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METABOLISM OF THE *CATHARANTHUS* ALKALOIDS: FROM *STREPTOMYCES GRISEUS* TO MONOAMINE OXIDASE B[1]

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ABSTRACT.—More than three decades after their discovery and implementation in medicine, essentially nothing is known about the metabolism or the implications of metabolism in mechanism of action or toxicity of the *Catharanthus* alkaloids. The frustrating paucity of information about pathways of metabolism has limited a major source of structure–activity relationship information and has blocked a critical avenue necessary for the logical development of new and more useful *Catharanthus* alkaloids. Microbial transformations, peroxidases, copper oxidases, mouse and rat cytochrome P-450 systems, and mouse brain and bovine liver monoamine oxidase (MAO) preparations have been explored in the study of *Catharanthus* alkaloid metabolism. In this report, we present results which have clarified the involvement of enzymatic and chemically catalyzed one-electron oxidations that yield nitrogen-centered cation radicals, iminium, and carbinolamine intermediates, all of which explain how new carbon-carbon and carbon-oxygen bonds form, or break and rearrange. The dimeric *Catharanthus* alkaloids are recalcitrant to oxidations catalyzed by monoamine oxidases and to both normal and induced P-450 rat microsomal preparations. However, the *Catharanthus* alkaloids appear to be selective reversible inhibitors of MAO-B. Chemical and biochemical aspects of the metabolic transformations of dimeric *Catharanthus* alkaloids are reviewed together with the implications of our findings.

The *Catharanthus* alkaloids (also commonly referred to as the *Vinca* alkaloids), vinblastine [**1**] (VLB) (1,2), vincristine [**2**] (VCR), and leurosine [**3**] (3,4) were originally isolated from the plant *Catharanthus roseus* G. Don. (Apocynaceae). The only structural difference between VLB [**1**] and VCR [**2**] is in the state of oxidation of the carbon atom on the indoline nitrogen atom as a Me or a CHO, respectively. Despite this subtle structural difference, VLB and VCR differ in their potencies, clinical applications, metabolic fate (in our labs) and toxicities. The physical/chemical and biological bases for these difference are not yet understood. Numerous aspects of their occurrence, physical and chemical nature, structure, syntheses, structure-activity relationships, pharmacology, and therapeutic uses have been extensively reviewed (5).

The *Catharanthus* alkaloids are of wide importance in clinical medicine. Their discovery during the 1950s prompted the wider search for natural products as antineoplastic drugs (6). The era of multiple agent combination chemotherapy owes its inception to the original uses in 1964 of combinations of VCR and prednisone together with another antileukemic drug or combination of drugs. Today, either VCR or VLB is found as an essential component of each of the standard chemotherapy regimens that are used as first-line treatments in at least four of the few malignancies that can be cured by chemotherapy (7). VLB and VCR have been indispensable components of virtually every curative regimen for metastatic malignancy, and they are widely used in adjuvant and neoadjuvant drug regimens (7,8).

TOXICITY AND MECHANISM OF ACTION.—The major mechanism of action attributed to the *Catharanthus* alkaloids is cellular metaphase arrest caused when the com-

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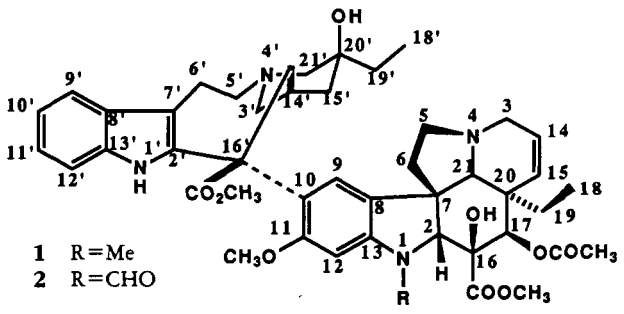

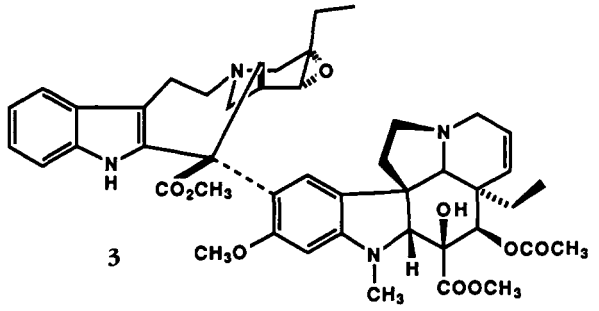

pounds disrupt cell microtubules (9–11). However, the precise mechanism of tubulin interaction remains to be determined (12). Measurable differences are observed in tubulin binding affinity and assembly for various alkaloids (8,13). The well-known interaction of *Catharanthus* alkaloids with tubulin has provided a basis for evaluation of the activities of synthetic (13) and semisynthetic *Catharanthus* alkaloid analogues (14).

In vivo tissue distribution of *Catharanthus* alkaloids appears to correlate highly with tissue tubulin concentrations (15,16). However, the cytotoxic effects of many *Catharanthus* alkaloids may not correlate with their abilities to arrest cells in metaphase (8,17), or to inhibit tubulin polymerization in vitro (18), suggesting unknown mechanisms of action (19,20) or differing pharmacokinetic properties of the various alkaloids.

VCR neutrotoxicity is reversible, dose-related, and cumulative (8,21–23), while VLB exhibits bone marrow suppression that precedes neurotoxicity (24). The neurotoxic side effects are complex (23). When administered, the compounds are taken up by axonal tissue (25); they exhibit specific effects on a choline uptake system (26) and influence neuromuscular junctions (27). The relationships of all of these to clinically observed neurotoxicity, and the reason for selective vulnerability of certain neuron types, are obscure (28). In vivo, VLB is distributed into tissues where it is found in the nuclear fraction >50%, mitochondria 19%, and the cytosol 22% (29). VLB is inserted into mitochondrial membranes, where it apparently interferes with the translocation system of the mitochondrial envelope. Mitochondrial effects caused by VLB do not seem to relate to its action on microtubuli (30). Isolated mitochondria from *Catharanthus*-alkaloid-resistant and -sensitive cells exhibit different respiratory capacities (31). The basis for this difference, and its implication in neurotoxicity, has not yet been explained. Unfortunately, after more than 30 years, there are still no satisfactory, simple, in vitro or in vivo neurotoxicity models for general use in evaluating new *Catharanthus* alkaloid analogues (8), and the search for such models continues (32).

THE ROLE OF DRUG METABOLISM IN DRUG DEVELOPMENT.—The structures of administered drugs are usually altered as they pass into the blood stream and through

metabolically proficient organs such as the liver. It is well accepted that metabolic transformation of drugs and other xenobiotics can produce biologically reactive intermediates of central importance in understanding the bases for mechanisms of action and/or toxicity. In fact, the toxicity of many organic chemicals is associated with their enzymatic conversion to toxic metabolites, a process termed bioactivation (33). Thus, it is important to investigate the structural changes of a drug catalyzed by drug metabolizing enzymes.

Drug metabolism studies are a crucial element in the drug development process. The discovery of any new biologically active natural product leads to predictable ancillary research, broadly designed to probe the structural features critical to drug activity. Research avenues usually include total or semi-synthesis efforts, alterations in the structures of prototype compounds, and drug metabolism investigations. All of these approaches afford new compounds for biological evaluation, the results of which contribute to our understanding of structure-activity relationships. For the *Catharanthus* alkaloids, many semi-synthetic derivatives of alkaloids such as VLB have been prepared and tested for biological activity. Biomimetic and total syntheses have also afforded the *Catharanthus* alkaloids and their derivatives for biological evaluation (5). There exists almost no information about the metabolic fate of the *Catharanthus* alkaloids at this time.

Figure 1 outlines the possible consequences of in vivo metabolic drug transformation. Bioactivation of VCR or VLB, for example, increases drug activity if it is converted to a more active metabolite. Alternatively, bioactivation can lead to undesirable side effects if the drug is converted either to a toxic metabolite or a chemically reactive intermedate. Bioinactivation, or conversion of the alkaloid into an inactive metabolite, is a deleterious process usually decreasing drug utility. In drug metabolism investigations, the isolation and identification of both active and inactive drug metabolites are regarded with equal importance. While the active drug metabolites provide clues to favorable structural alterations which may increase or improve drug utility, inactive drug metabolites indicate structural foci rich for alteration to afford derivatives recalcitrant to metabolism, thus improving drug utility. Furthermore, the structures of metabolites usually provide important clues to the biochemical pathways by which they are formed, and the possible involvement of chemically reactive intermediates.

CATHARANTHUS ALKALOID METABOLISM AND REACTIVE INTERMEDIATES.—Ample evidence indicates that the *Catharanthus* alkaloids are extensively metabolized in mammals. Six VLB metabolites were reported as spots on chromatograms in the first

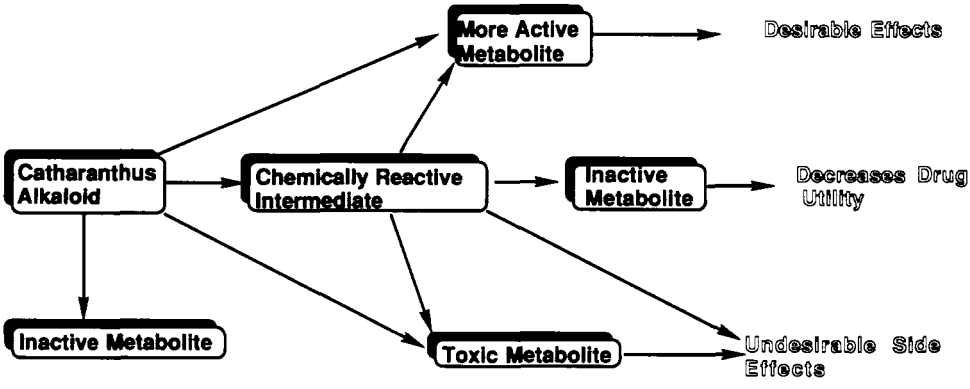

FIGURE 1. The consequences of metabolic transformations in vivo.

mammalian metabolism study in 1964 (34). Since then numerous other *Catharanthus* alkaloid metabolites have been observed by tlc or hplc. However, to date, the only identified VLB metabolite is desacetylvinblastine, found in human (35) and mouse tissue (36). Aside from this simple hydrolytic product, which was easily identified by comparison with the chemically prepared compound, the structures of other metabolites remain unknown.

Traditional approaches in which alkaloids have been administered to animals including humans have failed to solve the problem of *Catharanthus* alkaloid dimer drug metabolism. The paucity of information about *Catharanthus* alkakoid metabolism may be due to several converging factors, including structural complexities of the alkaloids, very low doses of compounds used in therapy, the need to obtain relatively large amounts of *Catharanthus* metabolites for complete structural analysis, the presumed instabilities of metabolites, and the difficulties encountered in using total synthetic approaches for the synthesis of presumed metabolites.

METABOLIC TRANSFORMATIONS OF NITROGEN HETEROCYCLES.—Figure 2 shows the structure of VLB [**2**] indicating the numerous types of potential metabolic transformations possible with this alkaloid. The reactions include hydroxylations (aromatic or aliphatic positions), ester hydrolyses, *N*- or *O*-dealkylation reactions, and either hydration or epoxidation of the isolated double bond. All of these biotransformation reactions are predictable based upon extensive knowledge of the metabolic disposition of other classes of drugs.

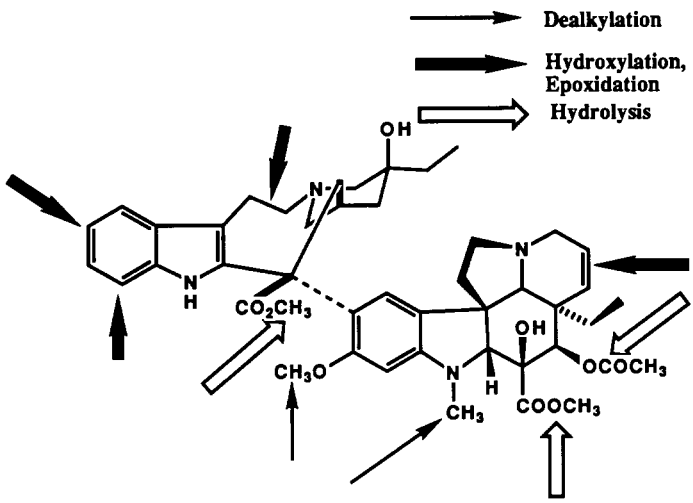

FIGURE 2. Typical metabolic transformations that can be predicted for the structure of vinblastine (VLB).

With nitrogen heterocyclic compounds like VLB, the prediction of metabolic fate is even more complicated. This alkaloid contains four different types of nitrogen atoms including the indole and tertiary nitrogen atoms or the upper of Iboga-type ring, and the dihydroindole and allylic nitrogens of the Aspidosperma-type ring system. As illustrated in Figure 3, each of these nitrogen atoms can be subject to a range of interesting and complicated biotransformation reactions leading to an array of new metabolic products. In Figure 3, **4** is intended to be generally representative of a nitrogen heterocyclic system.

Hydroxylation of **4**, by means of molecular oxygen and enzymes such as cytochrome P_{450} leads to the formation of carbinolamine **5**, which can reversibly cleave to give sec-

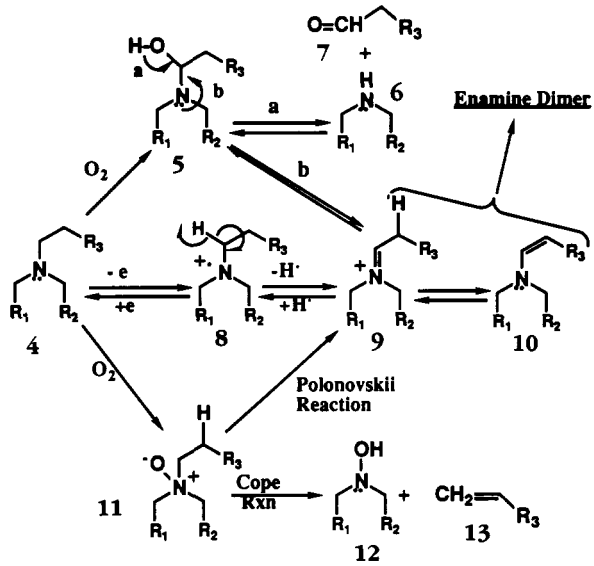

FIGURE 3. Possible pathways involved in nitrogen heterocyclic metabolism.

ondary amine **6**, and aldehyde **7** components. A second metabolic pathway typical of that catalyzed by cytochrome P_{450}, peroxidases, and monoamine oxidses involves direct one-electron oxidation of nitrogen to give nitrogen-centered, cation radicals such as **8**. Cation radicals like **8** are highly unstable intermediates which can be reversibly reduced back to **4**, or oxidized by loss of hydrogen to an iminium intermediate **9**. Iminium derivatives also are highly reactive and isomerize to yield enamines **10** which dimerize; or they may add H_2O to give carbinolamine **5** by a second path. Direct oxidation of nitrogen affords *N*-oxides like **11** which can give hydroxylamines such as **12** and olefins such as **13** via presently unknown metabolic Cope-type reactions, or iminium **9** after initial acetylation, phosphorylation or sulfation, and subsequent elimination of acetate, phosphate, or sulfate via biochemical Polonovskii-type reactions. Almost all of the reactions indicated for nitrogen are reversible, and the same metabolic products can arise by any of three completely different biochemical pathways. Cation radicals, iminium species, carbinolamines, and enamines are all examples of highly reactive intermediates, any of which may be directly or indirectly implicated in drug action or toxicity. The metabolic complexities illustrated in Figure 3 underline the need to combine chemical and biochemical approaches in solving problems of drug metabolism.

INITIAL INVESTIGATIONS OF VINDOLINE.—Extensive metabolism studies were first conducted with the monomeric *Catharanthus* alkaloid vindoline [**14**] (Figure 4). This alkaloid was selected for study because it is found essentially intact in the structures of dimers like VLB, and because nothing was known about the metabolism of this type of alkaloid. Microbial transformation of **14** with *Streptomyces griseus* gave the enamine dimer **15**, the structure of which was based upon its high resolution mass, ^{13}C-nmr, and ^{1}H-nmr spectral properties (37). The enamine structure of **15** raised interesting questions about the pathway by which the metabolite was formed and prompted a search for putative intermediates. By careful manipulation of conditions, **18** was isolated and characterized by ^{1}H-nmr, by mass spectrometric analysis of the enamine trapped by reduction with $NaBD_4$ in MeOD, and by observing the isolated enamine undergo dimerization to **15** by nmr (38). *S. griseus* (37), peroxidases (39), and copper oxidases (40,41) were all used as biocatalysts to produce the conjugated iminium de-

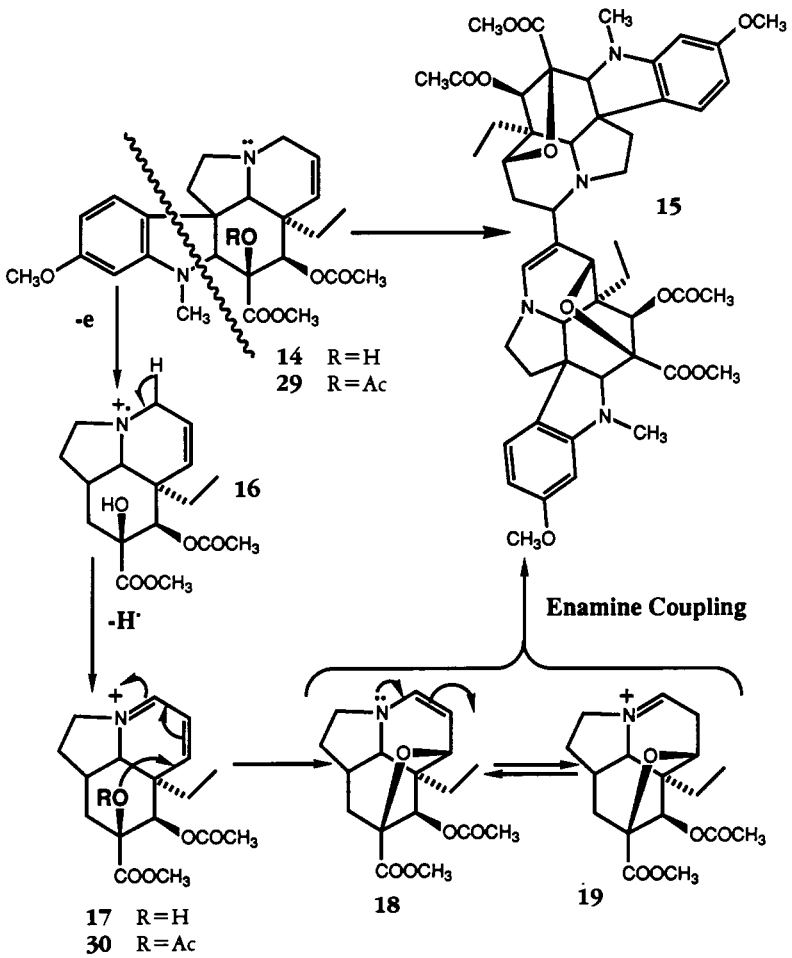

FIGURE 4. Metabolic transformations of vindoline [**14**].

rivative **30** as an intermediate in the oxidation pathway. With 17-*O*-acetylvindoline [**29**] as substrate, the reaction path to the enamine dimer **15** was blocked at **30** to permit isolation, nmr and mass spectral analysis, deuteration with $NaBD_4$ in MeOD, and chemical ester cleavage to demonstrate the conversion of the resulting iminium compound **17** to **18** and **15**. Further detailed mechanistic investigations using horseradish peroxidase clearly showed that the initial oxidation step occurred by one-electron oxidation of **14** to **15** as evidenced by direct spectral measurement of the reduction of horseradish peroxidase Compound I (39). The involvement of free radical, one-electron oxidation chemistry in vindoline biotransformations was confirmed by chemical (2,3-dichloro-5,6-dicyanobenzoquinone, DDQ) and photochemical oxidations of **29** using chloropromazine as a photosensitizing agent (42). These chemical and photochemical oxidizing systems mimicked copper oxidase enzymic biotransformations of vindoline, and led to quantitative conversions of acetylvindoline to **30**. Subsequent investigations, originating in our laboratory and elegantly pursued by Sariaslani and co-workers at DuPont, revealed that *S. griseus* strains contain a soybean-meal-inducible cytochrome P_{450} enzyme system (43) which catalyzes oxidation reactions with a host of xenobiotic compounds (44,45). Mouse microsomal cytochrome P_{450} preparations (46) also catalyzed the conversion of vindoline to **15**, implicating this additional type of one-electron oxidizing enzyme species in the observed bioconversion.

This extensive early work with vindoline provided a complete description of a metabolic pathway with one of the key alkaloid components of the more important *Catharanthus* dimeric alkaloids. In addition, we gained detailed working knowledge of the types of metabolic systems (peroxidase, copper oxidases, cytochrome P-450, bacteria) and chemical mimics (photochemistry and DDQ) that could be used to address metabolism studies with the much more complex dimeric alkaloid substrates. Furthermore, we learned to work with unstable enamine and iminium intermediates and discovered the value of trapping reactive intermediates by reduction with $NaBD_4$ for isolation and complete characterization. These investigations set the stage for investigations of the metabolism of leurosine, vinblastine, and vincristine.

LEUROSINE BIOTRANSFORMATIONS.—Leurosine [**3**] differs structurally from VLB and VCR by the presence of an epoxide moiety at the 15′,20′ positions in the Iboga-type ring. Two *Aspergillus* species, human serum ceruloplasmin, and DDQ converted leurosine into the relatively stable carbinolamine **22** (Figure 5) (47). Proof of the structure of the carbinolamine was complicated because it was necessary to determine which of five equivalent methylene functional groups adjacent to a nitrogen atom in the dimer structure had been converted metabolically or chemically into a carbinolamine methine carbon atom. ^{13}C-nmr revealed two new signals in **22** at 89.5 and 87.98 ppm and twin signals for each of the surrounding carbon atoms at positions 6′, 3′, 21′, and 15′. Twin signals suggested that the metabolite was actually a mixture of carbinolamine isomers. Unambiguous assignment of the position of the carbinolamine was achieved by reduction of **22** with $NaBD_4$ in MeOD and ^{13}C-nmr analysis of the deuterated product **23**. Replacement of the hydroxyl group by deuterium gave a simplified nmr spectrum with no twinning of signals and no signal for the carbon at position 5′, the deuterated carbon. Three phenomena converge to diminish the signal for

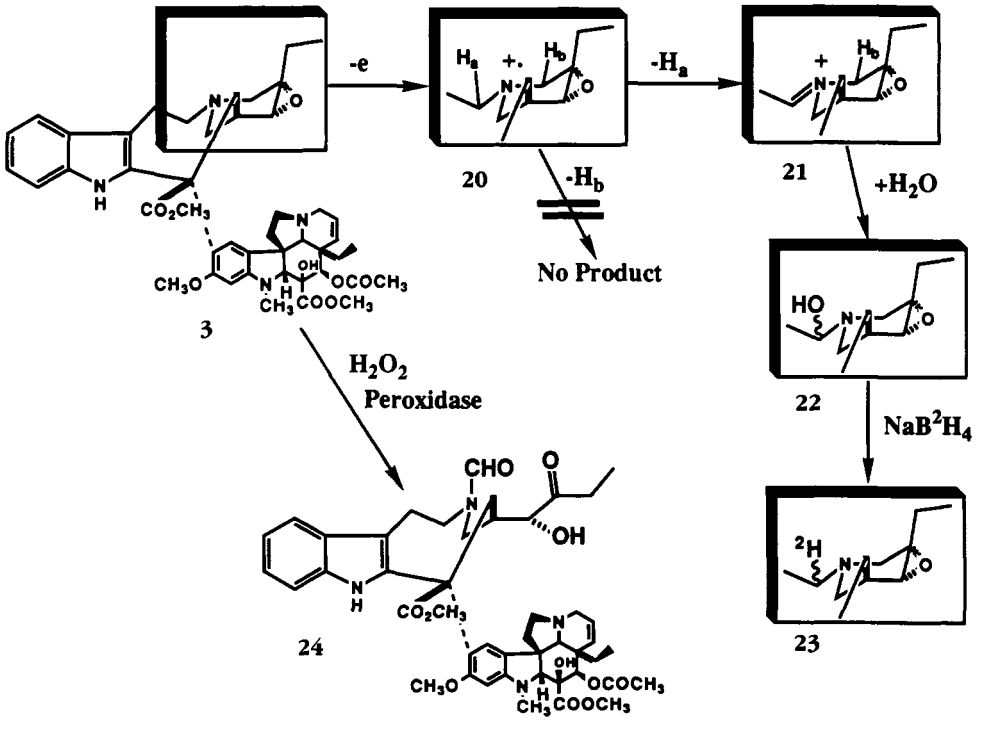

FIGURE 5. Copper-oxidase-mediated transformation of leurosine [**3**].

deuterium-bearing carbon atoms, including increased multiplicities due to C-D coupling, the increased relaxation time for C-D vs. C-H, and the loss of nOe.

The pathway to **22** illustrated in Figure 5 suggests that the likely first intermediate in the pathway of oxidation by copper oxidases, microorganisms, and chemical and photochemical oxidants is the cation radical **20**. Such an intermediate would appear to be capable of forming two products, depending upon which adjacent carbon atom would lose a hydrogen atom. Loss of H_a from **20** would form **21**, while loss of H_b from the unstable cation radical would result in formation of an iminium derivative in the smaller and more rigid six-membered epoxide-containing ring. The elimination of H_a is likely favored by the relative ease with which the iminiuim double bond can be accommodated within the more flexible 9-membered ring. The mixture of carbinolamine isomers **22** can be rationalized by nonspecific hydroxide addition to **21**.

IBOGA-TYPE RING FISSION REACTIONS CATALYZED BY PEROXIDASES.—Leurosine undergoes a completely different metabolic transformation reaction when subjected to oxidation by horseradish peroxidase (48). The novel reaction involves carbon-carbon bond fission between positions 20′ and 21′ as illustrated in Figure 5 to give **24**. The proof of structure for **24** was straightforward, relying upon ^{1}H- and ^{13}C-nmr, hrms, and comparisons with known compounds (49). In **24**, proton and carbon signals for the Aspidosperma-type (vindoline) ring of the metabolite were essentially identical to those for leurosine [**3**]. By ^{1}H nmr, an *N*-formyl group signal appeared at 7.31 ppm, and the signal for the Me-18′ group was shifted upfield by 0.3 ppm indicating structural change close to this carbon. By ^{13}C nmr, the 15′, 20′, and 21′ signals were all absent in the spectrum of **24** and were replaced by a ketone carbonyl signal at 210.9 ppm, a secondary hydroxyl group signal at 80.37 ppm, and an *N*-formyl carbon signal at 163.8 ppm (Table 1). The similarities to the nmr spectra of a known alkaloid, vinamidine [**28**] (also known as catharinine), were apparent in the final assignment of the metabolite structure as **24**.

The results obtained by enzymic transformation of leurosine with peroxidase were

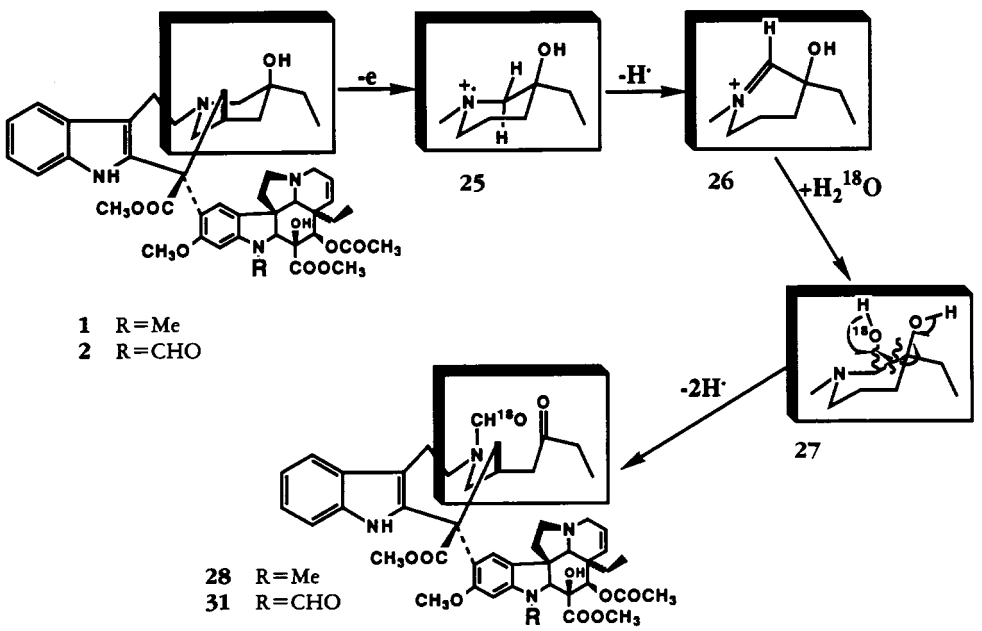

FIGURE 6. Peroxidase-mediated ring fission reactions with vinblastine [**1**] and vincristine [**2**].

unexpected. Instead of following a pathway similar to that observed with the copper oxidases, peroxidase oxidations involved the smaller, six-membered Iboga-type ring. With peroxidase, the initial oxidation step likely occurs by hydrogen abstraction from an epoxide-activated 21′ position, rather than by electron abstraction at nitrogen as with the copper oxidases. Thus, the former reaction appears to be subject to electronic control, while the latter reaction is subject to steric control. These observations with the metabolism of leurosine indicate that completely different types of reactions can be expected depending upon the nature of the oxidizing enzyme (48).

VLB [**1**], 14,15-dihydrovinblastine, and VCR [**2**] undergo identical biotransformation reactions with peroxidase (49) and with human serum cerulopasmin (50) (Figure 6). In each case, metabolites obtained by enzymic transformation were isolated and characterized by hrms (Table 2), ^{1}H nmr, and ^{13}C nmr (Table 1). In each case, high

TABLE 1. ^{13}C-nmr Spectral Comparisons of the Iboga (upper) Half of Vinblastine [**1**], Vincristine [**2**], Leurosine [**3**], 15-Hydroxyvinamidine [**24**], Vinamidine [**28**], and the Vinamidine Metabolite **31** Obtained from Vincristine.

Carbon	Compound					
	1	**28**	**2**[a]	**31**	**3**	**24**
C-2′	130.9	132.71	130.1	130.38	130.8	133.5
C-3′	47.5	49.46	49.1	49.85	42.28	52.3
C-5′	55.5	55.73	55.9	56.34	49.84	49.64
C-6′	28.7	25.28	30.7	25.23	24.89	24.77
C-7′	115.9	111.53	117.9	111.80	116.8	111.26
C-8′	129.0	128.37	129.9	125.80	129.2	127.94
C-9′	118.1	117.68	118.5	118.45	118.12	117.51
C-10′	122.2	122.5	123.9	123.45	122.2	122.49
C-11′	118.8	119.3	118.9	120.23	118.79	119.3
C-12′	110.2	110.78	110.6	111.80	110.3	110.88
C-13′	134.7	135.29	135.2	136.30	134.58	135.42
C-14′	29.2	29.59	30.0	30.83	33.49	32.04
C-15′	40.0	51.09	41.2 (42.2)	50.24	59.97	80.37
C-16′	55.3	55.52	52.7	53.16	55.23	55.86
C-17′	34.1	34.88	34.4	35.63	31.11	34.87
C-18′	6.7	7.89	6.7	7.3 (7.8)	8.5	8.25
C-19′	34.1	37.44	34.4	35.63	28.02	29.93
C-20′	68.6	210.38	69.1	211.25	60.29	210.91
C-21′	63.1	163.45	63.8	164.47	53.94	163.68
16′-CO	174.6	174.19	170.1	174.48	174.1	173.11
16′-OMe	52.0	52.35	52.3	53.16	52.2	52.89

[a]Spectral data are for 17-desacetylvincristine; no published data are available on vincristine itself (57).

resolution fabms analysis indicated that metabolite structures **28** and **31** were changed by the incorporation of one oxygen atom and the loss of two mass units consistent with the loss of two hydrogens. As in the case of leurosine conversion to **24**, ^{13}C-nmr properties were consistent with cleavage of the six-membered heterocyclic ring of the Iboga half of the molecule. Significant changes in the ^{13}C-nmr spectra of **28** and **31** occurred for positions 20′ (from 68.6 ppm to 210 ppm—ketone), and 21′ (from 63 ppm to 163.45 ppm—N-CHO).

A mechanism to account for the unusual carbon-carbon bond cleavage reaction observed with VCR and VLB is illustrated in Figure 6. Peroxidase is well-known to catalyze oxidations of nitrogenous substrates, and the first oxidation step with this enzyme may involve either the elimination of hydrogen from carbons adjacent to nitrogen

TABLE 2. High Resolution Mass Spectral Properties of Metabolites **24**, **28**, and **31** Obtained by Peroxidase/H_2O_2 Catalyzed Oxidation of **3**, **1**, and **2**, Respectively.

24	**28**	**31**
ei mass $[M]^+$ 840.394 Calcd for $C_{46}H_{56}N_4O_{11}$, 840.395	ci mass $[M+1]^+$ 825.409 Calcd for $C_{46}H_{57}N_4O_{10}$, 825.407	fab mass $[M+1]^+$ 839.387 Calcd for $C_{46}H_{55}N_4O_{11}$, 839.387

or the formation of a cation radical centered at nitrogen. The steps shown in Figure 6 are speculative, and no intermediates have yet been isolated and identified. However, probable intermediates would include an iminium species like **26**, with addition of H_2O to form the putative carbinolamine **27**. A periodate-like cleavage of **27** catalyzed by peroxidase would provide **24**, **28**, or **31** by a process analogous to that observed in the cytochrome P-450 mediated diol cleavage in cholesterol side-chain degradations and in the cleavage of diols with iron-porphyrin catalysts (48). The proposed pathway was verified by conducting incubations in the presence of ^{18}O-labeled H_2O where a single atom of ^{18}O was incorporated into the metabolite structure (49).

THE PEROXIDASE REDOX CYCLE AND *CATHARANTHUS* ALKALOID OXIDATIONS.—Much of the metabolic work described above relied on the isolation of intermediates and end products of metabolism and their subsequent identification by spectral means. Characterization of the initial steps involving either one-electron oxidation reactions at nitrogen or at adjacent carbon atoms or the identification of radical intermediates is much more difficult. Following methods which we established in our original work with vindoline (39), we utilized the well-defined spectral characteristics of the different forms of horseradish peroxidase to probe the one-electron nature of *Catharanthus* alkaloid dimeric transformations.

The structure of horseradish peroxidase (HRP) is well-defined as is the redox cycle involved in mechanisms of xenobiotic oxidation reactions (39). The native enzyme consists of a protein containing a protoporphyrin IX-heme system which coordinates iron in the ferric (Fe^{+3}) oxidation state (Figure 7). The heme component of the native enzyme exhibits a λ max at 403 nm in the visible spectrum. Oxidation of the native enzyme with hydrogen peroxide gives the most powerful oxidizing form of peroxidase, known as Compound I. In Compound I, the heme-iron is represented in abbreviated form in the Fe^{+5} oxidation state. In actuality, Compound I consists of an Fe^{+4} iron atom within the heme system which also contains a stabilized cation radical. Compound I exhibits a λ max at 410 nm. Addition of an alkaloid substrate which can reduce Compound I gives still another form of the enzyme known as Compound II. Compound II contains an Fe^{+4} heme iron atom, and it exhibits a λ max at 418 nm. These spectral differences which distinguish the native enzyme from Compounds I and II were used to assess the nature of the oxidation reaction involving *Catharanthus* alkaloid substrates.

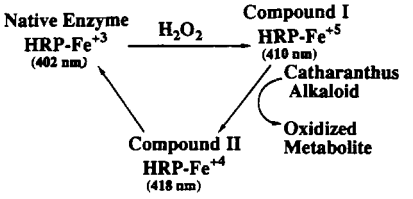

FIGURE 7. The redox cycle of horseradish peroxidase.

Figure 8 illustrates the actual spectral differences that can be observed among these three different forms of the enzyme.

By very careful experimental manipulation, it is possible to use these spectral differences to determine the one-electron oxidation nature of alkaloid transformation reactions, as well as reaction stoichiometries (i.e., moles of enzyme required to oxidize a single dimeric alkaloid substrate). The exact amount of peroxidase must first be determined spectrophotometrically before precisely titrating the enzyme with H_2O_2 to form Compound I. Excess peroxide causes enzyme cycling. Addition of sufficient amounts of

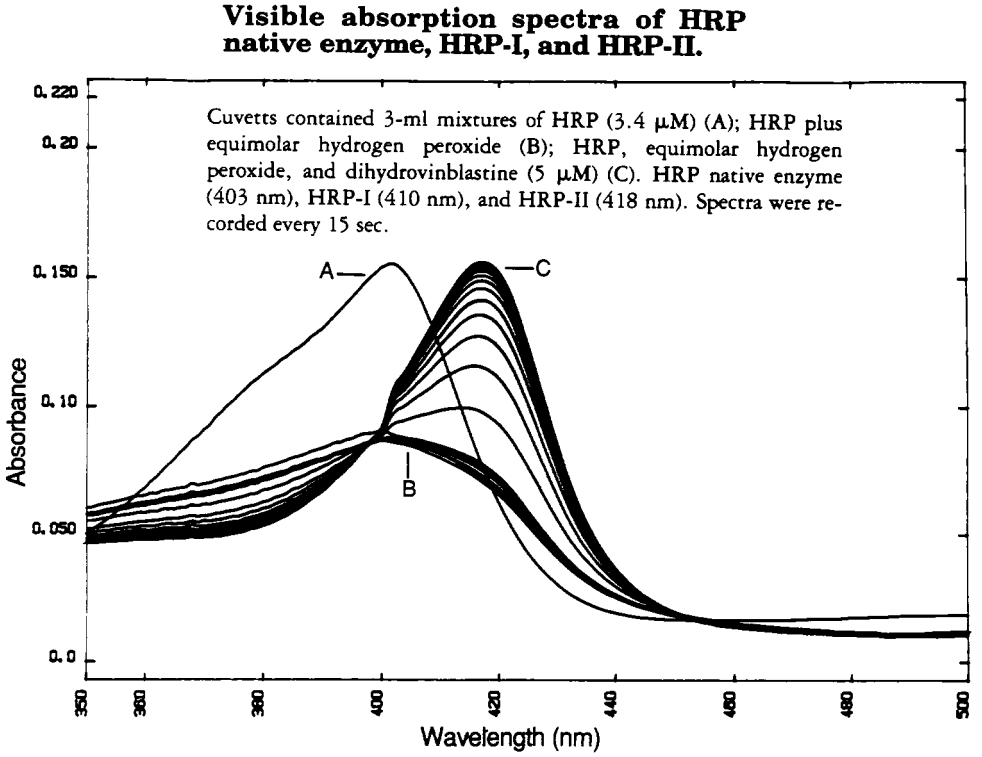

FIGURE 8. Absorption spectra for native horseradish peroxidase (A), Compound I (B), and Compound II (C).

alkaloid to convert all of Compound I to Compound II can be measured spectrophotometrically and quantitated. Using this technique enabled the ready determination of reaction stoichiometry in the peroxidative transformation of vindoline to the enamine dimer **15** (Figure 4) (39). With dihydrovinblastine as substrate, Compound I was clearly reduced to Compound II during the reaction. However, unlike our earlier findings with vindoline as a peroxidase substrate (39), preliminary results with 14, 15-dihydrovinblastine have not yet provided definite information on reaction stoichiometry.

ACTIVITIES OF METABOLITES AND RELATED *VINCA* DIMERS.—Leurosine metabolites were evaluated for activity vs. a tubulin polymerization assay and vs. the *Vinca*-alkaloid-sensitive CCRF-CEM cell culture system (48,50). The inhibitory 50% (I_{50}) values of leurosine and various products of leurosine vs. tubulin polymerization were compared. Leurosine is about ⅓ as active as VLB, and 5′-hydroxyleurosine [**22**] is

about as active as leurosine. 15′-Hydroxyvinamidine [**24**] is nearly three orders of magnitude less active than VLB. With the CRFF-CEM T-Cell leukemic cell line, similar results were observed with vinblastine metabolites. Vinamidine [**28**] is 77 times less active than VLB.

These results clearly indicate that the ring-fission reaction is a bioinactivation process. However, the observed biotransformation reactions involve free radicals, nitrogen cation radicals, and iminium intermediates, all of which are highly reactive and may be implicated in toxicity and/or mechanism of action. Furthermore, it is possible that products like vinamidine [**28**] and 15′-hydroxyvinamidine [**24**] may not be metabolic end products in vivo, where they may be subject to further metabolic transformation. Enzymatic cleavage of the *N*-formyl group, for example, would yield a new compound possessing a basic nitrogen atom in the Iboga-type ring, a requisite to *Catharanthus* alkaloid activity.

The results obtained with *S. griseus*, copper oxidases, and peroxidases also suggested the possibility that *Catharanthus* alkaloids could be substrates for other enzymes well-known to catalyze one-electron oxidation/reduction reactions with nitrogen heterocyclic compounds. Thus, attention was focused on cytochromes P-450 and monoamine oxidases.

METABOLISM OF VLB USING LIVER MICROSOMAL CYTOCHROME P-450.—The possible involvement of hepatic microsomal cytochromes P-450 in catalyzing the oxidation of VLB was investigated using hepatic microsomes from mice and rats. Activities of cytochromes P-450 in microsomal preparations were confirmed by determination of the aminopyrine demethylase activity using the method of Prough and Ziegler (51). The availability of analytical methods for expected metabolites such as vinamidine [**28**] and desacetylvinblastine rendered the examination of microsomal preparations relatively straightforward.

Microsomal preparations from both male and female CFI mice were incubated with concentrations of VLB ranging from 0.26 mM to 3.3 mM in the presence of NADPH (glucose-6-phosphate dehydrogenase, glucose-6-phosphate, and NADP+) at pH 7.35 and 37°. Results with both male and female CFI mice indicated no detectable amounts of known or unknown VLB metabolites. Pretreatment of mice for 3 days with daily injections of sodium phenobarbital (80 mg/kg in 0.9% saline, ip) induced microsomal aminopyrine demethylase activity as compared to saline-treated controls, but did not result in detectable levels of VLB metabolites. During the course of incubations, a steady decrease in levels of VLB could be measured (hplc). However, control experiments revealed that this most likely represented partitioning of lipophilic VLB into microsomes, and not metabolic activation and binding of the alkaloid to microsomes.

Microsomal preparations from male and female Sprague-Dawley rats were used in reaction mixtures containing 0.1 mM VLB under conditions identical to those described above for mouse incubations. No VLB metabolites were observed. Induction studies were carried out with male Sprague-Dawley rats with phenobarbital (daily ip injections in 0.9% saline at 80 mg/kg for 4 days) and with β-naphthoflavone (daily ip injections in corn oil at 45 mg/kg for 4 days). Incubations with 0.1 mM VLB and microsomes from rats treated with either phenobarbital or β-naphthoflavone gave no detectable metabolites by hplc. Control assays for aminopyrine demethylase activity indicated that the cytochrome P-450 monoxygenases were induced by both the phenobarbital and β-naphthoflavone treatments.

The results of the above experiments with mice and rats indicate that under the conditions explored, constitutive hepatic cytochrome P-450's, and those inducible by phenobarbital and β-naphthoflavone, in these species do not catalyze oxidation of VLB

to known metabolites, or to unknown peaks found by hplc. It is surprising that this is the only work yet reported in which microsomal P-450 systems have been examined for VLB metabolic transformations.

DISCOVERY THAT *VINCA* ALKALOIDS INHIBIT BRAIN AND HEPATIC MITOCHONDRIAL MONOAMINE OXIDASE.—Monoamine oxidase (MAO) catalyzes oxidations of endogenous neurotransmitter monoamines and various exogenous primary, secondary, and tertiary amines. MAO exists in two distinct forms, designated A and B. Within neural tissue, MAO-A activity has been correlated with noradrenergic activity and norepinephrine content of the cell type. MAO-B is predominantly localized in serotonin-rich regions of neural tissue as well as in extraneuronal cells. Both A and B forms of the enzyme are in the peripheral nervous system, a major site of VCR and VLB neurotoxicity.

Relationships between MAO and neurotoxicity are not established with the *Catharanthus* alkaloids. There is, however, a common structural feature between the vindoline portion of *Catharanthus* alkaloid dimers and the tertiary allylamine moiety within the piperidine ring of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin that causes Parkinsonian symptoms in primates. MAO-B catalyzes the oxidation of MPTP to a dihydropyridine derivative $MPDP^+$ and to the fully oxidized pyridinium derivative MPP^+. Since MPTP is both an inhibitor and a substrate for MAO-B, it was logical to examine whether *Catharanthus* alkaloids were either substrates or inhibitors for the enzyme (52).

MAO-A and MAO-B in mitochondria were assayed using the substrates kynuramine and benzylamine, respectively. Preliminary experiments with mitochondria from rat brain showed that all of the dimeric *Vinca* alkaloids tested were more potent inhibitors of benzylamine oxidation (MAO-B) than kynuramine oxidation (MAO-A). For oxidation of 0.1 mM benzylamine catalyzed by rat brain mitochondria, the relative extent of inhibition of MAO-B at 0.2 mM inhibitor was VLB = VCR > MPTP = MPP^+ > vindoline > $MPDP^+$ > 16α-carbomethoxycleavamine. For oxidation of 0.1 mM kynuramine catalyzed by rat brain mitochondria, the relative inhibition was MPP^+ > $MPDP^+$ > VCR > VLB > MPTP > vindoline = 16α-carbomethoxycleavamine. Preliminary experiments with highly purified beef liver mitochrondrial MAO-B (Figure 9) indicated that VCR and VLB were, however, slightly less effective inhibitors of the enzyme than MPTP. MAO-B inhibition was evaluated at 0.1 mM benzylamine and from 0 to 0.2 mM inhibitor, and the relative inhibitory potency was as follows: MPTP > VCR > VLB > leurosine > vindoline > 16α-carbomethoxycleavamine. Initial velocity experiments showed that VLB was a competitive inhibitor of MAO-B activity, with an estimated K_i of 77 μM (52). The inhibition was reversible and not time-dependent. In addition, no detectable metabolites were identified when purified MAO-B was incubated with VLB and the reaction mixture was analyzed by hplc and tlc.

Thus, while MPTP, VLB, and VCR are comparable in their abilities to inhibit the initial velocity of benzylamine oxidation catalyzed by MAO-B, VLB and VCR differ from the neurotoxic MPTP in that the two *Catharanthus* alkaloids are not substrates for the enzyme. The significance of our observation that *Catharanthus* alkaloids inhibit MAO-B and its potential importance to the neurotoxicity of these compounds remain to be established.

While the K_i value at 77 μM appears high relative to the actual doses of VLB or VCR used clinically, it is likely that the drugs become sufficiently locally concentrated within tissues to achieve inhibitory levels. Paracrystal formation, an accepted model for *Catharanthus* alkaloid interaction with tubulin, occurs when the drug is used at concentrations of 150 μM for neurotoxicity modeling (53). When tritiated VCR is used at

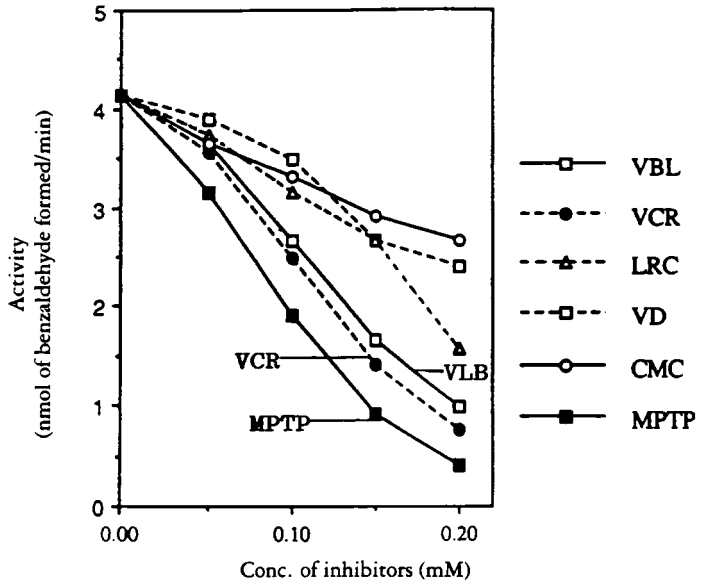

FIGURE 9. Inhibition of benzylamine oxidation by a purified bovine liver MAO-B.

doses of 1 mg/kg, the drug levels are found at 20–70 times blood levels within spleen adrenal, thyroid, and intestinal tissue (54). Furthermore, tissue concentrations of *Catharanthus* alkaloids are higher than plasma, and they appear to be closely tissue-related. Interestingly, in light of our observations with mitochondrial MAO-B inhibition, the subcellular distribution of VLB in mouse liver reveals that 18.7% of the drug within the cell is distributed within the mitochondrial fraction, a relatively small volume compartment within the cell (55).

SUMMARY AND CONCLUSION.—This work suggests further investigations on mitochondrial biology and the *Catharanthus* alkaloids. We are continuing our investigations on the mechanisms of nitrogen heterocyclic biocatalytic reactions with particular emphasis on the role of oxygen, H_2O_2, and H_2O during the course of *Catharanthus* dimer oxidations. Our findings with peroxidases and the involvement of free radical and other reactive intermediates may implicate the *Catharanthus* alkaloids along with other anticancer agents that form free radical intermediates during enzymatic activation in neurotoxicity, in the phenomena of drug cytotoxicity and drug resistance (56). Furthermore, our discoveries of the major structural entities that are subject to oxidative transformation with many enzymes provides new targets for molecular modification to prevent metabolism and thus to prolong drug half-lives in the cell.

It is a tragic irony that as this paper was in preparation for presentation at the International Congress on Natural Products Research in Chicago in July 1991, the announcement was made that Dr. Robert L. Noble, who was one of the discoverers of the *Catharanthus* alkaloids and their antineoplastic activities, died on December 11, 1990. In his excellent review (6) on the discovery of the *Vinca* alkaloids, Chemotherapeutic Agents Against Cancer, he drew from lines presented at his Terry Fox lecture to the British Columbia Medical Association about the discovery of valuable anticancer drugs as a result of investigating presumed hypoglycemic principles from the plant. He said, "Although the good ship *Vinca rosea*, launched by Dr. Johnston, sailed from Jamaica in search of the Isles of Langerhans, it encountered serendipitous western crosswinds and ended up discovering a new uncharted archipelago christened Cancer Phytochemo-

therapy." In light of our unexpected discovery of the MAO-B inhibitory properties of the *Catharanthus* alkaloids dimers, we are hopeful that Robert Noble would accept the following addition to his poetic thought. We believe that serendipitous winds continue to blow us through uncharted waters—but that somewhere there will lie calm seas and a new archipelago where new generations of *Catharanthus* alkaloid drugs—and those who need them—will live happily ever after.

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