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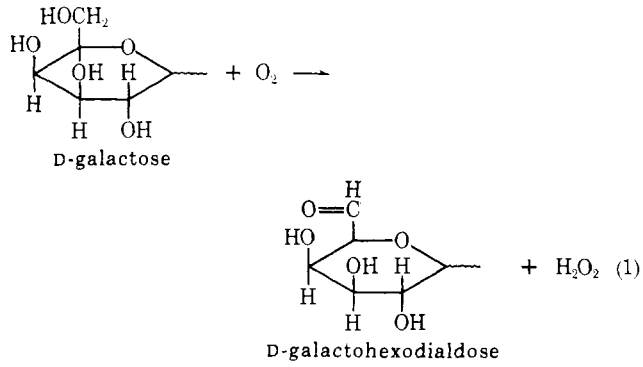
Circular Dichroism Spectra of the Copper Enzyme, Galactose Oxidase, in the Presence of Its Substrates and Products†

Murray J. Ettinger* and Daniel J. Kosman

ABSTRACT: Circular dichroism spectra were recorded for galactose oxidase in the presence of its substrates and products to determine which of these ligands interacts with the copper atom of the enzyme. Neither galactose nor oxygen has any detectable effect on protein conformation as evidenced by near- and far-ultraviolet circular dichroism spectra. In the absence of oxygen, D-galactose at concentrations an order of magnitude higher than its K_m causes a marked reduction of galactose oxidase copper optical activity. At the 314- and 610-nm copper extrema, the ellipticity is reduced to less than half of its value for the enzyme alone. On the other hand, removal or addition of oxygen to galactose oxidase solutions results in only small, barely significant, changes in copper optical activity which are in the same direction. In view of circular dichroism, kinetic, and electron spin resonance results, it is inferred that during the course of the enzyme reaction oxygen binds after galactose and interacts directly with reducing equivalents on the substrate rather than within the inner coordination sphere of the copper atom. In a manner similar to galactose, the aldehyde product of the enzyme reaction also interacts with the copper atom as evidenced by large changes in optical activity. Experiments with the other product, hydrogen peroxide, were somewhat ambiguous since this compound has effects on protein conformation in addition to its effects on copper optical activity. In addition to these effects on copper optical activity, large changes in near-ultraviolet circular dichroism spectra are observed with dihydroxyacetone and galactohexodialdose. These changes indicate that a tryptophan(s) residue(s) is at or near the active site of galactose oxidase.

This paper reports the effects of the substrates and products of the reaction catalyzed by D-galactose:O_2 oxidoreductase (EC 1.1.3.9) on the enzyme's pronounced copper and tryptophan optical activity (Ettinger, 1974a). Galactose oxidase catalyzes reaction 1. Michaelis constants which have been determined are: K_m = 20 mM for galactose at saturating oxygen concentrations; K_m = 0.6 mM for oxygen at 20 mM galactose; K_I = 13 mM for hydrogen peroxide at saturating concentrations of both galactose and oxygen; K_I is undetermined for galactohexodialdose (Kwiatkowski and Kosman, 1974). Although galactose oxidase is relatively specific for D-galactose when its activity with other hexoses is tested (Cooper *et al.*, 1959; Avigad *et al.*, 1962; Schlegel *et al.*, 1968), it also catalyzes the oxidation of nearly all aliphatic or aromatic primary alcohols (Hamilton *et al.*, 1973; Kwiatkowski and Kosman, 1974). In particular, dihydroxyacetone was found to be a better substrate for the enzyme than D-galactose in that k_{cat}/K_m for this substrate is fourfold greater than for D-galactose itself (Hamilton *et al.*, 1973; Zancan and Amaral, 1970). Both sequential and Ping-Pong kinetic schemes have been proposed for the galactose oxidase reaction (Hamilton *et al.*, 1973; Kwiatkowski and Kosman, 1974; Hamilton, 1969).



The objectives of this work were to demonstrate the utility of copper circular dichroism (CD) spectra as a probe of ligand interactions with copper proteins, to compare the spectral properties of the liganded and unliganded copper complex in galactose oxidase, to determine if the order of binding in the galactose oxidase bisubstrate reaction could be discerned from spectral measurements which were independent of

† From the Department of Biochemistry, State University of New York at Buffalo, Buffalo, New York 14214. *Received September 28, 1973.* This is a contribution of the Bioinorganic Group of the Departments of Biochemistry and Chemistry. Supported in part by U. S. Public Health Service, General Research Support, Grant RR05400 and a National Institutes of Health Biomedical Institutional Fund.

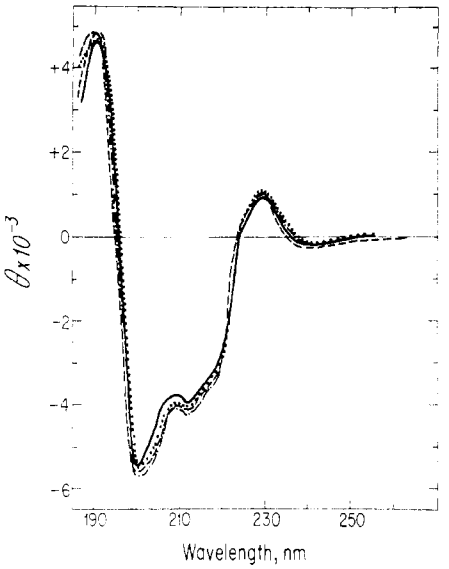


FIGURE 1: Far-ultraviolet CD spectra of galactose oxidase in 0.1 M sodium phosphate buffer at pH 7.0. Spectra were obtained with solutions which were: in air (——), deoxygenated (---), or oxygenated (-·-·-); or with deoxygenated solutions which contained 0.2 M D-galactose (· · · ·). Cell path lengths were 0.1 and 0.01 cm; protein concentration used was 1.5 mg/ml.

kinetic experiments, and to determine if any changes in protein group optical activity occurred with ligand binding that could be interpreted to suggest the nature of amino acid side chains in the vicinity of the galactose oxidase active site. Accordingly, CD spectra were recorded with deoxygenated or oxygenated galactose oxidase solutions, deoxygenated galactose oxidase solutions containing D-galactose, deoxygenated galactose oxidase solutions containing dihydroxyacetone, solutions of galactose oxidase which contained galactohexodialdose but no hydrogen peroxide, and deoxygenated galactose oxidase solutions containing hydrogen peroxide.

Experimental Section

Materials

Galactose oxidase was either purified from commercial sources (Miles) or purified from cultures of *Polyporus circinatus* as previously described (Kosman *et al.*, 1974). The CD spectra of the enzyme preparations from the two sources are identical. D-Galactose and dihydroxyacetone were obtained from Sigma; catalase was from Calbiochem. Hydrogen peroxide was a 30% solution of Baker reagent grade. Oxygen and nitrogen were Hi-Purity grade from Liquid Carbonic.

Protein Solutions in Air. All galactose oxidase solutions which were used to record control spectra in air were prepared as described previously (Ettinger, 1974a). Protein concentration was determined from absorbance measurements with a Zeiss PMQII spectrophotometer using the extinction coefficient which was determined by dry weight of $E^{1\%}_{1\ cm} = 15.4$ at 280 nm (Kosman *et al.*, 1974).

Protein Solutions in the Presence of Substrates or Products. To prepare deoxygenated solutions of galactose oxidase, a controlled atmosphere box was used. The box was first flushed for 1 hr with nitrogen which was passed through a water trap to avoid evaporation of solutions. Nitrogen was then bubbled through the galactose oxidase solution through a 26-gauge needle for an additional 30 min. The sample was then transferred to a cell within the box. Subsequently, the tightly stoppered cell was transferred to the CD cell compartment

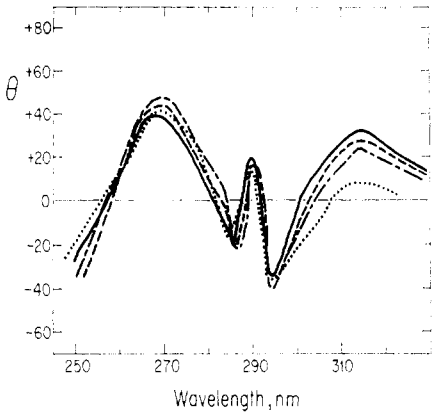


FIGURE 2: Near-ultraviolet CD spectra of galactose oxidase in 0.1 M sodium phosphate buffer at pH 7.0. Spectra were obtained with solutions which were: in air (——), deoxygenated (---), or oxygenated (-·-·-); or with deoxygenated solutions which contained 0.2 M D-galactose (· · · ·). Protein concentration was 1.5 mg/ml; cell path length was 1 cm.

which was also continually flushed with nitrogen. This procedure was judged to be adequate since the K_m for oxygen is approximately its air concentration in the presence of galactose at its K_m concentration (Kwiatkowski and Kosman, 1974). A similar procedure was sufficient to abolish enzymatic activity toward galactose. Fully oxygenated solutions were prepared by oxygen flushing in a similar manner. When they were used, galactose or dihydroxyacetone was placed in a calibrated tube which was then placed in a vacuum desiccator and transferred to the nitrogen box. After the galactose oxidase solution was deoxygenated, it was transferred into the calibrated tube containing substrate. The sample was then transferred to the cell and the CD instrument. When hydrogen peroxide was used, it was deoxygenated prior to addition to the galactose oxidase solution. The procedures used for bubbling gas through galactose oxidase solutions have no effect on its enzyme activity.

CD spectra were recorded and are expressed as previously described (Ettinger, 1974a). In the absence of enzyme, none of the ligands displayed any measurable optical activity.

Results

Far-Ultraviolet CD Spectra. Neither deoxygenation nor oxygenation has any significant effect on the far-ultraviolet CD spectra of galactose oxidase (Figure 1). Similarly, the far-ultraviolet CD spectrum of a deoxygenated galactose oxidase solution which contains an amount of D-galactose which is an order of magnitude higher than its K_m is indistinguishable from the spectrum obtained for a galactose oxidase solution in air (Figure 1). Thus the methods used to prepare the solutions for these experiments did not lead to any gross change in the protein conformation of galactose oxidase. Moreover, if galactose oxidase binds oxygen in the absence of galactose or galactose when no oxygen is present, the binding of either ligand has no detectable effect on its secondary structure.

Near-Ultraviolet CD Spectra. No significant change is detected in the galactose oxidase spectrum between 250 and 295 nm after deoxygenation, oxygenation, or addition of galactose to a deoxygenated solution of galactose oxidase (Figure 2). Neglecting for the moment the changes shown in Figure 2 above 295 nm, these spectra indicate that if either oxygen or galactose binds to galactose oxidase when only one substrate is present, neither ligand has a marked effect on the

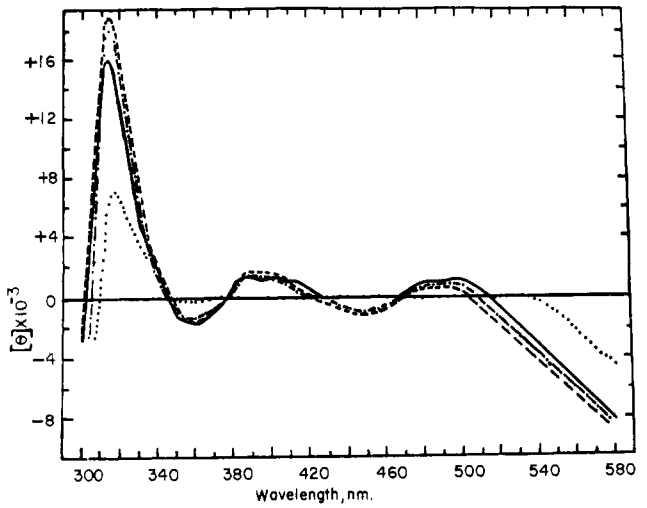


FIGURE 3: CD spectra from 300 to 580 nm of galactose oxidase in 0.1 M sodium phosphate buffer at pH 7.0. Spectra were obtained with solutions which were: in air (——), deoxygenated (---), or oxygenated (-·-·-); or with deoxygenated solutions which contained 0.2 M galactose (· · · ·). Protein concentration was 1.5 mg/ml; cell path length was 5 cm.

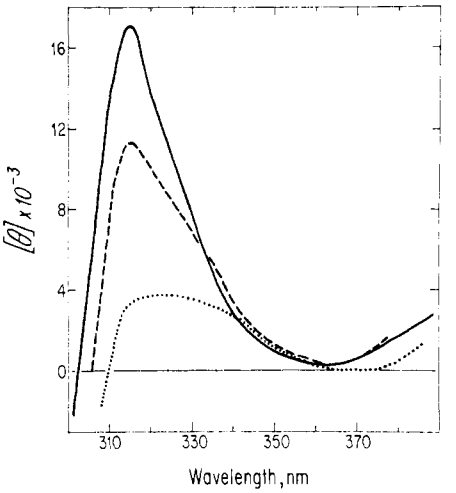


FIGURE 4: CD spectra from 300 to 380 nm of galactose oxidase solution in air (—); a deoxygenated solution of galactose oxidase which contained 0.2 M D-galactose (---); an oxygenated solution of galactose oxidase to which 0.2 M D-galactose and catalase has been added (· · · ·). Spectra were obtained with solutions at 1.5 mg/ml of galactose oxidase in 0.1 M sodium phosphate buffer at pH 7.0. A 5-cm path-length cell was used.

conformation of aromatic groups in the galactose oxidase molecule.

The spectra shown in Figure 2 from 295 to 330 nm were recorded with a 1-cm cell; therefore, they are somewhat less reliable than the spectra shown in Figure 3 which were obtained with a 5-cm cell under the same conditions. However, the spectra obtained with the 1-cm cell do appear to indicate that both oxygenation and deoxygenation result in a small reduction in the magnitude of the 314-nm extremum which is the peak of the largest copper CD band in the galactose oxidase spectrum (Ettinger, 1974a). Spectra recorded with the 1-cm cell also show that galactose in the absence of oxygen apparently induces a marked reduction in this band (Figure 2).

Copper CD Spectra. In contrast to what was observed in spectra recorded using a 1-cm cell, a small increase in the 314-nm CD band is recorded with either oxygenated or deoxygenated solutions of galactose oxidase in a 5-cm cell (Figure 3). On the other hand, the 314-nm band is again reduced to less than one-half of the magnitude obtained with the unperturbed enzyme when high concentrations of galactose are included in deoxygenated galactose oxidase solutions (Figure 3). The remainder of the CD spectrum of galactose oxidase associated with its copper optical activity reveals that galactose in the absence of oxygen abolishes detectable copper optical activity of galactose oxidase between 370 and 530 nm and leads to a marked diminution in the CD band which reaches its maximum at 610 nm[1] (Ettinger, 1974a) (Figure 3). Oxygenation or deoxygenation has no significant effect on the optical activity between 350 and 500 nm; both procedures lead to a slight increase in the 610-nm band (Figure 3).

Effects of Galactohexodialdose, Dihydroxyacetone, and Hydrogen Peroxide. To study the spectral effects of the galactohexodialdose product an anaerobic solution of galactose oxidase and galactose was oxygenated. Any possible effects of the hydrogen peroxide produced were avoided by the addition of catalase. After the solution stirred for a period long enough to allow the enzyme reaction to proceed to 90% completion (30 min), the CD spectrum was recorded in the vicinity of the 314-nm extremum. As evidenced by the further reduction in the 314-nm band, the aldehyde product of the galactose oxidase reaction appears to also interact at the copper locus of the enzyme (Figure 4). Under these same conditions, the near-ultraviolet CD spectrum between 250 and 295 nm, which is recorded within 1 hr after the addition of oxygen, is identical with that obtained with the native enzyme in the absence of substrates. However, large changes are recorded in this spectral region after the solution is permitted to stir overnight (Figure 5). The following changes are observed: the 314-nm extremum is absent; the 305-nm extremum that is also observed in the spectrum of the apoenzyme (Ettinger, 1974a) is present; a large increase in negative ellipticity is apparent in the 285- to 295-nm region; and a blue

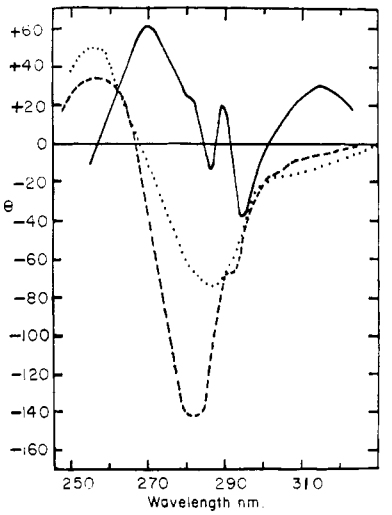


FIGURE 5: Near-ultraviolet CD spectra of galactose oxidase (——); a galactose oxidase solution which was allowed to stir in air overnight after 0.2 M D-galactose and catalase were added (----); and a deoxygenated solution of galactose oxidase which contained 0.2 M dihydroxyacetone (· · · ·). Spectra were obtained with solutions at 1.5 mg/ml of galactose oxidase in 0.1 M sodium phosphate buffer at pH 7.0. A 1-cm path-length cell was used.

[1] It was not feasible to extend these spectra beyond 580 nm due to the inability of the manufacturer to supply electrooptic plates that could withstand the high voltages necessary to yield circularly polarized light at high wavelengths.

shift occurs in the 270-nm extremum (Figure 5, dashed curve). Furthermore no optical activity can be detected above 340 nm. Apparently with time either the dialdehyde chemically modifies the enzyme, or a marked change in protein conformation occurs, or a relatively slow rearrangement of the copper–ligand complex occurs.

The near-ultraviolet CD spectrum of a deoxygenated solution of galactose oxidase which contains high concentrations of dihydroxyacetone has the identical features as that obtained with galactohexodialdose (Figure 5, dotted curve). From 340 to 560 nm, no galactose oxidase copper optical activity is detected in the presence of dihydroxyacetone. At 590 nm, the ellipticity of a deoxygenated dihydroxyacetone solution of galactose oxidase is approximately one-fifth the ellipticity of the free enzyme. These effects of dihydroxyacetone are completely reversed by anaerobic dialysis of the galactose oxidase–dihydroxyacetone solution.

A 0.5 M concentration of hydrogen peroxide is necessary to approach saturation of galactose oxidase. Although a large reduction in the magnitude of the 314-nm band is observed in the presence of hydrogen peroxide, concomitant large changes are also observed in both the far- and near-ultraviolet CD spectra. Therefore, it is impossible to distinguish between direct effects on copper optical activity ensuing from hydrogen peroxide interactions at the active site of galactose oxidase and indirect effects on copper optical activity as a result of chemical modification of protein groups or gross protein conformation changes.

Discussion

The results reported herein illustrate the sensitivity and utility of CD spectra for following the interactions of ligands with the copper chromophore in copper proteins. Results in our laboratories indicate that the binding of chloride, cyanide, or azide also leads to a diminution in the 314-nm CD extremum of galactose oxidase (Ettinger, 1974b).

Any interpretation of the changes in copper optical activity induced by galactose must be consistent with the fact that galactose does not significantly alter the electron spin resonance (esr) spectrum of galactose oxidase (Blumberg *et al.*, 1965; Kosman *et al.*, 1973). That galactose does bind directly to the copper atom is suggested by esr spectra which show that it competes with cyanide as a copper ligand (Giordano *et al.*, 1974). The esr result has been interpreted to indicate that when the 6-hydroxyl of galactose binds to the copper, it displaces a ligand of similar ionic character; namely, water or hydroxide. There is no evidence that galactose affects copper optical activity indirectly through changes in protein conformation. Galactose oxidase protein CD spectra are identical with and without galactose. Since galactose reduces the copper optical activity of galactose oxidase, one could infer that the geometry of the galactose–copper complex within galactose oxidase is geometrically less asymmetrical than the copper complex in the free enzyme. However, it is difficult to imagine any type of marked change in the geometry of the copper–chelate within galactose oxidase that would not be reflected by changes in both the CD and esr spectra. Perhaps the most likely basis for the changes in copper optical activity induced by galactose is that the copper optical activity transitions are affected by the electronic environment created by the bound galactose molecule. Groups other than the 6-hydroxyl group must be involved since any electronic effect of the 6-hydroxyl group would also be evidenced in the esr spectrum. The fact that galactose affects the intensity of the CD transitions rather than their position is consistent with this rationalization. The possibility remains that conformation changes that are detectable in the CD experiments at 27° are not detectable in esr experiments which involve rapidly bringing galactose oxidase solutions with and without galactose to 77°K. A similar discussion applies to any analysis of the spectral effects of galactohexodialdose since this ligand has no effect on the esr spectrum (Kosman *et al.*, 1973). Changes in the actual geometry of the copper locus may be associated with the changes in CD spectra that are induced by dihydroxyacetone since this ligand does affect the esr spectrum of galactose oxidase (Giordano and Bereman, 1974).

Dihydroxyacetone and galactohexodialdose have large effects on the near-ultraviolet CD spectra in addition to their effects on the CD spectra above 300 nm. It is unlikely that the time-dependent effects observed with the galactohexodialdose on the near-ultraviolet CD spectrum of galactose oxidase are due to some gross effect of the aldehyde on the general structure of the protein since strikingly similar effects are observed with the substrate, dihydroxyacetone. The effects of dihydroxyacetone are completely reversible. Both dihydroxyacetone and galactohexodialdose appear to interact with aromatic side chains which must be in close proximity to the active-site locus of galactose oxidase. Specifically, interactions with a tryptophan residue or residues are indicated by the marked changes in CD spectra from 290 to 295 mm. In addition, the blue shift of the 270-nm extremum is indicative of interactions with other aromatic amino acid(s) or disulfide bonds(s) as well. An alternative description of the large negative increase between 290 and 295 nm is that the positive 314-nm band in galactose oxidase undergoes a blue shift with inversion in sign in the presence of these ligands. There has been a suggestion that the 314-nm band may be a charge-transfer transition (Ettinger, 1974a) and charge-transfer transitions characteristically undergo large shifts with changes in environment (Kosower, 1968). However, for all ligands tested to date, when a decrease in ellipticity is detected above 340 nm, a parallel decrease in the 314-nm band is also detected. Since the copper optical activity above 340 nm is virtually abolished by either dihydroxyacetone or the galactohexodialdose, it is highly unlikely that a shift of the 314 nm occurs to give a transition of large magnitude at 290–295 nm.

That a tryptophan residue is in the vicinity of the active site of galactose oxidase can also be inferred from the apoholoenzyme difference spectrum (Ettinger, 1974a). Moreover, the enzyme is inactivated as two tryptophan residues are modified with *N*-bromosuccinimide (Ettinger, 1974b). However, the binding of galactose in the absence of oxygen did not result in any significant detectable changes in tryptophan optical activity. It is possible that a group on dihydroxyacetone is in juxtaposition to a tryptophan(s) side chain(s) while no such interaction can occur with galactose merely because of the differences in stereochemistry between the two compounds. Alternatively, galactose and dihydroxyacetone may both be in juxtaposition to a tryptophan(s) side chain(s), but the greater ionic character of the dihydroxyacetone may lead to a greater effect on the spectral properties of this tryptophan(s). Fluorescence experiments are in progress to clarify these interpretations.

From the CD results, several inferences can be made concerning the binding of substrates and products to galactose oxidase. The large effects of galactose on the copper optical activity of the enzyme are in marked contrast to the small effects of oxygen which are in the same direction whether it is removed or added to galactose oxidase solutions. These obser-

vations imply that galactose binds in the absence of oxygen, but oxygen does not bind in the absence of galactose. In view of the marked changes in copper optical activity caused by several other ligands, it is highly unlikely that oxygen, which causes no changes, binds directly to the metal. In addition to what is observed with galactose oxidase substrates, the CD results also show that the aldehyde product of the galactose oxidase reaction binds at the copper site in the absence of hydrogen peroxide. Moreover, hydrogen peroxide probably binds at the copper as well since it does abolish copper optical activity.

These results have a bearing on any kinetic scheme that is proposed for the galactose oxidase bisubstrate reaction. The CD results constitute evidence independent of kinetic data that the binding of the two substrates cannot be in a random order; galactose must bind prior to oxygen. Kinetic data for galactose oxidase have been interpreted to signify a Ping-Pong kinetic pathway for the enzymatic reaction (Hamilton *et al.*, 1973). In most proposed Ping-Pong schemes, galactose is assumed to bind prior to oxygen. Thus, the CD data provide nonkinetic evidence for this assumption. More recently a sequential kinetic scheme has been developed in our laboratories that is consistent with: the CD data for galactose and oxygen; the CD data with galactohexodialdose; reciprocal velocity–substrate plots; and product inhibition patterns with hydrogen peroxide (Kwiatkowski and Kosman, 1974). In this scheme, the CD and kinetic results suggest that first galactose binds directly to the copper atom. Subsequently, oxygen binds and within the ternary complex reacts directly with the reducing equivalents in the sugar alcohol rather than at the copper atom *per se*. That oxygen cannot bind within the inner coordination sphere of the copper atom after galactose binds is also suggested by esr experiments with cyanide. Cyanide induces marked changes in the galactose oxidase esr spectrum (Giordano *et al.*, 1974). Titration experiments indicate that only one cyanide molecule binds per copper atom. This esr result implies that only one site, presumably an axial one, is available to exogenous copper ligands.

Acknowledgment

We wish to express our appreciation for the excellent technical assistance of Mr. Lawrence Wilson.

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