Reversed-phase HPLC has been used for the separation of 6- and 7-methyl- (and-methanol-)2,4-diaminopteridines (9).

A nearly baseline separation of 6- and 7-methotrexate ($R$ = 1.4) was obtained using a gradient elution system illustrated in Fig. 5.

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# Microbial Transformations of Natural Antitumor Agents XVIII: Conversions of Vindoline with Copper Oxidases


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**Abstract** □ Vindoline occurs structurally intact in the clinically important *Vinca* alkaloids vinblastine and vincristine. It is oxidized by human ceruloplasmin and fungal and plant laccases into a reactive intermediate which undergoes intramolecular cyclization to an enamine which ultimately dimerizes. Transformations of vindoline by these copper oxidases are enhanced when enzyme incubations are performed with cofactors such as chlorpromazine. The role of copper oxidases in alkaloid metabolic interconversions and the possible implications of these reactions in *Vinca* alkaloid toxicity are discussed.



**Keyphrases** □ Vindoline—microbial transformations, natural antitumor agents, conversions with copper oxidase, *Vinca* alkaloids □ Alkaloids, *Vinca*—vindoline, microbial transformations, natural antitumor agents, conversions with copper oxidase □ Antitumor agents—vindoline, microbial transformations, natural antitumor agents, conversions with copper oxidase, *Vinca* alkaloids


Vincristine (II) and vinblastine (I) are widely used dimeric antitumor alkaloids obtained from species of *Catharanthus rosea*. These compounds differ structurally only in the oxidation state of one carbon atom which is attached to the dihydroindole nitrogen atom of the *Aspidosperma* portion of the molecule. Studies concerned with the metabolism of I and II are intended to reveal pathways of metabolic transformations which might ultimately be implicated in mechanism(s) of action and/or the toxicities associated with their use. Several attempts have been made to date (1–5) to study the metabolism of *Vinca* alkaloids, but no metabolites of these compounds have been isolated and fully identified. Low amounts of metabolites produced, very low doses of compounds employed, high molecular weights, and structural complexities probably rendered the identification of presumed metabolites difficult in previous studies.

Copper oxidases are widely occurring enzymes found in mammals, plants, and microorganisms. Enzymes from these different sources possess different physical characteristics including molecular weight, the number and oxidation states of copper, and the nature of copper ligands at presumed active sites (6, 7). Recognized similarities also exist among these enzymes, and direct comparisons between the catalytic capabilities of ceruloplasmin and laccases have been made previously (8, 9). The enzymes achieve the oxidation of substrates by the direct removal of substrate electrons and protons with the subsequent transfer of electrons to molecular oxygen *via* copper (6, 7, 10, 11). True substrates interact directly with copper oxidase enzymes to yield products, while pseudosubstrates require substances capable of interfacing between them and the enzyme during oxidations (6). The requirement for such cofactors has been noted primarily in work with ceruloplasmin in the oxidation of xenobiotics such as arylamines, phenols, and some centrally acting drugs and their analogs (6).

It was discovered that vindoline, a dihydroindole monomer found in the structure of I undergoes oxidation in the presence of copper oxidase enzymes including human ceruloplasmin, fungal, and plant laccases. This report describes the types of chemical transformations of vindoline catalyzed by the copper oxidases which result




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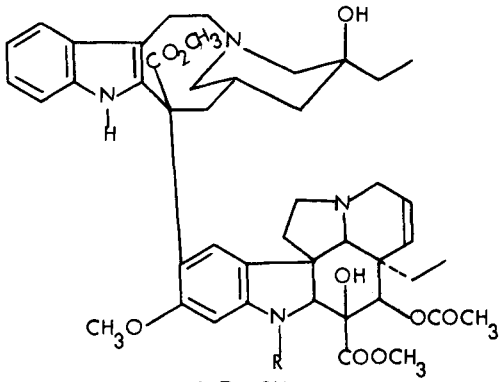


I: R = $CH_3$
II: R = CHO

in the formation of a reactive enamine intermediate which dimerizes.

## EXPERIMENTAL

**Compounds**—Vindoline (III)[1] was fully characterized (melting point, NMR, UV,and IR spectra) before being used in these experiments (12). Dihydrovindoline ether (IV) and the enamine dimer (V) were authentic standards from previous work (12, 13). These compounds were previously characterized by high resolution mass spectral, carbon-13 and proton NMR analyses.

**General**—IR spectra[2] were determined using potassium bromide disks, UV spectra[3] were measured in alcohol solution, proton NMR spectra[4] were obtained using deuterochloroform as the solvent and tetramethylsilane as an internal standard, and low resolution mass spectra[5] were obtained using a direct inlet probe.

**Chromatography**—TLC was performed on 0.25-, 0.50-, or 1.0-mm thick layers of silica gel $GF_{254}$[6] on glass plates. Prior to use, TLC plates were activated at 120° for 30 min. Solvent systems employed were: A, ethyl acetate–methanol (3:1); B, ethyl acetate–benzene (3:1); C, ethyl acetate–benzene (1:1); and D, ethyl acetate 100%. Compounds were detected on developed chromatograms by fluorescence quenching <254- or 365-nm UV light and were later visualized by spraying with cerium (VI) ammonium sulfate (1% in 50% $H_3PO_4$, v/v). Column chromatography was performed with silica gel[7] activated for 30 min at 120° prior to use. Columns were packed wet by slurrying silica gel in developing solvent. High-performance liquid chromatography (HPLC) was performed using a reversed-phase column[8] (0.4 × 30 cm), a septumless injector[9], a solvent delivery system consisting of a dual-piston pump[10], and a dual fixed wavelength 254-nm UV absorbance detector[11]. Separations were best achieved using acetonitrile and 0.1% $(NH_4)_2CO_3$–water (50:50) at an average flow rate of 1 ml/min, and an operating pressure of 2000 psi. Authentic standards were injected singly to establish individual retention volumes, and mixtures of metabolites were well-resolved. The identities of individual metabolite peaks were confirmed by spiking with analytical standards. Retention volumes of various compounds were: III, 6.59 ml; IV, 7.78 ml; and V, 23.95 ml.

**Enzymes**—Methods used in the production, isolation, and analysis of *Polyporus anceps* laccase enzyme were described previously (14). Laccase enzyme activities were determined using a simple colorimetric assay procedure based on the oxidation of syringaldazine (14–16). For these assays, a standard laccase unit was defined as the amount of enzyme catalyzing the oxidation of 1 μmole of syringaldazine to its quinone form ($\epsilon$ = 65,000 at 526 nm)/min at 25° in pH 5.0, 0.2 *M* phosphate ($K_2HPO_4$–$KH_2PO_4$) (14).

*Rhus vernicifera* laccase[12] enzyme activities were determined in pH 7.5, 0.1 *M* phosphate buffer using the syringaldazine method.

The purity of ceruloplasmin (human, type III)[13] was ascertained by determining the ratio of absorbances at 610/280 nm, which was measured at 0.046. Pure crystalline enzyme was reportedly measured at $A_{610}/A_{280}$ of 0.047 (17). Enzyme concentrations were calculated from the 610-nm absorption ($\epsilon$ = 10,900/*M*) (18) based on a molecular weight of 132,000 (19). Ceruloplasmin activity was standardized in terms of international units according to a previous procedure (20). A single variation of this procedure was the use of the sulfate rather than the oxalate salt of *N*,*N*-dimethyl-*p*-phenylenediamine for this determination. Based on these measurements, human ceruloplasmin contained a specific activity of 123 U/mg of protein. Ceruloplasmin activity was significantly lower at 25°, providing a measured specific activity of only 29 U/mg of protein.

**Enzyme Incubation Procedures**—Most incubations were conducted in 50-ml flasks which were shaken at 250 rpm[14].

Laccase incubations of *P. anceps* were conducted at 27°. Incubation mixtures were prepared by adding 2 mg ($4.39 \times 10^{-6}$ moles) of vindoline in 0.1 ml of methanol to 7 ml of phosphate buffer (pH 5.0, 0.2 *M*) containing 1.05 U of laccase.

Incubations with *R. vernicifera* laccase were also conducted at 27°, and incubation mixtures were prepared by adding 2 mg of vindoline in 0.1 ml of methanol to 5.2 ml of phosphate buffer (pH 7.0, 0.1 *M*) containing 0.019 U of *Rhus* laccase.

Incubations with human ceruloplasmin were conducted at 37°. These were prepared by adding 2 mg of vindoline in 0.1 ml of methanol to 7.05 ml of acetate buffer (0.2 *M*, pH 5.5) containing 54 U of ceruloplasmin activity.

Several cofactors were also employed in enzyme incubation mixtures. Where used, they were employed in molar ratios of 1:1 and 3:1 *versus* the substrate vindoline. Cofactors examined in this work were 3-hydroxy-4-methoxyphenethylamine; harmine; harmol hydrochloride; histamine; levodopa; syringaldazine; hydroquinone; *N*,*N*-dimethyl-*p*-phenylendiamine; and chlorpromazine. Cofactors were added to incubation mixtures in 0.1 ml of methanol.

Controls consisted of incubations containing enzyme alone, mixtures containing only substrates plus cofactors, and complete incubations containing boiled enzymes. No reactions were observable when boiled enzymes were used or when no enzymes were present in incubation mixtures.

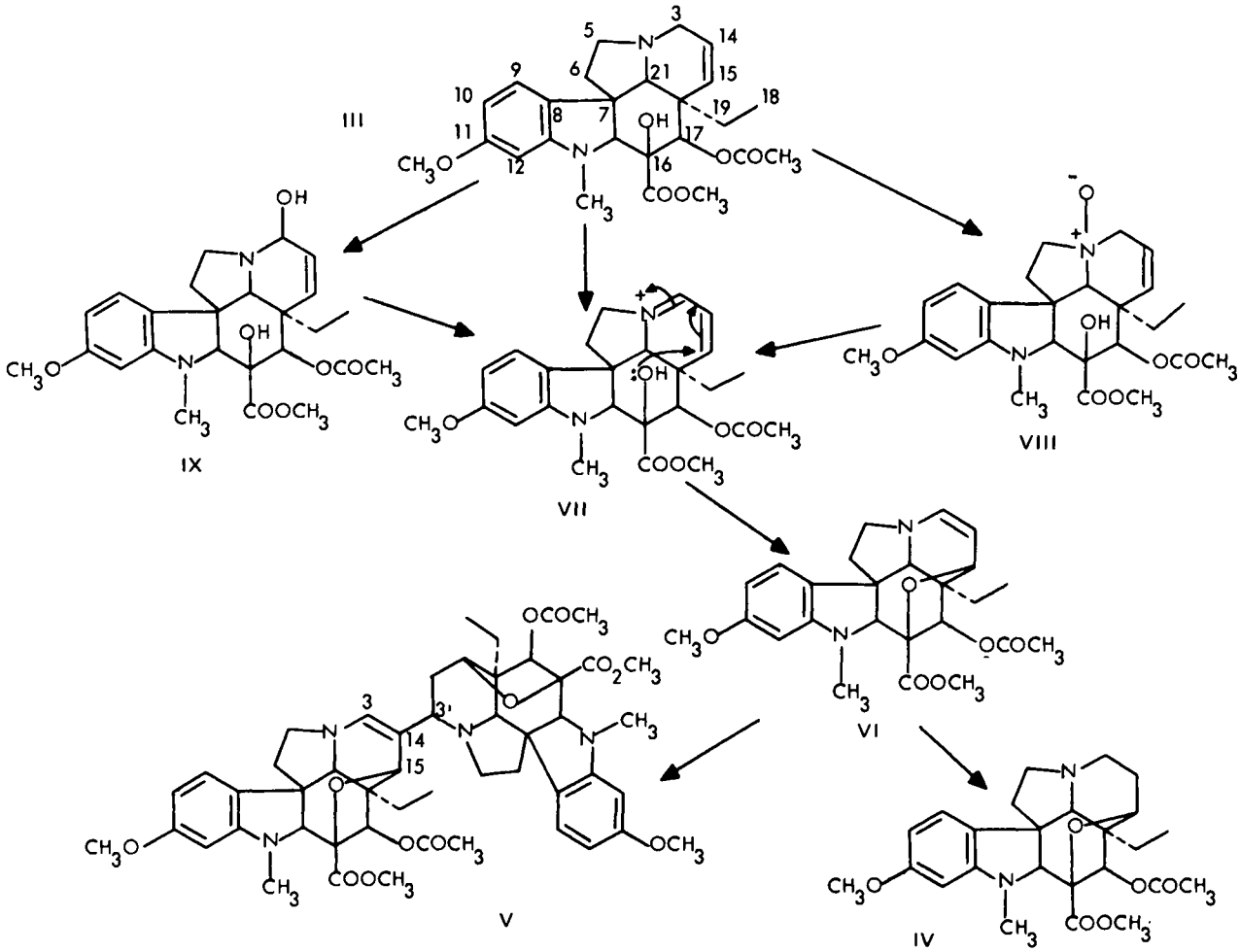
Rates of enzyme reactions were determined by measuring oxygen uptake with an electrode[15] connected to a stirred, water-jacketed reaction chamber 3.73 ml in volume (21). Incubations were conducted as described before using 54 U of ceruloplasmin, and 0.25 U of *P. anceps* laccase. Vindoline (1.02 mg, $2.24 \times 10^{-6}$ moles) was added to incubations in 0.05 ml of methanol, and chlorpromazine (9.4 mg, $2.65 \times 10^{-6}$ moles) was added as a cofactor for most incubations.

Analyses of enzyme incubation reactions were also performed by withdrawing samples of 1.0 ml at various time intervals. Reaction samples were adjusted to pH 10 with 58% $NH_4OH$ and extracted with 1 ml of ethyl acetate. Extracts were examined by TLC using solvent systems A and B or by HPLC.

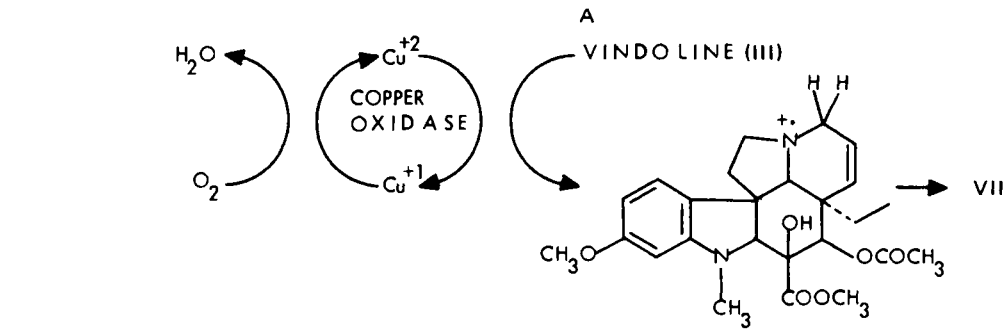
**Preparative Scale Production of the Dimer (V) Using *P. anceps* Laccase**—Vindoline (300 mg) dissolved in 25 ml of dimethylformamide was added to 300 ml of pH 5.0, 0.2 *M* phosphate buffer containing 66 units of *P. anceps* laccase enzyme and 3.2 mg of syringaldazine added in 2 ml of methanol. This mixture was incubated for 24 hr at room temperature to ensure a complete bioconversion reaction before being adjusted to pH 10 with 58% $NH_4OH$ and extracted four times with equal portions of ethyl acetate. The extracts were dried over anhydrous sodium sulfate and concentrated to a red oil (0.635 g). The oil was dissolved in 1 ml of ethyl acetate, applied to a silica gel column (3 × 33 cm, 100 g) and eluted with solvent system C at a flow rate of 2 ml/min while 10-ml fractions were collected. Fractions 269–300 contained the major reaction product, and these were combined, dried over anhydrous sodium sulfate, and concentrated to a brown solid (143 mg). This material was further purified by dissolving in 1 ml of acetone and applying to a 0.5-mm silica gel preparative layer TLC plate which was developed in solvent system B. The band at $R_f$ 0.39 was scraped from the plate, mixed with 10-ml portions of acetone (4X), stirred, and filtered. Evaporation of the combined filtrates yielded 81 mg of pure V as an amorphous glass.

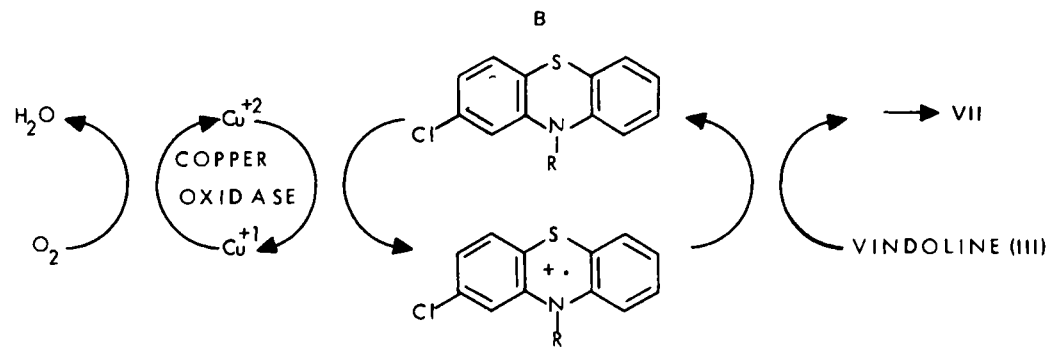
**Trapping a Reactive Enamine Intermediate (VI) by Reduction to IV with Sodium Borohydride**—Two milligrams of III dissolved in 0.1 ml of methanol was added to 7 ml of 0.2 *M* phosphate buffer, pH 6.5,

[1] Eli Lilly and Co., Indianapolis, Ind.
[2] Model 267, Perkin-Elmer, Norwalk, Conn.
[3] Model SP1800, Pye Unicam Ltd., Cambridge, England.
[4] Model EM360, Varian Associates, Palo Alto, Calif.
[5] Model 3200, Finnigan Corp., Sunnyvale, Calif.
[6] Merck and Co., Rahway, N.J.
[7] Baker 3405, J.T. Baker Chemical Co., Phillipsburg, N.J.
[8] μBondapak phenyl, Waters Associates, Milford, Mass.
[9] Model U6K, Waters Associates, Milford, Mass.
[10] Model 6000, Waters Associates, Milford, Mass.
[11] Model ALC/GPC 202, Waters Associates, Milford, Mass.
[12] H. B. Gray, California Institute of Technology, Pasadena, Calif.
[13] Sigma Chemical Co., St. Louis, Mo.
[14] Delong, Model G24, New Brunswick Scientific Co., Edison, N.J.
[15] Clark, YSI Co., Yellow Spring, Ohio.




***Scheme I**—Pathways for the oxidation of vindoline (III).*




***Scheme II**—Pathways for the flow of electrons in the oxidation of vindoline by the copper oxidases.*

containing 1.2 units of *P. anceps* laccase. The mixture was incubated for 2.5 hr before being adjusted to pH 9.6 with 58% $NH_4OH$ and extracted with 7 ml of ethyl acetate. TLC of the crude extract on silica gel plates (solvent system A) *versus* authentic standards revealed the presence of III $R_f$ 0.7, V $R_f$ 0.82, and the presumed enamine VI, $R_f$ 0.9. The extract was evaporated to dryness, redissolved in 1 ml of methanol and treated with 10 mg of sodium borohydride. After 30 min, this reaction mixture was evaporated to 1 ml, quenched with 5 ml of water and extracted with ethyl acetate. This extract was evaporated to dryness and TLC examination on silica gel $GF_{254}$ (solvent system A) revealed the presence of a substance with chromatographic mobility of $R_f$ 0.25, identical to known dihydrovindoline ether (IV). The extract was dissolved in ethyl acetate, applied to a preparative 0.5-mm silica gel plate, and developed with solvent system A. The band at $R_f$ 0.25 was removed and extracted from silica gel with acetone. TLC and HPLC (retention volume of 7.78 ml) determined that the eluted compound was identical to authentic IV.

## RESULTS AND DISCUSSION

The monomeric *Vinca* alkaloid III has been the subject of a previous metabolic study. This compound is the most abundant and available of the alkaloids of *C. rosea*, and it is found essentially intact in the dimeric structures of I and II. Enzymatic transformations observed with III also might be expected to occur in the appropriate functionalities of the dimeric alkaloids like I and II.

Early work with microorganisms as metabolic tools (22) resulted in the elaboration of a unique vindoline metabolic pathway (Scheme I) (12, 13). *Streptomyces griseus* whole cells oxidized vindoline to V and IV, both of which accumulated in incubation media in high yield. A reactive enamine VI was identified as a key intermediate in transformations of III (13). Although the enzyme systems of *S. griseus* responsible for the biotransformation reactions remain unknown, it is possible to postulate four different pathways by which III might undergo initial oxidation to VI (Scheme I). The first pathway involves initial *N*-oxidation of III to VIII, conjugation through the *N*-oxide and elimination of ROH to form VII; and subsequent intramolecular cyclization to VI. This pathway would represent a biochemical equivalent to the modified Polonovsky reaction observed in the chemical treatment of these alkaloids (12). A second path involves direct oxidation of vindoline to VII by the removal of two electrons and a proton. The third possibility would invoke a direct hydroxylation of vindoline at position 3 to form a carbinolamine (IX) which provides VII by elimination of a hydroxide ion. Finally, and perhaps least likely, initial epoxidation of the 14,15-double bond followed by intramolecular etherification and subsequent dehydration could also provide VI. The need to elaborate the precise steps involved in oxidations of III has stimulated a search for more highly defined enzyme systems such as the copper oxidases.

Since crude fungal laccase could be reproducibly obtained in relatively large amounts, it was employed in preparative scale incubations designed to afford sufficient amounts of metabolites for isolation and structure elucidation. Incubations of III with *P. anceps* laccase (14) produced two metabolites with similar TLC mobilities to III derivatives VI and V (12, 13). The major and stable metabolite produced by *P. anceps* laccase was obtained by column and preparative layer chromatography in 27% yield from 300 mg of III. This metabolite was identified as V by spectral and chromatographic comparison with an authentic sample of the dimer (12). Pertinent spectral properties included the UV spectrum in ethanol with $\lambda_{max}$ values at 252 nm (log $\epsilon$, 4.13) and 309 (log $\epsilon$, 3.84). Highly characteristic NMR signals were also obtained for the metabolite and included the following: ppm, 0.8 (6H, m, 2 overlapping 18H), 1.9 (3H, s, $COCH_3$), 2.0 (3H, s, $COCH_3$), 2.65 (3H, s, $NCH_3$), 2.75 (3H, s, $NCH_3$), 3.75 (12H, s, two overlapping $CO_2CH_3$ and $OCH_3$ groups), 4.05 (1H, m, 15′H), 4.2 (1H, s, 15H), 5.35 (2H, d, $J$ = 2 Hz, overlapping 17H), 5.99 (2H, d, $J$ = 2 Hz, overlapping 12H), 6.05 (1H, s, 3H), 6.25 (2H, q, overlapping 10H), 6.9 (2H, dd, overlapping 9H). Confirmation of the structure of the major and stable laccase metabolite as V is a significant finding, because the mechanism of copper oxidase reactions is reasonably well understood.

It was mechanistically important to confirm the involvement of the enamine (VI), in copper oxidase transformations of vindoline. The second and unstable laccase metabolite possessed similar TLC mobility to VI previously obtained from *S. griseus* incubations. Since the presumed enamine intermediate was considerably more stable at pH values near neutrality, enzyme incubations were conducted at pH 6.5 to favor its accumulation. Extracts of laccase incubation mixtures were concentrated, and reduced with sodium borohydride to convert the enamine to the known dihydrovindoline ether IV. This technique was employed earlier as a means of stabilizing and identifying the enamine VI (12, 13). Chromatographic (HPLC, retention volume 7.78 ml; TLC, $R_f$ 0.25) analysis of the reduced laccase extract clearly demonstrated the presence of IV. Thus, it has been shown that the copper oxidase oxidations of III result in the formation of VI as well as V.

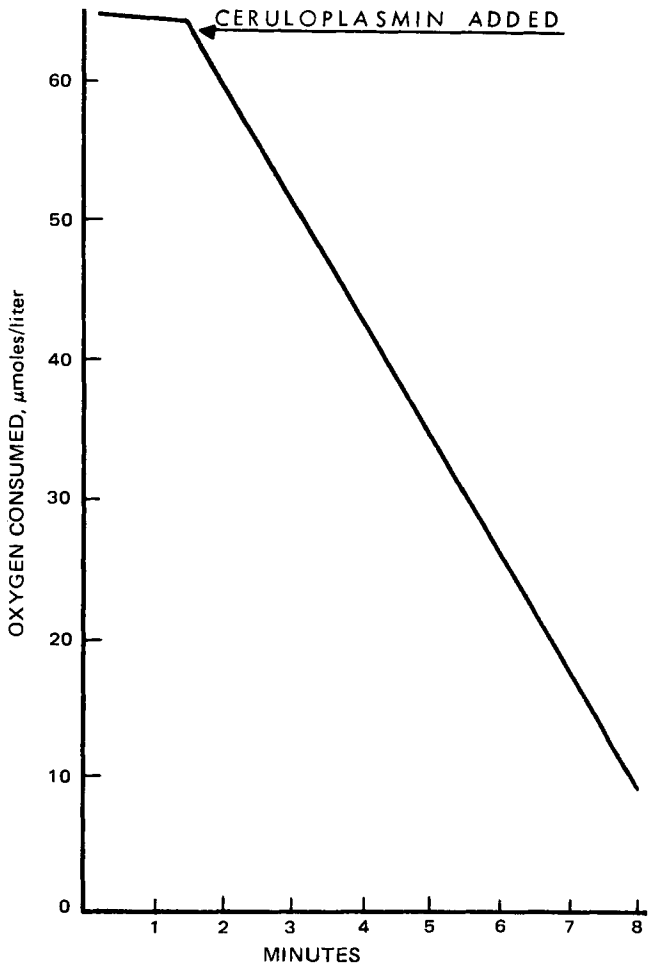



***Figure 1**—Rate of oxidation of vindoline by ceruloplasmin as shown by the uptake of molecular oxygen using a Clark oxygen electrode.*

The involvement of chlorpromazine and other cofactors in copper oxidase oxidations of III is illustrated in Scheme II. When III is a true substrate for ceruloplasmin, it is directly oxidized by the transfer of electrons through the copper oxidase enzyme to molecular oxygen (A). With chlorpromazine and syringaldazine, III is a pseudosubstrate which is oxidized directly by a chlorpromazine radical species which serves as a cycling intermediate between it and ceruloplasmin (Scheme IIB) (23). The oxidation of chlorpromazine to a radical species by ceruloplasmin has been associated with the formation of a red pigment with an absorption maximum at 529 nm by simultaneous optical-absorption, electron paramagnetic resonance measurements (23). With both laccase and ceruloplasmin, chlorpromazine forms a visible red pigment with an absorption maximum at 529 nm. Similar radical intermediates have been implicated in other oxidation reactions catalyzed by ceruloplasmin (23–26).

Human ceruloplasmin and *R. vernificera* laccase also oxidized III to the dimer (V). As with *P. anceps* laccase, III was an apparent true substrate for serum ceruloplasmin, forming products in the absence of added cofactors. However, reaction rates were increased sixfold when an ~1-mole equivalent of chlorpromazine was added to the reaction mixtures. A similar effect was observed when III was incubated with laccase alone or in the presence of laccase plus chlorpromazine. Results obtained using the oxygen electrode illustrate the rate enhancements obtained when chlorpromazine was added to enzyme incubation mixtures, and these are shown in Fig. 1. No reactions were observed at all with *Rhus* laccase unless cofactors were present.

Chlorpromazine clearly enhanced oxidation rates with all of the copper oxidases examined. Of the other cofactors examined, only syringaldazine, a known substrate for laccase (14), increased *Rhus* and *P. anceps* laccase oxidations. However, this compound had no measurable effect with ce-

ruloplasmin. Other substrates listed in the *Experimental* section had no effect on copper oxidase reactions. This was surprising since these compounds had previously been shown to facilitate oxidations of biogenic amine substrates with ceruloplasmin (23, 24, 27, 28).

This is the first report of any type of metabolic conversion of a *Vinca* alkaloid by mammalian enzymes, and the first demonstrating the oxidation of a nitrogen heterocycle of the *Aspidosperma* class of alkaloid by copper oxidases. It is an excellent example of the principle of microbial models for mammalian drug metabolism (22), and it clearly demonstrates the similarities with which microbial and mammalian enzymes achieve xenobiotic transformations. By their nature, copper oxidases most likely cause the elimination of two electrons and a proton from III as the first step in the biotransformation pathway. It appears that protons at position 3 of III are activated toward copper oxidase oxidations by virtue of their allylic nature and their proximity to the heterocyclic nitrogen atom. It can be expected that similar functionalities in other substrates will also be oxidized by the copper oxidases. While *N*-oxides, carbinolamines, and epoxides may be ruled out as possible intermediates in copper oxidase-mediated transformation of the *Vinca* alkaloids, it is possible that derivatives such as VIII and IX may be formed as a result of oxidation catalyzed by other types of enzymes such as the mixed function oxygenases found in mammalian liver (29–31).

This work provides a biochemical basis for understanding possible interactions of drugs like chlorpromazine and perhaps others with compounds like vindoline (III) and other copper oxidase substrates. The presence of cofactors that enhance conversions of their alkaloids and their congeners to chemically reactive species may have profound toxicological implications. The potential for chemically reactive imminium and enamine species to partake in reactions of pharmacological significance is enhanced by virtue of the stabilities of these substances in aqueous media (12, 13), which would allow for their facile distribution throughout the body. In this connection, ceruloplasmin levels are elevated in individuals afflicted with various types of cancer (32, 33). Further studies designed to elaborate enzymatic transformations of *Iboga* alkaloids representing the other half of dimers such as I and *Vinca* dimers themselves are in progress.

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