Trivalent Copper, Superoxide, and Galactose Oxidase[1]

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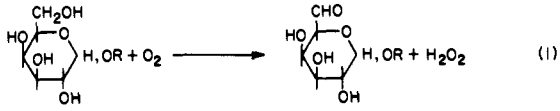
Abstract: In a detailed mechanistic investigation of the reaction catalyzed by the monocopper enzyme, galactose oxidase, the following observations (among others) have been made: (1) in kinetic experiments, O_2 uptake vs. time plots are frequently not linear but show induction periods or bursts depending on conditions, and enzyme inactivation by the product H_2O_2 occurs unless catalase is present; (2) the rate of O_2 uptake is inhibited by superoxide dismutase at pico- to nanomolar concentrations, and during this inhibition the intensity of the EPR signal given by the galactose oxidase Cu(II) *increases;* (3) ferrocyanide also inhibits the enzymic reaction and causes an increase in the intensity of the EPR signal given by the enzymic Cu(II); (4) the rate of the enzyme-catalyzed reaction of O_2 is increased by adding superoxide, or oxidants such as ferricyanide, chloroiridate, or manganic ions; (5) excess ferricyanide causes a complete disappearance of the EPR signal given by the enzymic copper but has no effect on the molecular weight of the enzyme; (6) in the presence of ferricyanide–ferrocyanide redox buffers the rate of O_2 uptake, and the intensity of the EPR signal given by the enzymic Cu(II), define a one-electron redox potential of 0.41 V above pH 7.5 but which increases by 0.06 V per pH unit at pHs below 7 (pK_a of the group involved is 7.25); (7) large changes in the optical spectrum of the enzyme are observed on adding oxidants, but the same spectrum (absorption maxima at 440 and 800 nm with molar extinction coefficients of ca. 4900 and 2800, respectively, and a large increase in absorption at 320 nm [$\Delta\epsilon \simeq 2800$]) is obtained irrespective of the oxidant used; (8) titration of galactose oxidase with hexachloroiridate indicates that the species giving the above spectrum is one electron further oxidized than the major species present in the resting enzyme; (9) with oxidants present the rate is maximal at pHs 6 to 7 but decreases only slowly at lower or higher pHs; (10) no exchange of radioactive aldehyde product into reactant alcohol occurs under several different conditions tried. The results constitute the first direct evidence for trivalent copper [Cu(III)] in an enzymic reaction. They also clarify the role of superoxide to a greater extent than has been possible with most other enzymic reactions. An overall mechanism consistent with all the data involves oxidation of the alcohol to the aldehyde by the Cu(III) enzyme with concomitant formation of a Cu(I) enzyme. Reoxidation of this species to the Cu(III) form by O_2 apparently occurs with a Cu(II) superoxide species as a fleeting intermediate. About once in several thousand turnovers superoxide leaks out from the catalytic cycle to form the inactive Cu(II) enzyme which can be converted to the catalytically active Cu(III) enzyme with one-electron oxidants. The probable role of superoxide in most oxidase and oxygenase reactions and the likelihood of Cu(III) involvement in other copper enzymes are briefly discussed.

Because of our interest in the mechanisms by which metalloenzymes catalyze reactions involving molecular oxygen,[2-4] we began several years ago a detailed investigation of the reaction (eq 1) catalyzed by galactose oxidase (D-galactose:



(1)

oxygen 6-oxidoreductase, EC 1.1.3.9).[5,6] This enzyme was chosen because of the simplicity of its overall reaction (eq 1), and because the enzyme exists as a single polypeptide chain (mol wt 68 000) with a single metal ion (copper) as its sole cofactor.[7-9] Most enzymes catalyzing reactions of O_2 are quite complex; they frequently require several metal ions and/or an organic cofactor, and they catalyze an overall complicated reaction. In such cases it has been difficult to obtain a detailed understanding of the role of the metal ion, and to obtain basic information concerning the initial steps in the reaction of O_2. It was anticipated that a study of the galactose oxidase reaction might provide such information, and the results reported here tend to bear this out. Thus, in this work we have obtained the first direct evidence for the involvement of Cu(III) in an enzymic reaction. In addition, although evidence for some role of superoxide has been obtained with several different enzymes,[10-12] its role in the galactose oxidase reaction has been clarified to a greater extent than has been the case for most other enzymic reactions. Preliminary communications of part of this work have appeared;[13,14] in the present article we describe additional experimental results and further document those previously communicated.

Galactose oxidase, an extracellular enzyme whose metabolic function is unknown, is produced by an organism which has been variously referred to as *Dactylium dendroides*[15] or *Polyporus circinatus*.[16] The enzyme is relatively nonspecific; although the nature and configuration of the substituent at C-4 are critical (glucose is not a substrate), a large number of α- and β-galactosides and compounds with other substituents at C-1, -2, and -3 are substrates.[6,16-18] Also, considerably simpler molecules, such as dihydroxyacetone,[19] glycerol, 1,3-propanediol, salicyl alcohol,[20] hydroxypyruvate, 2-methylene-1,3-propanediol, glycolaldehyde,[21] etc., are oxidized at reasonable rates. The primary alcohol group at C-6 of galactose is oxidized stereospecifically with removal of the *pro-S* hydrogen.[22]

Removal of the single copper ion of galactose oxidase gives an inactive apoenzyme, and activity can be restored by incubation with copper salts but not by other metal ions.[7] Early electron paramagnetic resonance (EPR) experiments[23] indicated that the copper in the resting enzyme exists largely (70 ± 30%) as Cu(II). The observation that no change in the intensity of the EPR signal given by the enzymic Cu(II) occurred on adding galactose either in the presence or absence of O_2 led these authors to suggest that the copper was not involved in the electron-transfer reaction. These experimental results can be completely confirmed[13] but in light of the present work the conclusions are invalid.

Experimental Procedures

Materials. The source of the galactose oxidase used for most of these experiments was a commercial preparation obtained from Worthington Biochemical Corp. and purified as indicated below. For comparison purposes, a few experiments were performed with a galactose oxidase preparation which had been purified by the method of Kosman et al.[9] and was kindly supplied by Dr. Kosman. Horseradish peroxidase (chromatographically purified lyophilized powder, 800 to 3200 IU/mg[24]), beef liver catalase (twice crystallized, 20 000 to 50 000 units/mg[25]), and superoxide dismutase (SOD, 2100 units/mg[26]) were obtained from Worthington Biochemical Corp. The SOD had a UV spectrum in good agreement with that for the pure enzyme; its con-

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centration was determined from the absorption at 258 nm assuming a molar extinction coefficient of 10 300.[26]

Unless stated otherwise, all chemicals were commercial materials which were used without further purification. D-Galactose (Mann Research Laboratories, M.A., or Sigma purified) was usually used as received but in a few cases it was recrystallized by dissolving in water, adding several volumes of ethanol, and allowing to stand at −10 °C. This material behaved no differently in kinetic assays. Each time a stock solution (10 to 11%) of galactose was prepared it was allowed to stand at least 12 h before use in assays so that mutarotational equilibrium was established. Stock solutions of $K_3Fe(CN)_6$ (Fisher Certified Reagent) and $K_4Fe(CN)_6$ (Baker and Adamson Reagent grade) were made fresh each day. Stock solutions of chloroiridate ($Na_2IrCl_6 \cdot 6H_2O$, Ventron) were prepared just prior to use. All H_2O used in this research was doubly distilled, the second time with a Kontes WS-2 glass still following percolation through Barnsted organic and ion exchange columns.

Methyl α-D- and β-D-galactopyranosides were prepared according to the procedure given by Frahn and Mills.[27] The same procedure was employed for the preparation of methyl α-D- and β-D-[1-^{14}C]galactopyranosides from D-[1-^{14}C]galactose (International Chemical and Nuclear Corp.). Methyl α-D-*galacto*-hexodialdo-1,5-pyranoside (MGDP) and methyl α-D-[1-^{14}C]-*galacto*-hexodialdo-1,5-pyranoside were prepared from the corresponding galactopyranosides by oxidation with O_2 catalyzed by galactose oxidase.[28] In a typical preparation, 0.22 g of the methyl α-D-galactopyranoside monohydrate was dissolved in 6 mL of solution containing 0.01 M bicine buffer (pH 7.8), 2.2×10^{-7} M galactose oxidase, and 50 μg of catalase. The solution (in two Warburg flasks) was shaken in the presence of pure O_2 until the utilization of O_2 stopped (ca. 24 h). After evaporation to dryness under reduced pressure, thin-layer chromatography indicated a small amount (ca. 2%) of unreacted starting material. This could be lowered to 0.6 to 0.9% by reincubation with galactose oxidase and O_2. The buffer salts and enzymes were separated from the product MGDP by passing a concentrated solution of the product mixture through a small column of Sephadex G-25 which was equilibrated and eluted with distilled water. The product MGDP could not be crystallized, but it showed behavior typical of aldehydes; it gives a positive Tollens test and is oxidized to its carboxylic acid by I_2 at pH 9.0. Proton and natural abundance ^{13}C NMR analysis[28] verified the general structure of the product, and showed that in aqueous (or D_2O) solution MGDP exists as a mixture of at least four isomers; ca. 5% as the free aldehyde, ca. 55% as the aldehyde hydrate, and ca. 40% as a mixture of the diastereomeric internal hemiacetals formed by cyclization of the C-3 hydroxyl with the C-6 aldehyde group. The radioactive product, [1-^{14}C]MGDP, had a specific activity of 8.73 μCi/mmol.

Purification of Galactose Oxidase. During the course of this research the enzyme was purified several times from various batches of the crude commercial preparation. In all cases the concentration of galactose oxidase in the commercial material was 5 to 10%. Various modifications of chromatographic procedures, some similar to those used by others[9,16] were found to give essentially homogeneous enzyme. The specific procedure outlined below gave enzyme of the highest purity and activity obtained. Although some of the chromatographic steps in early purifications were done at room temperature, enzyme of slightly higher specific activity is obtained if all operations are done at 4 °C.

In a typical purification, 0.8 to 1.0 g (ca. 70 000 Worthington units) of the commercial preparation was dissolved in 80 to 100 mL of 0.02 M sodium phosphate buffer (pH 7.1) and dialyzed (with two changes) vs. 2 L of the same buffer over a period of 2 days. The enzyme solution was added to a diethylaminoethylcellulose (Whatman DE-32) column (2.5 × 55 cm) and eluted with the same buffer. The fractions containing catalytic activity were pooled and subjected to ammonium sulfate (Schwarz/Mann special enzyme grade) fractionation. The enzyme fraction precipitating between 50 and 85% of saturation was dissolved in 15 mL of 0.02 M phosphate (pH 7.1) and dialyzed vs. 2 L of this buffer overnight. In a few cases catalase (9 μg/mL) was added to the dissolving buffer to prevent some enzyme inactivation during dialysis[7] but this was not always necessary. The dialyzed enzyme solution was applied to a Sephadex G-100 (Pharmacia) column (2.5 × 58 cm) and eluted with the same buffer. The pooled fractions (48 mL) with catalytic activity were dialyzed vs. 0.01 M phosphate (pH 7.0), applied to a carboxymethylcellulose (Schleicher and Shuell Type 20) column (2.5 × 42 cm), and eluted using a linear gradient derived from 750 mL of 0.01 M phosphate (pH 7.0) and 750 mL of 0.08 M sodium phosphate (pH 7.0). The fractions showing catalytic activity and only one band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis[29] or cathodic electrophoresis at pH 6.6[30] were combined and dialyzed vs. 0.01 M sodium phosphate (pH 7.0). For long-term storage, the enzyme was either diluted to ca. 0.1 mg/mL with the same buffer and stored at 4 °C, or concentrated to ca. 5 mg/mL and stored frozen at −20 °C. Concentration was effected by covering a dialysis tube containing the enzyme solution with dry Sephadex G-200 and leaving (with several changes of Sephadex) until the desired decrease in volume was accomplished.

In some cases when enzyme with a relatively low specific activity was obtained, its activity could be increased by adding copper under conditions (dialysis vs. 30 mM Tris buffer (pH 7.5) containing 3 mM $CuSO_4$) known to reactivate the apoenzyme.[7] However, for the very active preparations this treatment did not increase the specific activity.

Methods. Major Instrumentation. Electron paramagnetic resonance spectra were obtained at ca. 100 K using either a Varian E-9 or E-12 spectrometer under the following conditions: frequency, 9 to 9.15 GHz; modulation frequency, 100 kHz; microwave power, 20–30 mW; modulation amplitude, 10–20 G; time constant, 0.2 s; scanning rate, 125 G/min. Proton nuclear magnetic resonance (^{1}H NMR) spectra were measured using a Varian A60-A spectrometer and natural abundance ^{13}C NMR spectra were measured using a JEOL PS-100-FT spectrometer. Ultraviolet spectra were recorded with a Cary 14, 17, or 118 spectrometer; routine UV and visible assays were performed on Gilford Model 240 spectrophotometers. A Beckman Model E analytical ultracentrifuge equipped with UV optics was used for sedimentation coefficient determinations. A Packard Tri-Carb 3320 scintillation counter was utilized for radioactivity measurements.

Kinetic and Assay Methods. In some early experiments the catalytic activity during enzyme purification was monitored using a peroxidase–chromagen assay,[16] or the modification of this described in the Worthington Biochemical Corp. catalog. A Gilson Medical Electronics Model WB-3 Warburg apparatus and standard manometric procedures[31] were employed for manometric experiments. In most of the kinetic experiments the amount of oxygen consumed was measured polarographically using a Gilson Oxygraph Model K-IC equipped with a Clark electrode (Yellow Springs Model 5331) and Yellow Springs Model 5301 bath assembly for maintaining constant temperature.

Because of the effects of oxidizing and reducing agents on galactose oxidase, assays of enzyme activity are only quantitatively meaningful if the concentrations of these are strictly controlled. In the presence of 1 mM ferricyanide the catalytic activity is reproducible and at its maximum. Thus, the *standard* assay method now used for the enzyme is the polarographic method under the following conditions: temperature, 25 ± 0.1 °C; volume, 3 mL, air saturated; 0.10 M sodium phosphate buffer (pH 7.0); 0.10 M galactose; 1.0 mM ferricyanide; ca. 2 nM galactose oxidase. In the standard assay the reaction solutions without the enzyme are temperature equilibrated for at least 15 min prior to initiating the reaction by adding a 5-μL sample of a more concentrated enzyme solution. The amount of O_2 uptake is recorded until about 10% of the O_2 is reacted (usually about 1 min); over this period the uptake is linear with time.

Except when chloroiridate and Mn(III) were used as oxidants, the techniques were the same as the above for other kinetic experiments employing the polarographic method. For runs using these oxidants the reaction solutions, without the enzyme and oxidant, were temperature equilibrated, and the oxidant added in a small aliquot just prior to initiation by adding enzyme. This was done to minimize nonenzymic oxidation of the substrate by these oxidants.

Enzyme activities and specific activities are given in katals and katals/kilogram (kat/kg) as recently recommended by the International Union of Pure and Applied Chemistry and the International Union of Biochemistry.[32] One katal is equal to 1 mol of O_2 consumed per s. Enzyme concentrations were determined from the observed absorbances at 280 nm assuming a molar extinction coefficient of 104 900 and a mol wt of 68 000.[9]

When concentrations of oxygen equivalent to 100% O_2 in the gas phase were required, an H_2O-saturated stream of pure O_2 was passed over the vigorously stirred reaction solutions (for the polarographic assays), or through the vigorously shaking Warburg apparatus (for the manometric experiments), for a period of 15 to 30 min prior to reaction initiation. When other O_2 concentrations were used the same procedures were applied except that the gas stream was prepared by

mixing (using a three-way stopcock) air or O_2 and N_2 streams which had passed through calibrated Fisher gas–liquid flowmeters. In such cases the concentrations of O_2 in the stream and in the solutions were checked using a Beckman Oxygen Analyzer or the calibrated Gilson Oxygraph. Reproducibility in the O_2 content of better than 3% could be achieved using these procedures.

In those polarographic experiments utilizing a flavin-EDTA–light system to generate superoxide in situ,[33] darkness was maintained by cloaking the bath assembly with a black velvet cloth. The "light" conditions refer to room light which was admitted when the cloth was removed. In similar manometric experiments, the flasks were illuminated (through a Lucite bottom of the constant temperature bath) using a bank of 30-w reflector lamps located 10 cm below the reaction flasks.

In some kinetic experiments the amount of ferricyanide consumed was measured spectrally by following the decrease in absorbance at 420 nm. The molar extinction coefficient for ferricyanide at 420 nm is 1050 while that for ferrocyanide is 0.

Copper Analysis. In some cases the copper content of the enzyme solutions was determined from the absorption at 440 nm of the diethyldithiocarbamate complex. This reagent rapidly removes copper from galactose oxidase.[16] The molar extinction coefficient for the complex at 440 nm is 9650 (determined using standard $CuSO_4$ solutions). In other cases the copper content was obtained by atomic absorption using a Varian AA-5 spectrophotometer equipped with a Model 63 carbon rod atomizer and carbon tube furnace.

Exchange Experiments with Isotopically Labeled Products. All reactions were run in Warburg flasks attached to the Warburg apparatus, and were initiated by adding galactose oxidase (and catalase when present) from the side arm of the Warburg flask to the other components in the main flask compartment. Reactions were halted by adding 10 μL of 20 mM diethyldithiocarbamate. Prior to termination a 30-μL sample of each reaction solution was removed and analyzed for catalytic activity using a peroxidase assay. In the control everything was the same as in the regular runs except that the reaction was terminated within 1 min after initiation. Anaerobic conditions were achieved by passing N_2 through the system for 2 h prior to reaction initiation. The N_2 (from a gas cylinder) was made O_2 free and H_2O saturated by passing it successively through: (1) vanadium sulfate solutions in contact with a zinc-mercury amalgam, (2) reduced lumiflavin solutions, (3) a tube containing hot copper turnings, and (4) a tube containing water.

Following termination of the reactions and their controls, the amount of radioactivity in the reactant galactose (methyl α-D-galactopyranoside) was determined by the following procedure. The enzymes in each reaction mixture were removed by passage through a small column (1 × 20 cm) of Sephadex G-25 which was equilibrated and eluted with distilled water. The radioactive fractions were pooled and evaporated to dryness under reduced pressure. Because the reactant galactoside and product aldehyde (MGDP) gave poor separation on thin-layer chromatography, the aldehyde was quantitatively oxidized by alkaline–iodine solution to its carboxylic acid using a slight modification[28] of the procedure of MacLeod and Robinson.[34] Subsequent chromatography on Gelman ITLC plates of silica gel glass microfiber using a solvent system of methanol and chloroform (1:9, v/v) gave complete separation of the reactant galactoside (R_f 0.65) from the carboxylic acid derived from MGDP which remained at the origin. The distribution of radioactivity was determined by cutting the plate in 1-cm strips, placing each strip in a scintillation vial, adding 10 mL of scintillation cocktail,[35] and counting at 4 °C. Control experiments showed that the above iodine oxidation procedure did give quantitative oxidation of the aldehyde and none of the reactant galactoside is oxidized.

For the reactions performed in tritiated water, the analysis procedures were similar to the above except that, prior to the oxidation by iodine, the tritiated water and exchangeable tritium were washed out by 5 to 6 cycles of evaporation to dryness and dissolution in 5 mL of water. In each cycle the H_2O solution was left to stand for 20 h at room temperature to ensure that any exchangeable tritium would get into the water. After 5 to 6 cycles the radioactivity in the H_2O solution had reached a constant level.

EPR Spectra of Superoxide Dismutase (SOD) Inhibited Reactions. One milliliter of the reaction mixture containing 0.02 M phosphate buffer (pH 7.1), 0.10 M galactose, 2 μg/mL catalase, 1.03 μg/mL SOD, and 4.8 μM galactose oxidase was placed in a 500-mL three-necked round-bottomed flask maintained at 0 °C and continuously flushed with H_2O-saturated O_2 gas. To increase the diffusion of O_2 into the reaction solutions, the flask was continually swirled (to produce a thin film of the reacting solution on the inside of the flask). At the designated times, 200-250-μL samples were removed and frozen in liquid N_2 for subsequent spectral analysis.

Table I. Purification of Galactose Oxidase

Step	Vol, mL	Ca. amount of protein,[a] mg	Act.,[b] μkat	Sp. act.,[b] kat/kg	Yield, %
Dialyzed crude prep.	100	606	500	0.80	100
DEAE-cellulose	103	436	494	1.14	99
Sephadex G-100	48	87	436	5.0	87
CM-cellulose	78	26	200	7.8	40

[a] Estimated from the absorbance at 280 nm assuming the extinction coefficient ($E_{1cm}^{1\%}$ = 15.4) known for galactose oxidase.[9] [b] Determined polarographically using standard assay conditions (see Experimental Section) with 1 mM ferricyanide present.

Results

Enzyme Purification and Characterization. A typical purification of galactose oxidase from the commercial preparation is outlined in Table I. Overall yields of the order of 40–75% with a tenfold increase in specific activity are routinely obtained. The final preparation showed only one band on gel electrophoresis at pH 6.6[30] and on sodium dodecyl sulfate gel electrophoresis.[29] Ultracentrifugal analysis of other preparations purified by similar procedures indicated a homogeneous protein with an $s_{20,w}$ of 4.7 S in good agreement with that previously reported.[8,9] Analysis of various preparations for copper content gave values of 0.7 to 0.9 atom of copper per molecule (mol wt 68 000).

The specific activity of the purified material varies somewhat from preparation to preparation even though gel electrophoresis indicates the presence of only one protein band, or at most impurities which could account for less than 10% of the total protein. This variation in activity is not due to some of the enzyme being present as the apoenzyme because dialysis vs. copper ions under conditions known to regenerate the holoenzyme did not increase the specific activity of the less active preparations. The reason for the variation in activity is not known but it may be related to the irreversible inactivation by H_2O_2 (vide infra); possibly varying amounts of such inactivation occur during growth of the organism producing the enzyme or during the purification steps. The specific activity was usually constant throughout the activity peak in the final chromatography step. However, in one instance with an especially long column, the specific activity decreased somewhat on the tailing end of the peak even though only one band on gel electrophoresis was seen.

Most of the enzyme used in this research had a specific activity of 4 to 8 kat/kg but occasionally it was as low as 2 kat/kg. In all cases where they were checked with two or more enzyme preparations the same effects in kinetic and EPR experiments were observed regardless of the specific activity of the preparation used. Thus, the maximum rate obtained with a given preparation might be less than with another but the same relative effects of additives were always noted.

Because of the different assay conditions which have been used by others, and because of the now known effects of various additives on the enzymic rate (vide infra), it is difficult to compare quantitatively the specific activity of our purified preparations with those of others. The activities measured by the peroxidase assays used by Avigad et al.[6] and Kosman et al.[9] are very dependent on conditions as they themselves noted and as we confirm. The galactose oxidase reaction is inhibited by the chromagen (*o*-dianisidine) and is not linear in enzyme

concentration. Furthermore, the peroxidase–H_2O_2 system has an activating effect on the galactose oxidase reaction,[21,36] the extent of which varies with conditions. Our best efforts to duplicate the assay conditions of Avigad et al.[6] with our enzyme indicate that 1 kat/kg is equivalent to ca. 2700 of their units per mg. Therefore, their best preparation (8000 units/mg) would correspond to a specific activity of ca. 3 kat/kg. A sample of Kosman's enzyme, prepared and supplied by him, gave under our standard assay conditions a specific activity of 7.3 kat/kg. In summary then, the specific activities of our preparations appear similar to those of other apparently homogeneous preparations. In order to avoid the ambiguities of the peroxidase assay and the differing amounts of the inactive and active enzyme forms which result from uncontrolled redox conditions, it is strongly recommended that the future assays of the enzyme be conducted under strongly oxidizing conditions, for example, with 1 mM ferricyanide present as in our standard assay.

Galactose oxidase is relatively stable to long-term storage if precautions are taken. A dilute solution (ca. 0.1 mg/mL) in 0.01 M phosphate buffer (pH 7) can be stored for a year at 4 °C with only about 30% decrease in activity. At room temperature approximately 10% of the activity is lost over an 8-h period. More concentrated solutions (ca. 5 mg/mL) in the same buffer lose activity relatively rapidly (50% loss in 2 months) if stored at 4 °C. Concurrent with activity loss is the formation of a precipitate which cannot be redissolved in buffer. The concentrated solutions can be stored frozen at −20 °C without loss of activity over a period of months. Thawing and refreezing of such samples as many as 10 times over an 8-month period result in minimal loss of activity. The enzyme cannot be successfully stored at 4 °C in 0.01 M bicine buffer (pH 7.8). Over a 2-week period under such conditions essentially all its activity is lost.

All the above conditions refer to solutions in contact with air. Under anaerobic conditions (N_2) the enzyme appears to be much less stable. Although a systematic survey of the effects of buffer, pH, concentration, etc. was not undertaken, it was generally observed that any time the enzyme was left in the absence of O_2 for an extended period of time considerable loss of activity resulted.

General Kinetic Observations. It has been noted in earlier publications[13,20] that slight alterations in reaction conditions and the presence or absence of various additives frequently affect the rate and course of uptake of O_2 by large amounts. Some preliminary observations made in this early work include the following: (1) frequently O_2 uptake vs. time curves are not linear but show a slower initial rate (induction period); (2) the enzyme is inactivated by its product, H_2O_2; (3) ferricyanide causes a marked increase in the rate of the enzyme-catalyzed O_2 uptake; (4) EDTA under some conditions increases the enzymic rate; (5) superoxide dismutase (SOD) inhibits the enzymic reaction; (6) superoxide stimulates O_2 uptake. Among subsequent observations are the following: (1) sometimes O_2 uptake vs. time curves show an initial rapid reaction (burst phenomenon) followed by a slower phase, while under other conditions O_2 uptake is essentially linear with time in the initial stages of the reaction; (2) ferrocyanide inhibits the enzymic reaction; (3) chloroiridate and manganic ions have an activating effect similar to ferricyanide.

All of the above effects have now been studied in considerably more detail, and it is evident that complex interrelationships exist among them. In presenting these results an attempt will be made to isolate the effect of each additive as much as possible, but since the addition of a second (or third, etc.) additive affects the results obtained with the first, the various subsections will contain information relevant to the effects of other additives as well.

Induction Periods, Bursts, and Linear Initial Rates. These effects are seen using either the manometric or polarographic technique for following O_2 uptake. When oxidants and redox buffers (e.g., mixtures of ferricyanide and ferrocyanide) are present, a linear uptake of O_2 is obtained during the early stages of the reaction. If oxidants and redox buffers are not present, a linear initial rate is sometimes observed but this is probably coincidental (see Discussion). Induction periods tend to be observed under conditions which ultimately lead to a rapid uptake of O_2, while bursts are seen especially in those cases which ultimately give a relatively slow uptake of O_2. Depending on conditions, the induction period or burst may last for as short as a few seconds to as long as 30 min or more. Although the magnitude of these phenomena varies somewhat from one enzyme preparation to another, a typical set of conditions which usually shows induction periods is phosphate buffer, pH ca. 7, in the absence of oxidants or redox buffers.[20] Under these conditions the length of the induction period is increased by lower galactose or enzyme concentrations and by the presence of small amounts (up to 30 mg/mL) of catalase. Typical conditions under which bursts are observed are those which contain SOD.

The observation of induction periods and bursts suggests that the enzyme as stored exists as a mixture of at least two forms, one catalytically active and one not (or less active). If, under the conditions of catalysis, the ratio of these is gradually changed to a greater amount of the active form an induction period would result. On the other hand, a burst would be expected if the ratio changed to give a greater amount of the inactive (or less active) form during catalysis.

In those kinetic experiments reported below in which an induction period or burst is obtained (the general rule when oxidants and redox buffers are not present) the rate given is that obtained after the induction period or burst is completed. However, due to the curvature in these plots the rates reported cannot be considered as accurate as those (obtained when oxidants or redox buffers are present) which give an initial linear uptake of O_2 with time.

Inactivation by H_2O_2. When H_2O_2 is allowed to build up (absence of peroxidase and catalase) during active catalysis, galactose oxidase becomes inactive.[20] Some representative data illustrating the effect of various conditions on the inactivation are shown in Figure 1. After O_2 uptake has stopped, the enzyme is apparently irreversibly inactivated; no conditions have yet been found to reactivate the enzyme. For example, extended dialysis, or addition of catalase after the inactivation has occurred, does not lead to regeneration of catalytic activity. Also, the inactivation is not simply due to removal of the enzymic copper because subsequent dialysis vs. copper salts, under conditions known to regenerate the holoenzyme from the apoenzyme, does not reverse the inactivation.

As the data in Figure 1 indicate, the inactivation can be avoided by adding catalase (expt H) but occurs after less O_2 has reacted if H_2O_2 (1 mM) is added initially (expt F). More O_2 reacts when the O_2 concentration is increased (expt D) but the enzyme still becomes inactivated if the reaction is followed further than shown in Figure 1. At higher enzyme concentrations (expt C) more O_2 reacts before inactivation but less reacts at lower galactose concentrations (expt B). Experiment G of Figure 1 is particularly noteworthy. It indicates that the enzyme is quite stable in the presence of H_2O_2 in the absence of catalysis; the enzyme is only inactivated during active catalysis of its reaction. This result suggests that some enzymic species (reactive toward peroxide) is present during catalysis which is not present in the resting enzyme.

The inactivation by H_2O_2 has been observed using both the manometric technique (Figure 1) and the polarographic method for following O_2 uptake. Every preparation of galactose oxidase we have prepared shows this effect. Furthermore, the inactivation is also seen when the preparation supplied by

Table II. Effect of EDTA on the Rate of the Galactose Oxidase Catalyzed Reaction

Conditions[a]	Rate of O_2 consumption, kat/kg	
	No EDTA	0.5 mM EDTA
pH 7.0	0.20	0.72
pH 7.0, no catalase	0.16	1.10
pH 7.8, no catalase	0.18	0.74
pH 7.0 with ferricyanide (1.0 mM)	1.8	1.8
pH 7.0 with SOD (1.0 nM)	0.08	0.08

[a] Reaction conditions: volume, 3 mL; air saturated; 25 °C; 92 mM galactose; 1.6 nM galactose oxidase; 10 mM phosphate buffer at pH 7.0 and 10 mM bicine buffer at pH 7.8; 1.7 μg/mL catalase (840 kat/kg) except where indicated; rates determined polarographically.

Table III. Effect of Superoxide Dismutase on the Rate of the Galactose Oxidase Catalyzed Reaction[a]

[SOD], nM	Rate of O_2 consumption, kat/kg			
	pH 7.8[b]	pH 7.8[c]	pH 7.1[c]	pH 7.1[c,d]
0.0	0.54	0.45	0.61	1.8
0.021	0.33		0.47	
0.052	0.24		0.29	
0.25	0.13	0.11	0.13	
1.0	0.09	0.07	0.07	1.8
2.5	0.07	0.04		

[a] General reaction conditions: volume, 3 mL; air saturated; 25 °C; 0.5 mM EDTA; 1.7 μg/mL catalase (840 kat/kg); 10 mM bicine buffer at pH 7.8 and 20 mM phosphate buffer at pH 7.1; 1.6 nM galactose oxidase and 92 mM galactose in polarographic experiments, 16 nM galactose oxidase and 37 mM galactose in manometric experiments. [b] Determined manometrically. [c] Determined polarographically. [d] Ferricyanide (1.0 mM) present.

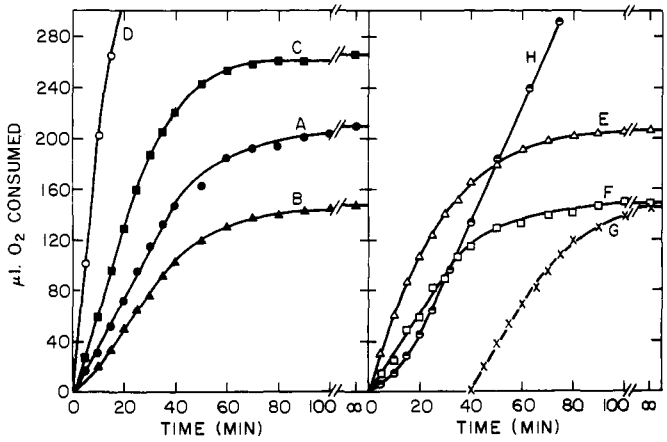


Figure 1. Effects of various additives and reaction conditions on the inactivation of galactose oxidase by its reaction product, H_2O_2. The O_2 uptake was followed manometrically. The reaction conditions for A (●) were as follows: 25 °C; volume, 3 mL; air atmosphere; 0.10 M phosphate buffer (pH 7.0); 0.10 M galactose; 25 nM galactose oxidase; reaction initiated by adding the enzyme. For the other runs the reaction conditions were the same as in A except as noted: B (▲) 0.02 M galactose; c (■) 50 nM galactose oxidase; D (○) 100% O_2 atmosphere; E (△) 0.5 mM EDTA also present; F (□) H_2O_2 (1.2 mM) present initially; G (×) the enzyme was incubated for 40 min with 1.2 mM H_2O_2 under the usual reaction conditions but with no galactose present, and then the reaction was initiated by adding galactose (to a final concentration of 0.10 M); H (⊖) 2.2 μg/mL catalase present.

Dr. Kosman is used. Thus, the inactivation is not an artifact of a particular preparation; it is a characteristic of the galactose oxidase reaction.

In kinetic runs using the polarographic technique the inactivation by H_2O_2 is not a problem because the disappearance of O_2 is followed to only 10% completion and the amount (25 μM) of H_2O_2 formed during this time is too small to have any significant effect. However, in manometric experiments only approximate rates can be obtained in the absence of catalase.

Kinetic Effects of EDTA. In Table II are summarized some representative data indicating that EDTA causes an increase in the rate of the galactose oxidase reaction when an oxidizing agent (ferricyanide) and superoxide dimutase (SOD) are not present. In other experiments it was found that lower concentrations of EDTA give less activation but higher concentrations give the same amount as 0.5 mM.[37] The actual magnitude (up to seven-fold or more) of the activation is not too meaningful because the rates in the absence of EDTA vary considerably (±20% or more) from day to day even with the same enzyme preparation. With 0.5 mM EDTA present the rates are reproducible within ±4% from day to day. As the results in Table II indicate, the EDTA activation is seen both in the presence and absence of catalase.

Since EDTA most likely exerts its influence by complexing with trace metal ions, these results indicate that trace metal ions inhibit the galactose oxidase reaction. Because there is no activation by EDTA when SOD is present, the trace metal ions in the absence of EDTA are probably causing inhibition by catalyzing the disproportionation of superoxide; i.e., they are functioning in the same way as SOD except less efficiently. Such as explanation is consistent with the observation that EDTA complexes of metal ions are not as effective as the uncomplexed metal ions in catalyzing the disproportionation of superoxide.[38]

Since EDTA has no effect (Table II) on the rate obtained with an oxidant (ferricyanide) or SOD present, rates obtained in the presence of such additives can be directly compared regardless of whether EDTA is present or not. In fact, EDTA can be used as a convenient buffer under such conditions. However, in considering the percent of inhibition caused by SOD, or the amount of activation given by ferricyanide, it is of prime importance whether EDTA is present or not. Thus, from the data in Table II, SOD causes a 60% inhibition in the absence of EDTA but an 89% inhibition in its presence. Under other conditions (presumably those giving more effective disproportionation by the trace metal ions) SOD has essentially no inhibitory effect in the absence of EDTA. Very likely this is the reason that only a small amount of inhibition by SOD was observed by Kwiatkowski and Kosman.[36]

Inhibition by Superoxide Dismutase. Summarized in Table III are some representative data showing the effective of SOD concentration on the inhibition of the galactose oxidase reaction under a number of conditions. The results shown in the table were obtained with catalase present but the magnitude of the inhibition with catalase absent is similar. Also, experiments done at different galactose and O_2 concentrations show similar effects.[37] For the reasons given in the previous section, EDTA is present in all these reactions.

One notable feature of the results in Table III is that SOD has no effect on the rate when ferricyanide is present (last column). This result is obtained for every set of conditions (different buffers and pHs, with and without EDTA and/or catalase, manometric or polarographic techniques, etc.) which have been examined. Since it now seems likely that the kinetic activation by peroxidase is due to an effect similar to that of ferricyanide (see later), it is thus not surprising that no inhibition by SOD is observed when peroxidase is present.[36]

Another significant aspect of the results shown in Table III is the extremely low level of SOD required to show inhibition. Even 20 pM (less than 1 ng/mL) SOD causes measurable inhibition, and greater than 50% inhibition is achieved with

Table IV. Effect of Superoxide on the Rate of the Galactose Oxidase Catalyzed Reaction

Conditions	[Galactose oxidase], nM	[Flavin],[a] μM	Rate of O_2 consumption, nkat
Manometric technique[b]			
Light	0	36	<0.1
Light	8.9	0	2.3
Light	8.9	36	4.8
Polarographic technique[c]			
Light	0	33	0.09
Dark	1.6	33	0.24
Light	1.6	33	0.59

[a] The flavin used was 3-carbethoxymethyl-10-methylisoalloxazine.[39] [b] Reaction conditions: volume, 3 mL; air saturated; 25 °C; 10 mM bicine buffer (pH 7.8); 0.1 M galactose; 6.6 μg/mL catalase (840 kat/kg); 11 mM EDTA; 200 μL of 5 N KOH present in reaction flask center well to remove CO_2 produced; light is a 30-W reflector light 10 cm from the bottom of the flask. [c] Reaction conditions: volume, 3 mL; air saturated; 25 °C; 10 mM phosphate buffer (pH 7.0); 92 mM galactose; 6 μg/mL catalase (840 kat/kg); 10 mM EDTA; light is room light.

Table V. Effect of Oxidants on the Rate of the Galactose Oxidase Catalyzed Reaction[a]

[Ferricyanide], μM	Rate of O_2 consumption, kat/kg pH 7.0[b]	pH 7.8[c]	[Chloroiridate], μM	Rate of O_2 consumption, kat/kg[d]
0	1.1	0.59	0	0.14
1	0.59	0.27	1.0	4.5
10	1.1	0.36	2.0	4.3
100	2.2	1.0	5.0	4.4
500	2.2	2.0	10	4.3
1000	2.3	2.2	*e*	4.4

[a] Rates measured polarographically starting with air-saturated solutions at 25 °C. [b] Reaction conditions: 92 mM galactose, 1.6 nM galactose oxidase, 0.5 mM EDTA, and 10 mM phosphate buffer. [c] Same conditions as footnote *b* except 10 mM bicine buffer instead of phosphate. [d] Reaction conditions: 100 mM galactose, 2.0 nM galactose oxidase (a different preparation from that in footnote *b*), 0.10 M phosphate buffer (pH 7.0). [e] Ferricyanide (1 mM) and no chloroiridate present.

50 pM (2 ng/mL) SOD. This is about 1/50 the amount required to show 50% inhibition in the xanthine oxidase–cytochrome *c* reaction,[26] and 3 to 4 orders of magnitude less than that required for most other enzymic reactions where SOD has been shown to have an effect.[10-12] Thus, under the right conditions (EDTA, but no oxidants present) the galactose oxidase reaction is very sensitive to SOD inhibition. This is considered strong evidence that superoxide is involved somehow in the galactose oxidase reaction.

A further characteristic of the SOD inhibition is that the inhibited rate is not obtained immediately on initiation of the galactose oxidase reaction (by adding the galactose oxidase). In all cases the reaction starts off at its usual rate (absence of SOD) but slows to the reported inhibited rate within a half-time of about 10 to 20 s. This occurs even though massive amounts of SOD are added. Since the turnover number for catalytically active galactose oxidase molecules is of the order of 200/s under the typical conditions of the inhibition experiments, the above phenomenon indicates that many turns of the catalytic cycle occur before superoxide leaks out, reacts with SOD, and gives a catalytically inactive form of galactose oxidase.

Kinetic Activation by Superoxide. Since removal of superoxide by SOD gives an inhibited enzyme, it is expected that addition of superoxide to the galactose oxidase system would enhance the enzymic activity. The results summarized in Table IV bear this out. In these experiments superoxide was generated in situ by the flavin–EDTA–light system described by Massey et al.[33] The rates obtained in the dark or with no flavin are measures of the enzymic rate without superoxide, while those with no galactose oxidase are measures of the amount of O_2 used in the generation of superoxide. It is particularly noteworthy that the rates obtained with light, flavin, and enzyme all present are greater than the sum of the other two rates. Thus, the addition of superoxide not only increases the enzyme-catalyzed O_2 reaction but it does so in a nonstoichiometric fashion; one molecule of superoxide reacting with the enzyme leads to the turnover of many molecules of O_2 in the usual catalytic reaction. The maximum rate obtained with the superoxide generating system on is very similar to the rate observed with excess oxidizing agents present (see next section).

Effect of Oxidants on the Enzymic Rate. As has been noted here and earlier,[13,20] the rate of O_2 uptake catalyzed by galactose oxidase is markedly enhanced when the reaction solutions contain ferricyanide. Similar effects are seen with other oxidants such as chloroiridate and Mn(III). Table V summarizes some results showing the magnitude of the activation as a function of ferricyanide and chloroiridate concentrations. The chloroiridate results were obtained with an enzyme preparation which was more active than that used for the ferricyanide experiments summarized on the left side of the table. With the same enzyme preparation the rate in the presence of 1 to 10 μM chloroiridate is the same as with 1 mM ferricyanide (last entry, right side of table). Micromolar concentrations of Mn(III) (as its EDTA complex) also activate the enzyme to the same extent.

The results in Table V indicate that, although ferricyanide activates the enzyme when present at 1 mM concentration, it is an inhibitor at 1 μM concentration. This inhibition shows characteristics similar to the inhibition by SOD; for example, the rate starts off at the same rate as in the absence of ferricyanide but slows to the reported rate after 1 to 2 min. Significantly, SOD has no effect on any of the rates with ferricyanide present, including the inhibited rate at 1 μM ferricyanide. The results suggest that ferricyanide acts as an inhibitor by reacting with superoxide, but this effect is completely countered at high ferricyanide concentration in the same way, and probably for the same reasons, that high ferricyanide concentrations counter completely the SOD inhibition (Table III). Since ferricyanide is an oxidant, one suspects that it is exerting its activating effect by oxidizing some enzymic group. The fact that chloroiridate and Mn(III) give the same amount of activation substantiates this conclusion. Lower concentrations of these other oxidants are probably required because of their higher oxidation–reduction potential. For example, the oxidation potential of chloroiridate is approximately 0.9 V[40] while that for ferricyanide is in the range of 0.4 V.[41] One suspects that other oxidants of sufficiently high oxidation potential should cause activation similar to that of ferricyanide, chloroiridate, and Mn(III). The peroxidase–H_2O_2–chromagen system generates intermediate radicals of high oxidation potential so almost certainly the observed activation[21,36] of galactose oxidase by this system is related to the ferricyanide activation.

The effects of 1 mM ferricyanide have now been studied under a vast array of conditions (with and without catalase, at various pHs, and with different buffers, etc.) and the results can be summarized briefly; under all conditions, 1 mM ferricyanide counters completely the effects of SOD and trace metal ions, and causes the maximum rate of O_2 uptake

achievable with any particular enzyme preparation. These results suggest that ferricyanide is able to perform the same function that superoxide usually does; it can somehow replace the superoxide which leaks out from the catalytic cycle and is trapped. In doing so, the amount of ferricyanide actually consumed is negligible relative to the amount of O_2 reaction it stimulates. For example, under typical reaction conditions with ferricyanide (1 mM), SOD, and EDTA present, less than 1 nmol (limit of detection by the spectral method) of ferricyanide is converted to ferrocyanide for each μmol of O_2 which reacts.[37] These are conditions in which ferricyanide stimulates the rate severalfold. Thus, the ferricyanide is obviously not reacting stoichiometrically.

The polarographic technique is preferable for studying the kinetics of the galactose oxidase reaction in the presence of ferricyanide (or other oxidants) because long-term contact of the enzyme with oxidants causes inactivation. With ferricyanide (1 mM) as oxidant, the inactivation is relatively slow and is not significant during the short time (1 to 3 min) required for polarographic assays; i.e., O_2 consumption is linear with time when one starts with air-saturated solutions. In manometric experiments, however, 1 mM ferricyanide causes curvature in the O_2 uptake vs. time plots in both the presence and absence of catalase. Higher O_2 concentrations protect the enzyme from the slow inactivation by ferricyanide but the inactivation occurs more rapidly at low O_2 concentrations. Thus, it is difficult even with the polarographic method to obtain good kinetics in the presence of ferricyanide at very low O_2 concentrations. High concentrations (>10 μM) of chloroiridate or Mn(III) also inactivate the enzyme but this inactivation is slow enough at concentrations less than 10 μM so that essentially a linear uptake of O_2 is observed.

Ferricyanide as a Substrate in the Reaction Catalyzed by Galactose Oxidase. Under anaerobic conditions, and with relatively high galactose oxidase concentrations, a slow enzyme-catalyzed conversion of ferricyanide to ferrocyanide is observed (followed spectrally at 420 nm) when galactose is present. Under the following conditions (10 mM phosphate buffer (pH 7.0), 92 mM galactose, 0.5 mM EDTA, 1 mM ferricyanide, 2 to 3 μg/mL catalase, 25 °C) the rate of O_2 uptake in the aerobic reaction (air, 1.6 nM galactose oxidase) is greater than 10^4 times the initial rate of ferricyanide consumption in the anaerobic reaction (300 nM galactose oxidase) when the different concentrations of enzyme are taken into account. These results are only approximate because, under anaerobic conditions, ferricyanide fairly rapidly inactivates the enzyme (progress curves are markedly curved). However, the results do indicate that ferricyanide can replace O_2, albeit poorly, as an electron acceptor in the enzymic reaction.

Some EPR Experiments with Galactose Oxidase. Several laboratories[21,23,42] including our own[13] have now reported EPR spectra of the resting state of galactose oxidase as isolated and stored at pH 7.0, and the spectra are all essentially the same. A strong Cu(II) signal with considerable superhyperfine structure is seen. As Blumberg et al.[23] noted, the intensity of the Cu(II) signal does not change appreciably on adding galactose.[13,42] However, since the foregoing kinetic results strongly imply that the enzyme does undergo oxidation and reduction changes, EPR spectra were taken under conditions known to give mainly catalytically inactive enzyme (with SOD present), and under others known to give completely active enzyme (ferricyanide present). In Figure 2 are illustrated the results from the SOD experiment. Because the inhibition by SOD is only evident after thousands of turnovers of the catalytic cycle have occurred, it was necessary to use low enzyme concentrations and special conditions (see Experimental Section) in order to minimize O_2 diffusion problems. Thus, the background noise in the observed spectra is considerable. Nevertheless, the results indicate that after 10-min reaction

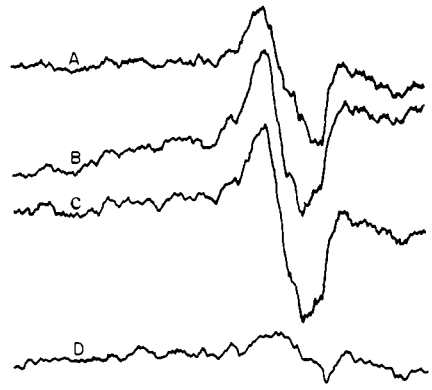


Figure 2. EPR spectral changes during the SOD inhibited galactose oxidase reaction. For reaction conditions see Experimental Section: (A) spectrum of the reaction solution (4.8 μM galactose oxidase) before initiation by adding galactose; (B) 1 min after initiation; (C) 10 min after initiation; (D) cavity signal given by the EPR spectrometer.

the intensity of the signal given by the enzymic Cu(II) increases about 30–50% above what it is initially. These are conditions which should have led to several thousand turnovers of the catalytic cycle and thus mainly inhibited enzyme. The result thus implies that the Cu(II) form of the enzyme is inactive catalytically. Furthermore, it indicates that the resting form of the enzyme is a mixture of at least two redox states of the copper.

Kosman and coworkers[42] have reported that SOD has no effect on the EPR spectrum given by galactose oxidase. However, in their experiments the galactose oxidase concentration was very high, and less than two turnovers of the catalytic cycle could have occurred before the O_2 in their system would be depleted. Since the kinetic effect of SOD is only observed after several thousand turnovers of the catalytic cycle, no effect of SOD would be expected under their conditions.

As will be shown later, 1 mM ferrocyanide, like SOD, also causes an almost complete inhibition of the galactose oxidase reaction. When 1 mM ferrocyanide is added to a solution of galactose oxidase, the intensity of the EPR signal given by the enzymic Cu(II) again increases 10–40% over what it is in the absence of ferrocyanide. This result not only confirms that the Cu(II) form of the enzyme is inactive catalytically, but also indicates that the resting form of the enzyme is a mixture of a Cu(II) state and a form more highly oxidized than the Cu(II) state.

As has been reported,[13] the addition of 1 mM ferricyanide to galactose oxidase causes the disappearance of the EPR spectrum given by the enzymic Cu(II). Since these are conditions which give fully active enzyme, the result indicates that a state of the enzyme more highly oxidized than the Cu(II) state is a catalytically active form. The same result is obtained under either aerobic or anaerobic conditions. Thus, the formation of the oxidized state does not depend on O_2 and the state therefore does not contain O_2.

The possibility was considered that the oxidized species which does not show an EPR spectrum is a Cu(II) superoxide complex. The only reasonable way in which this could be formed on oxidation of a Cu(II) species with ferricyanide is if H_2O_2 is also present. However, after extended dialysis (36 h) of a galactose oxidase solution (in 0.01 M phosphate buffer, pH 7.0) vs. a catalase solution, the subsequent addition of 1 mM ferricyanide to the galactose oxidase leads again to the complete disappearance of the enzymic Cu(II) EPR signal. Thus, it is unlikely that the EPR-silent and catalytically active oxidized enzyme is a Cu(II)–superoxide complex.

Extended contact of ferricyanide with galactose oxidase leads eventually to the slow appearance of a $g = 2$ EPR signal

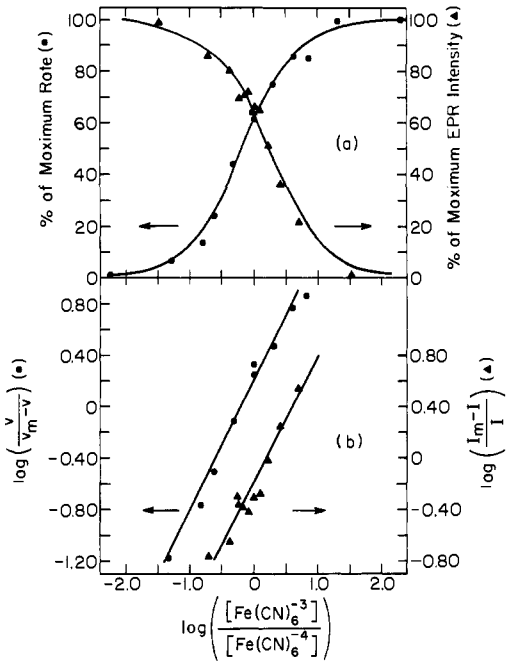


Figure 3. Effects of different ratios of ferricyanide to ferrocyanide concentrations on the rate (●) of the galactose oxidase reaction and on the intensity (▲) of the EPR signal given by the enzymic Cu(II). All solutions had 0.10 M phosphate (pH 7.0), 0.25 mM O_2, and had differing ferricyanide and ferrocyanide concentrations varying from 0 to 1 mM (total iron concentration 1 to 2 mM). The kinetic results were obtained polarographically using 0.10 M galactose and 2.7 nM galactose oxidase. For the EPR experiments no galactose was present and the galactose oxidase concentration was 30 μM. The ordinates for the lower figure are described in the text. The curves in the upper figure and the lines in the lower are theoretical ones calculated for a one-electron change and using the oxidation-reduction potentials given in the text.

apparently due to some organic radical. The identity of this radical is not known, but its formation may be related to the slow inactivation of galactose oxidase caused by ferricyanide. This signal is not seen (or is just barely detectable) when galactose oxidase–ferricyanide solutions are frozen soon after mixing and their EPR spectra taken immediately.

Sedimentation Coefficient of Galactose Oxidase in the Presence of Ferricyanide. To ensure that the effects of ferricyanide are not due to dimerization or polymerization of the enzyme, the sedimentation coefficients with and without ferricyanide present were determined from sedimentation velocity experiments. Under the following conditions (0.09 M phosphate (pH 7.0), 0.18 mM ferricyanide, 7.2 μM galactose oxidase, 16 °C) the observed sedimentation coefficient was found to be 4.45 S. In the absence of ferricyanide, but with all other conditions the same, the coefficient was measured to be 4.52 S. These compare favorably with the reported $s_{20,w}$ for the enzyme of 4.7 S.[8,9] Following the sedimentation velocity experiment, the enzyme sample containing the ferricyanide was assayed for catalytic activity and it still had 70% of its original activity. Since the concentration of galactose oxidase in the sedimentation experiment is in the range of that typically used for EPR experiments, and much higher than that used in kinetic experiments, it can be safely concluded that the observed effects of ferricyanide in both EPR and kinetic experiments cannot be due to dimerization or polymerization of the enzyme.

Enzyme Oxidation–Reduction Potential at pH 7.0. Not only does ferricyanide increase the rate of the galactose oxidase reaction but ferrocyanide inhibits the enzyme, and the effects are reversible. Thus, if the enzymic reaction is initiated with 1 mM ferricyanide present, the addition of 1 mM ferrocyanide a short time later causes a decrease in the rate of O_2 consumption. Correspondingly, if the reaction is initiated with 1 mM ferricyanide present a very slow uptake of O_2 is observed, but the addition of 1 mM ferricyanide increases the rate to the same final value as before. In Figure 3a are summarized the effects at pH 7.0 of different ferricyanide to ferrocyanide ratios on both the rate of the enzymic reaction and on the intensity of the EPR signal given by the enzymic Cu(II). These results dramatically substantiate the conclusion reached earlier that the Cu(II) form of the enzyme is inactive catalytically; the Cu(II) EPR signal and the catalytic activity appear under diametrically opposite conditions.

Table VI. Effects of Reactant Concentrations on the Kinetically Determined Enzymic Redox Potential[a]

$[O_2]$, mM	[Galactose], mM	v_l,[b] kat/kg	v_m,[c] kat/kg	E,[d] V
0.10	100	1.78	2.5	0.44
0.25	100	2.8	4.7	0.43
1.25	100	4.4	9.3	0.42
0.25	10	0.48	0.88	0.43
0.25	1.0	0.038	0.082	0.42
0.25	100	2.5[e]	4.7[e]	0.42[e]

[a] Reaction conditions: 25 °C; 100 mM phosphate buffer (pH 7.0); 2.2 nM galactose oxidase; total concentration of ferricyanide plus ferrocyanide is 1.0 mM except where indicated; rates determined polarographically. [b] Rate obtained with a 1:1 ratio of ferricyanide to ferrocyanide. [c] Rate obtained with ferricyanide alone. [d] Enzymic redox potential calculated using eq 2 as described in the text. [e] Total concentration of ferricyanide plus ferrocyanide is 0.2 mM.

The shapes of the curves in Figure 3a are suggestive of a typical redox titration. If such is the case then the data should fit eq 2:

$$E + \frac{0.059}{n} \log \frac{[\mathrm{Enz_{ox}}]}{[\mathrm{Enz_{red}}]} = 0.424 + 0.059 \log \frac{[\text{ferricyanide}]}{[\text{ferrocyanide}]} \quad (2)$$

(which is essentially a Nernst equation), where E is the redox potential of the enzymic group (under the particular conditions of the experiment) and n is the number of electrons involved in converting the catalytically inactive (but EPR visible) reduced form of the enzyme ($\mathrm{Enz_{red}}$) to the catalytically active (but EPR silent) oxidized form ($\mathrm{Enz_{ox}}$). For the kinetic data $[\mathrm{Enz_{ox}}]/[\mathrm{Enz_{red}}]$ is assumed to be given by $v/(v_m - v)$ where v is the rate obtained with the particular ratio of ferricyanide to ferrocyanide, and v_m the maximum rate obtained with excess (1 mM) ferricyanide alone. For the EPR data $[\mathrm{Enz_{ox}}]/[\mathrm{Enz_{red}}]$ is presumably given by $(I_m - I)/I$ where I is the observed Cu(II) EPR signal intensity obtained with the particular ferricyanide to ferrocyanide ratio and I_m is the maximum intensity obtained with excess (1 to 2 mM) ferrocyanide alone. The 0.424 in eq 2 is the estimated[41] oxidation–reduction potential for the ferricyanide–ferrocyanide couple for the particular conditions of these experiments.

As required by eq 2, the kinetic and EPR experimental data shown in Figure 3a, when plotted in log–log fashion (Figure 3b), give straight lines. Since the slope of each of these lines is one, the catalytically active (but EPR silent) oxidized form of the enzyme is one electron higher in oxidation state than the catalytically inactive Cu(II) state. The best estimate of the oxidation–reduction potential for the enzymic group calculated from these kinetic data is 0.41 V and that calculated from the EPR data is 0.44 V.

The kinetic results shown in Figure 3 were obtained using only one concentration of O_2 (0.25 mM) and galactose (0.10 M). However, the results given in Table VI illustrate that at pH 7.0 the same redox potential (within experimental error) is obtained using the above procedures even when the con-

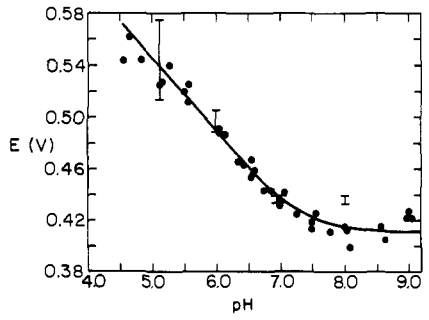


Figure 4. Effect of pH on the oxidation-reduction potential of the enzymic group. All solutions contained 0.01 M buffer (phosphate or EDTA; no effect of buffer was noted) and 1 to 2 mM total concentration of the iron hexacyanides. The kinetic data (●) were obtained polarographically at 25 °C using 0.10 M galactose, 0.25 mM O_2, and 2.04 nM galactose oxidase. The enzyme concentration for the EPR experiments (I) was 26 μM. The values of E were calculated from the primary data using eq 2 as described in the text. Most of the primary kinetic data at each pH were obtained using in separate experiments a 1:1 ratio of ferricyanide to ferrocyanide and 1 mM ferricyanide alone, but at some pHs data were obtained for several ferricyanide to ferrocyanide ratios and the point shown is an average one. In the EPR experiments the redox potential was calculated from the intensity of the Cu(II) signal obtained with a 3:1 ratio of ferricyanide to ferrocyanide relative to that obtained with 2 mM ferrocyanide alone. The illustrated line is a theoretical one (see text) calculated from the kinetic data.

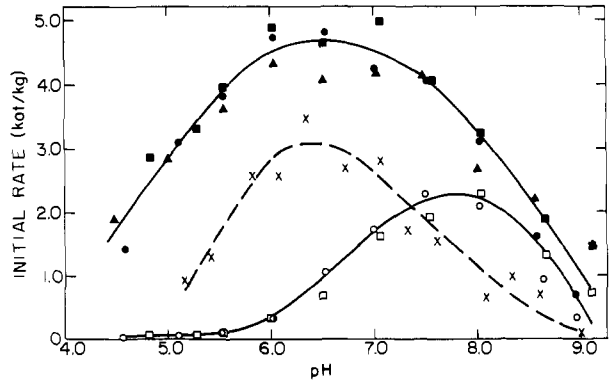


Figure 5. Effects of pH on the enzymic rate. General reaction conditions: 25 °C; 0.10 M galactose; 0.25 mM O_2; 2.04 nM galactose oxidase; rates determined polarographically. Specific conditions: (×) 10 mM EDTA buffer, no other additives; (○) 10 mM EDTA buffer, 0.5 mM ferricyanide, and 0.5 mM ferrocyanide; (□) 0.10 M phosphate buffer, 0.5 mM ferricyanide, and 0.5 mM ferrocyanide; (●) 10 mM EDTA buffer, 1.0 mM ferricyanide; (■) 0.10 M phosphate buffer, 1.0 mM ferricyanide; (▲) 0.10 M phosphate buffer, 4.0 μM chloroiridate. In all cases the pHs were measured at the end of the reaction but they changed by less than 0.1 unit during the reaction.

centrations of the two reactants are changed by a factor of 10 or more. In control experiments (not given in the table) it was shown that light also has no effect on these results. In the dark or in the presence of room light or strong light (275-W sunlamp within 10 cm of the reaction), the same rates and redox potentials are found when the kinetics are followed by the polarographic method. The results given on the last line of Table VI indicate, as they should if an oxidation-reduction is occurring, that the absolute concentrations of ferricyanide and ferrocyanide are unimportant in determining the rate and the resultant calculated redox potential. The observed rate depends only on the ratio of these concentrations. In EPR experiments the same was found to hold; the intensity of the enzymic Cu(II) EPR signal remains the same for a given ratio of ferricyanide to ferrocyanide when the total concentration of iron hexacyanides is varied from 0.5 to 2.0 mM.

Effect of pH on the Enzymic Redox Potential. The results shown in Figure 4 indicate that the enzymic redox potential varies with pH; at high pHs the redox couple is apparently that shown in eq 3, whereas at low pHs it is the reaction of eq 4. The

$$\mathrm{Enz_{ox}}^{+n} + e^- \rightarrow \mathrm{Enz_{red}}^{+n-1} \quad (3)$$

$$\mathrm{Enz_{ox}}^{+n} + H^+ + e^- \rightarrow \mathrm{EnzH_{red}}^{+n} \quad (4)$$

data can be used to calculate the redox potential (E_0) for the couple of eq 3 and an ionization constant (K_a) for the acid ionization of $\mathrm{EnzH_{red}}^{+n}$ by using the relation shown in eq 5.

$$E = E_0 + 0.059 \log \left(1 + \frac{[H^+]}{K_a}\right) \quad (5)$$

The best fit of the kinetic data gives $E_0 = 0.410 \pm 0.005$ V and $pK_a = 7.25 \pm 0.15$. The line through the data shown in Figure 4 was calculated using these values. The experimental kinetic values obtained at the extremes of pH (above pH 8.0 and below 5.5) can only be considered approximate because the enzyme is slowly inactivated under the conditions of the assays. The values of E were calculated from the best estimates of initial rates under such conditions.

The limited number of points obtained from EPR measurements appears to follow a curve similar to that given by the kinetic experiments except that at high pHs the limiting redox potential may be slightly higher. It would not be surprising if the enzymic redox potentials obtained by the two methods (kinetic and EPR) differ somewhat because the conditions under which they are obtained are not the same. Probably of primary importance is the fact that the substrate galactose is present in the kinetic experiments and not in the EPR experiments. Thus, at high pH the limiting redox potential of the enzymic group with galactose bound appears to be slightly lower than that for the free enzyme itself.

Effect of pH on the Enzymic Rate. In Figure 5 are illustrated the effects of pH on the rate of the enzyme-catalyzed O_2 consumption under several sets of conditions. For mechanistic considerations, the most meaningful data are those obtained under highly oxidizing conditions (closed symbols, 1 mM ferricyanide or 4 μM chloroiridate present), because under such conditions all of the enzyme should be in the catalytically active oxidized state; none will be in the inactive Cu(II) state. The results obtained with a 1:1 molar ratio of ferricyanide to ferrocyanide (open symbols) are shown to emphasize the role that the variation in redox potential with pH has on the general shape of the pH-rate curve. The results obtained in the absence of oxidants or redox buffers but with EDTA present (×'s) are included mainly for comparison purposes. Under these conditions the effect of pH on the rate is almost certainly some complex function of the effects of pH on the ratio of oxidized and reduced states of the enzyme established under the conditions of the experiment, and the effect of pH on the rate of catalysis by the active oxidized species. In the absence of EDTA (not illustrated) the rates are generally lower and scattered due to the unpredictable effects of trace metal ions. In general, these results emphasize that kinetics obtained under uncontrolled oxidation conditions are probably uninterpretable in terms of any detailed kinetic mechanism. This is true for essentially all kinetics with this enzyme presently in the literature.

The results summarized in Figure 5 show that in the presence of oxidants or redox buffers there is no effect of buffer on the rate; the same rates are obtained with both phosphate and EDTA buffers. Also, the results further emphasize that micromolar concentrations of chloroiridate have the same activating effect as millimolar concentrations of ferricyanide. Considering the results with excess oxidants present, the rate is at a maximum at pHs 6 to 7 and drops off slowly above and below these values. A specific interpretation of the reason for the slow decrease at the pH extremes cannot be given at this time because the data were obtained at only one set of substrate

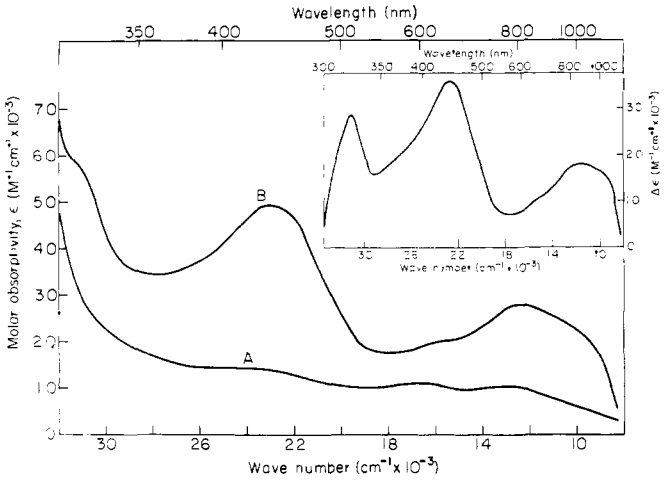


Figure 6. Optical spectra of the resting (A) and oxidized (B) galactose oxidase. The insert is the difference spectrum between B and A. The spectra were obtained using 22 μM (6.7 nmol in 300 μL) galactose oxidase in 0.10 M phosphate buffer (pH 6.0). To obtain spectrum A, galactose oxidase was dialyzed vs. the buffer for 2 days (two buffer changes) and then measured vs. the final dialysate as the blank. Spectrum B (corrected for the small volume change) was obtained immediately following the addition of 1.5 molar equiv (10.4 nmol) of hexachloroiridate to the enzyme solutions. The reduction product of chloroiridate, namely Ir(III), has no significant absorptions over the spectral region shown.

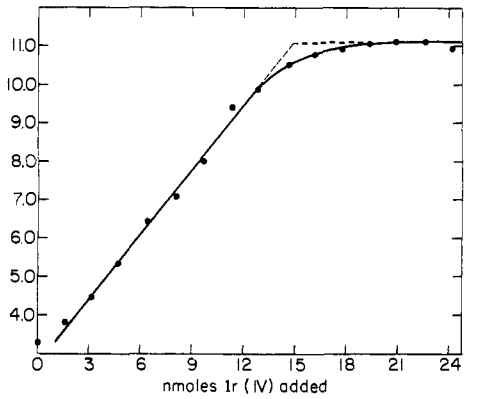


Figure 7. Titration of galactose oxidase with hexachloroiridate. To 13.6 nmol of galactose oxidase in 300 μL of 0.10 M phosphate buffer (pH 6.0) were added 5-μL aliquots of a 0.32 mM solution of hexachloroiridate, and the absorption was monitored at 800 nm. The entire titration required about 5 min. The absorbance values shown are corrected for the volume change which occurs on each addition.

concentrations, neither of which is apparently saturating. Thus, the pH effects are probably due to some complex function of the effects of pH on the K_ms and catalytic rate constants. A further complication is the previously mentioned slow inactivation of the enzyme at pHs below 5.5 and above 8 which makes the reported rates at these pHs subject to considerable error. The general conclusion which can be drawn from the data is that, from pH 5.5 to 8.0, there is very little effect of pH on the rate under conditions (highly oxidizing) where essentially all the enzyme is in the catalytically active oxidized state.

Optical Spectra of the Different Enzyme Forms. Shown in Figure 6 are the optical spectra of the resting and oxidized enzymes, as well as the difference spectrum (insert). The illustrated spectra were obtained at pH 6.0 because the oxidized enzyme is more stable at lower pHs, but essentially the same spectral features and molar absorptivities are observed at pH 7.

The spectrum (A) of the resting enzyme is very similar to that previously reported.[8,43] The illustrated spectrum of the oxidized enzyme (B) was obtained within a minute after adding a slight excess of hexachloroiridate to the resting enzyme. At pH 6.0 this spectrum changes by less than 1% over a period of 5 min at room temperature, but at higher pHs there is a more rapid general decrease in absorbance with time. If less than stoichiometric amounts of chloroiridate are added to the resting enzyme, the intensities of the various absorbances are less (see next section). When a large excess of chloroiridate is added, the intensities of the peaks above 500 nm remain the same as in Figure 6 but the absorbance in the 350-nm region increases. The absorptions due to the chloroiridate itself disappear fairly rapidly (within a minute or two) so the increased absorption in the 350-nm region, seen after the addition of a large excess of chloroiridate, must be due to further nonspecific oxidation of various organic groups on the protein. Since small amounts of chloroiridate activate the enzyme while a large excess causes inactivation, the oxidized species whose spectrum is shown in Figure 6 is presumably the catalytically active species, and that with increased absorption in the 350-nm region is a further oxidized inactive form of the enzyme.

When ferricyanide or Mn(III)·EDTA are used as oxidants, the *same* optical spectrum is given by the enzyme as that obtained with chloroiridate (Figure 6). The reaction of the enzyme with Mn(III)·EDTA is slower than with chloroiridate; the spectral features develop fully only after the reactants have been in contact for a few minutes. With ferricyanide, technical problems make it difficult to obtain as accurate a spectrum as with chloroiridate or Mn(III), but it is quite clear that the same absorption maxima with approximately the same extinction coefficients are seen.[14,37] Because ferricyanide has a lower redox potential, excess ferricyanide must be used to convert the enzyme completely to the oxidized form, and it has proved difficult to compensate exactly for the absorptions due to the reagent. Long-term contact of the enzyme with ferricyanide (for example, during dialysis to obtain a suitable blank) leads to slow formation of the same absorption in the 350-nm region as seen with excess chloroiridate. Since ferricyanide also slowly inactivates the enzyme, this is further evidence that the absorbance increase around 350 nm, which develops only when excess amounts of oxidants are used, is given by an inactive form of the enzyme.

Treatment of the resting enzyme with excess (1 mM) ferrocyanide leads to small decreases in absorbance throughout most of the spectral region above 300 nm. Because of difficulties in choosing a suitable blank it has not yet been possible to quantitate the magnitude of the decrease at each wavelength. However, the results are consistent with the hypothesis that the resting enzyme contains a small amount of oxidized species. The fact that the resting enzyme contains peaks of lower intensities in the same regions as the oxidized enzyme is further evidence for this conclusion.

As shown in Figure 6, the oxidized enzyme has extensive absorption throughout the entire region from 300 to 1200 nm with peaks at approximately 440 and 800 nm with molar extinction coefficients of ca. 4900 and 2800, respectively. There also appears to be a shoulder at about 1000 nm with an extinction coefficient of about 2200. The difference spectrum shows another absorption due to the oxidized enzyme at ca. 320 nm with a difference extinction coefficient of 2800.

Titration of Galactose Oxidase with Ir(IV). Because hexachloroiridate has a much higher oxidation–reduction potential than the enzyme it is possible to titrate the enzyme with this reagent. The results obtained by following the absorption change at 800 nm are shown in Figure 7. They indicate that it takes approximately 1.1 equiv of Ir(IV) to titrate the enzyme solution. Since other evidence (vide ante) indicates that the resting enzyme contains a small amount of the oxidized species, it is expected that only 0.8 to 0.9 equiv should have been necessary to titrate the copper site of the enzyme. The reason for the slightly higher value is undoubtedly because chloroiridate

Table VII. Representative Conditions under Which No Exchange of Isotopically Labeled Product into Substrate Is Observed[a]

Initial galactoside[b] concn, mM	Initial [1-^{14}C]MGDP concn, mM	Air or N_2 atm	Catal. act. at end of incubation,[c] %
100	25	N_2	46
25	25	N_2	28
0.25	25	N_2	67
2.5	25	N_2	36
0.25	2.5	N_2	77
100[d]	25[d]	Air[d]	*d*
2.5[e]	2.5[e]	Air[e]	*e*
2.5[f]	2.5[f]	Air[f]	0[f]
10	10[g]	N_2	12
1	1[g]	N_2	25

[a] Unless otherwise noted, all reaction solutions (3 mL) contained 10 mM phosphate buffer (pH 7.8), 53 nM galactose oxidase, 4.8 μg/mL catalase, and were incubated at 25 °C for 20 h. [b] Methyl α-D-galactopyranoside. [c] Given as a percent of that at the beginning of the incubation. [d] Incubation for only 3.9 h; the amount of O_2 consumed during the incubation indicated that 47% of the substrate initially present was oxidized. [e] Contained no catalase, had only 13 nM galactose oxidase, and was incubated for 2 h; the amount of O_2 consumed during incubation indicated that 48% of the substrate was oxidized. [f] Contained no catalase, had 13 nM galactose oxidase and 1 mM H_2O_2 present initially; incubated for 1.5 h; the amount of O_2 consumed during incubation indicated that 22% of the substrate was oxidized. [g] Unlabeled MGDP used; the solvent contained 10.3 mCi of tritiated water.

reacts to some extent with impurities in the enzyme solution and with other sites on the enzyme. It will be noted (Figure 7) that the first two additions of Ir(IV) cause less of an increase in absorbance than subsequent additions. This is presumably because small amounts of reactive impurities, whose oxidation products do not give absorption at 800 nm, are responsible for some of the Ir(IV) reaction. Also, since excess Ir(IV) does react even with the oxidized enzyme (see previous section), it is expected that during the titration of the copper site (Figure 7) some of the Ir(IV) will be used up in this nonspecific reaction. In any event, the fact that the number of equivalents of Ir(IV) required to titrate the enzyme is close to one indicates that the spectral species being observed is one electron higher in oxidation state than the major species present in the resting enzyme.

Irreversibility of the Alcohol Oxidation Step. The results summarized in Table VII indicate that under a variety of conditions the alcohol oxidation step of the galactose oxidase reaction is not reversible. Under anaerobic and aerobic (active catalysis occurring) conditions, with or without catalase, etc., no exchange of radioactive aldehyde product or tritiated water into the reactant galactoside is observed. The assay methods were sensitive enough that 0.5% exchange in the ^{14}C experiments and 0.05% exchange in the tritiated water experiments could have been detected. Although these experiments were not performed under all possible additive conditions, and although the product aldehyde exists in several forms in solution (see Experimental Section), it is felt that some exchange would have been seen under some of the conditions tried if the alcohol step were reversible.

The lack of exchange indicates that, if there is a half-reaction involving an oxidized form of the enzyme reacting with the alcohol to give aldehyde and an enzyme form reduced by two electrons, the oxidation-reduction potential for the enzymic group involved must be considerably more positive than that for the aldehyde-alcohol conversion (ca. −0.2 V[44]). This effectively eliminates the possibility that a disulfide-dithiol interconversion could be involved in the alcohol oxidation; the redox potential for disulfide-dithiol conversions is close to that for aldehyde-alcohol conversions[44] and some exchange would be expected if such a mechanism applied.

As well as the fact that the early kinetic data were obtained under uncontrolled oxidation-reduction conditions, the irreversibility of the alcohol oxidation step makes suspect any conclusions derived from double reciprocal plots of kinetic data obtained at various O_2 and galactose concentrations.[20,42] Thus, until considerably more work is done nothing can presently be said about the order of addition and release of reactants and products in this reaction.

Discussion

The Nature of the Oxidized Enzyme. The foregoing results constitute unequivocal evidence that a catalytically active form of galactose oxidase is a species which is one electron higher in oxidation state than the Cu(II) enzyme, and that the Cu(II) enzyme is catalytically inactive (or nearly so). The results also severely restrict the number of possible structures for the oxidized species. Since galactose oxidase contains only one atom of copper per molecule, and no polymerization of the enzyme is involved in forming the oxidized state, it must be a monocopper species. Because this species shows no EPR signal at ca. 100 K, the copper must be intimately involved in the oxidized state. Since the EPR changes can be observed under anaerobic as well as aerobic conditions, O_2 is apparently not an integral part of the oxidized species. Because H_2O_2 is not required for the conversion of the Cu(II) enzyme to the oxidized state in the presence of ferricyanide, the oxidized species is presumably not a Cu(II)-superoxide complex. The fact that under anaerobic conditions ferricyanide can serve as an electron acceptor in the galactose oxidase reaction is further evidence against O_2 or superoxide being a part of the oxidized species.

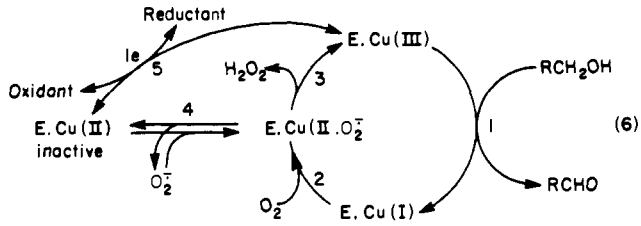
It has been suggested[21] that the observed effects of ferricyanide are due to binding of ferricyanide near to the enzymic Cu(II).[45] The following considerations among others eliminate this possibility: (1) the magnitude of the observed effects in both kinetic and EPR experiments depends not on the concentration of ferricyanide but only on the *ratio* of ferricyanide to ferrocyanide; (2) chloroiridate and Mn(III) at much lower concentrations cause the same kinetic effects as ferricyanide; (3) the same optical spectrum is given by the enzyme treated with any one of the three oxidants (ferricyanide, chloroiridate, or manganic ions) and this spectrum is different from that obtained in the absence of oxidants; (4) superoxide gives the same amount of activation as 1 mM ferricyanide; (5) since the copper appears to be intimately involved in the catalysis (see later), it is difficult to imagine a species with ferricyanide bound close enough to the enzymic Cu(II) to wipe out its EPR signal, being catalytically active.

All our results taken together indicate that the catalytically active oxidized species exists in a formal trivalent copper state. Whether the electronic structure of this state is better described as Cu(III) or Cu(II)·Ẋ, where X is some enzymic group complexed to the copper, is not known at present. However, Cu(II)·Ẋ and Cu(III)·X^- are just two limiting forms of a system which is expected to be a resonance hybrid, in which case the only question is which limiting structure contributes more to the hybrid. In the following discussion the oxidized species will be referred to as the Cu(III) state, but in doing so, the possibility that it is Cu(II)·Ẋ or a resonance hybrid of the two is implied.

Until recently, Cu(III) was considered to be a relatively esoteric valence state for copper, encountered in only a few special cases in inorganic chemistry. However, it is now apparent that Cu(III) is a readily accessible state; several stable

Cu(III) complexes involving organic ligands have now been well characterized,[47-54] and the involvement of this valence state in several copper ion catalyzed reactions has been suggested.[49,55-58] Of particular note are the relatively stable peptide complexes studied by Margerum and coworkers[53] which have redox potentials in the range of 0.5 to 0.6 V. This is not much different from the potential obtained for the enzymic group. On this basis, then, the possibility of the oxidized enzyme being a Cu(III) species seems eminently reasonable. Although suggestions have been made,[21,59,60] it is not known with certainty what ligands are involved in the bonding of copper to the enzyme. Indirect evidence[61] suggests a sulfhydryl group may be involved and this would certainly be expected to stabilize a Cu(III) state.[47,54]

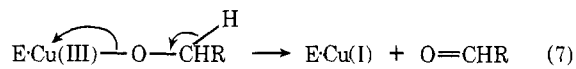
Overall Enzymic Mechanism. Illustrated in eq 6 is an overall



mechanism for the galactose oxidase reaction which is consistent with all the data now available. No specific sequence of addition of reactants and loss of products is meant to be implied in this mechanism; the illustrated steps merely outline the redox states of the enzymic species which oxidize or reduce the two reactants. In the usual catalytic cycle (steps 1, 2, and 3) it is suggested that the Cu(III) form of the enzyme reacts with the alcohol to give the aldehyde and a Cu(I) form. The Cu(I) enzyme is then reoxidized by O_2 to the Cu(III) form and H_2O_2 with an enzymic Cu(II) superoxide species as a fleeting intermediate.

The alcohol oxidation step (step 1) undoubtedly occurs without an organic free radical or an enzymic Cu(II) species being an intermediate. As has been discussed previously,[3] it is thermodynamically unreasonable to suggest that the alcohol oxidation could occur in one-electron steps. According to the spin conservation rule,[4] in order for step 1 to proceed by a direct two-electron reaction, E·Cu(III) and E·Cu(I) must have the same number of unpaired electrons. Since E·Cu(I) is a d^{10} system and has no unpaired electrons, the E·Cu(III) must be a low-spin d^8 system. This is certainly consistent with results obtained using model Cu(III) compounds[47-54] and with the observation that the oxidized enzyme shows no EPR signal.

The proposed oxidation of an alcohol to an aldehyde by the E·Cu(III) species is very similar to the conversion of an amine to an imine, a reaction known to occur in several nonenzymic Cu(III) systems.[49,55,56] The copper ion catalyzed autoxidation of methanol to formaldehyde[62] probably also involves such a step. A reasonable mechanism for step 1 is that shown in eq 7:



loss of a proton from the α carbon of the complexed alkoxide, and electron migration as shown, would give the products directly. Such a step is very similar to one which occurs in the oxidation of alcohols by chromate.[63-65] There is evidence[42,66] that galactose and other externally added ligands bind directly to the copper of the E·Cu(II) species. Also, since anions inhibit the enzymic reaction,[20] an alkoxide complex of the E·Cu(III) intermediate thus is reasonable.

Although there is considerable evidence that the Cu(III) form is in the usual catalytic cycle, there is no direct evidence that E·Cu(I) is. However, in addition to one's expectations (from the above considerations) that it is involved, the inactivation by H_2O_2 is indirect evidence for its existence. The H_2O_2 inactivation results (Figure 1) indicate that a species reactive toward H_2O_2 is present during active catalysis which is not present in the resting enzyme. In nonenzymic systems Cu(I) reacts very rapidly with H_2O_2 to generate the hydroxyl radical (HO·). Such a species formed near the active site of the enzyme would be expected to react nonspecifically with many different amino acid residues. It is proposed that this is what ultimately leads to enzyme inactivation. Consistent with the overall mechanism of eq 6 and the interpretation that H_2O_2 inactivates by reacting with E·Cu(I) is the observation that increased concentrations of O_2 dramatically protect the enzyme from inactivation by H_2O_2 (Figure 1).

The lack of exchange of isotopically labeled product into the reactant indicates that the alcohol oxidation step is irreversible. In terms of the proposed mechanism (eq 6 and 7), this means that the redox potential for the E·Cu(III)–E·Cu(I) couple is more positive than for the aldehyde–alcohol couple (ca. −0.2 V). The potential for the E·Cu(III)–E·Cu(I) couple is *not* known at present, but it is probably less than 0.2 V. The upper limit can be set because O_2 must be capable of converting E·Cu(I) to E·Cu(III) with formation of H_2O_2, and the redox potential for the O_2–H_2O_2 couple at pH 9 (where the enzyme is still active) is ca. 0.2 V.[67] Our experimental data are consistent with this upper limit; the measured limiting potential at high pHs for the E·Cu(III)–E·Cu(II) couple is 0.4 V and that for the E·Cu(II)–E·Cu(I) couple must be less than 0.2 V because excess ferrocyanide does not cause any appreciable reduction of the E·Cu(II). It seems likely that the redox potential of the E·Cu(II)–E·Cu(1) couple would probably be more negative than the potential for the O–$O_2^-\cdot$ conversion which is −0.33 V at pH 7.[67] Redox potentials of −0.4 V or less for the Cu(II)–Cu(I) conversion of various simple inorganic complexes are common.[68] Obviously it would be desirable to know the E·Cu(II)–E·Cu(I) potential, and experiments to determine this are planned.

The observation (inhibition by SOD and trace metal ions) that superoxide leaks from the catalytic cycle during catalysis with the concomitant formation of the catalytically inactive E·Cu(II) indicates that an E·Cu(II) superoxide species is probably a catalytic cycle intermediate. Such an intermediate is also expected on the basis of the spin conservation rule; if E·Cu(I) and E·Cu(III) are both spin-paired systems their interconversion involving triplet O_2 and singlet H_2O_2 must occur in a two-step mechanism involving radical intermediates. There is no direct evidence indicating whether the superoxide in the catalytic intermediate is bound directly to the copper or to other groups in the vicinity of the copper, i.e., whether inner sphere or outer sphere electron transfers are involved. However, the ease with which superoxide is lost from the enzyme might imply that superoxide is not in the inner coordination sphere of the copper.

In the overall reaction catalyzed by galactose oxidase no net uptake or release of protons from or to the solvent is involved. However, according to the mechanism of eq 6 and 7 both hydrogens are removed from the alcohol as protons and presumably given to the oxygen as protons. The details of this proton reshuffling remain to be elucidated, but the rapidity of the enzymic reaction probably precludes extensive exchange with the bulk solvent during turnover. The general lack of dependence of the rate on pH (Figure 5) at least from pH 5.5 to 8.0 indicates that any enzymic groups involved in proton transfers do not ionize in this pH region.

As indicated in the Results section, in the presence of EDTA and in the absence of external oxidants, the galactose oxidase reaction is more sensitive to inhibition by SOD than any other enzymic reaction known. It is so sensitive to trapping of superoxide that, in the absence of EDTA, metal ions, present even in only trace quantities, can cause inhibition comparable to that

of SOD.[69] These results can be readily understood in terms of the mechanism of eq 6; every time a superoxide molecule leaks from the catalytic cycle and is trapped, an inactive form of the enzyme (E·Cu(II)) is formed, and thus many turnovers of the catalytic cycle are prevented. The E·Cu(II) form of the enzyme is inactive because it is at the wrong oxidation level to react with either of the two substrates, O_2 or galactose. The other well-studied enzyme system known to involve superoxide, namely the xanthine oxidase–cytochrome *c* system,[26] is different from the galactose oxidase reaction in that each event of superoxide trapping does not give an inactive form of the enzyme but merely prevents the reaction of one molecule of cytochrome *c*. Thus, it is not surprising that comparable amounts of inhibition require higher concentrations of SOD in the xanthine oxidase–cytochrome *c* system than in the galactose oxidase reaction.

Superoxide apparently leaks out from the catalytic cycle only once in every 2000–5000 turnovers under usual assay conditions at pH 7 and 25 °C. This number is estimated from the turnover number under these conditions and the half-time (10–20 s) of the lag period before the SOD inhibition takes effect. Given this number and the mechanism of eq 6 one can readily understand how ferricyanide completely counters the SOD inhibition even though it does not react stoichiometrically (in relation to the amount of O_2 consumed). Each molecule of ferricyanide which converts E·Cu(II) to E·Cu(III) (step 5, eq 6) will lead on the average of 2000–5000 molecules of O_2 reacting before superoxide again leaks out of the catalytic cycle to give E·Cu(II) (step 4). The extensive activation by small amounts of superoxide (Table IV) is also explainable on the same basis; each reversal of step 4 leads to several thousand turns of the catalytic cycle.

It is not known why galactose oxidase leaks a molecule of superoxide once in every few thousand turnovers rather than once every turnover or not at all. Basically it is the competition between steps 3 and 4 (eq 6) which determines this number. Because step 4 does compete somewhat with step 3 in this enzymic reaction it has provided direct evidence that O_2 reacts by two simple one-electron transfers as expected from the spin conservation rule.[4] In almost all other enzymic reactions involving O_2 the same spin problem exists; in the overall reaction triplet O_2 reacts with singlet reactants to give singlet products. It seems probable that in most of these reactions the initial stages in the reaction of O_2 are very similar. If, in such cases, a step analogous to 4 (spin inversion) occurs more rapidly, or a step analogous to 3 (separation of superoxide from the enzyme) occurs more slowly, no evidence for superoxide as an intermediate would be obtained even though it is an intermediate. It would seem advantageous for most enzymes to behave in this manner because any leakage of superoxide would lead to an inactive form of the enzyme as in the case of galactose oxidase. Thus, it is not too surprising that for most oxidases and oxygenases direct evidence for superoxide as an intermediate is lacking even though it almost certainly is an intermediate. In those cases where direct evidence for superoxide has been obtained (effects of SOD), the main conclusion indicated is that the enzyme is leaky as in the case of galactose oxidase. One should not conclude that such enzymes catalyze the O_2 reaction by a mechanism basically different from that of enzymes not giving evidence for superoxide.

In the presence of ferricyanide–ferrocyanide redox buffers, thermodynamic equilibrium is apparently rapidly established and maintained between the E·Cu(II) and E·Cu(III) species even during active catalysis. Presumably the reason that the kinetically determined redox potentials do not vary with reactant concentrations (Table VI), and furthermore are similar to those determined by EPR, is because E·Cu(III) is the predominant catalytic cycle intermediate during turnover. This is expected because a large kinetic deuterium isotope effect (deuterium substituted for hydrogen at the carbon undergoing oxidation) is associated with alcohol oxidation;[22] i.e., the rate-determining step in the catalytic cycle is step 1 (eq 6).

In the absence of redox buffers there is apparently a much slower interconversion of E·Cu(II) and catalytic cycle intermediates but it apparently still occurs to some extent. This is evidenced by the fact that under any given set of conditions (not leading to irreversible enzyme inactivation) a pseudoequilibrium between catalytically inactive and catalytic cycle intermediates is eventually established; i.e., although the reaction may show an induction period or a burst, it eventually gives a linear uptake of O_2. The induction period and burst phenomena are almost certainly due to the position of the pseudoequilibrium eventually established during catalysis being different from the ratio of the catalytically inactive and catalytic cycle intermediates in the resting enzyme (the enzyme as isolated and stored in 0.1 M phosphate buffer, pH 7). A burst would be expected if the resting enzyme has a higher ratio of catalytic intermediates than the ultimately established pseudoequilibrium, and an induction period is expected if the opposite were the case. All our evidence indicates that the resting enzyme is a mixture of the E·Cu(II) and E·Cu(III) forms in a ratio of about 4:1, but the ratio appears to vary somewhat with method of isolation and storage. This ratio is estimated from (1) the observed increase in the intensity of the EPR signal due to the enzymic copper on adding excess ferrocyanide to the resting enzyme, (2) the intensity increase observed on SOD inhibition, and (3) the fact that the resting enzyme has absorbance maxima with molar extinction coefficients of ca. 1000 at the same positions that the fully oxidized enzyme has maxima with extinction coefficients of several thousand. Even in the earliest EPR experiments, the data of Blumberg and coworkers[23] indicated only 70% of the enzymic copper could be accounted for as Cu(II), but they assumed the accuracy of their measurement was only ±30%, and thus that all the copper was present as Cu(II). Their experiments were probably much more accurate than they claimed. It is not immediately clear why the resting enzyme is a mixture of the two forms with the above approximate ratio, but it seems possible that it may be controlled by the oxygen–hydrogen peroxide couple. Very low levels of H_2O_2 and an air atmosphere would give about the right redox potential.

Possible Involvement of Cu(III) in Other Enzyme Reactions. Since the Cu(III) state of galactose oxidase and various nonenzymic complexes can be attained so readily, it seems certain that the Cu(III) state will be shown to occur in some other cuproenzymic reactions as well. Apparently, the reason that large numbers of nonenzymic Cu(III) complexes have not, until recently, been characterized is not because the Cu(III)/Cu(II) oxidation–reduction potential is high (it is frequently lower than the Fe(III)/Fe(II) potential[47,53,54]), but rather that most Cu(III) complexes are kinetically unstable; i.e., oxidation of one of the ligands occurs.[49,55,56] This is exactly the property needed for a catalyst, so the involvement of Cu(III) in many cuproenzymic reactions, as well as in many nonenzymic copper ion catalyzed reactions, seems assured. The recent results of Aasa et al.[71] imply that a formal Cu(III) species is an intermediate in the reaction of reduced laccase with oxygen.

Most of the studies relating to the valence of copper in various copper enzymes have been done under static or nonturnover conditions. Thus, even though other valence states of copper may be shown to be the most stable ones it will still be unclear whether Cu(III) is involved during the catalytic reaction. Perhaps the greatest strength of the present work with galactose oxidase is that it has been possible to directly relate kinetic and EPR experiments, and thus come to definite conclusions concerning Cu(III) as a *catalytic* intermediate. Only when similar studies with other copper enzymes are done will

it be known if Cu(III) is involved. At present it is a possible intermediate in all of them.

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References and Notes

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