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Photosuicide Inactivation of Acetylcholinesterase by Nitrosamine Derivatives†

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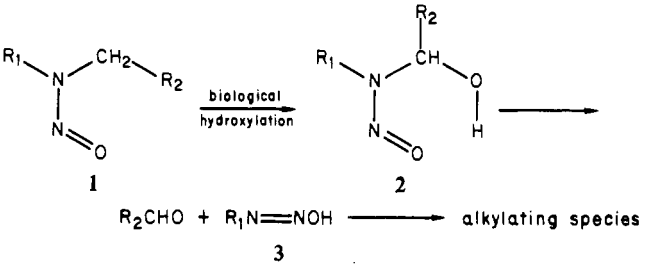
ABSTRACT: Methyl(acetoxymethyl)nitrosamine and methyl(butyroxymethyl)nitrosamine are respectively substrate (K_M = 10^{-2} M) and competitive inhibitor (K_i = 2 × 10^{-3} M) of electric eel acetylcholinesterase (EC 3.1.1.7). Irradiation of an incubation mixture of this enzyme with either nitrosamine leads to an irreversible loss of enzyme activity. The inactivation rates are dependent on photolysis wavelength, light intensity, and inhibitor concentration. Experiments where acetylcholinesterase was radioactively labeled by [^{14}C]-methyl(acetoxymethyl)nitrosamine show that the incorporation of 1 mol of radioactive label per active site is sufficient to cause complete enzyme inactivation irrespective of the reaction conditions used. Methyl(acetoxymethyl)nitrosamine shows no affinity for horse serum butyrylcholinesterase (EC 3.1.1.8) while methyl(butyroxymethyl)nitrosamine is a competitive inhibitor (K_i = 2 × 10^{-3} M), but no irreversible inhibition is induced by the action of light. We propose that a suicide type of inhibition [Bloch, K. (1969) *Acc. Chem. Res. 2*, 193–198] is responsible for the inactivation of acetylcholinesterase, based on photoactivation of nitrosamines only when associated with an acidic hydrogen of the active site.

Nitrosamines are among the most powerful carcinogenic chemicals known. They are easily converted after in vivo α-hydroxylation to alkylating species (Skipper et al., 1977), which are responsible for their carcinogenicity (Scheme I) [for a recent review, see Ingelman-Sundberg (1980)].

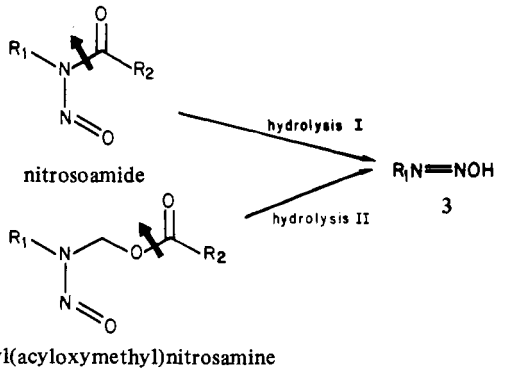
Diazoïc acid **3**, a precursor of carbonium ions, is the key intermediate in this transformation. One can use the alkylating power of those ions to inactivate enzymes. In addition, if precursor **3** of these carbonium ions is produced and decomposed at the active site of the enzyme, the conditions necessary for suicide inhibition of the enzyme have been fulfilled (Bloch, 1969; Rando, 1974, 1975; Abeles, 1976). Such a principle can be readily applied to the suicide inhibition of esterases. As a matter of fact, there are several ways to generate diazoïc acid **3** by using hydrolytic reactions (Scheme II).

Successful catalytic inhibition of chymotrypsin has been described by White et al. (1975, 1977a,b, 1978) on nitrosoamide hydrolysis. The use of nitrosamine derivatives (hydrolysis II) was of interest because this starting material is much more stable at physiological pH than the corresponding nitrosoamide and might allow studies of complex systems or in vivo experiments.

Scheme I



Scheme II



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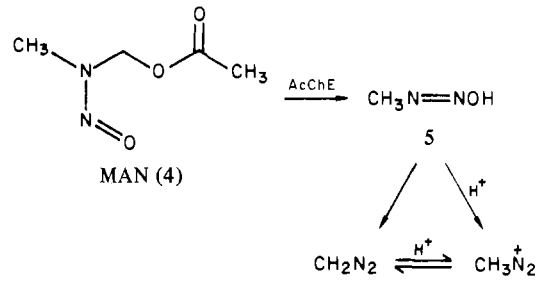
Scheme III



We studied the inhibition of acetylcholinesterase (EC 3.1.1.7) by this latter approach by using methyl(acetoxymethyl)nitrosamine (MAN, **4**) as substrate (Goeldner & Hirth, 1977) (Scheme III).

The irreversible inhibition of acetylcholinesterase by MAN[1] is too slow to be analyzed kinetically (M. Goeldner and C. Hirth, unpublished results). This result is in agreement with that obtained recently by Gold & Linder (1979) on the hog liver esterase. However, the inhibition of the enzyme by MAN becomes substantial when the incubation mixture is photolyzed ($\lambda > 300$ nm) (Goeldner & Hirth, 1977). We suggested that highly reactive species generated from photodecomposition of methyldiazoïc acid (**5**, **3** with R = CH_3) were responsible for enzyme inactivation. The results we report here show that this hypothesis is insufficient, and we propose a more complete mechanism based on selective photodecomposition of the nitrosamine in the enzyme–substrate complex.

Material and Methods

Chemicals and Buffers. All reagents used were of the best available grade except the compounds used for affinity chromatography: *m*-(dimethylamino)benzoïc acid pure (Fluka) and ethyl iodide pure (Prolabo).

Sepharose 4B was purchased from Pharmacia. Methyl(acetoxymethyl)nitrosamine and methyl(butyroxymethyl)nitrosamine were synthesized according to Roller et al. (1975).

O-Ethyl *S*-(diisopropylamino)ethyl methylphosphonothioate was a gift of Professor J. Massoulié.

The ^{14}C-labeled methyl(acetoxymethyl)nitrosamine was synthesized starting from [^{14}C]methylamine chlorohydrate (41.2 mCi mmol^{-1}) purchased from Commissariat à l'Energie Atomique. The specific activity of the labeled product was 4.5×10^8 cpm mmol^{-1}.

Sample solubilizer N.C.S. was from Amersham. Buffer A contained 50 mM potassium phosphate, pH 7.2. Buffer B contained 0.05 M $MgCl_2$, 0.01 M tris(hydroxymethylamino)methane, pH 7.2, and 0.15 M NaCl.

Enzymes. Electric eel acetylcholinesterase was either obtained from Sigma Chemical Co. (Grade V, 1400 units mg^{-1}) and used without purification or purified from *Electrophorus electricus* tissues according to Massoulié & Bon (1976).

Horse serum butyrylcholinesterase was obtained from Sigma Chemical Co. (Type X, 50–100 units mg^{-1}). Enzymes were assayed in buffer A in the presence of acetylthiocholine iodide (5×10^{-4} M) and 5,5′-dithiobis(2-nitrobenzoïc acid) (1 mg mL^{-1}). The liberation of thiocholine was followed spectrophotometrically by recording the absorption of thionitrobenzoate anion at 412 nm.

Acetylcholinesterase active sites were titrated with *O*-ethyl *S*-(diisopropylamino)ethyl methylphosphonothioate as the alkylating agent (Vigny et al., 1978).

[1] Abbreviations used: MAN, methyl(acetoxymethyl)nitrosamine; MBN, methyl(butyroxymethyl)nitrosamine; AcChE, acetylcholinesterase.

Table I[a]

Rate Equations for Scheme IV			
$d[E]/dt =$	$-k_1$ [E]	$+k_{-1}$ [ES]	$+k_3$ [Eπ]
$d[ES]/dt =$	k_1 [E]	$-(k_{-1} + k_2)$ [ES]	
$d[E\pi]/dt =$		k_2 [ES]	$-(k_3 + k_4)$ [Eπ]
$d[E']/dt =$			k_4 [Eπ]
Rate Equations for Scheme V			
$d[E]/dt =$	$-k_1$ [E]	$+k_{-1}$ [ES]	$+k_3$ [Eπ]
$d[ES]/dt =$	k_1 [E]	$-(k_{-1} + k_2 + k_4)$ [ES]	
$d[E\pi]/dt =$		k_2 [ES]	$-k_3$ [Eπ]
$d[E']/dt =$		k_4 [ES]	

[a] k_{-1}, k_2, k_3, and k_4 are first-order rate constants while $k_1 = k(S)$ ([S] >> [E]) is an apparent first-order rate constant.

Irradiation Experiments. The samples were irradiated with monochromatic light obtained from a 1000-W Xe–Hg lamp (Hanovia) connected to a grating monochromator (Schoeffel GM 250). The widths of the entrance and exit slits were maintained at 5 mm. The light intensity was regulated with an iris diaphragm placed after the condenser. The exit slit image (1 × 0.5 cm) was focused on the experimental cell (1-cm light path, 3-mL volume). The irradiated solutions were maintained at 20 °C and stirred magnetically. The incident light intensity was measured by actinometry (Hatchard & Parker, 1956) or by a thermopile (Kipp & Zohnen) coupled to a microvoltmeter. For example, at 350-nm light intensity, measurement of 1 mV corresponds to an incident energy of 2.17×10^{-6} einstein s^{-1} cm^{-2}.

Counting of Radioactive Samples. Radioactive samples were counted in 15 mL of Bray's solution (Bray, 1960) after partial solubilization in 0.2 mL of water and digestion by 1 mL of N.C.S. The counts were not corrected for quenching.

Stoichiometry of Inactivation of AcChE with Radiolabeled MAN. Solutions of the enzyme (3 mL; 0.15 mg mL^{-1}) in buffer B were photolyzed at 350 nm (light intensity 8 mV) in the presence of different concentrations of radiolabeled inhibitor until a chosen value of enzyme inactivation was reached. Successive additions of radiolabeled inhibitor (5×10^{-4} M) were necessary to obtain the higher values of enzyme inactivation. The reaction was stopped by freezing the samples, which were later dialyzed several times against 1% NH_4HCO_3 buffer and lyophilized. The incorporated radioactivity was determined as described above.

Explanation of the Kinetic Results. For the results of these kinetic experiments, we have presented the inactivation half-life as a function of the inhibitor concentration and the light intensity.

After the decrease in the amount of the active enzyme with time was taken into account, the inhibition reaction kinetics described by the system of differential equations (Table I) obtained from Schemes IV and V (see below) can *not* be solved in a steady-state approximation. We have resolved the differential equations shown in Table I by using Laplace transformations with only the realistic constraint that the pseudo-first-order constants k_1 and k_{-1} are large with respect to k_2, k_3, and k_4. This constraint leads to a solution with three "apparent" rate constants. Only one of them, the inactivation half-life, is measurable and appears in eq 1 and 2 corresponding to our proposed reaction Schemes IV and V.

The values of k_2 and k_3 were chosen to minimize the mean-square deviation of the inactivation half-life for all experiments.

$$\delta = \sum[(t_{1/2})_{\text{exptl}} - (t_{1/2})_{\text{calcd}}]^2$$

A linear least-squares fit was used to express k_4 as a function of the light intensity.

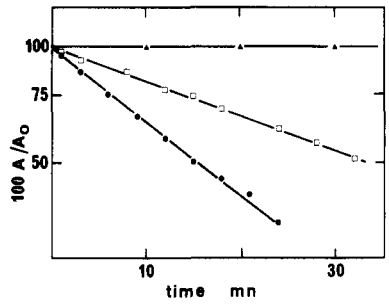


FIGURE 1: Inactivation of acetylcholinesterase by MAN at 20 °C in buffer A (semilogarithmic plot) in the presence of light (λ = 350 nm; light intensity = 5.8 mV). A_0 is the initial activity and A the residual activity at the indicated time. Concentrations of inactivator were (▲) 0, (●) 2.5×10^{-4} M, and (□) 2.5×10^{-4} M, plus 10^{-3} M of tetramethylammonium iodide.

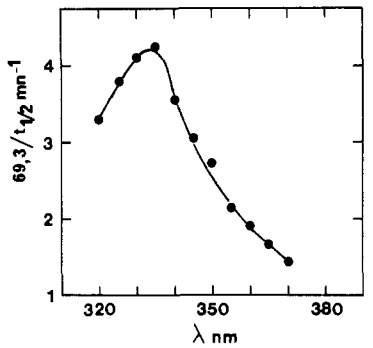


FIGURE 2: Inactivation of acetylcholinesterase as a function of wavelength in buffer A (MAN concentration 2.3×10^{-4} M). The incident energy was kept constant to 8.68×10^{-6} einstein s^{-1} cm^{-2} (i.e., 4 mV at 350 nm).

Results

Inactivation Kinetics of Acetylcholinesterase by MAN. Incubation of the enzyme with MAN (**1**) in the dark led to a gradual loss of enzyme activity. However, when the incubation mixture was irradiated with monochromatic light (λ = 350 nm), the rate of enzyme inactivation became substantial and obeyed a pseudo-first-order rate law (Figure 1). The enzyme remained stable when irradiated at this wavelength in the absence of MAN, regardless of the light intensity.

(*a*) *Influence of Wavelength.* Figure 2 represents the enzyme inactivation half-life as a function of the irradiation wavelength at fixed inhibitor concentration and light intensity. The inactivation rate was maximal at a photolysis wavelength of about 335 nm. This spectrum was independent of light intensity (results not shown).

(*b*) *Influence of Substrate Concentration.* The inactivation half-life of acetylcholinesterase as a function of the reciprocal of inhibitor concentration is shown in Figure 3. For three different light intensities at a fixed wavelength of 350 nm, the inactivation half-life was directly proportional to the reciprocal of inhibitor concentration.

In addition, the slopes of the lines and the intercepts on the ordinate increased with decreasing incident light intensities.

(*c*) *Influence of Light Intensity.* If we assume that the species responsible for acetylcholinesterase inactivation by MAN is a highly photosensitive species, the rate of enzyme inactivation for a given concentration of MAN should vary with the light intensity. Figure 4 shows the inactivation half-life as a function of light intensity for two concentrations of inhibitor. At weak light intensities, proportionately greater variation inactivation half-lifes were found.

Protection of Acetylcholinesterase against MAN Inactivation by Tetramethylammonium Iodide. Irradiation of acetylcholinesterase in the presence of a constant amount of MAN and increasing amounts of $Me_4N^+I^-$ (a competitive inhibitor of acetylcholinesterase) caused a net decrease in the rate of enzyme inactivation. The observed protection was directly proportional to the amount of $Me_4N^+I^-$ added (Figure 5). The equation defining this protection was fitted by linear least squares from the competitive inhibition data:

$$t_{1/2} = (15.5 \pm 0.3) + (15.4 \pm 0.4) \times 10^{-4} (N^+Me_4I^-)$$

Inactivation Stoichiometry. For experiments where acetylcholinesterase was labeled with [*methyl*-^{14}C]MAN, we used a partially purified enzyme, we determined that 30% of the total protein was enzymatically active (Vigny et al., 1978). These results are shown in Figure 6. The number of active

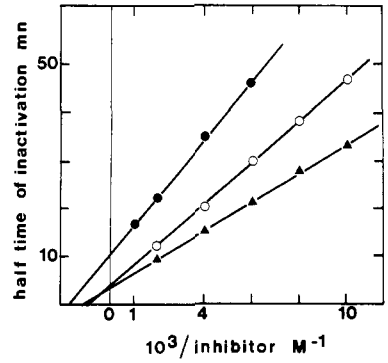


FIGURE 3: Half-time of inactivation vs. the inverse of the concentration of MAN at three different light intensities (λ = 350 nm): (▲) 5.8 mV; (○) 3.8 mV; (●) 3 mV.

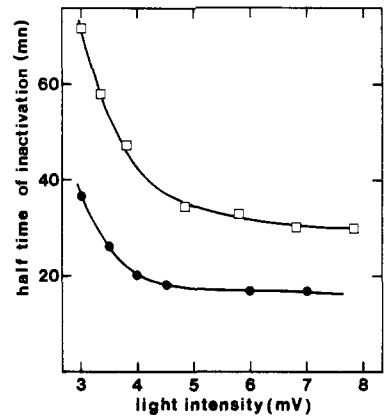


FIGURE 4: Half-time of inactivation of acetylcholinesterase by MAN vs. light intensity (λ = 350 nM). Concentrations of inactivator were (□) 10^{-4} M and (●) 2.5×10^{-4} M.

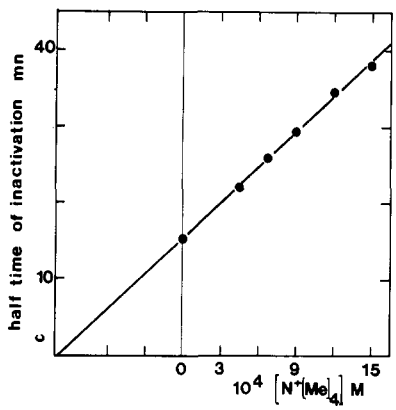


FIGURE 5: Protection against MAN inactivation by tetramethylammonium iodide. Half-time of inactivation as a function of tetramethylammonium iodide concentration. MAN concentration = 2.5×10^{-4} M; λ = 350 nm; light intensity = 5.8 mV.

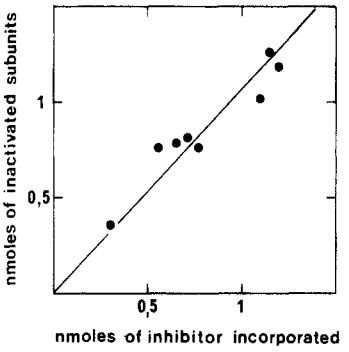


FIGURE 6: Stoichiometry of [^{14}C]MAN incorporation by acetylcholinesterase. Measurements were performed as described under Material and Methods.

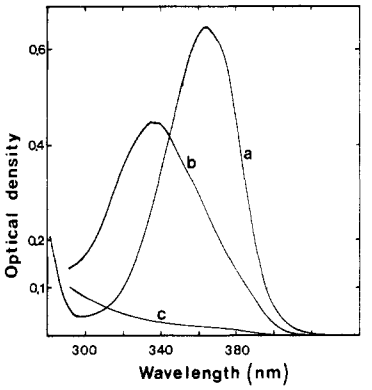


FIGURE 7: UV spectrum of MAN (6×10^{-3} M) in (a) heptane, (b) heptane in the presence of 0.9 M CF_3COOH, and (c) solution b after 2 min of irradiation (λ = 335 nm; light intensity 3.2 mV).

sites inhibited was directly proportional to the amount of radioactive inhibitor incorporated.

The incorporation of 0.95 mol of [^{14}C]methyl per mol of active site was required for complete inactivation of the enzyme. This stoichiometry was not dependent on light intensity. One should note that when the enzyme was 100% inhibited by prior reaction with *O*-ethyl *S*-(diisopropylamino)ethyl methylphosphonothioate and then incubated in the described conditions with [^{14}C]MAN, no radioactivity was incorporated.

Inactivation Selectivity. Table I contains the results of the inhibition of acetylcholinesterase and butyrylcholinesterase by MAN and MBN. It is clear that MBN as well as MAN irreversibly inhibits acetylcholinesterase in the presence of light. In the same conditions, neither MBN nor MAN irreversibly inhibits butyrylcholinesterase even though MBN is a competitive inhibitor (but not a substrate) of both enzymes.

Photostability of MAN. Even though MAN was photochemically relatively stable and showed maximal absorption at 350 nm in aqueous solution in the pH range 2–7.5, certain physicochemical conditions cause a considerable increase in its photosensitivity. In fact, the absorption maximum at 370 nm of the UV spectrum of MAN in a solvent with a low dielectric constant (chloroform or heptane) is shifted to 335 nm after the addition of proton donors. Irradiation of this acidic solution rapidly led to the disappearance of the absorption peak of nitrosamine (Figure 7). This result is in agreement with the results of Layne et al. (1963a,b) and Chow (1973).

Discussion

In this paper, we present results which confirm and extend preliminary results (Goeldner & Hirth, 1977), i.e., that MAN fulfills the requisite conditions of a photosuicide inhibitor for

Scheme IV

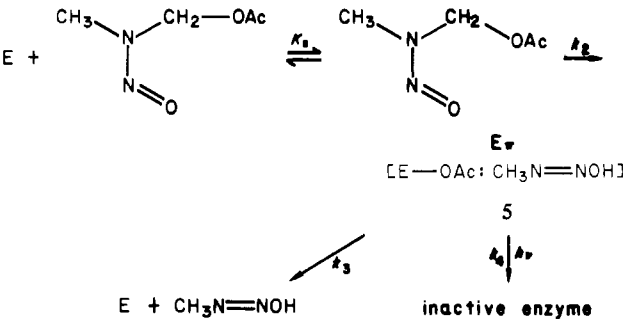


Table II

	acetylcholinesterase	butyryl-cholinesterase
MAN	poor substrate competitive inhibitor $K_i = 10^{-2}$ M irreversible inhibitor $K_{app} = 10^{-2}$ M $h\nu$: $I = 4.8$ mV $V_{max} = 0.693/t_{1/2} = 0.17$ min^{-1}	neither substrate nor inhibitor
MBN	not substrate competitive inhibitor $K_i = 2 \times 10^{-3}$ M irreversible inhibitor $K_{app} < 10^{-4}$ M $h\nu$: $I = 4.8$ mV $V_{max} = 0.693/t_{1/2} = 0.05$ min^{-1}	not substrate competitive inhibitor $K_i = 2 \times 10^{-3}$ M

acetylcholinesterase: (1) MAN is chemically and photochemically stable in our operating conditions. (2) The rate of enzyme inactivation varies with light intensity and wavelength. (3) Competitive inhibitors such as choline and tetramethylammonium iodide protect the enzyme against MAN photochemical inactivation. (4) An inhibition stoichiometry of 1 mol of inhibitor per active site was observed regardless of the incident light intensity or the degree of inhibition which indicates a good labeling selectivity.

According to these results, the simplest photosuicide mechanism which can be formulated is that shown in Scheme IV. According to this scheme, enzyme inactivation would be due to the inhibitor precursor methyldiazoic acid (**5**). From such a scheme, one can deduce eq 1, where K_s is the disso-

$$\frac{t_{1/2}}{0.693} = \frac{k_2 + k_3 + k_4}{k_2 k_4} + \left(\frac{k_3 + k_4}{k_2 k_4}\right)\frac{K_s}{[S]} \qquad (1)$$

ciation constant of the enzyme–substrate complex, [S] is the substrate concentration, and k_2, k_3, and k_4 are the first-order rate constants according to the proposed scheme. According to this equation, the inactivation half-time determined from experiments such as that shown in Figure 1 is directly proportional to the reciprocal of substrate concentration, and the intercepts of the ordinate and the abscissa are functions of k_4, thus of light intensity which was experimentally observed (Figure 3).

However, Scheme I does not take into account the following observations: First, light-induced irreversible inhibition of acetylcholinesterase by MBN shows the same rate saturation effect as by MAN whereas MBN is only a competitive inhibitor and not a substrate for the enzyme (Table II). Second, the photosensitive precursor responsible for enzyme inactivation has a UV absorption maximum at 335 nm (Figure 2) whereas the presumed precursor, that is, methyldiazoic acid (**5**), shows an absorption maximum at 360 nm (Müller et al., 1960).

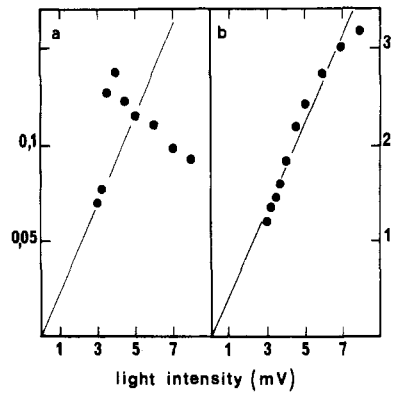
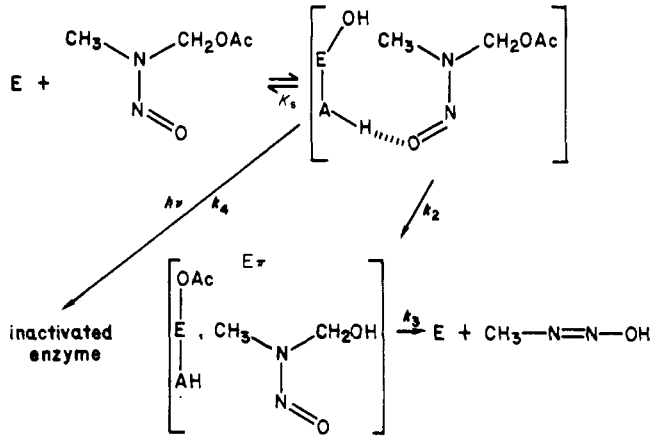


FIGURE 8: Variation of k_4 as a function of incident light intensity as calculated from Scheme IV (a) and from Scheme V (b).

Scheme V



Third, the reasonable assumption that k_4 is proportional to the incident light intensity, i.e., $k_4 = \alpha + \beta V$, is not confirmed by calculated variation of k_4 as a function of light intensity issued from experimental results shown in Figures 3 and 4 (Figure 8a).

If we now take into account these observations, we formulate a different kinetic scheme (Scheme V). This reaction mechanism implies that the starting nitrosamine would become photoactive as soon as it reaches the active site of the enzyme and then photodecomposes to an alkylating species. Such an assumption is not in contradiction with the relative photostability of the nitrosamine in water (at pH 3, 24% of MAN is degraded after 20 min at $\lambda = 350$ nm, $V = 4$ mV) even at acidic pH, but it implies that there are chemical requirements which induce photoactivation of nitrosamine when complexed to the active site. In fact, photoactivation of MAN occurs when dissolved in solvents of low dielectric constants in the presence of a proton donor. It is noticeable that the UV spectrum of MAN taken in these conditions shows a maximal absorption at the same wavelength (335 nm) than does the photosensitive precursor in the active site of acetylcholinesterase (Figure 8).

According to Scheme V, we can write eq 2. K_s, [S], k_2, k_3, and k_4 are the same as described for eq 1.

$$\frac{t_{1/2}}{0.693} = \frac{k_2 + k_3 + k_4}{k_3 k_4} + \frac{1}{k_4}\left(\frac{K_s}{[S]}\right) \qquad (2)$$

Experimental results agree well with the kinetic model on the following points: First, the enzyme half-life is directly proportional to the reciprocal of inhibitor concentration. Second, k_4 is directly proportional to the incident light intensity (V) (Figure 8b). The equation of the resulting straight line is $k_4 = \alpha + \beta V$ from which we found $\alpha = 0$ and $\beta = 0.41 \pm 0.02$. The calculated value of α was a small positive number not significantly different from zero. The rate constants k_2 and k_3 can be estimated as 1.2 min^{-1} and 0.2 min^{-1}, respectively.

Third, the competitive inhibitor tetramethylammonium iodide protects acetylcholinesterase against inactivation by MAN (Figure 5). According to Scheme V, $t_{1/2}$ must be directly proportional to the concentration of competitive inhibitor as expressed in eq 3, where K_i is the dissociation constant of

$$\frac{t_{1/2}}{0.693} = \frac{k_2 + k_3 + k_4}{k_3 k_4} + \frac{1}{k_4}\left(\frac{K_s}{K_i}\right)\left(\frac{[I]}{[S]}\right) \qquad (3)$$

the complex enzyme–tetramethylammonium iodide. From this equation, we calculated an affinity constant ($K_i = 1.5 \times 10^{-3}$ M) which agrees with previously reported values (Wilson & Alexander, 1962).

The validation of Scheme V implies the existence of two fundamental properties of the binding site of MAN on acetylcholinesterase.

First, there is at least one protonated residue in the enzyme active site which combines with the nitrosamine within the enzyme–substrate complex. Evidence for this comes from the displaced UV spectra of nitrosamine in the presence of trichloroacetic acid (Layne et al., 1963a,b) as well as from our results. This type of association has also been found for serine proteases where a hydrogen bond is formed between the carbonyl of the peptide bond to be broken and the enzyme (Blow, 1976). In addition, certain catalytic inhibitors requiring prior protonation to form an alkylating species are active with serine proteases (Erlanger et al., 1966; Rajogopalan et al., 1966), glutaminase (David et al., 1963; French et al., 1963; Buchanan, 1978), and β-galactosidase (Sinnot & Smith, 1976, 1978; Sinnot et al., 1978; Brockhaus & Lehmann, 1976).

Second, the acetylcholinesterase active site must have a hydrophobic nature. At least one tryptophan has been shown to be close to the active site (Rosenberry, 1975), and numerous cations which are competitive inhibitors of the enzyme have a hydrophobic character. Thus, when MAN binds to the enzyme, it passes from a hydrophilic to a hydrophobic environment. This change in environment is requisite for good binding of acetylcholine to the active site and has already been invoked as an important factor in enzyme catalysis (Jencks, 1975).

One should note that if MAN and MBN are photosuicide inhibitors of acetylcholinesterase, neither compound irreversibly inhibits butyrylcholinesterase, which implies important physicochemical differences between the active sites of these two enzymes.

With regard to specificity, this type of photosuicide inhibition has the advantage of combining catalytical (chemical property) and environmental (physical property) requirements.

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Amino Acid Sequence of a Carboxypeptidase Inhibitor from Tomato Fruit†

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ABSTRACT: The amino acid sequence of a 37 residue carboxypeptidase inhibitor from tomato fruit has been determined. The amino terminus was shown to be 2-oxopyrrolidine-5-carboxylic acid by digestion of reduced and S-carboxymethylated inhibitor with pyroglutamate aminopeptidase. The remainder of the sequence was assigned by analysis of peptides which had been generated by specific cleavage at the Asp_4–Pro_5 bond under acid conditions and by treatment with trypsin. The amino acid sequence of this inhibitor is identical with that of an analogous inhibitor from potatoes in 26 positions, and two of the replacements are highly conservative. The identification of the nonconservative replacements has been used to better define regions of the inhibitor which are not believed to contribute significantly to the free energy of association of the enzyme–inhibitor complex.

Although proteinaceous inhibitors of the serine proteinases are widely distributed in nature, polypeptides which specifically inhibit the pancreatic carboxypeptidases have only been found in roundworms (Homandberg & Peanasky, 1976), potatoes (Ryan et al., 1974), and, quite recently, in tomato fruit (Hass & Ryan, 1980). Since the tomato and potato plants are close phylogenetic relatives, it is not unexpected that the carboxypeptidase inhibitors from these sources exhibit similar physical and chemical properties. That the free energies of association of these two inhibitors with their target enzymes are virtually indistinguishable is, however, somewhat surprising (Hass & Ryan, 1980).

This report presents the amino acid sequence of the carboxypeptidase inhibitor from tomatoes to provide a means of comparison with the sequence of the inhibitor from potatoes (Hass et al., 1975). These data should be of particular utility both in assessing the extent of homology between these proteins and in identifying unusually important regions of these molecules.

Experimental Section

Materials. The carboxypeptidase inhibitor was prepared from ripened tomato fruit as described by Hass & Ryan

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