balance in certain patients (*3*). Thus, patient 5, who was responsive to this treatment, appears to retain a minor physicochemical change in receptor that lowers the receptor's affinity for the hormone without altering the capacity of the receptor to activate genes. In contrast, patients 6, 8, and 9 are unresponsive to treatment, and we predict that in their cases the receptors contain defects in the hormone-binding sites that lead to severely impaired function and consequent tissue resistance to $1,25(OH)_2D_3$. These newly clarified receptor defects must then be added to those previously characterized in VDDR II (*10, 11*), including nuclear transfer deficiencies (patients 1A and 2B) and post-receptor blocks, the latter evidenced here by lack of responsiveness to $1,25(OH)_2D_3$ (patient 7) in spite of apparently normal hormone-binding affinity and nuclear uptake capacity (Table 1).

The HB^- phenotype in VDDR II may have arisen genetically from receptor gene mutation or deletion, molecular abnormalities due to cellular synthesis or processing, or lack of important but unknown environmental principles. Our data rule out several mechanisms, including gene deletion, although they do not specify which of the alternative possibilities might be responsible for tissue resistance to $1,25(OH)_2D_3$. Similar findings will perhaps be observed in comparable tissue-resistant disease states for other steroid hormones (*6–9*). Our observations imply that the clinical manifestations of $1,25(OH)_2D_3$ resistance in VDDR II are rarely, if ever, due to the absence of receptor, but are probably due to internal changes in the molecule that prevents normal function. These aberrations might include molecular instability, lack of conformational changes inducible by $1,25(OH)_2D_3$, modifications in hormone affinity, or reduced capacity to bind to nuclear sites or DNA. Each of these possibilities requires further investigation.

The existence of biologically inactive receptors for $1,25(OH)_2D_3$ and the concomitant tissue resistance to the hormone seen in VDDR II lend strong support for the major $1,25(OH)_2D_3$–mediating role of this protein in normal cells and tissues and for the genomic mechanism of this sterol's action. As a consequence, cellular resistance to $1,25(OH)_2D_3$ leads to clinical symptoms similar to those of vitamin D deficiency, such as hypocalcemia, secondary hyperparathyroidism, and rickets. Presumably these symptoms are due to the lack of effect of $1,25(OH)_2D_3$ in traditional target tissues such as intestine, kidney, and bone, which are primary regulators of calcium homeostasis (*12*). Direct studies in VDDR II–derived fibroblasts have similarly shown these cells to be deficient in other putative receptor-mediated responses such as growth inhibition (*18*) and 25-hydroxyvitamin D_3-24-hydroxylase induction (*19–21*). Taken cumulatively, the present study defines more precisely the nature of the biochemical defects associated with VDDR II and provides further support for the concept that functional receptors are a requirement for the normal mediation of biological activity of $1,25(OH)_2D_3$.

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The Structural Gene for Tetanus Neurotoxin Is on a Plasmid

Abstract. *A pool of synthetic oligonucleotides was prepared based on the amino terminal amino acid sequence of tetanus toxin. This probe hybridized to plasmid DNA isolated from three toxigenic strains of* Clostridium tetani *but not to plasmid DNA from a nontoxigenic strain. These results show that the structural gene for the toxin is on the plasmid. The pCL1 plasmid from one of the toxigenic strains spontaneously deleted 22 kilobase pairs of DNA to form pCL2. Strains harboring this deleted plasmid are nontoxigenic. However, the probe mixture hybridized to pCL2, indicating that the DNA encoding the amino terminus of the toxin had not been deleted. Restriction endonuclease cleavage maps of pCL1 and pCL2 were constructed and indicate the approximate location and orientation of the structural gene for tetanus toxin.*

The symptoms of tetanus were linked to the production of a thermolabile toxin by *Clostridium tetani* in the late 19th century (*1*). Subsequently, much information has been compiled about the size, structure, and biological activities of this powerful neurotoxin, although the exact molecular action of the toxin has not been defined (*2*). The toxin is produced initially as a single polypeptide chain with a molecular weight of approximately 150,000. This chain is cleaved into two subunits with molecular weights of approximately 100,000 and 50,000 by the action of endogeneous proteases (*3*). The genetic mechanism controlling toxin production by *C. tetani* has not been investigated extensively. The genetic factor controlling the production of tetanus toxin is thought to be associated with an extrachromosomal element (*4*). Toxigenic strains of diverse origin have been found to contain a single large plasmid (*5, 6*). A number of nontoxigenic derivatives were isolated from these strains, and in each case the loss of toxigenicity correlated with the loss of the plasmid. However, the precise role of plasmids in toxin production was not elucidated. Plasmids may contain sequences coding for the toxin, or they may code for proteins involved with regulation of a

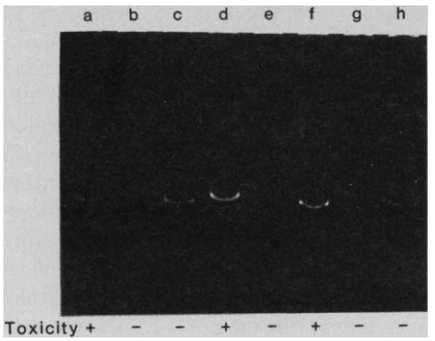

Fig. 1. Agarose gel electrophoresis of plasmid DNA from toxigenic and nontoxigenic strains of *C. tetani* after digestion with Bam HI. All plasmids examined have a single Bam HI site. Samples were run in 0.8 percent agarose, 89 m*M* tris-borate buffer (*p*H 8.2) for 3 hours at 120 V. DNA fragments were visualized by staining with ethidium bromide (0.4 μg/ml) and photographed in short-wave ultraviolet light. (Lane a), Strain BT100 (pCL1); (lane b), strain BT101; (lane c), strain BT102 (pCL2); (lane d), strain BT103 (pCL3); (lane e), strain BT104; (lane f), strain BT105 (pCL4); (lane g), strain BT106; and (lane h), strain BT107 (pCL5). Neurotoxic activity of fluids from cultures was assayed with N:GP (S) mice (13 to 18 g) (*10*). Cultures of *C. tetani* were grown for 96 hours at 34°C in BHI liquid medium. A 0.2-ml sample of culture fluid was diluted to 50 percent with phosphate-buffered saline containing 0.2 percent gelatin, filtered (0.45 μm, Millipore), and injected subcutaneously into the right inguinal fold of two animals. The animals were observed for at least 4 days following injection. (+) Death within 24 hours; (−) no effects observed.

chromosomally located structural gene. We now show that the structural gene encoding the toxin is on a plasmid. A single-stranded oligonucleotide fragment complementary to the DNA encoding the amino terminus of the toxin was synthesized and used as a hybridization probe. We also present a partial restriction endonuclease map of pCL1, a 75-kb plasmid isolated from the Massachusetts strain of *C. tetani*.

Plasmid DNA was isolated from all strains of *C. tetani* (Table 1) by a modification of the procedure described by Ish-Horowicz and Burke (*7*). All toxigenic strains that we examined contained a single large plasmid of approximately 75 kb (Fig. 1, lanes a, d, and f). The loss of toxigenicity correlated with either a complete loss of plasmid DNA (Fig. 1, lanes b, e, and g) (*5, 6*) or a 22-kb reduction in plasmid size as noted with pCL2 (Fig. 1, lane c). The size difference between pCL1 and pCL2 was not apparent with the gel conditions used in Fig. 1 but was determined by the summation of fragment sizes generated by digestion with Eco RI (Fig. 2B, lanes a and b) and by DNA–DNA heteroduplex analysis (Fig. 3A). To determine whether the structural gene for the toxin was plasmid encoded, we synthesized a group of oligonucleotides which were used as hybridization probes. The nucleotide sequences in the probe mixture were based on the amino terminal amino acid sequence of the toxin (*2*) isolated from the Massachusetts strain (*8*). Based on codon combinations for the bracketed amino acids shown in Fig. 2A, 96 different octadecamers were possible. These oligonucleotides were initially prepared as two pools containing 32 and 64 fragments each (F1A and F1B in Fig. 2A). Preliminary dot-blot hybridization experiments (data not shown) showed that the pool of 32 fragments (F1A) hybridized strongly to tetanus plasmid DNA. A subset of only eight oligonucleotides (F8 in Fig. 2A) hybridized to a 16.5-kb Eco RI fragment of pCL1 and pCL4 (Fig. 2B, lanes a′ and d′) and to a larger Eco RI fragment of pCL3 (Fig. 2B, lane c′). These data indicate that the structural gene of the toxin is on the plasmids from each of the toxic strains examined. The observations that the probe hybridized to Eco RI fragments from pCL1 and pCL4 of similar

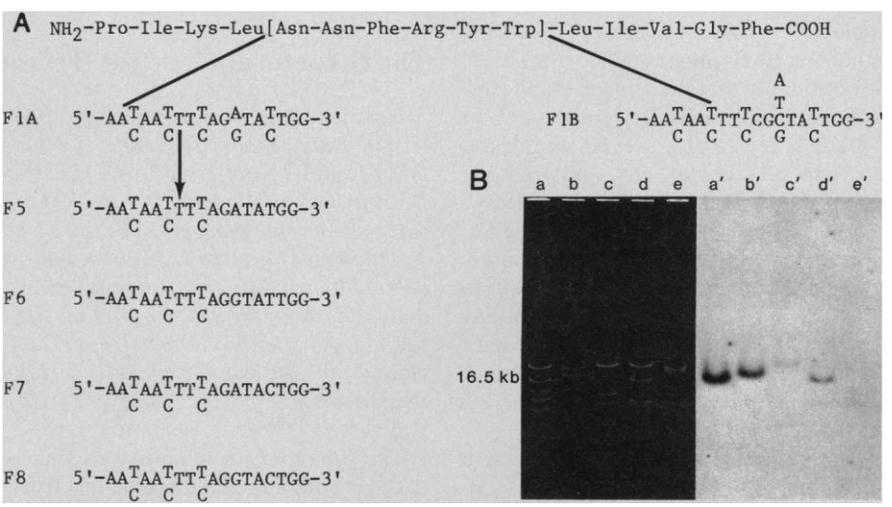

Fig. 2. (A) A portion of the amino terminal sequence of tetanus toxin (*2*). The bracketed sequence represents the six contiguous amino acids resulting in the least number of degenerate codons. The 96 possible oligonucleotides dictated by this amino acid sequence were prepared initially as two pools containing 32 and 64 possible sequences of octadecamers, respectively (F1A and F1B). Each pool was synthesized automatically by the phosphoramidite method (*11–13*) with an Applied Biosystems model 380A DNA synthesizer; mixed-coupling steps with equimolar mixtures of A and G, C and T, and A, G, C, and T (A, adenine; G, guanine; C, cytosine; T, thymine) phosphoramidite reagents were shown to give approximately 70:30, 50:50, and 20:15:35:30 ratios of the chain-extension reactions, respectively (*14*). The resultant 5′-dimethoxytrityl derivatives of the octadecamers were isolated as mixtures by reverse-phase liquid chromatography (Waters Micro/Bondapak C_{18}, 0.1*M* triethylammonium acetate, *p*H 7.8, 20 to 30 percent acetonitrile gradient) and then detritylated in dilute acetic acid solution (*p*H 2.5, 10 minutes). A 20-pmole sample of each oligonucleotide pool was labeled with ^{32}P with 10 units of T4 polynucleotide kinase in a 25-μl reaction volume containing 50 m*M* tris (*p*H 7.6), 10 m*M* $MgCl_2$, 5 m*M* dithiothreitol, 0.1 m*M* spermidine, and 0.1 m*M* EDTA. [γ-^{32}P]ATP was added (150 μCi at 3000 Ci/mmole), and the mixture was incubated at 37°C for 30 minutes, at which time 2 μl of 0.25*M* EDTA was added to stop the reaction. The labeled oligonucleotides were separated from unincorporated [γ-^{32}P]ATP by chromatography through a column (1 by 25 cm) of Sephadex G50 (Pharmacia-coarse grade) equilibrated in deionized H_2O. After initial dot-blot experiments had indicated that the F1A probe mixture hybridized strongly to pCL1 DNA (results not shown), four subset pools of eight oligonucleotides each were prepared (F5, F6, F7, and F8), labeled with ^{32}P, and used as probes. The pCL1 DNA was digested with Eco RI and the digestion products were separated by electrophoresis in 0.8 percent agarose. The DNA was transferred to nitrocellulose paper (*15*). The dried filter was incubated for 2 to 4 hours in six times standard saline citrate (SSC), 0.5 percent sodium dodecyl sulfate, five times Denhardt's, and 100 μg of sheared denatured salmon sperm DNA per milliliter (prehybridization fluid) at 30°C. The hybridizations were performed with 10^7 count/min of the appropriate probe mixture in prehybridization fluid containing 0.01*M* EDTA at 30°C for 40 hours. The filter was washed in six times SSC for 15 minutes at room temperature and twice more for 60 minutes. The filter was then dried and autoradiographed. The results (not shown) indicated that the F8 mixture contained material which hybridized strongly to a single 16.5-kb Eco RI fragment of pCL1. This probe was used in all subsequent hybridization experiments. (B) Electrophoresis in 0.8 percent agarose gels of restriction endonuclease digests of plasmid DNA and autoradiograms of filters obtained from the gels after hybridization with ^{32}P-labeled F8. Eco RI digest of (lane a), pCL1; (lane b), pCL2; (lane c), pCL3; (lane d), pCL4; and (lane e), pCL5. Autoradiograms made after hybridization with F8 (lanes a′ to e′). Hind III fragments of bacteriophage λ DNA were used as molecular weight standards.

Table 1. Strains of *Clostridium tetani* used.

Strain	Toxigenicity	Plasmid	Previous designation*	Source and/or comments
BT100	+	pCL1	Massachusetts C_2	Massachusetts State Biological Laboratories
BT101	−	None	Massachusetts C_{2a}	Spontaneous nontoxigenic derivative of BT100 isolated in our laboratory (*5*, *6*)
BT102	−	pCL2	None	Spontaneous nontoxigenic derivative of BT100 isolated in our laboratory (*9*)
BT103	+	pCL3	A552	Centers for Disease Control, Atlanta, Georgia
BT104	−	None	$A552_a$	Spontaneous nontoxigenic derivative of BT103 isolated in our laboratory (*5*, *6*)
BT105	+	pCL4	Tulloch II 127	Office of Biologics Research and Review, Food and Drug Administration
BT106	−	None	127_a	Spontaneous nontoxigenic derivative of BT105 isolated in our laboratory (*5*, *6*)
BT107	−	pCL5	NCTC 6336	National Collection of Type Cultures (NCTC) Central Public Health Laboratory, London, England

*Designations from (*5*, *6*).

size (16.5 kb) and that these plasmids also contain at least three additional Eco RI fragments that comigrate during agarose gel electrophoresis (Fig. 2B, lanes a and d) suggest that these plasmids may be related. The Eco RI digest of pCL3 bears little resemblance to that of pCL1 (Fig. 2B, lanes a and c).

The plasmid pCL2 (Fig. 2B, lane b) was generated by deletion of 22 kb of DNA from pCL1. Cells harboring pCL2 were nontoxigenic (Table 1). However, the F8 probe mixture hybridized to an 18-kb Eco RI fragment of pCL2 (Fig. 2B, lane b'), indicating that the DNA encoding the amino terminus had not been deleted. The lack of toxin production by strains carrying this plasmid (BT102) is probably due to deletion of DNA downstream from the sequence encoding the amino terminus of the toxin. The F8 probe did not hybridize to pCL5 (Fig. 2B, lane e'); strain BT107 harboring this plasmid was isolated as a nontoxigenic *C. tetani* (Table 1). The relation of this plasmid to others isolated from toxigenic strains is not known.

The 16.5-kb Eco RI fragment to which the F8 probe hybridized is located between the 49.5- and 66-kb coordinates (Fig. 3B). Cleavage at the Sst I site within this 16.5-kb Eco RI fragment generated 5.0- and 11.5-kb Eco RI–Sst I fragments (Fig. 3C). The F8 probe mixture hybridized to the 11.5-kb fragment (Fig. 3C, lane b'; 54.5 to 66 kb), while the deletion that led to the formation of pCL2 begins within the 5-kb Eco RI–Sst I fragment. These data suggest that a 4-kb gene coding for the toxin is oriented such that the sequence encoding the amino terminus lies near the Sst I end of the

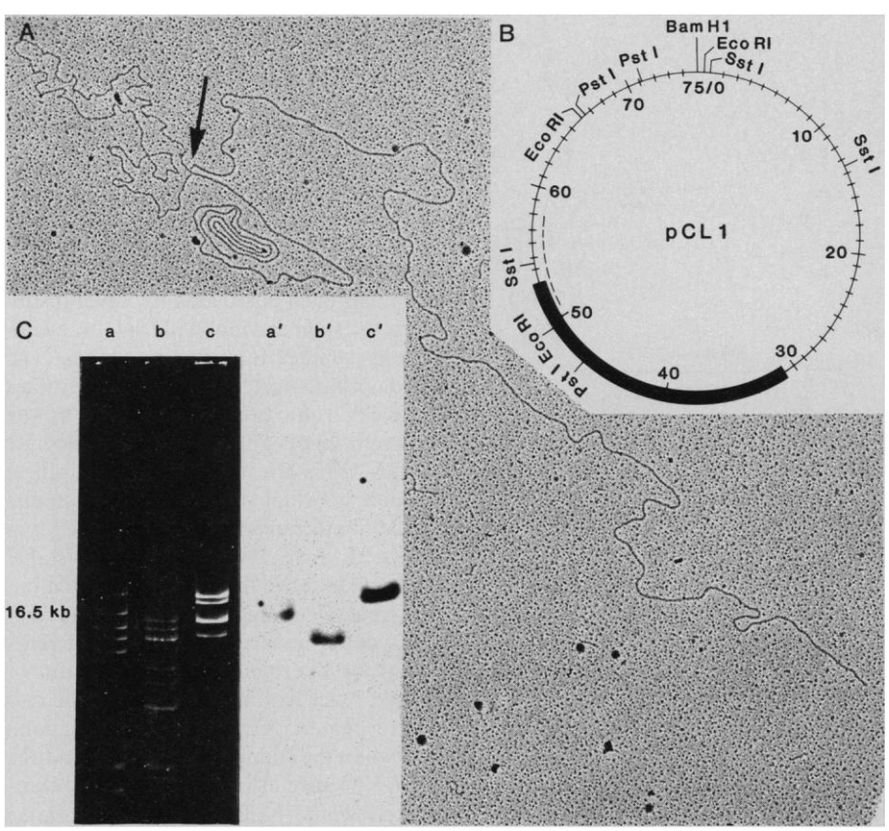

Fig. 3. (A) Electron micrograph of DNA–DNA heteroduplex between pCL1 and pCL2. Plasmids were cleaved with Bam HI and mounted for electron microscopy by the formamide technique (*16*). Grids were examined in a JEOL 100B electron microscope at 40 kV accelerating voltage. Electron micrographs were taken on Kodak Electron Image plates at a magnification of 8000. The magnification was calibrated for each set of plates with a grating replica (E. F. Fullam, catalog number 1000), and contour lengths were measured with a Numonics Graphics calculator. Arrow indicates position of deletion loop. (B) Restriction map of pCL1. A partial restriction endonuclease cleavage map of pCL1 (75 kb) was determined by analyzing the electrophoretic patterns of the DNA fragments generated after digestion with the appropriate combinations of restriction enzymes. The enzymes were used according to the recommendations of the supplier (Bethesda Research Laboratories and New England BioLabs). Restriction mapping of pCL2 (53 kb) and heteroduplex analysis were used to determine the location of the region deleted from pCL1 during the formation of this plasmid. The deleted region is indicated by the thick shaded portion of the map. The dashed line on the inside (extending from the 50- to 56-kb region) denotes the approximate location of the 4-kb toxin structural gene. (C) Southern hybridization analysis. The pCL1 DNA was digested with Eco RI (lane a), Eco RI and Sst I (lane b), and Sst I (lane c). An autoradiogram of a blot prepared from the gel after hybridization was performed using F8 as the probe (lanes a' to c').

11.5-kb Sst I–Eco RI fragment and the sequence encoding the carboxy terminus lies within the 5-kb Eco RI–Sst I fragment (Fig. 3B, dashed line).

Studies of the molecular genetics of toxin formation will not only provide valuable tools for the study of toxin structure and function but may also lead to the development of nontoxic cross-reacting proteins and synthetic vaccine candidates.

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7. A 0.1-ml sample of a culture grown overnight in brain-heart infusion broth (BHI) at 34°C was used to inoculate a Columbia blood agar plate (England Laboratories, 100 by 15 mm) containing 5 percent sheep blood and 2 percent agar. The cultures were incubated anaerobically (Gas Pak System, Baltimore Biological Laboratories) at 34°C for 20 to 24 hours. The cells were then removed with cotton swabs and resuspended in 50 m*M* glucose, 25 m*M* tris (*p*H 8.0), and 25 m*M* EDTA (solution 1), with 1 ml of buffer for each plate. Plasmid DNA was isolated from 1 ml of this suspension as described by D. Ish-Horowicz and J. F. Burke [*Nucleic Acids Res.* **9**, 2989 (1981)].
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Reactive Metabolites from the Bioactivation of Toxic Methylfurans

Abstract. *An important mechanism of toxicity of furans involves the cytochrome P-450 monooxygenase–catalyzed bioactivation of the compound in situ directly within the target tissues to highly reactive electrophilic products. The unsaturated aldehydes acetylacrolein and methylbutenedial have been identified as the principal reactive intermediates of 2- and 3-methylfuran, respectively, that are produced and bound covalently to tissue macromolecules in hepatic and pulmonary microsomal systems in vitro.*

Reduced nicotinamide adenine dinucleotide phosphate–dependent (NADPH) and oxygen-dependent activation of toxic furan compounds catalyzed by cytochrome P-450–dependent microsomal monooxidases results in the formation of highly reactive metabolites that bind covalently to tissue macromolecules (*1–4*). Epoxidation of the furan ring has been thought to be involved in the activation, a process analogous to the major mechanism of metabolism for benzenoid compounds. In addition, aflatoxin B_1 is almost certainly activated by epoxidation of the dihydrofuran ring in its structure (*5–7*).

Evidence that a furan epoxide is the intermediate in mixed-function oxidase metabolism of simple furans is lacking:

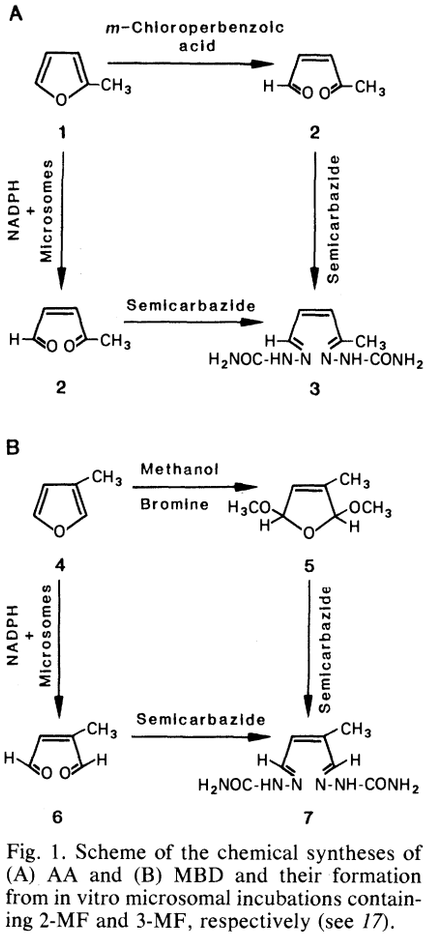

Fig. 1. Scheme of the chemical syntheses of (A) AA and (B) MBD and their formation from in vitro microsomal incubations containing 2-MF and 3-MF, respectively (see *17*).

no furan epoxides have been isolated and no metabolites resulting from attack of nucleophiles (for example, water to form dihydrodiols) have been identified. Although evidence exists for formation of glutathione adducts of 4-ipomeanol (*8*), the structures of the adducts remain to be determined.

Indirect evidence for epoxidation of the furan-containing drug furosemide has been reported. Addition of 1,2-epoxy-3,3,3-trichloropropane (TCPO) to mouse liver microsomal incubation mixtures has been found to result in a nearly twofold increase in covalent binding (*9*). The increase was attributed to the inhibition of epoxide hydrolase by TCPO, resulting in higher concentration of the active metabolite in the microsomes. Addition of TCPO has scant effect on the covalent binding of either 4-ipomeanol (10) or 2-(*N*-ethylcarbamoylhydroxymethyl)furan (*11*). Either an epoxide is not formed in the latter two cases, or an epoxide is formed but is not a substrate for the hydrolase, or an epoxide is formed which is so reactive that it undergoes alternate reactions before reaching the hydrolase enzyme.

Other evidence indicates that cytochrome P-450–catalyzed oxidations may proceed by discrete, one-electron steps. Oxidation by removal of a hydrogen atom or an electron as an initial step in cytochrome P-450–catalyzed metabolism has been postulated for several substrates, such as vinyl halides (*12*), cyclopropyl amines (*13*), organosulfides (*14*), and norbornane (*15*). The result of such a one-electron oxidation of a furan by the cytochrome P-450 perferryl intermediate [Fe(V)O] is the formation of a radical cation in which the radical or cationic site or both can be stabilized by the furan oxygen. Such a species may be stable enough to leave the site of formation but too reactive to migrate far. Cationic radicals could undergo radical-radical reactions or hydrogen atom abstraction or could react with nucleophiles at the cationic center. A radical-radical reaction between the furan radical cation and the Fe(IV)O species (which has radical character) would be facile, and the cationic