} mol protein, then

$$\frac{23.6 \times 10^{-7} \text{ mol copper}}{5.93 \times 10^{-7} \text{ mol protein}} = \begin{matrix} 4.0 \text{ mol copper} \\ \text{per mol protein} \end{matrix}$$

6. Absorption Spectrum of Hexose Oxidase

The visible and uv absorption spectrum of hexose oxidase was recorded with a Cary Model 15-spectrophotometer. The spectrum (Figure 26) revealed a featureless visible spectrum from 800 nm to 390 nm. From 390 nm extending into the ultraviolet there is a broad absorption shoulder which is centered at 330 nm with an ε_{330} of 1.47×10^4 $M^{-1}cm^{-1}$/Cu. There is absorption due to aromatic amino acids in the region of 260 nm to 300 nm, with an absorption maximum at 274 nm. The

Figure 26: Visible and uv absorption spectrum of hexose oxidase. The enzyme, 0.083 mg/ml with a specific activity of 36 U/mg was dialysed exhaustively against 0.01 M sodium phosphate, pH 6.2.



ratio of absorbance at 274 nm to that at 330 nm is 4.0.

7. EPR Spectrum of Hexose Oxidase

The EPR spectrum of the highly purified hexose oxidase as recorded using a Varian E-9 Spectrometer is shown in Figure 27. The signal observed at 77°K consisted of a single absorption derivative having no discernable hyperfine lines. From the resonance position of the external standard, DPPH, the midpoint g value, g_m, was calculated to be 2.075. The absorption derivative was found to have a narrow peak-to-peak line width derivative, σ, equal to 79 Gauss and there was a shoulder found in the middle of the transition. Figure 28 illustrates this shoulder more clearly as the scan range was decreased by a factor of five causing the line to spread. The EPR spectra shown did not have coherent baselines due to the broad paramagnetic absorption of oxygen dissolved in the liquid nitrogen.

The EPR spectrum of hexose oxidase taken after deaereation by bubbling with N_2 followed by glucose addition is shown in Figure 29. The magnitude of the signal intensity was found to decrease by 47% (36 ± 1.0 from 68 ± 2.4 mean ± SEM of 3 determinations) compared with the same hexose oxidase solution which had been scanned under aerobic conditions. In addition to the decreased intensity of the signal at g = 2.075, a new signal, centered at g = 2.003, having a narrow line width (< 10 Gauss) was also observed under these conditions.

Samples of purified hexose oxidase also contained another EPR transition at 1550 Gauss centered at g = 4.26 (Figure 30). This signal was power saturated at microwave powers of 60 to 80 mW at 77°K. Atomic adsorption analysis confirmed that this signal was due to iron,

Figure 27: EPR spectrum of hexose oxidase. The EPR spectrum of a sample of hexose oxidase which was 2.5 mg/ml and 23 U/mg was determined at 77°K using a Varian E-9 EPR spectrometer. The microwave power was 40 mW at 9.248 GHz at a modulation frequency of 100 K Hz. Receiver gain was 1.25×10^4 and a time constant of 3.0 sec. The scanning rate was 66 G/min. The inset is the transition position of the external standard DPPH, which has a g value of 2.0036. The amplitude is in arbitrary units.

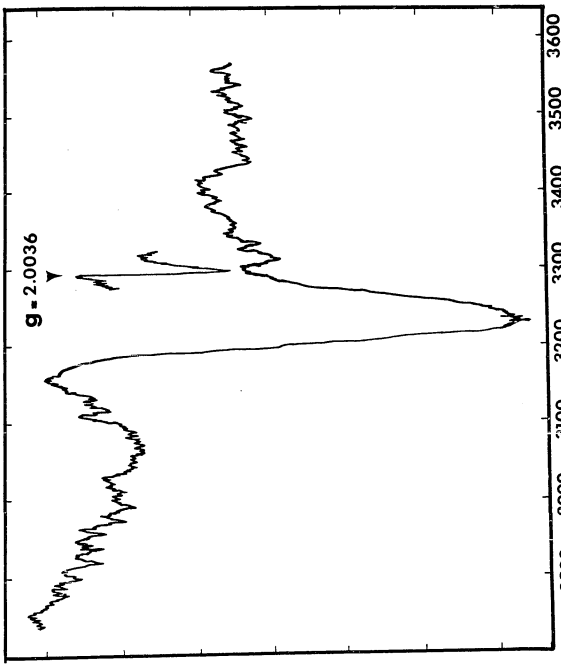


Figure 28: EPR spectrum of hexose oxidase. The same enzyme preparation as used for the EPR spectrum in Figure 27 was scanned over a smaller range at 13 G/min. The receiver gain was $6.3 \times 10^{+3}$ at a time constant of 10 sec. The microwave power was 40 mW at 9.248 GHz frequency with a modulation frequency of 100 K Hz. Temperature was 77°K. DPPH transition at 2.0036 (arrow) was used as a g marker. The arrow points up to the shoulder found near the midpoint of the transition.

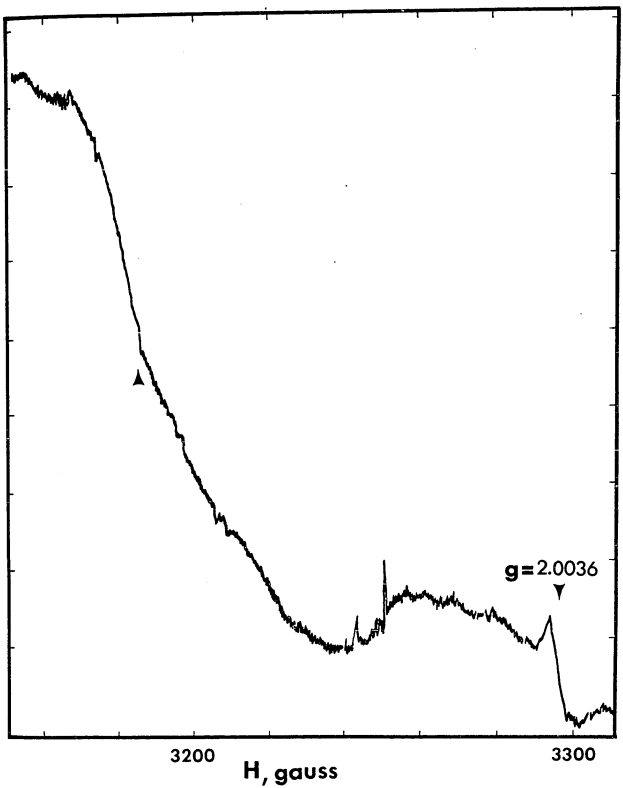
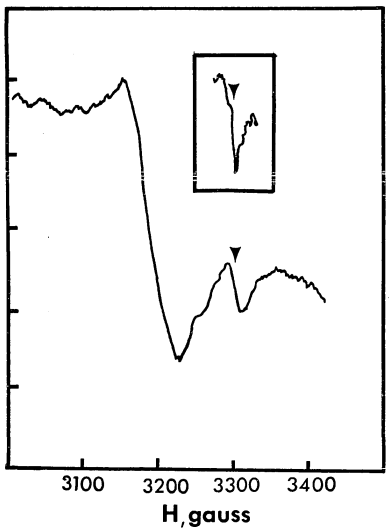
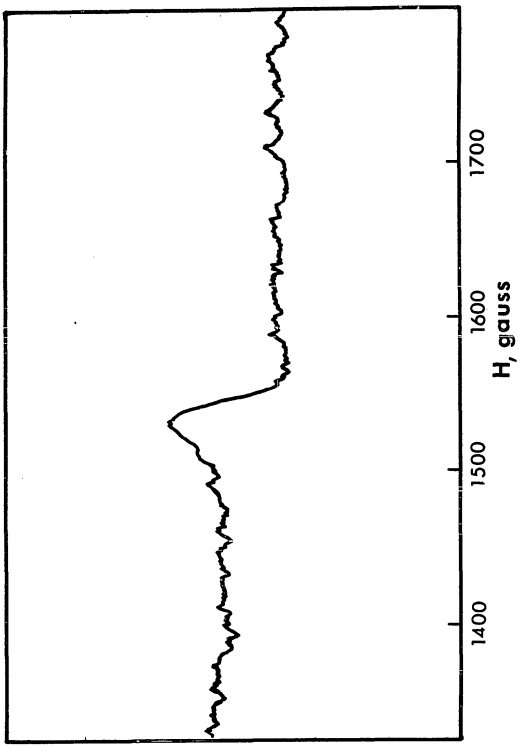


Figure 29: EPR spectrum of hexose oxidase after deaereation by bubbling with N_2 followed by glucose addition. The enzyme used contained 23 U/mg and was at a concentration of 2.5 mg/ml. The spectra was taken at 77°K at a microwave power of 40 mW and frequency of 9.268 GHz with a modulation frequency of 100 KHz. The receiver gain was 1.25×10^4 at a time constant of 10 sec. Scan rate was 33 G/min, except in the inset where the spectra was scanned at 8.3 G/min. The g value for the new transition (arrow) was 2.003 as determined from its position relative to the known copper maximum of g = 2.075.



found at a concentration of 5.3 g atoms per mol protein. This signal did not significantly diminish when the hexose oxidase solution was treated with glucose under anaerobic conditions (22 and 20 arbitrary intensity units, respectively).

Figure 30: EPR spectrum of hexose oxidase at 77°K at half field. The sample of hexose oxidase used in this analysis was the same as in Figure 29. Microwave power was 40 mw at 9.268 GHz with a modulation frequency of 100 KHz. The recorder gain was 1.25×10^4 with a time constant of 3 sec and a modulation amplitude of 6.3 G. The g value for the midpoint of the transition was calculated to be 4.26 from its position with respect to DPPH.



DISCUSSION

A. Purification of Hexose Oxidase

Precipitation of carrageenan from extracts of Chondrus crispus by $CaCl_2 \cdot 2H_2O$ produced a coprecipitation of hexose oxidase. The pH at which this precipitation was carried out was found to influence the recovery of the enzyme, with an optimal yield prevailing at pH 6 to 7 (Figure 4). Other agents capable of precipitating carrageenan, such as alcohol and KCl (Whistler and Smart, 1953) (Figure 5) were used but each failed to precipitate the enzyme. Because of the good recovery and small purification found with this step, precipitation with 2% $CaCl_2$ at pH 6.8 was used as the first step in the purification of hexose oxidase (Table II).

In the next step, a brief wash with 0.1 M sodium phosphate, pH 6.8 removed some easily solubilized protein from the $CaCl_2$ precipitate without dissolving hexose oxidase. Hexose oxidase and carrageenan were dissolved with 0.1 M sodium phosphate which contained 2 M NaCl (Table II). Carrageenan was removed from this solution by the addition of 0.2% cetyltrimethylammonium bromide (Table I). The high sodium chloride concentration was necessary in preventing precipitation of hexose oxidase by the cationic detergent. The data in Table II show that 0.2% CTAB eliminated 120 out of 125 mg of carbohydrate with only a 2% loss in enzyme activity.

Ion-exchange chromatography was used to fractionate the protein solution further. A linear NaCl gradient and a pH gradient (Table II) were used to recover an enzyme preparation (36 U/mg) which showed one

band when subjected to disc gel electrophoresis (Figure 6). The recovery of enzyme from these two DEAE steps was poor, with a 98% loss of activity from the preceding step.

B. Physical Chemical Characterization of Hexose Oxidase

The native molecular weight of hexose oxidase was determined to be 140,000 by gel filtration. SDS polyacrylamide gel electrophoresis revealed one protein species which corresponded to a molecular weight of 70,800. Thus, the native enzyme consists of two subunits, each with a molecular weight of approximately 70,000. Other copper oxidases from plant and animal sources are of similar size and structure. Ascorbic acid oxidase from squash has a molecular weight of 132,000-140,000 and contains two identical subunits (Lee and Dawson, 1973). Plasma ceruloplasmin is similar in size but the subunit structure is unknown at present (Deutsch, 1960).

Highly purified hexose oxidase was found to contain approximately 4% carbohydrate. This is considerably less than the value of 70% first reported by Sullivan (1973) in the original study of this enzyme. Failure to completely remove carrageenan from solution could have caused this high measurement. Hexose oxidase did not stain for carbohydrate with periodic acid-Schiff's stain of polyacrylamide gels, which confirms the low content of carbohydrate found in the enzyme preparation. It is possible that the little carbohydrate which is detected may be due to a minor carrageenan contaminant. Or, hexose oxidase may be a glycoprotein like some other copper oxidases. For example, ceruloplasmin contains 8% by weight carbohydrate (Deutsch, 1960), ascorbic acid oxidase, 10% (Lee and Dawson, 1973), and laccase,

45% (Reinhammer, 1970).

The isoelectric point of hexose oxidase was found to be pH 4.40 by isoelectric focusing. This value indicated a predominance of acidic over basic and neutral amino acids. Sullivan (1973) determined the amino acid composition of hexose oxidase and found a low amount of Lys, His and Arg - a total of 9 residues - to Glx and Asx - a total of 40 normalized residues, which suggests such an acidic over basic predominance.

Atomic absorption analysis for trace metals showed that purified hexose oxidase contained both copper and iron. The enzyme was found to contain 0.18% copper or 4.0 g atoms of copper per molecule. In the original study by Sullivan (1973) a value of 12 copper atoms was found per molecule of 130,000 daltons, and no iron was detected by emission spectral analysis. Additional support for copper as a cofactor in the enzyme came from the inhibition observed by metal binding agents such as sodium diethyldithiocarbamate. This chelating agent, which has a great selectivity for copper (II) ions (Hallaway, 1959), markedly inhibited the enzyme, even at 10^{-5} M concentrations (Sullivan, 1973).

The participation of iron in hexose oxidase action is not so well established. Atomic absorption measurements showed 0.21% iron or the equivalent of 5.3 g atoms of iron per mole protein. However, studies on the effect of metal inhibitors and chelating agents on the activity of hexose oxidase found that sodium azide, which inhibits iron-enzymes strongly (Hallaway, 1959) was approximately a thousand times less effective in inhibiting the activity of the enzyme than was sodium diethyldithiocarbamate (Sullivan, 1973).

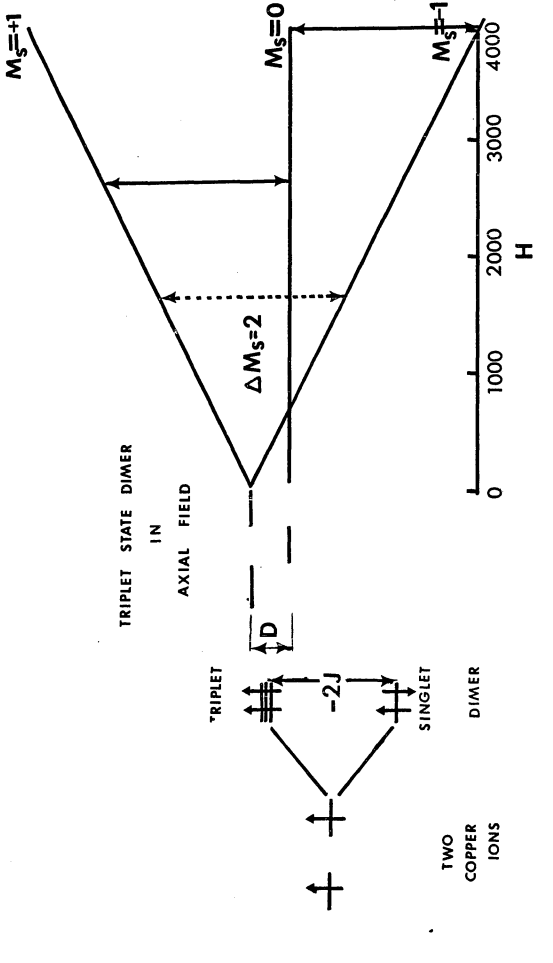
Most copper-containing proteins, like the blue oxidases, are intensely colored, but hexose oxidase is colorless. The uv spectrum of hexose oxidase consisted of typical aromatic amino acid side chain absorbances plus a broad shoulder which was centered at 330 nm. Another copper oxidase, tyrosinase, is pale green in color and it too contains a 330 nm absorbance shoulder similar to that found in uv spectra of hexose oxidase (Bouchilloux, et al. 1963). Several other copper oxidases including the blue oxidases laccase, ascorbic acid oxidase, and ceruloplasmin, as well as the oxygen carrier hemocyanin, have absorbance bands in the near ultraviolet centered at 330 nm to 340 nm (Dawson, 1966; Ehrenberg, et. al., 1962; Levin, 1966; Lontie and Vanquickenborne, 1974). From CD, EPR, and anaerobic titration studies of some of these enzymes, it was found that the 330 nm absorption band was a characteristic of a copper dimer structure in these proteins (Mason, 1976). Each dimer in these proteins is made of a pair of magnetically-coupled copper ions which is diamagnetic. It has been suggested that the dimer functions by undergoing two-electron redox reactions (Fee et. al., 1969). Model copper dimers, such as alkaline copper tartrate also possess an absorption at 330 nm (Chasteen and Belford, 1970). The 330 absorption band for hexose oxidase may be diagnostic of a copper dimer arrangment. Caution must be made in this assignment, however, because other interpretations are possible: the absorption may be due to iron or absorption from a contaminant like phycoerythrin, which absorbs in this region (Bennett and Bogorad, 1971).

Electron paramagnetic resonance techniques (EPR) were used to study the state of copper in hexose oxidase. An X-band spectrum at

g = 2.075 was typically seen in each enzyme preparation studied, varying from 4.2 to 23 U/mg. The spectrum consisted of a single absorption derivative with a lineshape unlike that normally seen with Cu(II) complexes in that there was a lack of hyperfine structure. A g_m value of 2.075 was calculated for this transition. This spectrum is commonly observed for axial Cu(II) in the perpendicular region with the g_m value and Gaussian peak-to-peak line width, σ, of 79 G. A closer inspection of the transition at g = 2.075 revealed that the line contained a shoulder at the center of the descending line (Figure 28, arrow). This was consistent with what is found in EPR spectra of model copper dimers in which the shoulder divides a pair of absorption derivatives predicted for the copper pair in the perpendicular region of the spectra. A dimer structure for the copper in hexose oxidase with a line width of 79 Gauss at a g value of 2.075 is not unreasonable when compared with the copper dimer complexes studied by Price, et al., 1970; Boas, et al., 1969; and Pilbrow, et al., 1970.

In theory, magnetically-coupled copper ions may produce an EPR spectrum when the unpaired electrons of each ion are antiferromagnetically coupled. When two copper (II) ions approach each other as a consequence of ligand binding, the unpaired electrons from each ion may pair up, and as a result the electron spins may be aligned or opposed as shown in Figure 31. If the spins of each electron are opposed to each other, a singlet state exists and the dimer is diamagnetic; no EPR signal is possible. When the spins are aligned, a triplet state is evident, since S=1 and the multiplicity, 2S+1=3. The dimer in this state is paramagnetic. The singlet state differs from

Figure 31: Energy diagram and transition probability for copper dimer structure.



the triplet state by an energy of -2J, where J is referred to as the exchange interaction constant. The copper ions are said to be antiferromagnetically coupled if the singlet state is the ground state and the triplet state is the excited state; J in this case will be negative.

If the copper ions are situated in an axial field, the triplet state will be split into two energy states, $m_s=0$ and $m_s=\pm 1$ which is separated by D, the zero field splitting parameter. In the presence of an applied magnetic field, H, the magnetic moment of the electrons interact with this magnetic field and the multiplet state splits (the Zeeman effect). An EPR resonance signal occurs when an electromagnetic wave of energy $h\nu$ irradiates the compound in the direction perpendicular to the magnetic field causing a magnetic dipolar transition or "flipping of spins" to occur between the split states separated by quantum number $\Delta m_s=1$. With a spin S=1, a transition corresponding to a double jump in the electronic magnetic quantum number may sometimes occur. This is referred to as a forbidden transition because it disobeys the selection rules for EPR as it corresponds to $\Delta m_s=2$. In addition, anisotropy in g will affect the position and intensity of the lines as the direction of the axis of symmetry of the dimer is changed with respect to the direction of the magnetic field. Figure 31 diagrams this situation occurring with two copper ions and shows the transitions which will occur for a dimer with a symmetry axis parallel to the magnetic field direction.

Changes in D, the zero-field splitting parameter, can dramatically change the appearance of the EPR spectra of copper dimers. Price, et al. (1970) have studied this effect by computer simulations with a hypothetical dimer. They found that if D was less than half the

energy difference of the resonance condition--about 0.15 cm^{-1} for X-band--the transition probability for forbidden lines occurring at half field was very slight. Instead, a doublet would be seen in the g=2 region. The D value for hexose oxidase copper was calculated, assuming that the shoulder divided the two perpendicular transitions and a value of 29 G or 2.81 x 10^{-3} cm^{-1} was calculated from g value of 2.075. This D value is at least 10 to 20 times less than values of typical model copper dimer systems (Chasteen and Belford, 1970; Price, et al., 1970). A D value as low as that observed in the spectrum of hexose oxidase may be caused by a large distance separating the two coupled copper ions, or to exchange coupling at a distance greater than 5 Å, or to dipole-dipole interactions that are too small to have a noticeable effect in the spectral line width (Price, et al., 1970). Assuming that the interaction between the two copper ions is solely dipole-dipole and that the axis of symmetry between the coppers is perpendicular to the magnetic field direction, then the dipole equation can be used to estimate the distance separating the two copper ions (Chasteen and Belford, 1970):

$$D_{dd} = \frac{3}{4} g^2 \beta^2 \left(1 - \frac{3 \cos^2 \theta}{r_{12}^3}\right)_{max}$$

where D_{dd} is the zero field splitting parameter for the dipole-dipole interaction equal to 5.58 x 10^{-19} ergs, g is the gyromagnetic ratio for the transition, β is the Bohr magneton, equal to 0.92731 x 10^{-20}, and θ is the angle between the r_{12} (the interelectron distance) direction and magnetic field direction. Thus, r was calculated to be 7.9 Å for the distance between the two copper atoms in hexose oxidase.

The copper in hexose oxidase may be exchange coupled through the overlap of the electrons of each copper separated by a distance greater than 5 Å. Cu(II) cyclopentanetetracarboxylic acid chelate was found to have exchange coupling parameters which are in line with those found for hexose oxidase: g=2.07-2.09 and σ of 60 G. The D value for this chelate however, 0.10-0.23 cm^{-1} was much greater than that found for copper in hexose oxidase, so the two systems are not directly comparable. It is risky to compare small dimeric copper species with one found in an unresolved protein environment, due to the many factors which contribute to line width. Bleaney and Bowers (1952) have outlined the major factors which contribute to the line width of an EPR transition:

1. Magnetic interaction between electron spins of neighboring paramagnetic units.
2. Spin-lattice relaxation.
3. Magnetic interaction between the electron spins and the nuclear magnetic moments of the neighboring diamagnetic atoms.
4. Unresolved components of the hyperfine structure.

Certainly the copper in hexose oxidase may be affected by the protein environment, perhaps in ways different from the environment of the small copper chelate. So, the signal observed for hexose oxidase may be that of a pair of copper(II) ions coupled through exchange interactions or weak dipole interactions in a protein matrix. The spectrum may also be interpreted as due to separate, bound copper (II) ions which are in a specific environment in the protein, such that the hyperfine normally seen for divalent copper is not discernable. The lack of hyperfine might be attributable also to the low level of

copper present in concentrated samples of hexose oxidase (about 0.75 ppm Cu) which comes close to the detection limit of the spectrometer for this metal (0.5 ppm).

In addition to the line found at 3200 G, another transition was always observed at about 1550 G (g=4.26) (Figure 30). It was thought at first that this line was a forbidden transition but atomic absorption analysis provided evidence that the signal was due to iron. Also, the signal could be power saturated at 60-80 mW at 77°K, a property which is not expected for forbidden transitions owing to their small transition probability (Schoot Witerkamp and Mason, 1973). The large amount of iron that was found in the 23 U/mg sample, even after dialysis against 18 megohm water, suggested that the iron was an integral part of hexose oxidase, a cofactor like copper. Alternatively, the iron may be a contaminant, bound to residual polyanionic carrageenan. This is likely since Sullivan (1973) did not find iron in emission spectral analysis of pure hexose oxidase solutions. Since carrageenan is in ion-exchange equilibrium with sea water, iron and other transition metals are to be expected in greater than trace amounts. It is noteworthy that the EPR spectra of a dialyzed NaCl-resuspension of a hexose oxidase fraction produced iron, manganese and copper ion transitions. Further studies using methods to remove loosely bound metals and carrageenan completely should resolve this question of iron involvement.

The EPR spectrum of hexose oxidase following deaereation by saturation with nitrogen, and subsequent glucose addition, produced a line form similar to that of oxygenated samples but with the amplitude diminished by 47% (Figure 29). Also with this treatment, a new line was detected at g=2.003. The narrow line width (<10 Gauss) and g

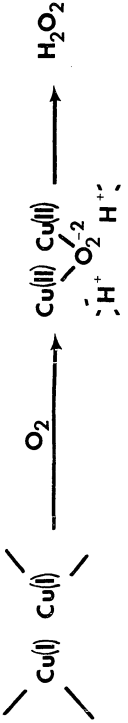
value close to that of a free electron (g=2.0023), suggests the presence of a radical. However, the appearance of a free radical signal is not sufficient evidence for invoking a one-electron step mechanism of glucose oxidation. The low field line, however, which was due to iron, showed a decrease in intensity of only 15%, which is considered insignificant in view of the noise produce by the high receiver gain (1.25×10^4). The interpretation was that the iron signal remained essentially undiminished while a certain quantity of paramagnetic species which have been attributed to copper in the g=2 region was lost upon reduction of hexose oxidase. The experiment suggested that Cu(II) changed to diamagnetic Cu(I) by this treatment, with the formation of a free radical.

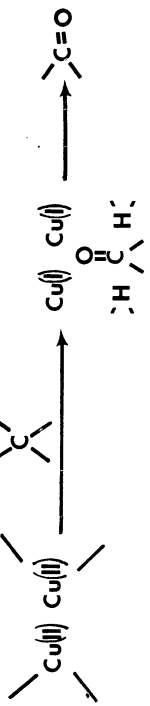
C. Mechanism of Action of Hexose Oxidase

It was established by kinetic analysis that hexose oxidase acts by alternating the addition and release of substrate and product in a typical ping pong bi bi fashion. Integrating this mechanism of action with the results of physical-chemical studies of the enzyme structure allowed a unified picture of hexose oxidase action to be postulated.

One mechanism which can be proposed calls for copper to exist in binuclear clusters within the enzyme which act to transfer pairs of electrons (Figure 32). Since EPR data suggests the possibility of exchanged or dipole coupled ions which undergo reduction with glucose in the absence of oxygen, perhaps the copper in the enzyme exists in the reduced state as pairs of Cu(I) ions. With the introduction of oxygen, the peroxo anion is formed in a complex with copper, forming the paramagnetic ternary $[Cu_2\ (II)\ O_2]^{-2}$ complex. Model copper (I)

Figure 32: Postulated mechanism of action of hexose oxidase





salts have the capacity to bind oxygen reversibly in aqueous solution. O'chiai (1977) has reported the free energy changes which accompanies O_2 binding to aqueous or ammoniacal Cu (I) pairs:

$$2\ Cu\ (I) + O_2 \longrightarrow [Cu_2\ (II)\ O_2]^{-2}$$

The standard free energy change was a favorable-4.8 and-12.6 kcal/mole, respectively. This same scheme is relevant to the action of oxygen-carrying capacity of the hemocyanins in molluscs (Lontie and Vanquickenborne, 1974).

With the formation of peroxo ion, the ping pong mechanism predicts that hydrogen peroxide be formed and released. This may occur with ionizable groups in the protein supplying the needed protons. With the formation and subsequent release of hydrogen peroxide, the copper ions would be in the divalent state. Glucose would add to the enzyme at this stage and the Cu (II) dimers would be reduced to pairs of Cu (I) by this substrate. This scheme of the mechanism is supported by EPR experiments in which the paramagnetism was reduced under these same experimental conditions. D-glucono-δ-lactone would be formed as the protons from glucose are replaced to the groups in the protein.

An alternate mechanism may be proposed which doesn't require contiguous copper pairs. If the copper in the enzyme is considered to be isolated Cu (II) ions, a step-wise mechanism involving one electron transfer may be predicted. This theory is supported by the free radical signal which appears in the EPR spectrum of hexose oxidase under reducing conditions. However, formation of a superoxide radical from oxygen in a one electron addition is thermodynamically unfavored, costing 10.4 kcal/mole at pH 7 and 25°C. Yet, a one electron trans-

fer mechanism has been implicated in xanthine oxidase action (Van Heurelen, 1976) but this enzyme contains Mo, iron and FAD, the latter stabilizing the free radical intermediate through resonance stabilization. Perhaps iron is a real cofactor in hexose oxidase, storing electrons in the same way as the iron-sulfur centers in xanthine oxidase. Unfortunately, not enough information is available to postulate a complete mechanism involving iron in the action of hexose oxidase.

From the physical-chemical study of hexose oxidase, taken with the kinetically-determined mechanism, the copper in hexose oxidase may be interpreted as conduits for electrons shuttling from glucose to oxygen. The coppers may work in tandem, transferring a pair of electrons, or as individual centers, with the transfer occurring in electron steps. Thus the state of copper in hexose oxidase is intimately involved in the mechanism of action of hexose oxidase.

BIBLIOGRAPHY

1. Andreasson, L. E., and Vänngård, T. (1970) Evidence of a Specific Copper (II) in Human Ceruloplasmin as a Binding Site for Inhibitory Anions. Biochem. Biophys. Acta 200, 247

2. Bean, R. C., and Hassid, W. Z. (1958) Carbohydrate Oxidase from a Red Alga, Iridophycus Flaccidum. J. Biol. Chem. 218, 425

3. Beers, R. F., Jr., and Sizer, I. W. (1952) A Spectrophotometric Method for Measuring the Breakdown of Hydrogen Peroxide by Catalase. J. Biol. Chem. 195, 133

4. Bennett, A., and Bogorad, L. (1971) Properties of Subunits and Aggregates of Blue-Green Algal Biliproteins. Biochemistry 10, 3625

5. Bleaney, B., and Bowers, K. D. (1952) Anomalous Paramagnetism of Copper Acetate. Proc. Roy. Soc. 214, 451

6. Boas, J. F., Pilbrow, J. R., and Smith, T. D. (1969) An Electron Spin Resonance Study of Dimer Formation of Copper (II) Protoporphyrin IX. J. Chem. Soc. (A), 721

7. Bouchilloux, S., McMahill, P., and Mason, H. S. (1963) The multiple forms of mushroom tyrosinase. J. Biol. Chem. 238, 1699

8. Broman, R. L., Malmström, B. G., Aasa, R., and Vänngård, T. (1962) Quantitative Electron Spin Resonance Studies on Native and Denatured Ceruloplasmin and Laccase. J. Mol. Biol. 5, 301

9. Butler, M. R. (1936) Seasonal Variations in Chondrus crispus. Biochem. J. 30, 1338

10. Byers, W., Curzon, G., Garbett, K., Speyer, B. E., Young, S. N., and Williams, R. J. P (1973) Anion-Binding and the State of Copper in Ceruloplasmin. Biochem. Biophys. Acta 318, 38

11. Chasteen, N. D., and Belford, R. L. (1970) Triplet State of a Binuclear Copper dl- Tartrate and Electron Paramagnetic Resonance Spectra of the Copper Tartrates. J. Inorg. Chem. 9, 169

12. Cleland, W. W. (1963) The Kinetics of Enzyme-Catalyzed Reactions with Two or More Substrates or Products. I. Nomenclature and Rate Equations. Biochem. Biophys. Acta 67, 104

13. Cleland, W. W. (1977) Determining the Chemical Mechanisms of Enzyme-catalyzed Reactions by Kinetic Studies. Adv. in Enz. 45, 273

14. Davis, B. S. (1964) Disc Electrophoresis II. Methods and Applications to Human Serum Proteins. Ann. N.Y. Acad. Sci. 121, 404

15. Dawson, C. R. (1966) Ascorbate Oxidase-A Review, in The Biochemistry of Copper. (Peisach, J., Aisen, P. and Blumberg, W. E., eds) p. 305, Academic Press, New York

16. Deinum, J., Reinhammer, B., and Marchesini, A. (1974) The Stoichrometry of the Three Different Types of Copper in Ascorbate Oxidase from Green Zucchini Squash. Fed. Eur. Biochem. Soc. Lett. 42, 241

17. Deutsch, H. F. (1960) The Preparation of Crystalline Ceruloplasmin from Human Plasma. Arch. Biochem. Biophys. 89, 225

18. Dixon, M., and Webb, E. C. (1964) Enzymes, 2nd Ed, p. 1, Longman Green, London

19. Dixon, M. (1953) The Determination of Enzyme Inhibitor Constants. Biochem. J. 55, 170

20. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956) Colorometric Method for Determination of Sugars and Related Substances. Anal. Chem. 28, 350

21. Ehrenberg, A., Malmström, B. G., Broman, L. and Mosback, R. (1962) A Magnetic Susceptibility Study of Copper Valence in Ceruloplasmin. J. Mol. Biol. 5, 450

22. Fee, J. A., Malkin, R., Malmström, B. G., and Vänngård, T. (1969) Anaerobic Oxidation-Reduction Titrations of Fungal Laccase: Evidence for Several High Potential Electron-Accepting Sites. J. Biol. Chem. 244, 4200

23. Frieden, E., and Hsieh, H. S. (1976) Ceruloplasmin: The Copper Transport Protein with Essential Oxidase Activity, in Advances in Enzymology 44, 187

24. Gomori, G. (1955) Preparation of Buffers for Use in Enzyme Studies, in Methods in Enzymology, 1, 138

25. Haglund, H. (1971) Isoelectric Focusing in pH Gradients. A Technique for the Fractionation and Characterization of Ampholytes, in Methods of Biochemical Analysis 19, 1

26. Hallaway, M. (1959) Metal Chelating Agents, in Data for Biochemical Research. (Dawson, R. M. C., Elliott, D. C., Elliott, W. H., Jones, K. M., eds) p. 155, Clarendon, Oxford

27. Hamilton, G. A., Dyrkacz, G. R., and Libby, R. D. (1976) The Involvement of Superoxide and Trivalent Copper in the Galactose Oxidase Reaction, in Advances in Exp. Med. and Biol. 74, 489

28. Haus, A. (1974) Algal Polysaccharides, in MTP International Review of Science 11. (Northcote, D. H., ed) Butterworths, University Park Press, Baltimore

29. Hayaishi, O. and Hashimoto, Z. (1950) Pyrocatecase: A New Enzyme Catalizing Oxidative Breakdown of Pyrocatechin. J. Biochem. (Tokyo) 37, 371

30. Ingraham, L. L. (1966) Enzymatic Activation of Oxygen, in Comprehensive Biochemistry 14, (Florkin, M. and Stotz, E. H., eds) Elsevier, New York

31. Kertesz, D. (1966) The Copper of Polyphenoloxidase, in The Biochemistry of Copper. (Peisach, J., Aisen, P. and Blumberg, W. E., eds) p. 359, Academic Press, New York

32. Lee, M. H., and Dawson, C. R. (1973) Ascorbate Oxidase: Further Studies on the Purification of the Enzyme. J. Biol. Chem. 248. 6596

33. Levine, W. G.(1966) Laccase, A Review, in The Biochemistry of Copper (Peisach, J., Aisen, P. and Blumberg, W. E., eds) p. 371, Academic Press, New York

34. Lontie, R., and Vanquickenborne, L. (1974) The Role of Copper in Hemocyanins, in Metal Ions in Biological Systems (Sigel, H., ed) Marcel Dekker, New York pp. 183-197

35. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein Measurement with the Folin Phenol Reagent, J. Biol. Chem. 193, 265

36. Malmström, B. G. (1965) In Oxidases and Related Redox Systems (King, T. E., Mason, H. S. and Morrison, M., eds) Wiley, New York

37. Malmström, B. G., Reinhammer, B., and Vänngård, T. (1968) Two Forms of Copper (II) in Fungal Laccase: Biochem. Biophys. Acta 156, 67

38. Malmström, B. G., Andreasson, L. E., and Reinhammer, B. (1975) Copper-Containing Oxidases and Superoxide Dismutase, in The Enzymes Vol. XII, Third Ed. (Boyer, P. D., ed) Academic Press, New York, p. 507-574

39. Malkin, R., Malmström, B. G., and Vänngård, T. (1969) The Reversible Removal of the Specific Copper (II) from Fungal Laccase. Eur. J. Biochem. 7, 253

40. Manwell, C., and Baker, C. M. A. (1963) Starch Gel Electrophoresis of Sera from Some Marine Arthropods: Studies on the Heterogeneity of Hemocyanin and on a Ceruloplasmin-like Protein. Comp. Biochem. Physiol. 193, 208

41. Mason, H. S. (1976) Binuclear Copper Clusters as Active Sites for Oxidases; Adv. in Experimental Med. and Biol. Vol 74, p. 646

42. Nakamura, T. (1976) Oxidation and Reduction of Copper Ions in Catalytic Reactions of Rhus laccase; Adv. in Exp. Med. and Biol. Vol. 74, p. 408

43. Ochiai, E. I. (1977) Bioinorganic Chemistry. An Introduction, p. 1, Allyn and Bacon, Boston

44. Pilbrow, J. R., Carr, S. G., and Smith, T. D. (1970) An Electron Spin Resonance Study of the Formation of Mixed-Ligand Chelates of Copper (II) with Amino-acids and Certain 3,4,-Dihydroxy Phenols. J. Chem. Soc., (A), 723

45. Price, J. H., Pilbrow, J. R., Murray, K. S. and Smith, T. D. (1970) Electron Spin Resonance Study of Exchange and Dipole-Dipole Coupling in Copper (II) Chelates of Cyclopentanetetra-carboxylic Acid and Related Systems. J. Chem. Soc. (A), 968

46. Reinhammer, B. (1970) Purification and Properties of Laccase and Stellacyanin from Rhus Vernicifera. Biochem. Biophys. Acta 205, 35

47. Schoot Witerkamp, A. J. M., and Mason, H. S. (1973) Magnetic Dipole-Dipole Coupled Cu (II) Pairs in Nitric Oxide-Treated Tyrosinase: A Structural Relationship Between the Active Sites of Tyrosinase and Hemocyanin. Proc. Nat. Acad. Sci. (USA) 70, 993

48. Scott, J. E. (1960) Aliphatic Ammonium Salts in the Assay of Acid Polysaccharides from Tissues, in Methods of Biochemical Analysis 8, 145

49. Sullivan, J. (1973) A Purification and Characterization of Hexose Oxidase From the Red Alga Chondrus crispus. Ph.D. Dissertation. University of New Hampshire, Durham, New Hampshire

50. Vallee, B. L., and Williams, R. J. P. (1968) Metalloenzymes: The Entatic Nature of Their Active Sites. Proc. Nat. Acad. Sci. (USA) 59, 498

51. Van Heurelen, A. (1976) Electron Transport in Xanthine Oxidase: A Model for Other Biological Transport Chains. Biophys. J. 16, 939

52. Weber, K., and Osborn, M. (1969) The Reliability of Molecular Weight Determination by Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. J. Biol. Chem. 244, 4406

53. Whistler, R. L., and Smart, C. L. (1953) Polysaccharide Chemistry Academic Press, New York, p. 218