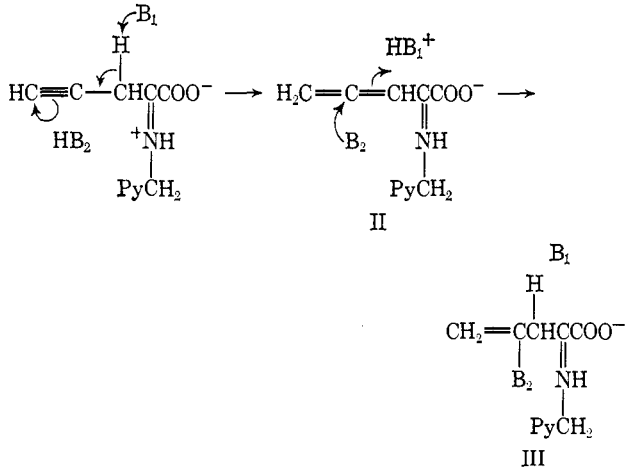
an active site residue. The exact nature of the linkage is now under investigation.

Studies were conducted with propargylglycine in intact mice. Groups of three mice were injected intraperitoneally with 2 and 5 μmol of D,L-propargylglycine, respectively, while three control mice received the same volume of saline solution. After 24 hr, the mice were sacrificed, and the livers were removed and assayed for γ-cystathionase. The livers from the treated mice had 10–20 and 0%, respectively, of the enzyme levels from control livers. Propargylglycine inactivation of the enzyme *in vivo* indicates induction of a condition similar to that found in the genetic defect cystathionuria[10] in which liver γ-cystathionase is absent or defective.

An essential feature of the mechanism of action of γ-cystathionase is the abstraction of a proton from the β position.[6] We, therefore, tentatively propose the mechanism in Scheme I for the inactivation by propargylglycine. The allene formed after proton abstraction is in conjugation with the ketimine and should be capable of ready Michael addition by an enzyme active site nucleophile to produce covalent labeling of the active site (III). (B_1 and B_2 are active site basic groups and PyCHO is pyridoxal-P.)

Scheme I



In the proposed mechanism, the acetylenic linkage is essential. This is consistent with finding that allylglycine (Sigma Chemical Co.) (2-amino-4-pentenoic acid) does not inactivate γ-cystathionase. Also, only those pyridoxal-dependent enzymes which abstract substrate β-hydrogens should catalyze their own destruction by propargylglycine. Preliminary experiments[11] with threonine deaminase and transaminase show no inactivation by propargylglycine.

In summary, then, we have shown for the first time that an acetylenic amino acid will irreversibly inactivate a pyridoxal-P dependent enzyme, rat liver γ-cystathionase, *in vitro* and *in vivo*, presumably *via* a reactive allene intermediate. This extends our recent observations that several flavine coenzyme-dependent enzymes[3,12] are irreversibly inactivated by acetylenic substrates. It confirms the expectation that, in general, acetylenic substrate analogs should be potent and specific irreversible inactivators in enzymatic catalyses where the carbon-bound hydrogen adjacent to the acetylenic moiety is abstracted as a proton.

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Highly Specific Enzyme Inhibitors. Inhibition of Plasma Amine Oxidase

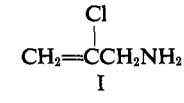
Sir:

We have recently suggested[1] that substrate analogs containing an acetylenic group adjacent to a carbon-bound hydrogen should be generally useful as enzyme inactivators when this hydrogen is abstracted as a proton during catalysis.

Based upon earlier work[2] from the laboratory of Bloch, it was suggested that this inactivation proceeds through rearrangement of the acetylene to the allene which then reacts with a nucleophile at the active site. Results obtained in our studies with beef plasma amine oxidase[3] suggest that an early step in the oxidation involves proton abstraction[4] from the carbon atom which is oxidized. Therefore, this enzyme might be susceptible to inactivation by appropriate acetylenic substrates.

As reported below, we have found that 1-amino-2-alkynes are capable of inactivating plasma amine oxidase. Furthermore, if the proposed mechanism of inactivation is correct, other substrates which can form allenes during the catalytic process should also inactivate the enzyme.

2-Chloroallylamine (I), which could undergo allene



formation by expulsion of chloride after the C-1 hydrogen is abstracted as a proton, is found to inactivate the enzyme irreversibly.

(1) R. H. Abeles and C. T. Walsh, *J. Amer. Chem. Soc.*, **95**, 6124 (1973).
(2) M. Morisaki and K. Bloch, *Biochemistry*, **11**, 309 (1972).
(3) Plasma amine oxidase is a nonflavoprotein oxidase, which catalyzes the oxidation of certain primary amines according to the equation $RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + H_2O_2 + NH_3$. The enzyme is believed to contain Cu^{II} and pyridoxal phosphate: H. Yamada and K. T. Yasunobu, *J. Biol. Chem.*, **237**, 3077 (1962); **238**, 2669 (1963). We feel, however, that the presence of pyridoxal phosphate is not firmly established. The enzyme used in these studies was purified and assayed according to the procedure of H. Yamada and K. T. Yasunobu, *J. Biol. Chem.*, **237**, 1511 (1962).
(4) R. Hevey and R. H. Abeles, unpublished results.

(10) G. W. Frimpter, *Science*, **149**, 1095 (1965); F. C. Brown and P. H. Gordon, *Biochim. Biophys. Acta*, **230**, 434 (1971).
(11) W. Washtien and R. H. Abeles, unpublished experiments.
(12) C. T. Walsh R. H. Abeles, and H. R. Kaback, *J. Biol. Chem.*, 247, 7858 (1972).

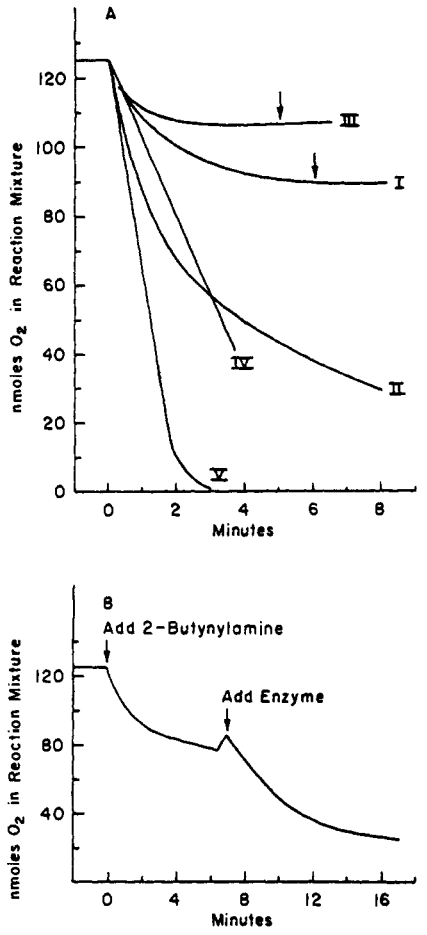


Figure 1. Oxygen uptake with 2-chloroallylamine (I), 2-butynylamine (II), 2-propynylamine (III), benzylamine (IV), and allylamine (V). The reaction for I, II, IV, and V contained 34 μmol of sodium phosphate (pH 7.2) and 100 units of plasma amine oxidase of specific activity 70, reaction temperature 25° (activity units defined according to ref 3). To the solution preequilibrated with air in the oxygen electrode was added 2 μmol of substrate or inhibitor. The final volume in the cell was 0.5 ml. (In the reaction with I, 2 μmol of benzylamine was added at the point indicated by the arrow. In reaction with III, the preequilibrium mixture was the same except that only 0.19 μmol of inhibitor was used. After 5 min, 2 μmol of allylamine was added at the point indicated by the arrow.)

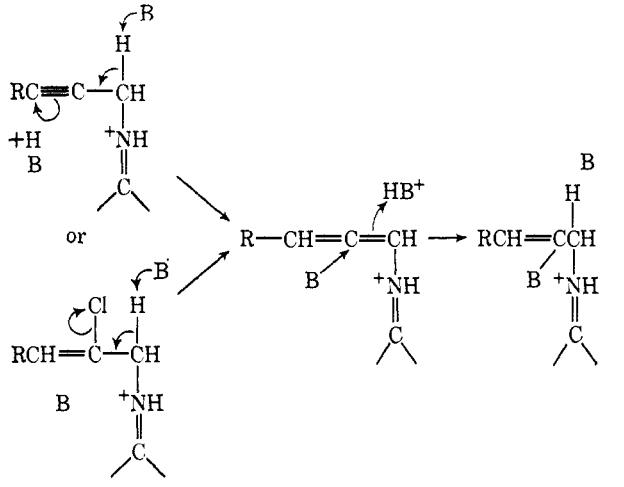
Figure 1 summarizes the results obtained when the enzyme is allowed to react with I, 2-butynylamine (II), 2-propynylamine (III), benzylamine (IV), and allylamine (V).[5] All compounds consume O_2 and are, therefore, substrates for amine oxidase. With I, II, and III, but not IV or V, O_2 uptake ceases before all of the O_2 is depleted and is not reinitiated by the addition of IV or V at the points indicated in Figure 1. The rate of inactivation is reduced in the presence of benzylamine. If more enzyme is added (Figure 1B) after O_2 consumption has ceased, O_2 consumption resumes, initially, at the uninhibited rate. Inactivation is, therefore, not due to accumulation of an inhibitory substance. When inactivated enzyme is dialyzed for 12 hr against 30 mM potassium phosphate buffer (pH 7.2) or is passed through Sephadex G-25 to remove small molecules, enzyme activity is not restored. These results show that I, II, and III are substrates as well as irreversibly inactivators of plasma amine oxidase. Only those substrates which may react to form an allene appear to be inactivators.

When amine oxidase is inactivated with 2-propynyl-[3-^{3}H]amine[6] and passed through Sephadex G-25, the protein peak contains radioactivity, 7.3×10^3 cpm/mg of protein. When the pooled peak fractions were dialyzed 16 hr against 30 mM potassium phosphate buffer (pH 7.2), some radioactivity was lost. The protein, after dialysis, contained 4.0×10^3 cpm/mg of protein or 2.0 mol of inhibitors/mol of protein.[7] Dialysis of the radiolabeled inactivated enzyme without prior treatment with Sephadex G-25 results in a stoichiometry similar to that observed after dialysis of the Sephadex G-25 treated labeled protein. Exposure of the labeled enzyme for 30 min to 0.1 N NaOH–0.1 N HCl or for 11 hr to 8.0 M urea followed by extensive dialysis does not reduce the amount of radioactivity associated with the protein. These results indicate that the inhibitor is covalently bound to the protein. Furthermore, the stability of the radioactivity to 0.1 N NaOH indicates that the acetylenic linkage is no longer present in the inactivator–enzyme adduct, since in compound III the C-3 proton exchanges rapidly under basic conditions.

When the enzyme is inactivated by reaction with $NaBH_4$[8] or with I and then exposed to 2-propynyl-[3-^{3}H]amine, no radioactivity is incorporated into the protein indicating that enzymic activity is necessary for covalent bond formation between enzyme and III.

We tentatively propose that the inactivation occurs through the sequence of reactions shown in Scheme I.

Scheme I



The inactivator is bound to the enzyme as a Schiff base.[9] A basic group (B) at the active site removes a proton leading to the allene intermediate which subsequently adds a nucleophile from the enzyme to form the observed covalent adduct.

(5) Compounds III, IV, and V were purchased from Aldrich Chemical Co. and recrystallized as the HCl salts. The other compounds were prepared as follows: (I) A. J. Speziale and P. C. Hamm, *J. Amer. Chem. Soc.*, **78**, 2556 (1956) as modified by A. T. Bottini, V. Dev, and J. Klinck, *Org. Syn.*, **43**, 6 (1963); (II) E. K. Schulte and K. P. Reiss, *Chem. Ber.*, **87**, 964 (1954); H. R. Ing and R. H. F. Manske, *J. Chem. Soc.*, 2348 (1926).

(6) Prepared by exchange in T_2O according to C. Eaborn, G. A. Skinner, and D. R. M. Walton, *J. Chem. Soc. B*, 922 (1966); final specific activity of the amine was 1.2×10^6 cpm/μmol.

(7) This calculation is based on the assumption that the molecular weight of the enzyme is 1.7×10^5 [H. Yamada, P. Gee, M. Ebata, and K. T. Yasunobu, *Biochim. Biophys. Acta*, **81**, 1965 (1964)] and the specific activity is 500.

(8) H. Yamada and K. T. Yasunobu, *J. Biol. Chem.*, **238**, 2669 (1963).

(9) Some evidence exists for the presence of a cofactor, possibly pyridoxal phosphate, at the active site (see ref 8).

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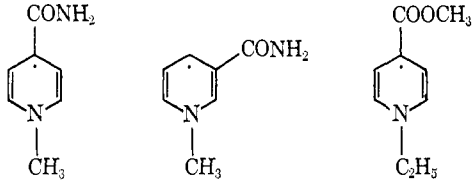
Bimolecular Disappearance of Pyridinyl Radicals in Water

Sir:

The 1-ethyl-4-carbamidopyridinyl radical is stable



4a 3a 4e

for hours in acetonitrile.[1] We now report that the related 1-methyl radical (**4a**) disappears in water at a rate which is proportional to the square of the radical concentration and has the unusual feature of being strongly pH dependent.

Pyridinyl radicals were generated through one-equivalent reduction of the corresponding pyridinium ion[2-4] in aqueous buffers containing the appropriate combinations of sodium formate and formic acid, phosphate, or sulfuric acid, using 2–5 μsec pulses of 10 MeV electrons.[5] Optical transmission was monitored with an EMI 9558 photomultiplier. Dosimetry was based on the absorptions due to **3a**.[3]

The **4a** radical was found to disappear by second-order kinetics with rate constants k (defined by $d[Py\cdot]/dt = -k[Py\cdot]^2$) at pH 6.9 and 4.0 of 2.5×10^4 and 6.8×10^8 M^{-1} sec^{-1}, respectively. Rate constants measured for intermediate pH values suggested a second-order dependence on $[H^+]$, as illustrated in Figure 1. The rate constant for the disappearance of **3a** was 6.7×10^7 M^{-1} sec^{-1}, in agreement with the previous determination,[2] and was independent of pH in the range over which k_{4a} varied.

The absorption spectrum of **4a** was the same at pH 6.9 and 4.0 and exhibited a narrow peak with a maximum close to 305 nm and a broader peak with a maximum at 405 nm. Extensive protonation at pH 4.0 is therefore excluded. In more acid solutions (below H_0 of about -2) spectra were observed in which the 405-nm peak was somewhat lower in intensity and shifted to 425 nm, perhaps because of protonation of the amide group, as reported for benzamide in the same acidity

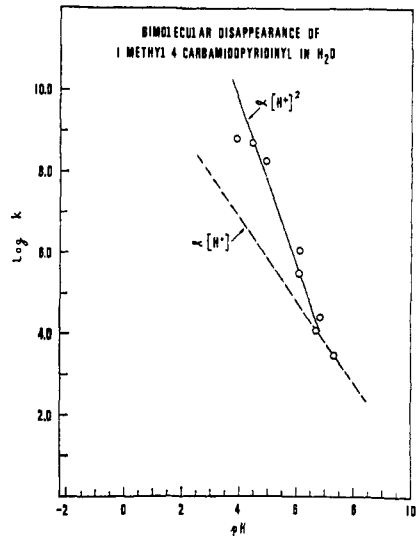


Figure 1. Log k, bimolecular rate constant for disappearance of 1-methyl-4-carbamidopyridinyl radical in water solution, plotted against pH. The dashed line represents a process linear in $[H^+]$ and the solid line a process proportional to $[H^+]^2$.

range.[6] The products of the reaction of **4a** radicals could not readily be determined, but the pyridinyl radical **4e** can conveniently be prepared in a pure form[7-9] and has previously been found to react fairly rapidly in water. By mixing small amounts of dilute acetonitrile solutions of **4e** with an aqueous buffer in a quartz cell in a Cary spectrophotometer, the radical has now been found to disappear in a second-order reaction with a rate constant at pH 8.0 of 3.8×10^3 M^{-1} sec^{-1}. Addition of modest amounts of acetonitrile dramatically decreased the rate of disappearance. Using larger quantities of **4e**, the products of the reaction have been found to be the corresponding pyridinium ion and the hydrolysis products of the dihydropyridine in *ca.* 1:1 ratio, based primarily on nmr spectra. The pH-dependent disappearance of the **4a** radicals can be explained by Scheme I. This mechanism accounts

Scheme I

$$Py\cdot + Py\cdot \rightleftharpoons [Py\cdot, Py\cdot \rightleftharpoons Py^+, Py^-] \quad (a)$$

$$[Py^+, Py^-] + H^+ \rightleftharpoons [Py^+, PyH] \quad (b)$$

$$[Py^+, PyH] \longrightarrow Py^+ + PyH \quad (c)$$

$$[Py^+, PyH] \xrightarrow{H^+} Py^+ + PyH_2^+ \quad (d)$$

$$PyH \xrightarrow[H_2O]{H^+} \text{products} \quad (e)$$

$$PyH_2^+ \xrightarrow{H_2O} \text{products} \quad (f)$$

for the products of the reaction of the **4e** radicals, which are taken to be like those of **4a**. It also explains the stability of the radicals in less-polar solvents, since the electron-transfer equilibrium shown in the first step would be shifted to the left by amounts which could readily be estimated from the Z values for the solvents.[9-11]

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(2) A. J. Swallow, *Biochem. J.*, **54**, 253 (1953).
(3) E. J. Land and A. J. Swallow, *Biochim. Biophys. Acta*, **162**, 327 (1968).
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(5) J. P. Keene, *J. Sci. Instrum.*, **41**, 493 (1964).
(6) R. B. Moodie, P. D. Wade, and T. J. Whaite, *J. Chem. Soc.*, 4273 (1963).
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(9) M. Mohammad and E. M. Kosower, *J. Amer. Chem. Soc.*, **93**, 2709, 2713 (1971).
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(11) **4e** is stable indefinitely in a 0.05 M solution in acetonitrile at 5°.