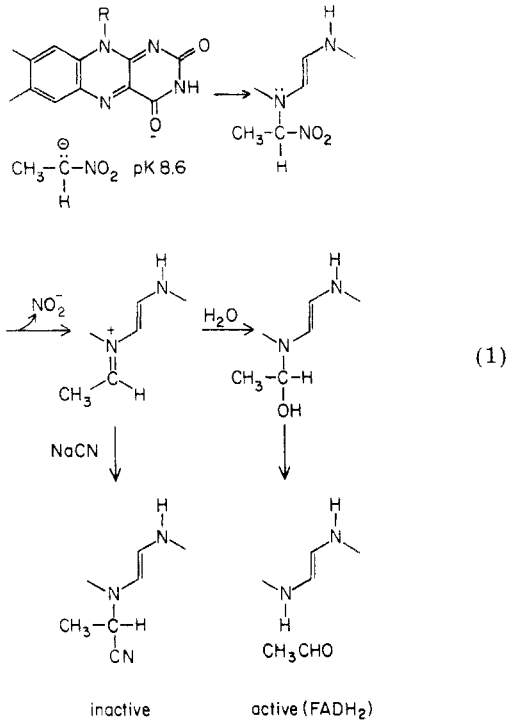
Enzyme Inhibition by Nitro and Nitroso Compounds

THEODORE A. ALSTON,* DAVID J. T. PORTER,* and HAROLD J. BRIGHT*

Department of Biochemistry and Biophysics, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Received February 8, 1983 (Revised Manuscript Received June 29, 1983)

Our group became interested in mechanisms by which nitro and nitroso compounds may inhibit enzymes when one of us (D.J.T.P.) found that nitroalkanes such as nitroethane are oxidized as substrates by renal D-amino acid oxidase.[1] We were able to deduce the reaction mechanism in good chemical detail because an FAD–substrate covalent intermediate could be intercepted with cyanide and structurally characterized (eq 1). The inactivation of D-amino acid oxidase



(1)

by nitroethane in the presence of cyanide may be viewed as an example of "mechanism-based" or "suicide" inactivation of an enzyme. Suicide inactivators are substrate analogues that are converted by target enzymes to bound species that then react irreversibly with the active site. Suicide substrates do not form stable linkages to an active site unless the catalytic forces of that active site mediate the reaction. The superposition of the high specificity of enzymatic catalysis upon that of substrate binding to form the Michaelis complex thus permits suicide substrates to be highly specific inactivators. Hence, the term "suicide substrate" was coined by Abeles and co-workers to emphasize active catalysis by enzymes of their own inactivation, and examples other than nitro and nitroso compounds have been discussed in previous Accounts.[2] Our work with D-amino acid oxidase and nitroethane has prompted us to examine additional mechanisms by which nitro and nitroso compounds may serve as specific suicide inactivators of enzymes. Furthermore, some nonreactive nitro and nitroso compounds are not suicide inactivators but, rather, are substrates converted to highly reactive products that are released free in solution.

While suicide substrates are pharmacologically interesting as agents capable of highly selective enzyme inactivation, compounds that are enzymatically converted to reactive free products are pharmacologically interesting as agents that can prove selectively toxic to organisms or tissues possessing the requisite activating enzymes. Only the latter type of these nitro compounds is in clinical use at present. We have found in other cases that nitro and nitroso compounds may serve as reversible inhibitors that do not bind covalently to enzymes. In particular, the ionizable nitro group closely resembles the carboxylate group, and nitro analogues of carboxylic substrates are often potent inhibitors, especially in those cases where the ionized nitro compounds appear to function as "transition-state analogues". Although nitro compounds such as chloramphenicol are valuable drugs, no drugs yet exploit the analogy of the nitro group to the carboxylate group, and this and other features of the nitro group may permit the rational design of additional pharmacologically valuable inhibitors of enzymes.

Suicide Enzyme Inactivators

Stopped-flow kinetic analysis, as well as the iminium-trapping experiment outlined in eq 1, established that the oxidation of nitroethane by D-amino acid oxidase proceeds via a substrate adduct at the N^5 position of the flavin cofactor. Consideration of this mechanism enabled us to predict that a nitroalkane bearing a second leaving group would inactivate the enzyme without the requirement of cyanide or other additional reagents. Thus, 1-chloro-1-nitroethane (II) inactivates the enzyme in a suicide reaction in which the flavin cofactor becomes acylated, rather than alkylated, at the N^5 position.[3] Interestingly, the suicide substrate is competitively oxidized to acetate in a noninactivating enzyme turnover at about one-half the frequency of enzyme suicide (eq 2). This flavin-acylation reaction clearly requires active catalysis by the enzyme; other flavoenzymes such as lactate oxidase (*Mycobacterium*

Theodore A. Alston was born in Philadelphia and received his bachelor's degree as well as M.D. and Ph.D. from the University of Pennsylvania. He is currently Resident in Surgery at the Yale-New Haven Hospital.

David J. T. Porter is Research Associate at the University of Pennsylvania. He was born in Vancouver, Canada, and received his bachelor's degree from Washington State University and his Ph.D. from Pennsylvania.

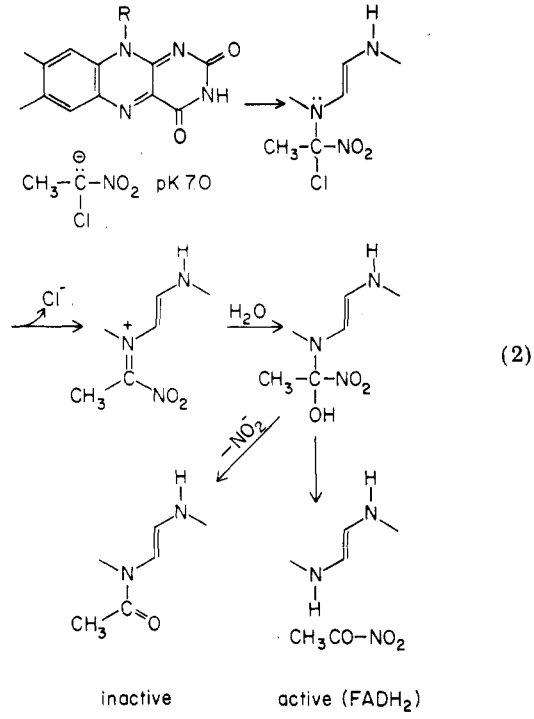
Harold J. Bright is Professor of Biochemistry and Biophysics at Pennsylvania. He was born in Salisbury, England, and received his B.A. from Cambridge University. Working with Lloyd L. Ingraham, he received his Ph.D. from the University of California at Davis. His work has dealt chiefly with mechanisms of enzyme action.

(1) D. J. T. Porter, J. G. Voet, and H. J. Bright, *J. Biol. Chem.*, 247, 1951 (1972); 248, 4400 (1973).

(2) K. Bloch, *Acc. Chem. Res.*, 2, 193 (1969); R. R. Rando, *ibid.*, 8, 281 (1975); R. H. Abeles and A. L. Maycock, *ibid.*, 9, 313 (1976); T. A. Alston, *Pharmacol. Ther.*, 12, 1 (1981).

(3) T. A. Alston, D. J. T. Porter, and H. J. Bright, *J. Biol. Chem.*, 258, 1136 (1983).

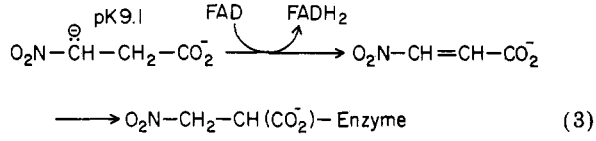
0001-4842/83/0116-0418$01.50/0



(2)

smegmatis), glycolate oxidase (spinach), glucose oxidase (*Aspergillus niger*), and succinate dehydrogenase (bovine heart) are not subject to inactivation by 1-chloro-1-nitroethane. Of these, glucose oxidase accepts nitroethane as a substrate, but the oxidation of nitroethane by glucose oxidase is not subject to inactivation by cyanide and may occur in part via a free-radical-mediated mechanism.[4] For that matter, the reactions depicted as purely ionic in eq 1 and 2 may ultimately prove further dissectable into one-electron-transfer steps. In any event, the highly selective inactivation of renal D-amino acid oxidase by 1-chloro-1-nitroethane demonstrates that suicide substrates may sometimes be designed on the basis of nonphysiological side reactions catalyzed concomitantly with the ordinary reactions of target enzymes.

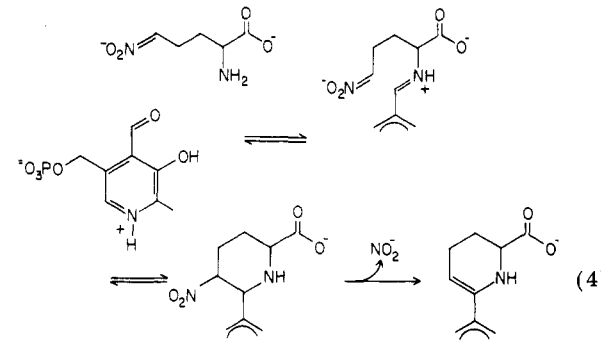
Some nitroalkyl compounds occur naturally. The first of these to be noted was 3-nitropropanoic acid, bovinocidin (III), discovered because of the toxicity of *Indigofera* and *Astragalus* species to grazing animals. 3-Nitropropanoate is an isoelectronic analogue of the Krebs cycle intermediate succinate and is an inhibitor of cellular respiration. Our experiments with nitroethane and the flavoenzyme D-amino acid oxidase prompted us to test 3-nitropropanoate as an inactivator of the flavoenzyme succinate dehydrogenase. Bovinocidin proved to be a potent suicide inactivator of the dehydrogenase.[5] It is probably oxidized by the enzyme to 3-nitroacrylate, which alkylates a nucleophilic group of the apoenzyme without diffusing from the active site (eq 3).



(3)

(4) D. J. T. Porter and H. J. Bright, *J. Biol. Chem.*, **252**, 4361 (1977).
(5) T. A. Alston, L. Mela, and H. J. Bright, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 3767 (1977); C. J. Coles, D. E. Edmondson, and T. P. Singer, *J. Biol. Chem.*, **254**, 5161 (1979); D. H. Gould and D. L. Gustine, *Neuropath. Appl. Neurobiol.*, 8, 377 (1982).

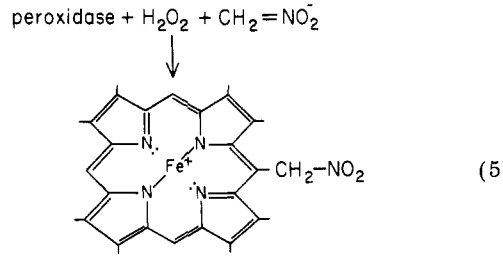
In addition to vitamin B_2 dependent enzymes, vitamin B_6 dependent enzymes are subject to inactivation by nitro compounds. Derivatives of alanine bearing a leaving group such as sulfate, chloride, fluoride, acetate, or carbamate in the β-position are known to inactivate several pyridoxal phosphate-dependent enzymes in suicide reactions.[2,6] We have prepared 3-nitroalanine (IV) and found that it inactivates alanine and aspartate aminotransferases, probably by a mechanism analogous to that of 3-fluoroalanine.[7] The enzymes catalyze the elimination of nitrite or fluoride to yield a reactive 2-aminoacrylate Schiff base. By a different mechanism, these enzymes are also inactivated by 5-nitro-L-norvaline (V), the second higher homologue of 3-nitroalanine.[7] This δ-nitro amine can condense with the formyl group of the pyridoxal cofactor to afford a stable six-membered cyclic adduct (eq 4). Similarly, γ-nitro



(4)

amines should afford five-membered cyclic adducts. We have thus synthesized 3-nitro-1-propanamine (VI) as an isoelectronic analogue of the neurotransmitter γ-aminobutyrate and confirmed that the analogue inactivates GABA aminotransferase.

Compounds such as nitroalkanes and nitrosaminoalkanes bearing acidic α-carbon atoms are generally good substrates for heme-dependent oxidases and peroxidases. Oxidation of nitroalkanes by horse radish peroxidase was reported by Little in 1957.[8] We have found that this peroxidase initiates the free-radical-mediated autoxidation of nitromethane and, in so doing, suffers inactivation secondary to nitromethylation of its heme prosthetic group (eq 5).[9]



(5)

Other inactivation reactions of relevant interest have been described in other laboratories. For instance, Westheimer has reported that acetoacetate decarboxylase is potently inhibited by 5-nitrosalicylaldehyde.[10] This reagent forms a metastable Schiff base with the active-site lysyl residue, which ordinarily

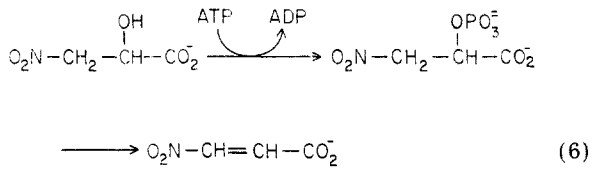
(6) T. A. Alston, D. J. T. Porter, L. Mela, and H. J. Bright, *Biochem. Biophys. Res. Commun.*, **92**, 299 (1980); T. A. Alston, H. Muramatsu, T. Ueda, and H. J. Bright, *FEBS Lett.*, **128**, 293 (1981).
(7) T. A. Alston and H. J. Bright, *FEBS Lett.*, **126**, 269 (1981).
(8) H. N. Little, *J. Biol. Chem.*, **229**, 231 (1957).
(9) D. J. T. Porter and H. J. Bright, *J. Biol. Chem.*, **258**, 9913 (1983).
(10) F. H. Westheimer, *Methods Enzymol.*, 14, 231 (1969).

condenses with the carbonyl group of acetoacetate. Thymidylate synthetase is another enzyme that employs covalent catalysis, and Wataya et al. have reported that 5-nitro-2′-deoxyuridylate forms a stable adduct with that enzyme.[11] Nitronate esters are highly electrophilic, and Schorstein et al. have shown that 3-chloro-1-nitropropane cyclizes to an ester capable of covalently inactivating enzymes.[12] *N*-Nitrosamides spontaneously decompose into products reactive with amino acid residues of proteins. However, White et al. have described such a reagent that exploits covalent catalysis by chymotrypsin.[13] The enzyme attacks an *N*-nitroso lactam with its nucleophilic active-site seryl residue to afford a diazonium alkylating agent bound in ester linkage to the seryl residue. The bound diazonium functionality then reacts with an adjacent active-site residue. Enzymes such as aldolase and transketolase that generate carbanionic reaction intermediates are subject to inactivation by tetranitromethane and other oxidants in a process that Christen has termed "paracatalytic enzyme modification".[14] Givot et al. have reacted the nitromethane anion with an electrophilic group at the active site of histidine ammonia-lyase in another interesting enzyme inactivation that, similarly, is also not strictly a suicide reaction.[15]

Compounds Enzymatically Converted to Reactive Products

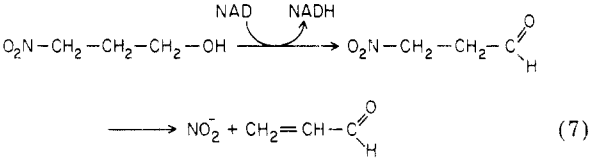
A number of intrinsically reactive nitro and nitroso compounds enjoy limited biochemical or pharmacological application. Examples include Sanger's reagent, Ellman's sulfhydryl reagent, chloropicrin, mutagens such as *N*-methyl-*N*-nitroso-*N*′-nitroguanidine, and antineoplastic nitrosourea drugs such as streptozotocin and carmustine (BCNU). Vascular smooth muscle relaxants such as nitroprusside, nitrates, and nitrites are intrinsically reactive but cause activation rather than inhibition upon reaction with their apparent target enzyme guanylate cyclase,[16] whereas inorganic nitrite inactivates hemoglobin and other metalloproteins. Some organisms produce intrinsically reactive nitro and nitroso compounds to their ecological advantage. For instance, streptomycetes elaborate the reactive antibiotics enteromycin,[17] aureothin,[18] and streptozotocin,[19] while certain soldier termites spray reactive 1-nitro-1-alkenes at predators.[20] A more subtle strategy is practiced by streptomycetes that elaborate azomycin. This structurally simple antibiotic, 2-nitroimidazole, has only low-grade intrinsic reactivity[21] until it undergoes enzymatic reduction by susceptible organisms to a number of highly reactive products. It and its artifical congeners such as the clinically important drug metronidazole thus exhibit highly selective toxicity toward H_2-evolving anaerobic microorganisms including certain pathogenic eukaryotes.[22] The dramatic clinical efficacy of the antimicrobial nitroheterocyclic "wonder drugs" has been one inducement for us to examine other mechanisms by which nitro and nitroso compounds may be enzymatically converted to reactive products.

The suicide inactivation of mitochondrial succinate dehydrogenase by bovinocidin (eq 3) prompted us to consider other mechanisms to enzymatically generate conjugated nitroalkenes such as 3-nitroacrylate. We found no evidence for dehydration of 3-nitrolactate (VII) to 3-nitroacrylate by fumarase. However, like glycolate and lactate, 3-nitrolactate is readily phosphorylated by mammalian pyruvate kinase, and the product spontaneously eliminates phosphate to afford 3-nitroacrylate. Pyruvate kinase is thus inactivated by 3-nitrolactate in an ATP-requiring reaction (eq 6).[23]



(6)

In addition to promoting α,β-eliminations, the nitro group may itself serve as a leaving group to afford an electrophilic Michael acceptor. For instance, locoweeds and poison vetches of the Astragalus genus produce 3-nitropropanoate but are generally richer in 3-nitro-1-propanol (miserotoxin aglycon), which in some cases is more toxic than 3-nitropropanoate. Upon oxidation by hepatic alcohol dehydrogenase, 3-nitro-1-propanol affords inorganic nitrite and highly cytotoxic acrolein (eq 7).[24] 3-Nitropropanoate might similarly yield a



(7)

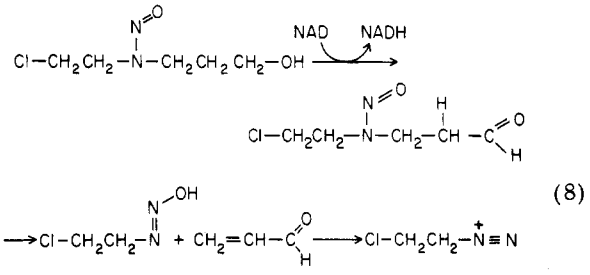
reactive acrylyl thio ester after succinic thiokinase catalyzed esterification of coenzyme A.

As in the case of the suicide inactivation of D-amino acid oxidase by 1-chloro-1-nitroethane (eq 2), nitro compounds may be enzymatically converted to acylating agents as well as alkylating agents. In addition to the flavin-dependent oxidase, heme-dependent enzymes such as horse radish peroxidase and probably others[25] catalyze the oxidation of 1-chloro-1-nitroethane to

(11) Y. Wataya, A. Matsuda, and D. V. Santi, *J. Biol. Chem.*, **255**, 5538 (1980).
(12) D. E. Schorstein, C. J. Suckling, R. Wrigglesworth, *J. Chem. Res., Synop.*, 264 (1978).
(13) E. H. White, D. F. Roswell, I. R. Politzer, and B. R. Branchini, *Methods Enzymol.*, **46**, 216 (1977).
(14) P. Christen, *Methods Enzymol.*, **46**, 48 (1977).
(15) I. L. Givot, T. A. Smith, and R. H. Abeles, *J. Biol. Chem.*, **244**, 6341 (1969).
(16) P. J. Lad, M. A. Liebel, and A. A. White, *Biochem. Biophys. Res. Commun.*, **103**, 620 (1981).
(17) S. E. DeVoe, M. McCrae, and L. A. Mitscher, *Antimicrob. Agents Chemother.*, 105 (1964).
(18) R. Cardillo, C. Fuganti, D. Ghiringhelli, D. Giangrasso, P. Grasselli, and A. Santopietro-Amisano, *Tetrahedron*, **30**, 459 (1974).
(19) P. Schein, R. Kahn, P. Gorden, S. Wells, and V. T. DeVita, *Arch. Intern. Med.*, **132**, 555 (1973).
(20) S. G. Spanton and G. D. Prestwich, *Tetrahedron*, **38**, 1921 (1982).
(21) P. Goldman and J. D. Wuest, *J. Am. Chem. Soc.*, **103**, 6224 (1981).
(22) S. D. Nelson, *J. Med. Chem.*, **25**, 753 (1982); B. C. Millar, *Biochem. Pharmacol.*, **31**, 2439 (1982); R. C. Knight, I. M. Skolimowski, and D. I. Edwards, *Biochem. Pharmacol.*, **27**, 2089 (1978); L. A. Poirier and J. H. Weisburger, *Biochem. Pharmacol.*, **23**, 661 (1974).
(23) D. J. T. Porter, D. E. Ash, and H. J. Bright, *Arch. Biochem. Biophys.*, **222**, 200 (1983).
(24) T. A. Alston, S. P. Seitz, and H. J. Bright, *Biochem. Pharmacol.*, **30**, 2719 (1981); T. A. Alston, L. Mela, and H. J. Bright, *Arch. Biochem. Biophys.*, **197**, 516 (1979).
(25) Nitroalkane-oxidizing enzymes are widely distributed in nature. They have been found in extracts from microorganisms, nitroalkane-grown microorganisms, plants, mammalian liver, buttermilk, and reptile venom; see H. N. Little, *J. Biol. Chem.*, **193**, 347 (1951); T. Kido, K. Soda, T. Suzuki, and K. Asada, *J. Biol. Chem.*, **251**, 6994 (1976); M. R. Dahawale and U. Hornemann, *J. Bacteriol.*, **137**, 916 (1979); T. Kido, K. Hashizume, and K. Soda, *ibid.*, **133**, 53 (1978); V. Ullrich, G. Hermann, and P. Weber, *Biochem. Pharmacol.*, **27**, 2301 (1978); D. J. T. Porter, J. G. Voet, and H. J. Bright, *Z. Naturforsch. B*, **27**, 1052 (1972).

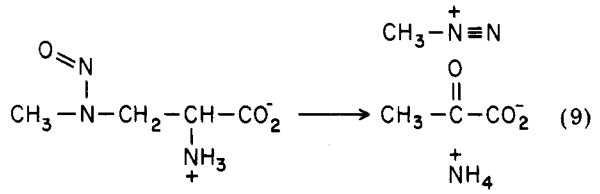
N-acetyl nitrite. A similar enzymatic reaction may be involved in the antifungal activity of *gem*-bromo nitroalkanes.[26] *N*-Acetyl nitrite is a potent acetylating agent and may rearrange to *O*-acetyl nitrite, a strong nitrosating agent.[27]

Oxidation of secondary nitrosamines by heme-dependent enzymes is responsible for the carcinogenic action of these environmental hazards to human health. Dialkylnitrosamines are oxidized by cytochrome P-450 to α-hydroxy compounds that decompose into primary nitrosamines and thence to diazonium alkylating agents.[28] The secondary nitrosamines exhibit very low reactivity under physiological conditions, whereas their diazonium metabolites are extremely powerful electrophiles. Little work has appeared on the deliberate design of secondary nitrosamines that could be converted to primary nitrosamines by enzymes other than cytochrome P-450. The efficacy of the nonenzymatically activated antineoplastic nitrosourea drugs should spur such investigation. One approach might involve γ-oxidation rather than α-oxidation. For instance, *N*-nitroso-β-(methylamino)isobutyl methyl ketone, $CH_3N(NO)C(CH_3)_2CH_2COCH_3$, has been employed as a reagent for the generation of diazomethane. Because of the γ-carbonyl group, this secondary nitrosamine decomposes into a labile primary nitrosamine in a base-catalyzed reaction.[29] Strongly alkaline conditions are generally employed to generate diazomethane from the nitrosamino ketone, but we have found the reaction to proceed, albeit slowly, under mild conditions. As judged by N_2 evolution followed manometrically, the nitrosamino ketone has a decomposition rate of 0.7%/day at 25 °C in water buffered at pH 7.4 with 1.0 M potassium phosphate. This slow reaction ought to occur more rapidly at body temperature in the cases of aldehydes bearing electron-withdrawing halogen substituents. Thus, the (2-chloroethyl)diazonium ion is a cross-linking agent produced by spontaneous hydrolysis of the nitrosourea drugs carmustine (BCNU) and loumstine (CCNU). That species could also be generated, for instance, in an alcohol dehydrogenase dependent reaction proposed in eq 8. This scheme is



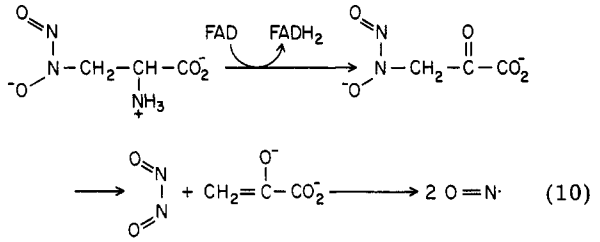
(8)

reminiscent of the alcohol dehydrogenase prompted evolution of diazomethane from the azoxy carcinogen cycasin[30] as well as the enzymatic conversion of miserotoxin aglycon to acrolein (eq 7). The reaction proposed in eq 8 should be generalizable to other secondary nitrosamines and to other enzymes. Furthermore, lyases acting on secondary nitrosamino analogues of substrates might actively catalyze the elimination of reactive diazoates. As a hypothetical example, tryptophanase catalyzes the elimination of indole from tryptophan and might release diazomethane from the compound shown in eq 9.



(9)

Tryptophanase has been reported to catalyze the decomposition of alanosine,[31] an antineoplastic antibiotic, which is shown in eq 10 and which resembles the amino acid proposed in eq 9. In the case of alanosine, tryptophanase catalyzes the release of nitrous oxide (N_2O) rather than diazomethane (N_2CH_2). Nitrous oxide is sufficiently inert to be widely employed as an anesthetic drug in humans, but the compound is capable of inactivating vitamin B_{12} dependent enzymes such as methionine synthetase, and this reaction of nitrous oxide may be clinically significant.[32] We have found with D. A. Cooney that alanosine may also be enzymatically converted to other oxides of nitrogen.[33] Metabolism of this analogue of aspartate to the analogue of oxalacetate, as by the action of L-amino acid oxidase, permits its decomposition into nitric oxide, a highly reative cytotoxic agent (eq 10).



(10)

Alanosine exhibits sufficient antineoplastic activity to have warranted clinical trials in humans. Its mechanism of antineoplastic activity probably does not involve nitric oxide evolution but, rather, inhibition of purine biosynthesis from aspartate. In particular, alanosine is conjugated to 5-aminoimidazole-4-carboxylic acid ribonucleotide by the action of phosphoribosylaminoimidazole-succinocarboxamide (SAICAR) synthetase, and the resultant nucleotide is a potent reversible inhibitor of adenylosuccinate synthetase.[30,34] Alanosine also inhibits pyrimidine biosynthesis.[35] Since *N*-hydroxy-*N*-nitrosamines bind zinc avidly[36] and since dihydroorotase is subject to inactivation by zinc-chelating agents,[37] it is tempting to spectulate that the

(26) I. Schmeltz and A. Wenger, *Food Cosmet. Toxicol.*, 17, 105 (1979).
(27) A. B. Kyte, R. Jones-Parry, and D. Wittaker, *J. Chem. Soc., Chem. Commun.*, 74 (1982).
(28) E. C. Miller and J. A. Miller, *Adv. Exp. Med. Biol.*, 136, 1 (1982); D. Y. Lai and J. C. Arcos, *Life Sci.*, 27, 2149 (1980); G. A. Digenis and C. H. Issidorides, *Bioorg. Chem.*, 8, 97 (1979).
(29) C. E. Redemann, F. O. Rice, R. Roberts, and H. P. Ward, "Organic Syntheses"; Wiley, New York, 1955, Collect Vol. III, p 244.
(30) A. Feinberg and M. S. Zedeck, *Cancer Res.*, 40, 4446 (1980).
(31) H. N. Jayaram, A. K. Tyagi, S. Anandaraj, J. A. Montgomery, J. A. Kelley, J. Kelley, R. H. Adamson, and D. A. Cooney, *Biochem. Pharmacol.*, 28, 3551 (1979).
(32) K. Yasuhiko, S. Sakamoto, K. Sakuraya, T. Kubota, K. Hida, K. Suda, and F. Takaku, *Cancer Res.*, 41, 4698 (1981); I. Chanarin, *J. Clin. Pathol.*, 33, 909 (1980).
(33) T. A. Alston and H. J. Bright, Biophys. *Res. Commun.*, 105, 560 (1982).
(34) J. M. Kelley, R. H. Adamson, D. A. Cooney, H. N. Jayaram, and S. Anadaraj, *Cancer Treat. Rep.*, 61, 471 (1977); H. N. Jayaram and D. A. Cooney, *ibid.*, 63, 1095 (1979); S. J. Anadaraj. H. N. Jayaram, D. A. Cooney, A. K. Tyagi, N. Han, J. H. Thomas, M. Chitnis, and J. A. Montgomery, *Biochem. Pharmacol.*, 29, 227 (1980).
(35) G. R. Gale, W. E. Ostrander, and L. M. Atkins, *Biochem. Pharmacol.*, 17, 1823 (1968).
(36) G. Powis and J. S. Kovach, *Biochem. Pharmacol.*, 30, 771 (1981).

inhibition of pyrimidine biosynthesis by alanosine involves inactivation of dihydroorotase by the *N*-carbamyl derivative of alanosine (synthesized by aspartate transcarbamylase, the preceding enzyme in the pyrimidine biosynthetic pathway). Furthermore, *N*-hydroxy-*N*-nitrosamines bear chemical analogy to hydroxamic acids and may thus also prove to inactivate hydoxamate-sensitive enzymes such as ribonucleotide reductase.[38] In this regard, we have observed that, like hydroxamic acids,[39] alanosine and other hydroxynitrosamines are readily oxidized by peroxidases to nitroxide free radicals; this reaction provides another mechanism for enzymatic generation of nitric oxide from hydroxynitrosamines.

Nitroaryl and nitro heterocyclic compounds are readily reduced to toxic products by anaerobic microorganisms. To a lesser extent such reduction can occur in mammalian tissues via heme-dependent enzymes such as cytochrome P-450 and via molybdenum-dependent enzymes such as xanthine oxidase.[19] Hypoxia favors metabolic reduction of nitro compounds. Since malignant tumors frequently outgrow their blood supply and thus contain hypoxic cells that are resistant to radiation and to drugs that kill rapidly dividing normoxic cells, nitro compounds have been designed as enzyme-activated chemotherapeutic agents for hypoxic tumor cells. One approach to the design of so-called "bioreductive alkylating agents"[40] is illustrated by *p*-nitroaniline mustard, $p\text{-}O_2NC_6H_6N(CH_2CH_2Cl)_2$.[41] The electron-withdrawing nitro group retards cyclization of this mustard to a reactive aziridinium ion, but enzymatic reduction of the nitro group to an electron-donating amino group facilitates aziridinium ion formation. 1,3-Dichloro-2-methyl-nitropropane has also been synthesized as a potential mustard-type bioreductive alkylating agent.[42]

Another approach involves *o*-nitrobenzyl carbamates, $o\text{-}O_2NC_6H_4CH_2O_2CNR_2$, which are enzymatically reduced to *o*-aminobenzyl carbamates. These products spontaneously eliminate carbamate to afford reactive methide compounds.[43] However, nitroaromatic compounds need not undergo complete bioreduction to amino compounds in six-electron redox reactions in order to yield reactive species. The intermediate aromatic free radicals, nitroso compounds, and hydroxylamines can themselves covalently damage cellular constituents.[22] Although aromatic nitroso compounds are intrinsically reactive with protoplasmic nucleophiles, their reactivities can be enzymatically enhanced. For instance, *p*-nitrosoanilines are reduced by NADH in an alcohol dehydrogenase catalyzed reaction to yield *p*-(hydroxyamino)anilines, which lose hydroxide to afford reactive quinonediimine cations.[44] Similarly, 4-(dimethylamino)-1-nitrosobenzene reacts with the "active acetylaldehyde" form of pyruvate decarboxylase to yield *N*-[4-(dimethylamino)phenyl]acetohydroxamic acid, $p\text{-}Me_2NC_6H_4N(OH)COCH_3$, which then loses hydroxide to afford a reactive *N*-acetylquinonediimine cation.[45] Although hydrolysis of the nitrosourea drugs to reactive diazonium ions occurs spontaneously, that process may also be enzymatically facilitated.[13] For instance, diazomethane evolution from streptozotocin, *N*-(methylnitrosocarbamyl)glucosamine, might be promoted by the enzymes that ordinarily catalyze hydrolysis of *N*-acetylglucosamine.

Competitive Inhibitors and Substrates

The nitro group closely resembles the carboxylate group, and we have found that nitro analogues of carboxylic substrates generally bind well to enzymes. The similarity to carboxylates is increased upon ionization of nitroalkanes to the nitronate state. However, nitroalkanes are weak carbon acids that are predominantly not ionized at equlibrium at physiological pH.[46] The acidity of nitroalkanes is enhanced by electron-withdrawing substituents, though, so that the pK of 1-chloro-1-nitroethane is 7.0, while that of nitroethane is 8.6 in water at 25 °C.[46] *N*-Nitro compounds (nitramines) may also serve as carboxylate analogues and are more acidic than the corresponding *C*-nitro compounds.[47] Thus, the second ionizations of succinate, nitraminoacetate (VIII), and 3-nitropropanoate (III) have pK values of 5.6, 6.6, and 9.1, respectively. *N*-hydroxy-*N*-nitrosamines, on the other hand, are fairly strong acids. For instance, the hydroxynitrosamino group of alanosine (eq 10) has a pK of 4.8. Cooney and co-workers have shown that alanosine and its metabolites thus interact with the active sites of numerous enzymes involved in the metabolism of aspartate and glutamate.[31,34]

Our findings that succinate dehydrogenase and γ-aminobutyrate aminotransferase are inactivated by the respective suicide substrates 3-nitropropanoate and 3-nitro-1-propanamine prompted us to examine the corresponding *N*-nitro compounds as inhibitors. Nitraminoacetate (VIII) is a toxic antibiotic from *Streptomyces noursei*,[48] and 2-nitraminoethylamine (IX) is produced by the mushroom *Agaricus silvaticus*.[49] The former compound is a strong competitive inhibitor of the dehydrogenase,[47] and the latter is oxidized as a substrate by the aminotransferase.[50] Our experiments with *C*-nitro and *N*-nitro analogues as competitive inhibitors and substrates of enzymes acting on carboxylic substrates are summarized in Table I. There are, incidentally, many examples of nitroaromatic compounds

(37) R. I. Christopherson and M. E. Jones, *J. Biol. Chem.*, **255**, 11381 (1980).
(38) B. van't Riet, L. B. Kier, and H. L. Elford, *J. Pharm. Sci.*, **69**, 856 (1980).
(39) I. Aviram, *Arch. Biochem. Biophys.*, **212**, 483 (1981).
(40) A. J. Lin, R. S. Paradini, L. A. Crosby, B. J. Lillis, C. W. Shansky, and A. C. Sartorelli, *J. Med. Chem.*, **16**, 1268 (1973); H. W. Moore, *Science*, **197**, 527 (1977); K. A. Kennedy, B. A. Teicher, S. Rockwell, and A. C. Sartorelli, *Biochem. Pharmacol.*, **29**, 1 (1980); B. A. Teicher, J. S. Lazo, and A. C. Sartorelli, *Cancer Res.*, **41**, 73 (1981); A. C. Sartorelli, *Adv. Enzyme Regul.*, **20**, 233 (1982).
(41) K. C. Tsou and H. C. F. Su, *J. Med. Chem.*, **6**, 693 (1963).
(42) R. Preussman, *Arzenim. Forsch.*, **8**, 638 (1958).
(43) K. A. Kennedy, B. A. Teicher, S. Rockwell, and A. C. Sartorelli, "Molecular Actions and Targets for Cancer Chemotherapeutic Agents", A. C. Sartorelli, J. S. Lazo, and J. R. Bertino, Eds., Academic Press, New York, 1981, p 85.
(44) S. C. Koerber, P. Schack, A. M.-J. Au, and M. F. Dunn, *Biochemistry*, **19**, 731 (1980).
(45) M. D. Corbett and B. R. Corbett, *Bioorg. Chem.*, **11**, 328 (1982).
(46) Nitronates are sluggish to reprotonate, however, so that nitronates that are not in equlibrium with the parent nitro compounds can be applied to enzymes in experiments in vitro; see R. G. Pearson and R. L. Dillon, *J. Am. Chem. Soc.*, **75**, 2439 (1953).
(47) T. A. Alston, S. P. Seitz, D. J. T. Porter, and H. J. Bright, *Biochem. Biophys. Res. Commun.*, **97**, 294 (1980).
(48) Y. Miyazaki, Y. Kono, A. Shimazu, S. Takeuci, and H. Yonehara, *J. Antibiot.*, **21**, 279 (1968).
(49) W. S. Chilton and C. P. Hsu, *Phytochemistry*, **14**, 2291 (1975).
(50) T. A. Alston, D. J. T. Porter, S. P. Seitz, and H. J. Bright, *Biochem. Biophys. Res. Commun.*, **103**, 1077 (1981); T. A. Alston, D. J. T. Porter, D. M. S. Wheeler, and H. J. Bright, Biochem. *Pharmacol.*, **31**, 4081 (1982).

Table I
Representative Examples of Nitro Analogues of Carboxylic Substrates

compound	activity[a]	enzyme	ref
$^-O_2N{=}CHCH_3$ (I)	S	D-amino acid oxidase (porcine kidney), EC 1.4.3.3	1
$^-O_2N{=}CClCH_3$ (II)	X	D-amino acid oxidase	3
$^-O_2N{=}CHCH_2CO_2^-$ (III)	X	succinate dehydrogenase (rat liver, bovine heart), EC 1.3.99.1	5
	S	succinic thiokinase (porcine heart), EC 6.2.1.4	*b*
	I	aspartase (*Bacterium cadaveris*), EC 4.3.1.1	52
	I	fumarase (porcine heart), EC 4.2.1.2	52
	I	isocitrate lyase (*Pseudomonas indigofera*), EC 4.1.3.1	56
$^-O_2N{=}CHCH(^+NH_3)CO_2^-$ (IV)	X	aspartate aminotransferase (porcine heart), EC 2.6.1.1	7
	S	adenylosuccinate synthetase (*Azotobacter vinelandii*), EC 6.3.4.4	58
	S	D-amino acid oxidase	*b*
	I	aspartase	52
$^-O_2N{=}CHCH_2CH_2CH(^+NH_3)CO_2^-$ (V)	X	alanine aminotransferase (porcine heart), EC 2.6.1.2	7
	X	aspartate aminotransferase	7
	X	GABA aminotransferase (*Pseudomonas fluorescens*, porcine brain), EC 2.6.1.19	7
$^-O_2N{=}CHCH_2CH_2{}^+NH_3$ (VI)	X	GABA aminotransferase	*b*
$^-O_2N{=}CHCHOHCO_2^-$ (VII)	S	malate dehydrogenase (rabbit muscle), EC 1.1.1.37	52
	I	fumarase	52
	I	malic enzyme (chicken liver), EC 1.1.1.40	52
$^-O_2N{=}NCH_2CO_2^-$ (VIII)	S	succinic thiokinase	*b*
	I	aspartase	47
	I	fumarase	47
	I	succinate dehydrogenase	47
$^-O_2N{=}NCH_2CH_2{}^+NH_3$ (IX)	S	GABA aminotransferase	50
$^-O_2N{=}CHCOH(CO_2^-)CH_2CO_2^-$ (X)	S	ATP-citrate lyase (rat liver), EC 4.1.3.8	*b*
	S	citrate lyase (*Enterobacter aerogenes*), EC 4.1.3.6	*b*
	I	aconitase (porcine heart), EC 4.2.1.3	55
$^-O_2N{=}C(CH_2CO_2^-)CHOHCO_2^-$ (XI)	I	aconitase	55
	I	isocitrate dehydrogenase (porcine heart), EC 1.1.1.41	*b*
$^-O_2N{=}CHCOCO_2^-$ (XII)	S	citrate synthetase (pigeon liver), EC 4.1.3.7	*b*
	S	malate dehydrogenase	52
	I	pyruvate kinase/oxalacetate decarboxylase (rabbit muscle), EC 2.7.1.40	*b*
$^-O_2N{=}CH_2$	S	carbamate kinase (*Streptococcus faecalis*), EC 2.7.2.2	*b*
$^-O_2N{=}CHCO_2^-$	I	succinate dehydrogenase	47
$^-O_2N{=}CClCH_2CO_2^-$	I	succinate dehydrogenase	3
$^-O_2N{=}NCH_2CHO$	S	succinic semialdehyde dehydrogenase (*Pseudomonas fluorescens*), EC 1.2.1.16	50
$^-O_2N{=}CHCHOHCH_3$	S	3-hydroxybutyrate dehydrogenase (*Pseudomonas lemoignei*), EC 1.1.1.30	*b*
$^-O_2N{=}C(CH_3)CHOHCH_3$	I	3-hydroxybutyrate dehydrogenase	*b*
$^-O_2N{=}NCH_2CH(^+NH_3)CO_2^-$	S	glutamate dehydrogenase (bovine liver), EC 1.4.1.3	*b*
	I	glutamine synthetase (ovine brain), EC 6.3.1.2	*b*
$^-O_2N{=}NCH(CO_2^-)CH_2CO_2^-$	I	aconitase	*b*
$^-O_2N{=}CHCH(AMP)CO_2^-$	I	adenylosuccinate lyase (brewers yeast), EC 4.3.2.2	58

[a] I = reversible inhibitor, S = substrate, X = suicide inactivator. [b] Unpublished observation.

that have been employed as chromogenic substrates for enzymes,[51] but these compounds are not accepted by the enzymes by virtue of structural analogy to carboxylic substrates.

Transition-State Analogues

In view of the suicide inactivation of succinate dehydrogenase by 3-nitroacrylate generated enzymatically from the nitronate of 3-nitropropanoate (eq 3), we have tested the nitronates of 3-nitrolactate (VII) and 3-nitroalanine (IV) as 3-nitroacrylate-generating substrates of fumarase and aspartase, respectively.[52] No production of 3-nitroacrylate was detectable, but the nitronates proved to be potent reversible inhibitors of those enzymes. The 3-nitrolactate nitronate binds 900 times more tightly than malate binds to fumarase, and the 3-nitroalanine nitronate binds 1600 times more tightly than aspartate binds to aspartase.[52,53] As discussed elsewhere,[54] since enzymes must stabilize transition-state species in order to catalyze their reactions, then it is reasonable to expect that structural analogues of transition-state species should bind more tightly than substrates or products to enzymes. Since enzymes accelerate reaction rates by orders of magnitude, so-called transition-state analogues can theoretically bind more tightly than substrates by orders of magnitude. The nitronate inhibitors are plausible transition-state analogues for fumarase and aspartase because nitronates are isoelectronic with α-carbanions of carboxylates. Those enzymes probably abstract protons from the C-3 position of their substrates to transiently generate such α-carbanions. The aconitase reaction is similar to that of fumarase, and aconitase is exquisitely sensitive to the nitronate analogues of citrate and isocitrate.[55] The nitronate of 2-hydroxy-3-nitropropane-1,2-dicarboxylic acid (X) binds 2700 times more tightly than citrate binds to aconitase, and the nitronate of 1-hydroxy-2-

(51) Y. Wataya and D. V. Santi, *Anal. Biochem.*, 112, 96 (1981).
(52) D. J. T. Porter and H. J. Bright, *J. Biol. Chem.*, **255**, 4772 (1980).
(53) A naturally occurring nitro compound that may similarly inhibit aspartate-utilizing enzymes is 1-amino-2-nitrocyclopentanecarboxylic acid, a phytopathic antibiotic; see B. F. Burrows and W. B. Turner, *J. Chem. Soc. C*, 255 (1966).
(54) G. E. Lienhard, *Science*, **180**, 149 (1973); R. Wolfenden, *Methods Enzymol.*, **46**, 15 (1977).
(55) J. V. Schloss, D. J. T. Porter, H. J. Bright, and W. W. Cleland, *Biochemistry*, **19**, 2358 (1980).

nitropropane-1,3-dicarboxylic acid (XI) binds 72 000 times more tightly than isocitrate. Interestingly, the onset and release of inhibition of aconitase by the nitronate analogue of isocitrate is not instantaneous. The analogue rapidly associates with the enzyme to form a loose complex that rearranges to a tight complex in a first-order reaction with a rate constant of 6.6 min^{-1}. The inhibition is only slowly reversible as the tight complex rearranges back to the loose complex in a first-order reaction with a sluggish rate constant of only 0.063 min^{-1}. The apparent K_i value for the inhibitor is 6.8×10^{-10} M, and such hysteretic behavior is commonly seen with extremely tight-binding inhibitors of enzymes.

It is tempting to speculate that the nitronate analogue of isocitrate functions as a suicide substrate for aconitase. The enzyme might catalyze transformation of the inhibitor to a nitroalkene analogue of aconitate, which then alkylates an active-site nucleophilic group in a slowly reversible Michael reaction. However, the structural resemblance of the nitronate of 1-hydroxy-2-nitropropane-1,3-dicarboxylic acid to a plausible transition-state species in the aconitase reaction suggests that noncovalent forces may entirely account for the remarkably tight binding. In another case, the nitronate of 3-nitropropanoate binds 65 000 times more tightly than succinate to isocitrate lyase, and the dissociation rate is less than 0.0011 min^{-1} in that instance.[56] Nitro compounds that are not strict nitro analogues of carboxylic substrates might also serve as transition-state analogues. For instance, 3-nitropyruvate (XII) binds much more tightly than pyruvate to pyruvate kinase. In that case the nitro substituent may facilitate binding of the oxo acid to the active site by promoting enolatization and thus resemblance to the enolate of pyruvate. However, pyruvate kinase actively catalyzes the decarboxylation of oxalacetate as a side reaction,[57] and the dianion of 3-nitropyruvate is an eminently plausible transition-state analogue for that reaction.

(56) J. V. Schloss and W. W. Cleland, *Biochemistry*, 21, 4420 (1982).

Concluding Remarks

The rich chemistry of the nitro and nitroso groups should permit the rational design of numerous potent and selective inhibitors of enzymes. In particular, ionizable nitro and related compounds may serve as close analogues of carboxylic substrates for enzymes. The nitro group is more reactive than the carboxy group, though, and this reactivity may result in enzyme inactivation in special cases. Examples in this Account include reactions in which the nitro or nitroalkyl group functions as a reductant,[1,5,9,25] oxidant,[22,41-43] nucleophile,[1,3,7] electrophile,[5,12,21,23] and leaving group.[1,3,24] There are no nitroalkyl, halonitroalkyl, nitramino, dialkylnitrosamino, nor *N*-hydroxy-*N*-nitrosamino compounds currently in clinical use as drugs. We anticipate that further work along the lines described herein will fruitfully add such compounds to the armamentarium of medicinal chemistry.

Work from our laboratory was supported by Research Grant GM 11040 from the National Institutes of Health. T.A.A. has received support from the Medical Scientist Training Program, GM 07170, NIH.

(57) D. J. Creighton and I. A. Rose, *J. Biol. Chem.*, **251**, 69 (1976).
(58) D. J. T. Porter, N. G. Rudie, and H. J. Bright, *Arch. Biochem. Biophys.*, **225**, 157 (1983).