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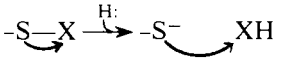
Studies on the Mechanism of Action of Plasma Amine Oxidase†

Robert H. Suva‡ and Robert H. Abeles*

ABSTRACT: The product released when plasma amine oxidase catalyzes the oxidation of *p*-hydroxybenzylamine is *p*-hydroxybenzyaldehyde. No free imine can be detected. However, when this oxidation is carried out in the presence of NaB^3H_4, stereospecifically tritiated *S*-[α-^{3}H]-*p*-hydroxybenzylamine can be isolated. We concluded that oxidation of the substrate leads to the formation of an enzyme-bound imine, which is then hydrolyzed by the enzyme prior to release. In the presence of $NaBH_4$, some of the enzyme-bound imine (approximately 10%) was trapped. When the enzyme is reduced under anaerobic conditions with ethylglycinate in ^3H_2O or ethyl[α-^{3}H]glycinate in H_2O, no nonexchangeable ^{3}H is introduced into the reduced enzyme. The failure to incorporate nonexchangeable hydrogen into the reduced enzyme and the intermediate formation of an imine make the proposal very unlikely that pyridoxal-phosphate, or a structurally related molecule, is a cofactor which is directly reduced by the substrate. When the enzyme is inactivated by bromoethylamine, a suicide inactivator which probably reacts with the reduced enzyme, a number of functional groups, including a cysteine SH group, are labeled. When the enzyme is first reduced by substrate and then denatured, one cysteine SH group can be labeled in the nonreduced enzyme. A peptide map of the NEM-labeled enzyme shows that a single peptide is predominantly labeled in the reduced enzyme. We concluded that, upon reduction of the enzyme, one SH group is released per subunit. In the oxidized enzyme, the SH group is bonded to an as yet unidentified molecule (X). The reduction of the enzyme involves:



Plasma amine oxidase catalyzes the oxidation of primary amines:

$$RCH_2NH_3^+ + O_2 + H_2O \rightarrow RCHO + NH_4^+ + H_2O_2 \quad (1)$$

When substrate is added to the enzyme in the absence of O_2, a stoichiometric amount of product is formed (Oi et al., 1970; Reed & Swindell, 1969), and the enzyme becomes reduced as indicated by the altered absorption spectrum of the enzyme (Yamada & Yasunobu, 1962). The reduced enzyme also differs from the oxidized enzyme in that it is unaffected by several "suicide" inactivators such as 2-bromoethylamine (Neumann et al., 1975) and phenylglycinate (Maycock et al., 1975; Suva, 1978).

The identity of the group at the active site which becomes reduced is presently unknown. Since the enzyme contains Cu^{2+}, it appears possible that Cu^{2+} is reduced, but ESR studies show that the oxidation state of Cu^{2+} is unchanged in the reduced enzyme (Yamada et al., 1963). It has also been frequently proposed (Von Werle & Von Rechman, 1949; Yamada & Yasunobu, 1963) that pyridoxal functions as electron acceptor. This proposal, first made in 1949, is based on the spectral properties of the enzyme and the sensitivity of the enzyme to carbonyl reagents. However, pyridoxal cannot be resolved from the enzyme and all attempts to isolate a pyridoxal derivative from the enzyme have been unsuccessful. This negative evidence has cast doubt on the conclusion that plasma amine oxidase is a pyridoxal enzyme (Yasunobu et al., 1976; Tsurushiin et al., 1975). The possibility remains that a structurally modified form of pyridoxal, possibly covalently attached to the protein, is involved and therefore cannot be readily identified.

In this paper, we described a number of experiments designed to test the pyridoxal mechanism and to elucidate the structure of the active site.

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Experimental Section

Reagents and Substrates

Pronase (B grade) was purchased from Calbiochem. [^{3}H]H_2O, [^{3}H]$NaBH_4$, and *N*-[^{14}C]ethylmaleimide were purchased from New England Nuclear. [U-^{14}C]Ethanolamine and *N*-ethyl[1,4-^{14}C]maleimide were purchased from Amersham/Searle. All other chemicals were research grade and purified if necessary. Mitochondrial monoamine oxidase was a gift from Dr. Thomas Singer.

Synthetic Procedures

p-Hydroxybenzylamine Hydrochloride. *p*-Cyanophenol (0.6 g) was dissolved in 10 mL of dioxane and added dropwise to 0.3 g of $LiAlH_4$ in 10 mL of refluxing dioxane. Refluxing was continued for 1 h. H_2O (5 mL) was slowly added to the cooled solution and the resulting precipitate removed by centrifugation. HCl (1 N) was added dropwise until the dark blue solution became orange. The solvent was removed by rotary evaporation and the amine hydrochloride crystallized from ethanol–acetone, mp 191–193 °C, lit. mp 191–192 °C (Sekiya et al., 1961).

p-Hydroxybenzaldimine. *p*-Hydroxybenzaldimine was prepared using the procedure of Moffett & Hoehn (1947) for related imines. To a refluxing solution of 12 g of *p*-hydroxybenzaldehyde in 30 mL of benzene was added gaseous NH_3. H_2O, which formed in the reaction, was removed by azeotropic distillation and collected in a Dean-Stark trap. When the H_2O volume reached 1 mL, the reaction was cooled and the benzene decanted. The solid residue was triturated in ether several times. The excess solid was removed by filtration, and the imine was precipitated by bubbling dry HCl through the ether. The imine hydrochloride was rapidly filtered and immediately stored in vacuo over P_2O_5. The imine was dissolved in dry dimethylformamide before use. The material was extremely hygroscopic and was used without further purification. The following criteria demonstrate the purity of the *p*-hydroxybenzaldimine hydrochloride. The imine hydrochloride was reacted with phenylhydrazine to give *p*-hydroxybenzaldehyde phenylhydrazone (Pasto & Johnston, 1969), mp 180–183 °C; the authentic phenylhydrazone prepared by the same procedure had mp 182–184 °C. Hydrolysis of a known amount of the imine (by weight) at pH 9 resulted in quantitative formation of *p*-hydroxybenzaldehyde as determined spectrophotometrically. In aqueous solution the imine has an absorption peak at λ_{max} = 365 nm, $\epsilon = 1.2 \times 10^4$ M^{-1} cm^{-1}, pH 9. This peak disappears with time, with the concomitant appearance of the *p*-hydroxybenzaldehyde peak (λ_{max} = 330 nm). Both processes are first order and have the same rate constant (k = 0.3 min^{-1}). Bromo[4-^{14}C]ethylamine was prepared as described by Neumann et al. (1975) and purified by chromatography on Dowex-50 (H^+), 0.6 × 10 cm, in 1.5 N HBr/50% MeOH. The bromoethylamine ran as a symmetrical peak at V_e/V_0 = 5; the specific activity was constant through the peak. Final specific activity = 1.1×10^7 dpm/μmol. Concentration of the amine was determined by ninhydrin assay.

S-(2-Hydroxyethyl)cysteine (Carson & Wong, 1964), *S*-(2-aminoethyl)cysteine (Cavallini et al., 1955), and *S*-succinylcysteine (Calam & Waley, 1963) were synthesized using standard literature procedures and all products were obtained as crystalline solids with satisfactory melting points.

Enzyme Purification and Assay. Plasma amine oxidase was purified by a modification of the procedures of Yamada & Yasunobu (1975). A final purification was achieved by chromatography on aminohexyl-Sepharose according to Toraya et al. (1976). The enzyme was assayed spectrophotometrically by the procedure of Tabor et al. (1954). The enzyme was added to 1 mL of 50 mM KPO_4, pH 7.4, containing 4 mM benzylamine hydrochloride at 25 °C. The production of benzaldehyde was monitered at λ 250 nm: the extinction coefficient of benzaldehyde was taken as 12 500 M^{-1} cm^{-1}. The activity is expressed in international units; 1 unit catalyzes the formation of 1 μmol of benzaldehyde per min under the reaction conditions.

Previous publications have expressed the enzyme activity in spectrophotometric units, with 1 unit defined as the amount of enzyme causing an OD change of 0.001/min in a 3-mL assay. One international unit = 4250 spectrophotometric units. Enzyme activity was also measured in a Clark-type oxygen electrode. Enzyme was equilibrated in the electrode with 0.5 mL of the appropriate buffer, and reactions were initiated by the addition of substrate.

Experimental Procedures

^{3}H Incorporation into Reduced Enzyme from ^3H_2O and Ethyl[2-^{3}H]glycinate. The reaction was carried out in a two-compartment vessel capped with serum cap. One compartment contained 0.35 U of plasma amine oxidase in 0.4 mL of 0.1 M K^+, PO^{3-} buffer, pH 7.4. The vessel was made anaerobic by repeated flushing with argon. Ethylglycinate hydrochloride (0.1 μmol) in 50 μL was then injected to reduce the enzyme. The reduced enzyme was denatured by the addition of 0.35 g of guanidine hydrochloride from the side arm, followed by 10 μL of mercaptoethanol. The solution was then incubated at 37 °C for 30 min. Air was admitted and the enzyme was separated from small molecules (i.e., ^3H_2O or ethyl[2-^{3}H]glycinate) by chromatography on Sephadex G-25 (1.0 × 40 cm column) in 6 M guanidine hydrochloride at 45 °C. Two separate experiments were carried out. In one experiment ethyl[2-^{3}H]glycinate (7.0×10^5 cpm/μmol) was added. In the other experiment, nonisotopic ethylglycinate was used and the reaction was carried out in ^3H_2O (6×10^5 cpm/μmol).

Imine Detection. The oxidation of *p*-hydroxybenzylamine was followed spectrophotometrically. The assignment of the absorption peak at λ = 365 nm to the *p*-hydroxybenzaldimine is based on the absorption of the synthetic material. In addition, the kinetics of appearance of this peak in the monoamine oxidase reaction are consistent with its assignment to the imine. If the imine is produced by the enzyme and released into solution, then the amount of imine in solution is described by:

$$\text{amine} \xrightarrow[k_1]{\text{enz}} \text{imine} \xrightarrow[k_2]{H_2O} \text{aldehyde} + NH_3$$

The formation of the imine is zero order and the hydrolysis is first order so that the amount of imine in solution is:

$$[I] = (k_1/k_2)(1 - e^{-k_2 t})$$

This predicts that the imine will approach a steady-state concentration in a first-order process with rate constant k_2, the rate hydrolysis of the imine. Experimentally, the OD_{365} approaches a steady state in the monoamine oxidase reaction in a first-order process with rate constant of 0.25 min^{-1}. Under these conditions, the nonenzymatic hydrolysis rate of the chemically synthesized imine was 0.30 min^{-1}. Also, the rate of aldehyde appearance in this reaction gradually increases and approaches a constant rate in a first-order process described by:

$$V = k_2[I] = k_1(1 - e^{-k_2 t})$$

Again, the experimentally observed rate of aldehyde appearance in the monoamine oxidase reaction approaches the con-

stant rate in a first-order reaction with rate constant of 0.26 min^{-1}. The peak at λ = 365 nm therefore corresponds to *p*-hydroxybenzaldimine.

Imine Trapping Experiment. [^{3}H]$NaBH_4$ (10 mCi) and 30 mg of $NaBH_4$ were dissolved in 0.3 mL of 0.1 N NaOH, and 0.1 mL of this solution was added to a solution of 2 μmol of *p*-hydroxybenzylamine in 2.0 mL of 0.1 N $Na_2B_4O_7$, pH 8.6. The experiment was started by adding 0.5 U of plasma amine oxidase and stopped 60 s later by the addition of 1 mL of 15% Cl_3CCOOH. After H_2 evolution had stopped, the excess $^3H_2$ was removed under vacuum. Cl_3CCOOH was removed by extraction with ether (3 × 5 mL). The aqueous layer was evaporated under reduced pressure, dissolved in H_2O, and applied to Dowex 50 (H^+) in H_2O (0.6 × 2 cm). The column was washed with 10 mL of H_2O, 10 mL of 0.1 N NaOAc, and 10 mL of H_2O. The *p*-hydroxybenzylamine was eluted with 30 mL of 1 N NH_3 in 65% EtOH. The ammonia eluate was evaporated and dissolved in 1 mL of 0.05 M $Na_2B_4O_7$, pH 8.6, 50% EtOH. The solution was applied to an Amberlite CG-50 column (Kakimoto & Armstrong, 1962), 0.6 × 17 cm, equilibrated with 0.5 N $Na_2B_4O_7$, pH 8.6. The column was eluted with the same buffer, and about 1.3-mL fractions were collected. The OD_{277} was measured and 50-μL aliquots were removed for scintillation counting. *p*-Hydroxybenzylamine was quantitated by oxidation to completion with plasma amine oxidase (in 0.1 M Tris-HCl, pH 8.9), and the aldehyde absorbance measured and compared with the aldehyde produced by addition of a standard solution of *p*-hydroxybenzylamine in the same cuvette.

The control reaction contained 2.0 μmol of *p*-hydroxybenzylamine and 2 μmol of NH_3 in 2 mL of the same borate buffer. An identical amount of the same [^{3}H]$NaBH_4$ solution used above was added and the reaction initiated by the addition of 1 μmol of *p*-hydroxybenzaldehyde. The reaction was stopped after 1 min with Cl_3CCOOH as above and the *p*-hydroxybenzylamine was purified by exactly the same procedures. As a standard, a sample (about 10 mg) of chemically synthesized *p*-hydroxybenzaldimine was reduced for 1 min in the borate buffer using the same [^{3}H]$NaBH_4$ solution used above and purified by the same procedures.

Inactivation by Bromoethylamine. Enzyme (1–2 U) in 1 mL of 70 mM KPO_4, pH 6.7, containing 0.015 mg catalase was inactivated by the addition of four equal aliquots of bromo[U-^{14}C]ethylamine (total = 1.2 μmol/U). Oxygen consumed in the reaction was replaced by the addition of H_2O_2 (up to 0.6 μmol/U). When the enzyme activity had decreased to 95% of the original value, the reaction was chilled on ice and immediately chromatographed on Sephadex G-25f (1 × 40 cm). Fractions were monitored for protein and ^{14}C and the peak protein fractions were assayed for enzyme activity. Stoichiometry of inactivation was calculated from the ^{14}C incorporated per unit of enzyme inactivated assuming that pure enzyme has a specific activity of 0.36 U/mg and a molecular weight of 170 000/dimer.[1]

For further analysis, the pool of labeled protein fractions was mixed with 10–15 mg of $NaBH_4$, and guanidine hydrochloride was added to a final concentration of 6 M. One drop of antifoam emulsion was added to control foaming. The solution was incubated at 0 °C for 6 h. Excess borohydride was destroyed by dropwise addition of 2 N HCl to pH 2–3. The solution was then dialyzed vs. 4 × 100 mL of 0.05 M Tris-HCl, pH 8.1.

[1] Previously published calculations (Neumann et al., 1975) of the stoichiometry of bromo[^{14}C]ethylamine-labeling were based on a specific activity of 0.14 U/mg, and an erroneous value for the absorbance of the enzyme at 280 nm. Accordingly, these values should be corrected by a factor of 2.5.

Analysis of the ^{14}C-Labeled Dialysate. Aliquots (1 mL) of the first dialysate of the ^{14}C-inactivated reduced enzyme above were applied to Dowex 50 (H^+) in H_2O (0.6 × 5 cm) and the columns were washed with H_2O. Fractions (1 mL) were collected directly into scintillation vials and counted. The main body of the dialysate was passed through Dowex 50 (H^+) (2.6 × 21 cm) in H_2O. The ^{14}C-containing eluate was concentrated by bulb-to-bulb distillation and dissolved in 3 mL of H_2O. An aliquot (100 μL) of this solution was passed through Dowex 1 (OH^-) as above.

Periodate Oxidation. To 0.5 mL of the ^{14}C-containing solution was added 60 μL of 1 M ethylene glycol, 10 mL of H_2O, and 10 mL of fresh 70 mM $NaIO_4$. After 20 min at room temperature, 3 drops of 1 N HCl and 1 mL of 10% KI were added and the I_2 which formed was tritiated with 10% $NaAsO_3$. To the solution was added 6 mL of 1% dimedone in 0.5% Na_2CO_3, and the resulting suspension was refrigerated overnight. The derivative was filtered, dried, and recrystallized from ethanol–H_2O to constant specific activity.

Peptide Maps. The [^{14}C]NEM-labeled protein (0.7 mL, 1 mg) was mixed with 0.6 mL of 1.44 M Tris-HCl, pH 8.6, 0.8 mL of 5% EDTA, 960 mg of urea and 20 μL of 2-mercaptoethanol. The sample was made anaerobic by repeated evacuation and flushing with argon and was incubated 5.5 h at room temperature under a stream of argon. Iodoacetic acid (54 mg in 0.2 mL of 1 N NaOH) was added and the reaction incubated in the dark for 20 min. The solution was dialyzed vs. 4 L of 0.1 M ammonium formate, pH 8.1, 1 mM $CaCl_2$ in the dark overnight. To the reduced carboxymethylated protein was added trypsin-TPCK (40 μL of 1 mg/mL) and the solution was incubated at 37 °C for 6 h; after this time, a second aliquot of trypsin was added and the incubation continued overnight. The digest was lyophilized and dissolved in H_2O (0.45 mL). An insoluble residue was removed by centrifugation before chromatography.

Purification of S-Succinylcysteine from Reduced Enzyme Labeled with [^{14}C]NEM. To the substrate reduced [^{14}C]-NEM-labeled enzyme (1.0×10^5 cpm) was added 10 μmol of *S*-succinylcysteine. The solution was made 6 N in HCl, purged with nitrogen, and sealed and incubated at 120 °C for 48 h. HCl was removed by rotary evaporation and the sample dissolved in 1 mL of H_2O. Half of the solution (0.5 mL) was applied to a Dowex 1 column (0.6 × 50 cm), equilibrated with 0.5 N acetic acid. The column was washed with 30 mL of 0.5 N acetic acid, 30 mL of 1 N acetic acid and the succinylcysteine was eluted with a linear gradient make by adding 100 mL of 1 N HCl to 100 mL of 1 N acetic acid. Fractions (1.5 mL) were collected and 50-mL aliquots were counted for ^{14}C. Fractions 125–136 were evaporated in vacuo and the residue was dissolved in 0.25 mL of H_2O. An aliquot (50 μL) was removed from each fraction and counted for ^{14}C and the remainder assayed for *S*-succinylcysteine with ninhydrin.

Results

Hydrogen Incorporation into the Reduced Enzyme. If the functional group at the active site is a carbonyl group, then reduction of the enzyme by substrate might result in the incorporation of a nonexchangeable hydrogen into the reduced enzyme from either the substrate or the solvent. To test the first possibility, the enzyme was reduced anaerobically with ethyl [α-^{3}H]glycinate (7.0×10^5 cpm/μmol) in a Thunberg type reaction vessel. The reduced enzyme was denatured under anaerobic conditions by the addition of guanidine hydrochloride from the side arm. The denatured enzyme was separated from excess substrate by Sephadex chromatography and the radioactivity associated with the protein was determined. The

enzyme contained 10 cpm/mg. If ^3H were transferred quantitatively from the substrate to the reduced enzyme, the enzyme should contain 1.6×10^3 cpm/mg. Thus, no significant hydrogen transfer from substrate to reduced enzyme could be detected. It is possible that this lack of tritium incorporation is due to isotope selection. It would correspond to an isotope effect of 160 which seems very large. In the oxidation of [α-^{3}H]ethylglycinate, no tritium isotope effect was observed.[2] We, therefore, believe the lack of tritium incorporation indicates that no hydrogen is transferred to the reduced enzyme.

An analogous experiment was also done in which the enzyme was reduced with nonradioactive substrate in ^3H_2O. The purified protein incorporated 160 cpm/mg, while in a control experiment carried out under identical conditions, except that reduction by substrate was omitted, 140 cpm/mg was incorporated. Incorporation of one atom of ^3H per subunit would have resulted in a difference of 1.4×10^3 cpm/mg between the experiment and control. It is unlikely that the low incorporation is due to isotope discrimination since an unreasonably high discrimination factor would be required. Thus, the reduction of the enzyme by substrate also does not incorporate hydrogen from the solvent. From these experiments it can be concluded that the group on the enzyme which is reduced by the substrate is probably not a carbonyl group, or any other group in which the hydrogen introduced in the reduction process is not readily exchangeable.

Structure of Oxidation Product. Experiments were carried out to determine if the product released into solution by plasma amine oxidase is the imine or the aldehyde. The oxidation of *p*-hydroxybenzylamine was studied since the corresponding imine can be readily determined spectrophotometrically ($\epsilon = 1.2 \times 10^4$ M^{-1} cm^{-1}, $\lambda_{max} = 365$ nm, see Experimental Section). When the enzyme is mixed with *p*-hydroxybenzylamine, no imine can be detected (Figure 1). The spectra show only the accumulation of the aldehyde, $\lambda_{max} = 330$ nm. In the control experiment, plasma amine oxidase is replaced with mitochondrial monoamine oxidase, a flavoenzyme which is known to produce the imine (Patek et al., 1972). In this case, one sees the initial appearance of the imine in solution (peak at 365 nm). Hydrolysis of this imine results in the subsequent appearance of the aldehyde peak (330 nm) (Figure 1). Thus, plasma amine oxidase releases the aldehyde into solution and not the imine.

It is possible that the imine is an intermediate in the reaction but that the enzyme catalyzes its hydrolysis. If plasma amine oxidase produces an enzyme-bound imine, then reduction with [^{3}H]$NaBH_4$ might trap this imine before hydrolysis; this reaction would regenerate substrate which contains ^3H. This was tested in the following experiment. Enzyme (20 mU) was added to a solution containing 2 μmol of *p*-hydroxybenzylamine and excess [^{3}H]$NaBH_4$ as described in Experimental Section. After 1 min, the reaction was stopped by the addition of trichloroacetic acid; about 10% of the amine had been oxidized. If any imine had been formed, it would be detected as *p*-hydroxy[α-^{3}H]benzylamine. The pure amine contained 3.0×10^4 cpm of $^3H/\mu$mol. This result suggests that an intermediate imine was reduced by the [^{3}H]$NaBH_4$.

Since both NH_3 and the aldehyde are produced by the enzyme in the above experiment, it is possible that these products condense nonenzymatically to form the imine in solution, and it is this imine that was reduced by the [^{3}H]$NaBH_4$. Therefore, a control experiment was done in which 1 μmol of *p*-hydroxybenzaldehyde was slowly added to a solution containing 1 μmol of NH_3 with the same buffer, substrate, and [^{3}H]$NaBH_4$ as above. This reaction was also stopped after 1 min and the amine purified as before. The pure amine contained 1×10^3 cpm/μmol, 1/30 the value found in the enzyme experiment. In addition, the aldehyde and NH_4^+ concentrations used in this control were at least five times higher than that present in the experiment. Thus, less than 1% of *p*-hydroxy[α-^{3}H]benzylamine isolated in the enzyme experiment was the result of nonenzymatic imine formation.

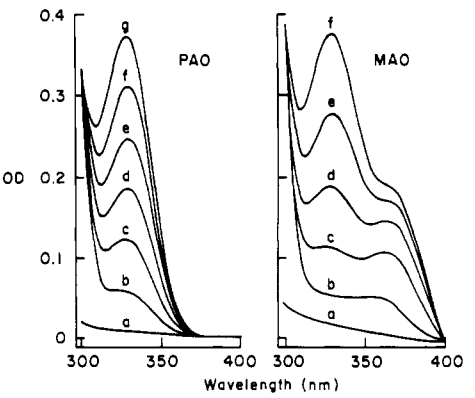


FIGURE 1: Spectral analysis for imine production by plasma amine oxidase. Reactions were carried out in a cuvette containing 1 mL of 0.1 M Tris, pH 8.9, and either plasma amine oxidase (PAO) or mitochondrial monoamine oxidase (MAO) sufficient to oxidize 1 nmol of *p*-hydroxybenzylamine per min. (a) Spectrum of enzyme before addition of substrate; (b–g) spectra initiated at 75-s intervals after the addition of 1 μmol of *p*-hydroxybenzylamine hydrochloride. Scan speed: 100 nm/min.

It is not possible to directly determine the amount of imine trapped in these experiments since it is difficult to determine the specific radioactivity of [^{3}H]$NaBH_4$ or the isotope effect which occurs in the reduction of the imine. To enable us to estimate the amount of [^{3}H]amine found, we reduced a synthetic sample of *p*-hydroxybenzylimine with [^{3}H]$NaBH_4$ under the same conditions under which the enzymatically formed imine was reduced and determined the specific activity of the resulting amine. The specific activity of the amine formed in the nonenzymic control was 2.7×10^6 cpm of $^3H/\mu$mol. We then assumed that the specific activity of the *p*-[^{3}H]hydroxybenzylamine formed in the enzyme experiment is identical with that in the nonenzymic control. One can then calculate that 0.02/μmol (5.4×10^4 cpm) of imine was reduced in the enzyme experiment. Since 0.2 μmol of the substrate was oxidized, about 10% of the oxidation product was trapped.[3]

Since the imine is not released into solution, it is probably that the hydrolysis of the imine is enzyme catalyzed. This implies that reduction of the imine by $NaBH_4$ occurs on the enzyme surface. If this is the case, then this reaction should be stereospecific, producing chirally labeled *p*-hydroxy[α-^{3}H]-benzylamine The chirality of this [^{3}H]amine was determined by oxidation of the amine to completion by plasma amine oxidase and determination of the ^3H distribution in the products. Although no stereochemical data concerning plasma amine oxidase has been published,[4] it is likely that proton removal from the substrate is stereospecific. The results of this experiment are shown in Table I. When a sample of [^{3}H]amine formed in the trapping reaction is oxidized, 70% of the ^3H is isolated as ^3H_2O and 30% is found in the product aldehyde.

[2] Suva, R. H., & Abeles, R. H., unpublished results.

[3] It is possible that some of the [^{3}H]-*p*-hydroxybenzylamine found on the enzyme was oxidized prior to release. If this happens, the amount of imine trapped would be underestimated.

[4] The stereochemistry of the oxidation of benzylamine by plasma amine oxidase has been investigated in the laboratory of J. P. Klinman. It was found that the enzyme is specific for the *pro-S* hydrogen (personal communication).

TABLE I: Chirality of *p*-Hydroxy[α-^{3}H]benzylamine from Trapping Reaction.[a]

substrate	^3H_2O (cpm)	[^{3}H]aldehyde (cpm)
[^{3}H]amine from $NaBH_4$ trapping reaction	904	390
racemic [^{3}H]amine	47 000	49 500
p-hydroxy-(*R*)-[α-^{3}H]-benzylamine	2 050	33 540

[a] Aliquots of *p*-hydroxy[α-^{3}H]benzylamine were incubated with 0.02 unit of plasma amine oxidase and 10^4 units of catalase in 0.5 mL of $NaBO_4$ buffer, pH 8.9. Aldehyde production was followed spectrophotometrically, and, when the reaction was complete, the samples were frozen. ^3H_2O was removed by bulb-to-bulb distillation (2×). The residue was dissolved in water and 50 μmol of HCl, and the [^{3}H]-aldehyde was extracted into ether. *p*-Hydroxy-(*R*)-[α-^{3}H]benzylamine was prepared from racemic [^{3}H]amine as described in the text.

When racemic [^{3}H]amine is oxidized, the distribution of ^{3}H in the aldehyde and the H_2O is equal as expected. This shows that the ^{3}H is introduced by the [^{3}H]$NaBH_4$ to a considerable extent stereospecifically and in the same position from which the enzyme normally abstracts a proton. We conclude from these results that the immediate oxidation product is the imine. The imine is not released from the enzyme, but is hydrolyzed to the aldehyde and NH_4^+ which are the actual products released by the enzyme.

Stereochemistry of the Plasma Amine Oxidase Reaction. In order to establish the absolute stereochemistry of the $NaBH_4$ reduction, it is necessary to determine the absolute stereochemistry of plasma amine oxidase reaction. This was done by reference to mitochondrial monoamine oxidase which is known to remove the R hydrogen from the substrate (Belleau et al., 1962). A sample of racemic *p*-hydroxy[α-^{3}H]benzylamine was incubated with monoamine oxidase for 4 h which resulted in oxidation of 75% of the [^{3}H]amine as determined by the isolation of oxidation products ([^{3}H]aldehyde + ^3H_2O). However, spectrophotometric determination of the amount of aldehyde produced showed that over 90% of the amine had been oxidized. Therefore, the remaining amine was enriched in ^{3}H, due to a discrimination against ^{3}H by the enzyme. This implies that the residual amine is tritiated in the R position since the enzyme normally removes this hydrogen. When the R-labeled amine was incubated with plasma amine oxidase, 94% of the ^{3}H was found in the aldehyde (Table I). This result shows that plasma amine oxidase removes the *pro-S* hydrogen from *p*-hydroxybenzylamine. It can also be concluded that, during the reduction of the imine on the enzyme by $NaBH_4$, the hydride is preferentially introduced to the *pro-S* face of the imine, producing *p*-hydroxy-(*S*)-[α-^{3}H]benzylamine.

Microbiological Pyridoxal Analysis. Enzyme samples were either digested with Pronase, hydrolyzed in 0.05 M H_2SO_4 or reduced by substrate and hydrolyzed in 6 N HCl prior to analysis. Each sample contained enzyme equivalent to 10 μg of pyridoxal if pyridoxal was a cofactor on the enzyme. The samples were then analyzed for pyridoxal by a microbiological assay. (We wish to thank Professor E. E. Snell for carrying out the assay.) No significant pyridoxal was found in any sample while the control (no enzyme) contained 0.18 μg. Thus, less than 1% of the expected amount of pyridoxal was found.

Reaction of Plasma Amine Oxidase with Bromoethylamine. Bromoethylamine is a "suicide" inactivator of plasma amine oxidase. The properties of this inactivator suggest that it covalently labels the enzyme at or near the active site (Neumann et al., 1975; Suva, 1978). We hoped that further investigation of the mechanism of this inactivation may elucidate the structure of the active site. It was proposed that in the course of inactivation, a two-carbon aldehyde, derived from the inactivator, becomes attached to an active site nucleophile (Neumann et al., 1975). We, therefore, decided to reduce the inactivated enzyme with $NaBH_4$ prior to degradation of the enzyme, since $NaBH_4$ reduction of the aldehyde group should prevent reaction with amino groups liberated upon hydrolysis of the enzyme. The enzyme was with inactivated bromoethylamine and then reduced with $NaBH_4$ (see Experimental Section). The reduced enzyme was dialyzed 24 h against 1% NH_4HCO_3 buffer. During dialysis, 30% of the radioactivity associated with the enzyme was lost. The material released from the enzyme during dialysis was a neutral molecule since it was not absorbed on either cation- or anion-exchange resins. Carrier ethylene glycol was added to the radioactive material followed by $NaIO_4$. The formaldehyde produced was isolated as the dimedone adduct. The adduct contained 80–90% of the radioactivity present in the reaction mixture. It was concluded that the material released from the bromoethylamine inactivated enzymes is probably ethylene glycol.

The remaining ^{14}C-labeled enzyme was stable to dialysis from pH 2.5–11. This labeled enzyme was hydrolyzed in 6 N HCl to determine the identity of the labeled group. Chromatography of the hydrolysate on Dowex 50 (Schroeder, 1972) revealed that at least 12 different ^{14}C-labeled compounds were formed. A similar result was obtained when the labeled enzyme was digested with Pronase instead of the acid hydrolysis. One of the ^{14}C-containing compounds isolated from the labeled enzyme (by both acid or Pronase hydrolysis) was identified as *S*-(2-hydroxyethyl)cysteine by cochromatography with authentic *S*-(2-hydroxyethyl)cysteine in butanol/acetic acid/H_2O, 4/1/5 (R_f 0.28), 80% aqueous phenol (R_f 0.67), and coelectrophoresis at pH 8.9 (R_{asp} = 0.65). In addition, the electrophoretic mobility of the ^{14}C-containing material and *S*-(2-hydroxyethyl)cysteine were altered identically by oxidation with 10% H_2O_2 (20 °C, 15 s).

Since Dowex chromatography showed that hydroxyethylcysteine was only one of several labeled compounds, the fraction of ^{14}C present in the labeled enzyme present as hydroxyethylcysteine was determined. The ^{14}C-labeled, reduced enzyme (containing 7.2×10^4 cpm) was mixed with 20 μmol of *S*-(2-hydroxyethyl)cysteine and the mixture hydrolyzed in 6 N HCl for 24 h. The *S*-(2-hydroxyethyl)cysteine was repurified and contained 340 cpm of ^{14}C per μmol. Thus, 10% (7000 cpm) of the starting ^{14}C was attached to cysteine residues.

Bromoethylamine appears to label a number of functional groups on the enzyme. This could occur in one of two ways. Oxidation of bromoethylamine will produce bromoethylimine, a reactive alkylating agent. This molecule while still bound to the enzyme has sufficient freedom of motion to react with a number of functional groups at or near the active site. Alternatively, the molecule initially reacts with one functional group, but the covalent linkage formed is labile and the inactivator is transferred to other functional groups when the enzyme is denatured. Precedence for migration of an active site directed alkylating agent is provided by the inactivation of triose phosphate isomerase by bromohydroxyacetone phosphate (Coulson et al., 1970). Here, migration of the label from a carboxyl group to a tryosine hydroxyl group occurs.

Bromoethylamine does not appear to be a useful reagent for the identification of active site residues and, therefore, the mode of action of this inactivator was not further pursued. It is surprising, however, that a cysteine group was labeled by the inactivator since plasma amine oxidase does not contain a re-

TABLE II: Reaction of Plasma Amine Oxidase with [^{14}C]NEM.[a]

expt	enzyme act. (U/mg)	mol of [^{14}C]NEM/mol of enzyme		
		reduced enzyme	control	difference
1	0.23	2.3	0.9	1.4
2	0.18	2.7	0.9	1.7
3	0.26	1.8	0.5	1.3
4	0.32	2.7	0.8	1.9

[a] Enzyme (5–6 mg in 0.4 mL of 50 mM KPO_4, pH 6.6) was made anaerobic by repeated evacuation and purging with argon, leaving a final positive pressure of argon. The enzyme was reduced by adding 0.3 μmol of anaerobic ethyl glycinate. After incubation for 10 min at 20 °C, the enzyme was mixed with guanidine hydrochloride from a side arm (890 mg per mL), and [^{14}C]NEM was added to 10^{-3} M. The solution was incubated at 37 °C for 90 min (30 min for experiment 4), air was admitted, and the solution was chromatographed on Sephadex G-25 (f) in 6 M urea (1 × 40 cm). The stoichiometry is based on ^{14}C bound per unit of enzyme inactivated based on pure enzyme of 0.36 U/mg. The control does not contain substrate.

active –SH group (Wang et al., 1968). The results obtained with bromoethylamine suggested that an –SH group may become available for reaction upon reduction of the enzyme.

Sulfhydryl Content of the Reduced Enzyme. To determine whether a sulfhydryl group becomes available when the enzyme is reduced, ethyl glycinate was added to 5–6 mg of enzyme under anaerobic conditions. Addition of ethyl glycinate causes an immediate bleaching of the pink enzyme. The reduced enzyme was denatured under anaerobic conditions by the addition of guanidine hydrochloride. [^{14}C]NEM was then added and the anaerobic reaction mixture was maintained at 37 °C for 90 min. Excess NEM was removed by gel filtration and the radioactivity of the protein was determined. A control experiment was carried out in which substrate was omitted. The results are summarized in Table II. The data show that reduction of the enzyme makes available 1.4–1.9 functional groups per mole of enzyme which can react with NEM. It is probably that these are SH groups. To confirm the identity of the group labeled by NEM, the ^{14}C-labeled protein from the above experiment was mixed with a known amount of *S*-succinylcysteine (the cysteine-NEM adduct is converted to succinylcysteine by acid hydrolysis). The mixture was hydrolyzed in 6 N HCl and chromatographed on Dowex 1. The purified succinylcysteine contained ^{14}C with a specific activity 80% of the value predicted if all of the ^{14}C were present as succinylcysteine.

We believe that these data indicate that two –SH groups become available when the dimeric enzyme is reduced. It is extremely difficult to maintain totally anaerobic conditions and therefore it is not surprising that we failed to incorporate two molecules of NEM per dimer. It is important to note that, in all experiments, the number of molecules incorporated per dimer exceeds one.

We next determined whether a specific cysteine residue is labeled by NEM. The ^{14}C-labeled enzyme and control from above were reduced, carboxymethylated, and digested with trypsin. The resulting peptides were mapped using two dimensional electrophoresis and chromatography. Staining of these maps with fluorescamine revealed that 40–50 peptides were separated. From the published amino acid analysis (Yasunobu et al., 1976), 63 tryptic peptides should be formed. The location of the radioactivity was determined by autoradiography; the autoradiograms of both maps are shown in Figure 2. Both maps showed several weakly radioactive spots.

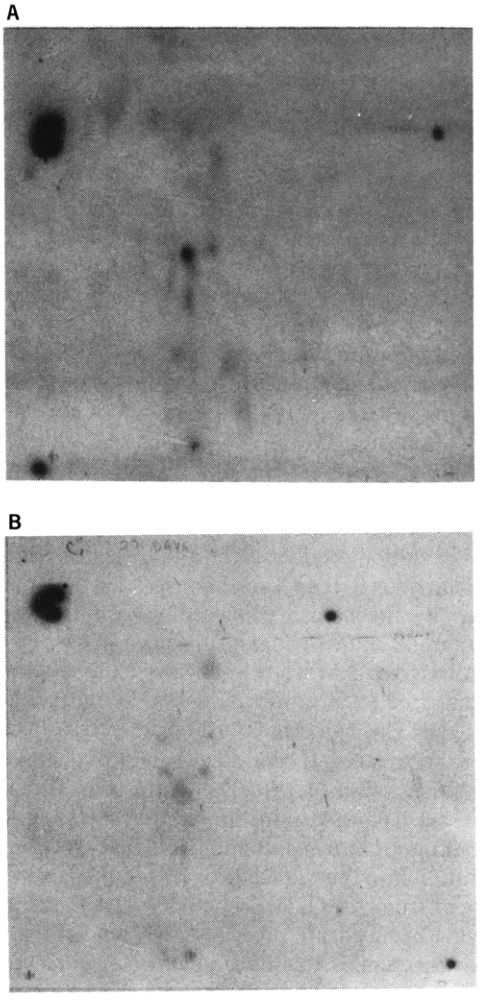


FIGURE 2: Autoradiography of peptide maps prepared from [^{14}C]-NEM-labeled enzyme. The [^{14}C]NEM-labeled enzyme was reduced, carboxymethylated, and digested with trypsin as described in Methods section. Aliquots containing 1000 cpm were spotted on Analtech silica gel plates. The plates were chromatographed in butanol/acetic acid/H_2O (4/1/1) and air dried. Pyridine–acetate buffer was sprayed on the plates (0.24 M, pH 6.4) and they were electrophoresed at 400 V for 2 h. After drying, the peptides were visualized with fluorescamine, and the location of the ^{14}C was determined by autoradiography with Kodak X-Omat-R film for 21 days. (A) Enzyme reduced with ethyl glycinate prior to reduction with mercaptoethanol. (B) Ethyl glycinate omitted.

However, the map of the reduced labeled enzyme had one strongly radioactive spot not present on the control map. This shows that a specific cysteine residue is labeled by [^{14}C]NEM in the reduced enzyme. Since the enzyme is a dimer of identical subunits, it is likely that an identical cysteine residue is labeled in each subunit.

Discussion

It has been proposed that pyridoxal-phosphate (Von Werle & Von Rechman, 1949; Yamada & Yasunobu, 1963), or possibly a pyridoxal-like compound, is the immediate electron acceptor in the reaction catalyzed by plasma amine oxidase. The presence of pyridoxal-phosphate has, however, never been directly demonstrated. We have now analyzed plasma amine oxidase with a sensitive microbiological assay and failed to detect pyridoxal-phosphate. It can, therefore, be concluded that pyridoxal-phosphate is not the cofactor of plasma amine oxidase. That then leaves the possibility that the cofactor might be similar to pyridoxal, but sufficiently modified so that it cannot be detected by the microbiological assay.

The mechanism proposed (Blashko, 1962) for the participation of pyridoxal is a two-step process represented by

$$\text{E-Pyr-CHO} + \text{RCH}_2\text{NH}_2 \rightarrow \text{E-Pyr-CH}_2\text{-NH}_2 + \text{R-CHO}$$

$$\text{E-Pyr-CH}_2\text{NH}_2 + \text{O}_2 \rightarrow \text{E-Pyr-CHO} + \text{H}_2\text{O}_2 + \text{NH}_3 \quad (2)$$

According to this mechanism, a transamination first takes place between the substrate and enzyme-bound pyridoxal or possibly some other cofactor containing an aldehydic group which gives rise to the product aldehyde and converts the enzyme bound aldehydic group to an amino group. In the second step, the amino group is oxidized by O_2 and the aldehydic group is regenerated. A modification of this mechanism has also been proposed by Hamilton which does not require the transamination step (Hamilton, 1971). According to that mechanism, a Schiff base is initially formed between the substrate-amine and enzyme-bound pyridoxal. Cu^{2+} mediated electron transfer then takes place from the Schiff base to O_2. The final oxidation product is the aldehyde derived from the amine and pyridoxal. It appears, however, that under anaerobic conditions, the mechanism proposed by Hamilton essentially becomes similar to the mechanism shown in eq 2.

According to eq 2, when the enzyme-bound aldehydic carbon is converted to the amine, a nonexchangeable hydrogen is introduced. This hydrogen must be derived from either the solvent or the substrate. Therefore, when the reaction is carried out with either tritium-labeled substrate, or in ^3H_2O, tritium should be incorporated into the reduced enzyme. We have carried out these experiments and found no significant tritium incorporation into the reduced enzyme, either from tritiated substrate or from ^3H_2O. We concluded that the reduction of the enzyme by substrate probably does not involve a transamination reaction. In addition, any mechanism involving the direct reduction of an enzyme carbonyl group by substrate is inconsistent with these results. Other functional groups which acquire a nonexchangeable hydrogen atom upon reduction are also excluded.

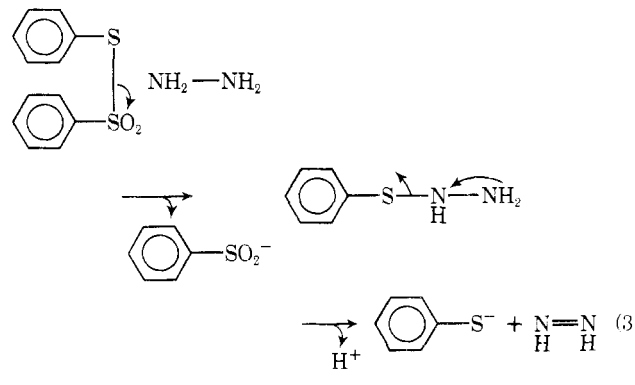
The transamination mechanism (eq 2), as well as the modified version of that mechanism, requires that the immediate product of the amine oxidation is the aldehyde and not the imine. We have obtained evidence that the amine is initially converted to the imine rather than the aldehyde. This result is also inconsistent with the proposed mechanism.

Previously, a different mechanism, shown in Scheme I, was proposed for the reaction catalyzed by plasma amine oxidase (Benitez & Allison, 1974; Neumann et al., 1975; Abeles & Maycock, 1976). According to this mechanism, the enzyme contains a bond between a cysteine sulfur and a group "X". The reaction with substrate involves addition of the amine across this bond to produce a sulfenamide (I in Scheme I) and the "X" anion. Proton removal from the α carbon of the sulfenamide results in the elimination of the thiol anion and the formation of the enzyme-bound imine. This imine is hydrolyzed to give ammonia and the aldehyde. The reduced enzyme is then reoxidized by oxygen. This mechanism is consistent with the lack of nonexchangeable hydrogen incorporation into the reduced enzyme and also with the previously reported observation that the enzyme can catalyze proton abstraction from the substrate. The intermediate formation of the substrate derived imine is also in accord with the proposed mechanism. A salient feature of the mechanism shown in Scheme I is that the reduced enzyme contains a free -SH group, while the oxidized enzyme does not. We have now presented several lines of evidence supporting the presence of an -SH group in the reduced enzyme. Initial indications for the presence of an -SH group were provided by experiments in which the enzyme was covalently labeled with bromoethylamine, a reagent which probably reacts with the reduced enzyme. Bromoethylamine labels a number of functional groups including a cysteine -SH group. It is known that the oxidized enzyme does not contain any free -SH group (Blashko, 1962). The results obtained with bromoethylamine, therefore, suggest that, in the reduced enzyme, an -SH group becomes available. More conclusive evidence for the presence of an -SH group in the reduced enzyme was obtained from experiments in which reduced and nonreduced denatured enzymes were treated with [^{14}C]NEM. The reduced enzyme incorporated from 1.3 to 1.9 mol of NEM more than the oxidized enzyme. A peptide map showed that NEM was distributed among many peptides in the oxidized enzyme, whereas it was found predominately in a single peptide in the reduced enzyme.

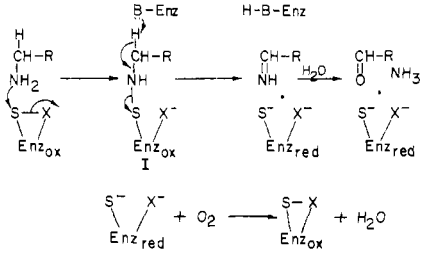
These experiments establish that a unique -SH group, probably one per subunit, is generated when the enzyme is reduced. Therefore, in the oxidized enzyme, the -SH group must be bonded to another structure. This structure is designated as X in Scheme I.

Previously, it had been proposed (Benitez & Allison, 1974) that "X" is oxygen, i.e., a sulfenic acid residue. We consider that proposal unlikely since such a structure would be highly unstable. Furthermore, it is unlikely that reduction of a cysteine-sulfenic acid would produce the observed spectral changes. At this stage, the nature of X is unknown. Identification of that group is crucial to the understanding of the mechanism of action of plasma amine oxidase.

A novel step in the proposed mechanism involves the synthesis and breakdown of the sulfenamide (I, Scheme I). These reactions are not without chemical precedents. Similar sulfenamides have been made by the nucleophilic attack of amines on sulfenyl chlorides (Heimer & Field, 1970), sulfenyl thiolsulfonates (Dunbar & Rogers, 1966), and disulfides (using metal ion catalysis) (Bentley et al., 1971). A redox reaction very similar to that shown in the proposed enzyme mechanism has also been demonstrated. Kice (1974) has shown that hydrazine attacks benzene phenylthiosulfonate to form phenylsulfenyl hydrazide; this breaks down with the loss of a proton to yield the thiol and diimide:

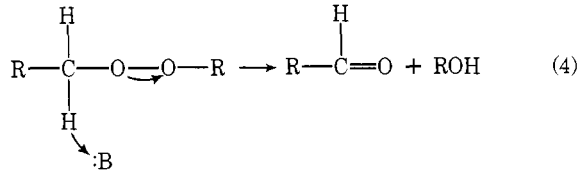


SCHEME I: Mechanism of Action of Plasma Amine Oxidase.



In the enzyme reaction, the α proton of the sulfenamide is much less acidic than the hydrazide proton above, and its abstraction would require enzyme catalysis. There is evidence to support proton removal from the substrate during the enzyme reaction (Neumann et al., 1975; Suva, 1978).

A nonenzymic model reaction for the breakdown of the sulfenamide is provided by the conversion of peroxides to aldehydes:

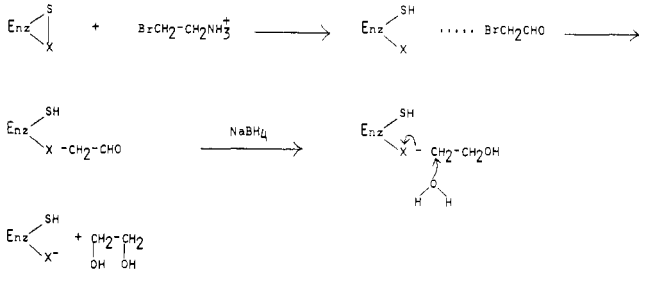


(4)

The proposed mechanism may also help to explain the inhibition of the enzyme by carbonyl reagents. The reaction of the enzyme with compounds such as hydrazine was originally interpreted as evidence for the presence of pyridoxal on the enzyme (Von Werle & Von Rechman, 1949; Yamada & Yasunobu, 1963). However, Reed (1970) has shown that hydrazines are chemically altered by the enzyme. When the enzyme is inhibited by benzylhydrazine, it eventually regains activity and releases benzaldehyde (Reed, 1970). This evidence suggests that hydrazines do not act as simple carbonyl reagents. The mechanism shown in Scheme I predicts that hydrazines would be oxidized to diimides, as in Kice's reaction above. The reactive diimide would then become covalently attached to the enzyme (thus, hydrazines would qualify as "suicide" inactivators) (Abeles & Maycock, 1976). It has been shown that phenylhydrazine inactivates mitochondrial monoamine oxidase via the intermediate formation of phenyldiimide (Patek & Hellerman, 1974).

The verification of the sulfenic enzyme mechanism in Scheme I rests on the identification of the group "X" which is bonded to cysteine in the oxidized enzyme. Although the structure of this group is not known, several of its properties can be inferred from the data. First, the "X" group is probably chromophoric when bonded to sulfur in the oxidized enzyme. Second, the results of model studies of sulfenamide formation reactions suggest that "X" is a good leaving group; this is also consistent with the lack of ^3H incorporation into the reduced enzyme discussed above. Since bromoethylamine inactivation alters the enzyme absorbance spectrum (Suva, 1978), and since it appears to label a very reactive group, this inactivator may in fact react with the "X" group. This would also provide an explanation for the formation of ethylene glycol when the enzyme inactivated by bromoethylene is hydrolyzed. If bromoethylamine labels a good leaving group, it could then be displaced by water as shown in Scheme II.

SCHEME II: Mechanism of Ethylene Glycol Formation.



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