Table I.[a] Inhibition of Isopentenyl Pyrophosphate Isomerase by Substrate Analogues

compound[b]	K_i, M
isopentenyl pyrophosphate	3.5×10^{-5} (K_m)
3-bromo-3-butenyl pyrophosphate	4.5×10^{-5}
methyl pyrophosphate	7×10^{-5}
isoamyl pyrophosphate	4×10^{-4}
2-(dimethylamino)ethyl phosphate	2.0×10^{-3}

[a] Assay mixtures contained 0.1 M sodium maleate pH 6.3, 10 mM $MgCl_2$, [4-^{14}C]isopentenyl pyrophosphate (10–100 μM), inhibitor, and isomerase (0.5 units) in 0.25-mL total volume. Assays were initiated by addition of enzyme. After 6 min at 37 °C, 15 μL of 5 N HCl was added and the amount of product formed was determined.[8] In all cases, K_i values were determined from the slope replots of $1/v$ vs. 1/[IPP] plots. Three inhibitor concentrations and four substrate concentrations were used. [b] 2-(Dimethylamino)ethyl phosphate was synthesized by a published procedure[9] and purified by paper electrophoresis at pH 8.9. 3-Bromo-3-butenol was synthesized according to ref 10. Isopentenyl phosphate was synthesized according to ref 11. Isopentenyl pyrophosphate was synthesized by using the diphenyl phosphorochloridate coupling method.[12] All other pyrophosphate monoesters were synthesized by the above procedures and purified by chromatography on QAE Sephadex using a linear triethylammonium bicarbonate gradient from 0.0 to 0.6 M. The purity of all phosphate and pyrophosphate monoesters was determined by ^{1}H NMR and paper electrophoresis at pH 3.5 and/or 8.9. Samples from paper electrophoresis and paper chromatography were visualized with ammonium molybdate spray.[13] Concentrations of substrate and inhibitor samples for use in enzyme assays were determined by phosphate analysis.[14]

a slow event occurs subsequent to formation of enzyme–inhibitor complex. Time dependence of the inhibition was demonstrated more directly by incubating **3** with IPPI. At intervals, aliquots were withdrawn and assayed for enzyme activity. Results are shown in Figure 1b. Loss of catalytic activity is a first-order-order process at all inhibitor concentrations tested. Over the range of concentrations tested, k_{obsd} is proportional to enzyme concentration. No evidence for saturation was seen. Figure 1b also shows that substrate reduces the rate of inactivation, indicating that **3** probably interacts with the active site. When IPPI was incubated with **3** without Mg^{2+} in the presence of 5 mM EDTA, no inhibition occurred. The inhibition is, therefore, Mg^{2+} dependent, as is the catalytic process. Several analogues of **3** were also investigated. All compounds tested were competitive inhibitors, but no time-dependent inhibition was observed. The K_i values are listed in Table I. To determine whether the reaction of IPPI with **3** is reversible, 6.67 μM **3** was added to enzyme (11 units) in the assay buffer in a total volume of 150 μL. After 10 min at 37 °C, it was determined, by assaying an aliquot, that the enzyme was >95% inactivated. The reaction mixture was then subjected to rapid gel filtration[4] to remove excess inhibitor. No enzyme activity was detected after filtration nor was any activity detected 90 min after gel filtration. A parallel experiment was carried out in which enzyme and inhibitor were mixed and subjected to gel filtration within 10 s after mixing. Immediately after filtration the enzyme had 67% of its original activity and 50% after 90 min.

The result presented here shows that inhibition of isopentenyl pyrophosphate isomerase by **3** is time dependent. From the data available so far, it cannot be determined whether dissociation of the enzyme inhibitor complex is extremely slow ($t_{1/2}$ for dissociation >10 h) or whether irreversible modification of the enzyme occurs. Possibly irreversible inactivation could occur by methyl transfer from **3** to a nucleophile at the active site.

We believe that inhibition of isopentenyl pyrophosphate isomerase by **3** provides strong evidence for a carbonium ion mechanism. There are now several examples in which substitution of a carbon atom, which acquires carbonium ion character in the transition state, by a positively charged nitrogen leads to compounds that are very good inhibitors, presumbly transition-state analogues.[5] It is surprising that an ammonium ion can take the place of a carbonium ion, since the geometry of the two structures is quite different. The fact that these compounds are inhibitors indicates that electrostatic interactions and not geometry are of overwhelming importance. It is likely that this interaction occurs with a negatively charged group on the enzyme, which in the catalytic process stabilizes the carbonium ion. Some evidence exists that in glycosidases, where carbonium ion mechanisms are involved, a carboxylate group stabilizes the carbonium ion.[6] The nature of the negatively charged group at the active site of isopentenyl pyrophosphate isomerase is not known. Possibly, it is a sulfhydryl group, since it has been established that the enzyme is sensitive to sulfhydryl reagents.[7]

Acknowledgment. This work was supported in part by National Science Foundation Grant PCM-80-07670, Shell Development Corporation Grant 81-0411, and National Institutes of Health Postdoctoral Fellowship F32 GM 09307-02 (J.E.R.).

(4) Penefsky, N. S. *Methods Enzymol.* **1979**, *56*, 527.
(5) (a) Narula, A. S. *J. Am. Chem. Soc.* **1981**, *103*, 2408. (b) Sandifer, R. M.; Thompson, M. D.; Gaughan, R. G.; Poulter, C. D. *J. Am. Chem. Soc.* **1982**, *104*, 7376.
(6) (a) Parsons, S M.; Jao, L.; Dahlquist, F. W.; Borders, C. L.; Groff, T.; Racs, J.; Raftery, M. A. *Biochemistry* **1964**, *8*, 700. (b) Legler, G. *Z. Physiol. Chem.* **1966**, *345*. (c) Voet, J. G.; Abeles, R. H. *J. Biol. Chem.* **1980**, *245*, 1020.
(7) Agranoff, B. W.; Eggerer, H.; Henning, U.; Lynen, F. *J. Biol. Chem.* **1960**, *235*, 326.
(8) Banthorpe, D. V.; Doonan, S.; Gutowski, J. A. *Arch. Biochem. Biophys.* **1977**, *184*, 381.
(9) Ansell, G. B.; Spanner, S. *J. Neurochem.* **1962**, *9*, 253.
(10) Cousseau, J. *Synthesis* **980**, 805.
(11) Cornforth, R. H.; Popjak, G. *Methods Enzymol.* **1969**, *15*, 359.
(12) Michelson, A. M. *Biochim. Biophys. Acta* **1964**, *91*, 1.
(13) Hanes, C. S.; Isherwood, F. A. *Nature (London)* **1949**, *64*, 1107.
(14) Ames, B. N.; Dubin, A. T. *J. Biol. Chem.* **1960**, *235*, 769.

Molecular Mechanics Calculations on *cis*-$[Pt(NH_3)_2\{d(GpG)\}]$ Adducts in Two Oligonucleotide Duplexes

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The anticancer drug *cis*-diamminedichloroplatinum(II) (*cis*-DDP) binds predominantly to d(GpG) sequences in DNA.[1] Since *cis*-DDP binding to DNA destroys substrate recognition for nucleases[2] and polymerases,[3] shortens the duplex,[4,5] and unwinds the double helix,[5] substantial changes in DNA structure, including base pair disruption, have been suggested. On the other hand, NMR studies of platinated octa- and decanucleotide duplexes, while confirming d(GpG)–Pt binding, were interpreted in terms of base-paired structures up to 28 °C.[6]

Molecular mechanics calculations on oligonucleotide chains have been used to explore possible structures and have been shown to predict correctly relative energies.[7] We have applied this method

(1) For a review, see: Pinto, A. L.; Lippard, S. J. *Biochem. Biophys. Acta Rev. Cancer*, in press.
(2) (a) Cohen, G. L.; Ledner, J. A.; Bauer, W. R.; Ushay, H. M.; Caravana, C.; Lippard, S. J. *J. Am. Chem. Soc.* **1980**, *102*, 2487–2488. (b) Tullius, T. D.; Lippard, S. J. *J. Am. Chem. Soc.* **1981**, *103*, 4620–4622. (c) Royer-Pokora, B.; Gordon, L. K.; Haseltine, W. A. *Nucl. Acids Res.* **1981**, *9*, 4595–4609. (d) Ushay, H. M.; Tullius, T. D.; Lippard, S. J. *Biochemistry* **1982**, *21*, 3744–3748.
(3) Pinto, A. L.; Lippard, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, in press.
(4) Macquet, J.-P.; Butour, J.-L. *Biochim.* **1978**, *60*, 901–914.
(5) Cohen, G. L.; Bauer, W. R.; Barton, J. K.; Lippard, S. L. *Science (Washington, D.C.)* **1979**, *203*, 1014–1016.
(6) (a) den Hartog, J. H. J.; Altona, C.; van Boom, J. H.; van der Marel, G. A.; Haasnoot, C. A. G.; Reedijk, J. *J. Am. Chem. Soc.* **1984**, *106*, 1528–1530. (b) Van Hemelryck, B.; Guittet, E.; Chottard, G.; Girault, J. P.; Huynh-Dinh, T.; Lallemand, J. Y.; Igolen, J.; Chottard, J. C. *Ibid.* **1984**, *106*, 3037–3039.

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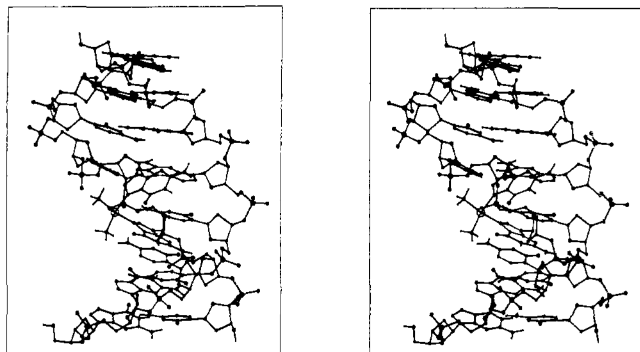

Figure 1. Stereoscopic view of oligonucleotide duplex **1**, with *cis*-$\{Pt(NH_3)_2\}^{2+}$ forming the intrastrand d(GpG) cross-link. The starting B DNA model had a modified ϕ value of 18° for the G5 nucleotide and the refined model had ϕ values of −2° and 155° for G5 and G6, respectively.[8,11,13,14]

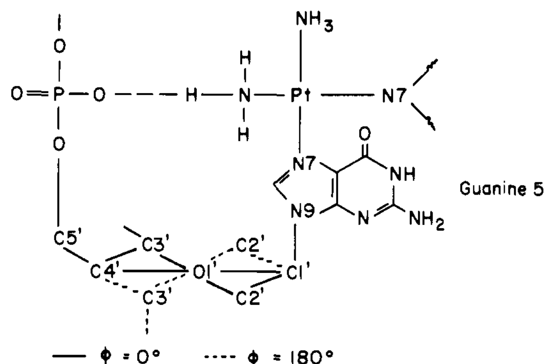

Figure 2. Schematic drawing of the ring closed by formation of the new hydrogen bond discussed in the text. The dashed line depicts C(2′)-endo, C(3′)-exo and the solid line C(3′)-endo, C(2′)-exo.

to two d(GpG)-containing oligonucleotide duplexes and their *cis*-DDP adducts. The major findings are the following: (i) The 5′-end-coordinated guanine is predicted to tilt out of the base stack, destroying one of the amino hydrogen bonds involved in GC base pairing and either weakening the imino hydrogen bond (A DNA) or forming a weak bifurcated hydrogen bond at this position (B DNA). (ii) A hydrogen bond is suggested to occur between one ammine ligand on platinum and the 5′-phosphate group of the d(pGpG) unit both in A and B DNA. This hydrogen bond closes a ring in which the 5′-sugar is constrained to either of two twisted conformations with phase angles[8] close to 0° or 180°. (iii) In B DNA models the coordination of *cis*-$\{Pt(NH_3)_2\}^{2+}$ on a d(GpG) unit switches the sugar pucker of the 5′-guanosine to C(3′)-endo.

Calculations were performed on two double-stranded oligonucleotides in both free and *cis*-DDP platinated forms. The sequences examined were [d(GGCCG*G*CC)-d(GGCCGGCC)] (**1**), the unplatinated structure of which has been studied by single-crystal X-ray diffraction,[9] and [d(TCTCG*G*TCTC)-d-(GAGACCGAGA)] (**2**), which has recently been investigated by NMR spectroscopy.[6a] Using the program AMBER,[10] we refined A and B DNA structures[11] for both duplexes as well as altered models with repuckered sugar rings on one or both of the G* guanosines, where the asterisk designates a platinum binding site. In a second series, refinements were carried out on the same structures with a *cis*-$\{Pt(NH_3)_2\}^{2+}$ unit attached to the N(7) atoms of G* bases.[12]

The major structural change predicted to occur by platinum coordination is a tilting of G5 off the base stack with an increase of the interplanar angle between G5 and G6 to 50 ± 8°.[13] In B DNA models, the surrounding base stack remains roughly unchanged so that GC base pairing to G5 is severely impaired (Figure 1). The imino H(1) proton of G5 is predicted to form a bifurcated hydrogen bond to the two N(3) atoms of C12 and G13 (**1**) or C16 and G17 (**2**). In platinated A DNA models, the bases flanking the coordination site move in such a manner that the interplanar angles between them and the coordinated guanines do not exceed 35°. Thus the loss in pairing and stacking energy is more evenly distributed among several base pairs compared to the platinum-modified B DNA structures. Another important feature arising from the calculations is the occurrence of a hydrogen bond from coordinated ammonia to the 5′-phosphate group of the G5-G6 dinucleotide in all platinated structures (Figure 2).

Conformational energies of the refined platinated structures as well as the final phase angles ϕ^8 of the sugars of guanosine residues G5 and G6 are available in Table S1.[14] For both platinated oligonucleotides, structures with C(3′)-endo on G5 show uniformly lower energies than those where no such repuckering has occurred. Analysis of atom–atom and group–group interactions disclosed the following five factors favoring the C(3′)-endo conformation of G5: The electrostatic attraction between the *cis*-$\{Pt(NH_3)_2\}^{2+}$ moiety and the phosphate group connecting nucleosides G5 and G6 becomes stronger if G5 has a C(3′)-endo sugar pucker. The ammine–phosphate hydrogen bond (Figure 2) is shorter if the sugar conformation on G5 is C(3′)-endo. In B DNA structures, the C(3′)-endo conformation on G5 diminishes the strain caused by platinum coordination to the d(GpG) unit. A hydrogen bond between one ammine ligand and O(2) of C4 further stabilizes the C(3′)-endo conformation of G5 in B DNA structures. In oligonucleotide **2**, a hydrogen bond from the other ammine to O(4) of T7 also stabilizes the C(3′)-endo conformation of G5 in platinated B DNA models. While the first three points are expected to be valid generally, the latter two are sequence specific.[15]

The model building calculations reveal several points that are important with respect to NMR spectroscopic investigations of oligonucleotide adducts of *cis*-DDP. They offer a rationale for the C(3′)-endo, C(2′)-endo sugar conformations observed for oligonucleotides modified with *cis*-$\{Pt(NH_3)_2\}^{2+}$ at d(GpG) sequences;[15–17] they demonstrate that detection of an imino hydrogen bond involving N(1) of guanine in NMR spectra of platinated oligonucleotides does not prove normal Watson–Crick base pairing (Figure 1), and they predict a specific ammine–phosphate hydrogen bond (Figure 2) which should be detectable by NMR spectroscopy. The persistence of this hydrogen bond in all model structures studied here is also interesting in view of the fact that

(7) (a) Tilton, R. F., Jr.; Weiner, P. K.; Kollman, P. A. *Biopolymers* **1983**, *22*, 969–1002. (b) Kollman, P. A.; Weiner, P. K.; Dearing, A. *Ibid.* **1981**, *20*, 2583–2621. (c) Rao, S. N.; Kollman, P. A. *J. Am. Chem. Soc.* **1985**, *107*, 1611–1617.

(8) Cremer, D.; Pople, J. A. *J. Am. Chem. Soc.* **1975**, *97*, 1354–1358.

(9) Wang, A. H. J.; Fujii, S.; van Boom, J. H.; Rich, A. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 3968–3972.

(10) Weiner, P.; Kollman, P. *J. Comput. Chem.* **1981**, *2*, 287–303.

(11) (a) Arnott, S.; Campbell-Smith, P.; Chandresekharan, P. *CRC Handb. Biochem.* **1976**, *2*, 411–414. (b) Saenger, W., "Principles of Nucleic Acid Structure", Springer-Verlag: New York, 1984, pp 220–241.

(12) In order to rule out possible artifacts of the calculations we performed numerous control refinements using a different potential, different weighting parameters, and different dielectric constants. These variations influenced the total energies but changed only marginally relative energies and final geometries of the refined structures. The results of these refinements and computational details will be given in the full paper.

(13) The numbering scheme employed here is as follows: **1**, d[G1·G2-C3-C4-G5-G6-C7-C8]·d[G9-G10-C11-C12-G13-G14-C15-G16]; **2**, d[T1-C2-T3-C4-G5-G6-T7-C8-T9-C10]·d[G11-A12-G13-A14-C15-C16-G17-A18-G19-A20].

(14) Supplementary material.

(15) Calculations were also performed on the single-stranded d(AGGCCT) hexanucleoside pentaphosphate and its d(GpG) adduct with *cis*-$\{Pt(NH_3)_2\}^{2+}$, both of which have been studied thoroughly by NMR spectroscopy.[16] The results suggest that, as in double-stranded oligonucleotides, the platinated strand tends to repucker the 5′-guanosine sugar to the C(3′)-endo conformation and to form the ammine–phosphate hydrogen bond described above.

(16) Caradonna, J. P.; Lippard, S. J.; Gait, M. J.; Singh, M. *J. Am. Chem. Soc.* **1982**, *104*, 5793–5795. (b) Caradonna, J. P.; Lippard, S. J., manuscripts in preparation.

(17) (a) den Hartog, J. H. J.; Altona, C.; Chottard, J. C.; Girault, J. P.; Lallemand, J. Y.; de Leeuw, F. A. A. M.; Marcelis, A. T. M.; Reedijk, J. *Nucleic Acids Res.* **1982**, *10*, 4715–4730. (b) den Hartog, J. H. J.; Altona C.; van Boom, H. J.; Reedijk, J. *FEBS Lett.* **1984**, *176*, 393–397. (c) den Hartog, J. H. J.; Altona, C.; van Boom, J. H.; van der Marel, G. A.; Haasnoot, C. A. G.; Reedijk, J., unpublished results.

the antitumor activity of *cis*-[Pt(ammine)$_2$X$_2$] complexes decreases markedly along the series $NH_3 > NH_2R \geq NHR_2 >> NR_3$.[18]

In closing, we wish to emphasize that the present conclusions are based on a theoretical analysis with the program AMBER. They show what is possible and do not necessarily reveal the actual structures. Further theoretical and experimental studies of platinated oligonucleotides are in progress.

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Supplementary Material Available: Table S1 summarizing conformational energies and G5,G6 phase angles for platinated oligonucleotides **1** and **2** (1 page). Ordering information is given on any current masthead page.

(18) Cleare, M. J.; Hoeschele, J. D. *Bioinorg. Chem.* **1973**, *2*, 187–210.

Cytochrome P-450 Catalyzed Oxidation of Quadricyclane. Evidence for a Radical Cation Intermediate[1]

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The question of whether oxygen transfer occurs in a single two-electron step or is preceded by electron transfer from the substrate to the activated oxygen complex is one of the more elusive aspects of the catalytic mechanism of cytochrome P-450. The large isotope effect and the epimerization associated with the cytochrome P-450 catalyzed hydroxylation of tetradeuterated norbornane,[2] and the scrambling of regiochemistry observed in the allylic hydroxylation of unsaturated hydrocarbons,[3] require an intermediate (probably a radical) in hydrocarbon hydroxylation reactions. The available data,[4,5] particularly the finding that the cytochrome P-450 catalyzed oxidation of 4-alkyl-1,4-dihydropyridines results in release of the 4-alkyl groups as free radicals,[4] furthermore suggest that electron transfer is the initial event in the oxidation of nitrogenous substrates. We now report evidence that quadricyclane (**1**) is oxidized by cytochrome P-450 to a radical cation that is captured in a distinct step by the activated oxygen species.

The strain in the quadricyclane structure and the extremely low oxidation potential (0.9 V) inherent in that strain[6] make quadricyclane a unique hydrocarbon. Quadricyclane, as a result, is isomerized to norbornadiene by metal catalysts[7] and readily undergoes oxidative addition reactions.[8] The quadricyclane radical cation, the probable intermediate in the oxidative addition reactions, is in equilibrium with the norbornadiene radical cation.[9]

(1) This research was supported by NIH Grants GM 25515 and GM 32488.

(2) Groves, J. T.; McCluskey, G. A.; White, R. A.; Coon, M. J. *Biochem. Biophys. Res. Commun.* **1979**, *81*, 154–160.

(3) Groves, J. T.; Subramanian, D. V. *J. Am. Chem. Soc.* **1984**, *106*, 2177–2181.

(4) Augusto, O.; Beilan, H. S.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1982**, *257*, 11288–11295.

(5) Guengerich, F. P.; Willard, R. J.; Shea, J. P.; Richards, L. E.; Macdonald, T. L. *J. Am. Chem. Soc.* **1984**, *106*, 6446–6447.

(6) Gassman, P. S.; Yamaguchi, R.; Koser, G. F. *J. Org. Chem.* **1978**, *43*, 4392–4393.

(7) Schwendiman, D. P.; Kutal, C. *J. Am. Chem. Soc.* **1977**, *99*, 5677–5682.

(8) Baggaley, A. J.; Brettle, R.; Sutton, J. R. *J. Chem. Soc., Perkin Trans. 1* **1975**, 1055–1059.

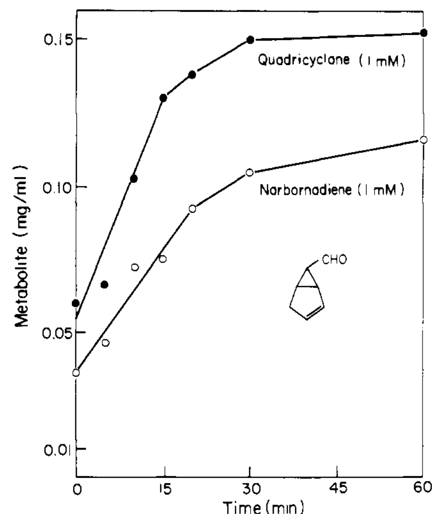

Figure 1.

Incubation of quadricyclane (1 mM) and an NADPH regenerating system with liver microsomes at 37 °C for 30 min, followed by extraction with ether and analysis by gas–liquid chromatography, provides nortricyclanol (**2**) and rearranged aldehyde **3** as the principal metabolites.[10] Quantitative studies with 5-norbornen-2-ol as an internal standard and studies with inhibitors demonstrate that the formation of nortricyclanol is independent of cytochrome P-450 whereas that of aldehyde **3** depends strictly on catalytic turnover of the enzyme (Table I). The oxidation of quadricyclane to nortricyclanol is equally well catalyzed by reduced and unreduced microsomes, aqueous $FeCl_3$, aqueous $FeCl_2$ plus H_2O_2, or 0.1 M $NaKPO_4$ (pH 7.4) buffer,[11] but not by double glass distilled water. The oxygen in nortricyclanol, as shown by studies with ^{18}O-labeled water and oxygen, derives from the medium. The aldehyde, in contrast, is only formed if microsomes, oxygen, and NADPH are present, although oxygen and NADPH can be replaced by iodosobenzene (Table I).

The oxidative microsomal metabolism of norbornadiene, the valence tautomer of quadricyclane, yields **3** but no nortricyclanol. The possibility that quadricyclane isomerizes to norbornadiene before the hydrocarbon is oxidized to aldehyde is ruled out by the finding that (a) more aldehyde is formed from 1 mM quadricyclane than from 1 mM norbornadiene (Figure 1),[12] (b) norbornadiene, as shown by NMR analysis of extracts, does not accumulate in incubations of quadricyclane (1 mM) with the microsomal system, (c) the spectroscopically determined binding constants (K_s) for quadricyclane and norbornadiene are both approximately 100 μM, and (d) the aldehyde is not formed in detectable amounts with low (μM) concentrations of norbornadiene.

Autooxidation of quadricyclane to nortricyclanol with incorporation of oxygen from the medium is explained by addition of water to the radical cation to give the nortricyclyl radical that abstracts a hydrogen or is reduced and protonated (Figure 2). The rearranged aldehyde in the cytochrome P-450 catalyzed oxidation, in contrast, is most reasonably explained by oxidation of quadricylane to the radical cation followed by reaction with the concomitantly generated equivalent of a hydroxyl radical to give the nortricyclyl *cation*, which is known to rearrange to aldehyde **3**.[13] Alternatives such as concerted exo addition to give the epoxide of norbornadiene or insertion of oxygen into the carbon–carbon bond to give an oxetane have no chemical or bio-

(9) Roth, H. D.; Schilling, M. L. M.; Jones, G. *J. Am. Chem. Soc.* **1981**, *103*, 1246–1248.

(10) The chromatographic analysis was carried out at 125 °C on a glass column packed with 10% Carbowax 20M on 120/140 mesh gaschrome Q. The structures of the metabolites were confirmed by mass spectrometry.

(11) Traces of iron and other metal ions are present in phosphate buffers.

(12) Product is present at the "zero" time point because it takes approximately 1–2 min to quench the reaction.

(13) Meinwald, J.; Labana, S. S.; Chadha, M. S. *J. Am. Chem. Soc.* **1963**, *85*, 582–585.

0002-7863/85/1507-4081$01.50/0 © 1985 American Chemical Society